# THE ROLE OF B-VITAMINS – GENE INTERACTIONS IN COLORECTAL CARCINOGENESIS

## A MOLECULAR EPIDEMIOLOGICAL APPROACH

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Dit onderzoek is uitgevoerd binnen de onderzoeksschool VLAG.

# THE ROLE OF B-VITAMINS – GENE INTERACTIONS IN COLORECTAL CARCINOGENESIS

## A MOLECULAR EPIDEMIOLOGICAL APPROACH

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**Proefschrift**
**ter verkrijging van de graad van doctor**
**op gezag van de rector magnificus**
**van Wageningen Universiteit,**
**Prof. dr. M.J. Kropff,**
**in het openbaar te verdedigen**
**op dinsdag 13 december 2005**
**des namiddags te half twee in de Aula**

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The role of B-vitamins – gene interactions in colorectal carcinogenesis –
A molecular epidemiological approach

Thesis Wageningen University – With references – With summary in Dutch

ISBN 90-8504-328-X

# ABSTRACT


## The role of B-vitamins–gene interactions in colorectal carcinogenesis – A molecular epidemiological approach

*PhD thesis by Maureen van den Donk, Division of Human Nutrition, Wageningen University, The Netherlands, December 13, 2005.*

Folate deficiency can affect DNA methylation and DNA synthesis. Both factors may be operative in colorectal carcinogenesis. Many enzymes, like methylenetetrahydrofolate reductase (MTHFR), thymidylate synthase (TS), methionine synthase (MTR), and serine hydroxymethyltransferase (SHMT), are needed for conversions in folate metabolism. Flavin adenine dinucleotide, a metabolite of vitamin B2, is a cofactor for MTHFR; vitamin B6 is a cofactor for SHMT; and vitamin B12 is a cofactor for MTR. Polymorphisms exist in most of the genes encoding the enzymes that play a role in folate metabolism. Therefore, genetic variation might influence DNA methylation and synthesis processes and thus colorectal carcinogenesis. This thesis describes studies that have been conducted to clarify the role of folate, related B-vitamins, and genetic variation in colorectal carcinogenesis.

In a meta-analysis of human observational studies on the association between folate intake and risk of colorectal adenomas, including data from 4 cohort studies and 10 case-control studies, pooled relative risks (95% confidence interval (CI)) for highest *vs.* lowest exposure category of 0.85 (0.71;1.01) for dietary folate intake and 0.75 (0.61;0.93) for total folate intake were found.

In a Dutch case-control study, including data of colorectal adenoma cases (n=768) and endoscopy controls with no history colorectal polyps (n=709), a slightly positive association between folate and colorectal adenoma risk (odds ratio (OR) highest *vs.* lowest tertile 1.32, 95% CI 1.01;1.73), and an inverse association between vitamin B2 intake and colorectal adenoma risk (OR highest *vs.* lowest tertile 0.51, 95% CI 0.36;0.73) was found, especially among those with *MTHFR 677 TT* genotype. A null association was found for vitamin B6 and vitamin B12. Furthermore, the combined intake of B-vitamins might be important: the positive association between folate intake and colorectal adenomas seemed to be more pronounced among those with a low vitamin B2 intakes. The polymorphisms in the folate metabolism studied (*MTHFR*, *TS* and *SHMT1*) did not seem to influence colorectal adenoma risk when dietary factors were not taken into account. Furthermore, relatively high folate intake (>212 μg/day) was mildly inversely associated with promoter methylation of six selected tumor suppressor and DNA repair genes in adenoma tissue as compared with low folate intake (<183 μg/day), with statistically non-

significant ORs ranging from 0.54 to 0.86. This effect was mainly restricted to those carrying the *MTHFR 677 TT* genotype.

In a randomized, controlled intervention study including 86 subjects with a history of colorectal adenomas, a high dosage of synthetic folic acid (5 mg/day) and vitamin B12 (1.25 mg/day) for six months seemed to increase uracil incorporation ($\Delta_{intervention} - \Delta_{placebo}$ 0.45, 95% CI -0.19;1.09) and promoter methylation of six selected tumor suppressor and DNA repair genes ($OR_{upmethylation}$ 1.67, 95% CI 0.95;2.95), both biomarkers measured in DNA from rectal mucosa biopsies. Again, the effect seemed more pronounced in those with the *MTHFR 677 TT* genotype.

The results of these relatively small studies suggest that a potential adverse effect of folic acid should be considered, especially when administered after colorectal neoplastic lesions have been established. However, these results need to be confirmed by larger studies among other populations with similar relatively low intakes of vitamin B2, in which the *MTHFR C677T* genotype or other polymorphisms in folate metabolism should be taken into account.

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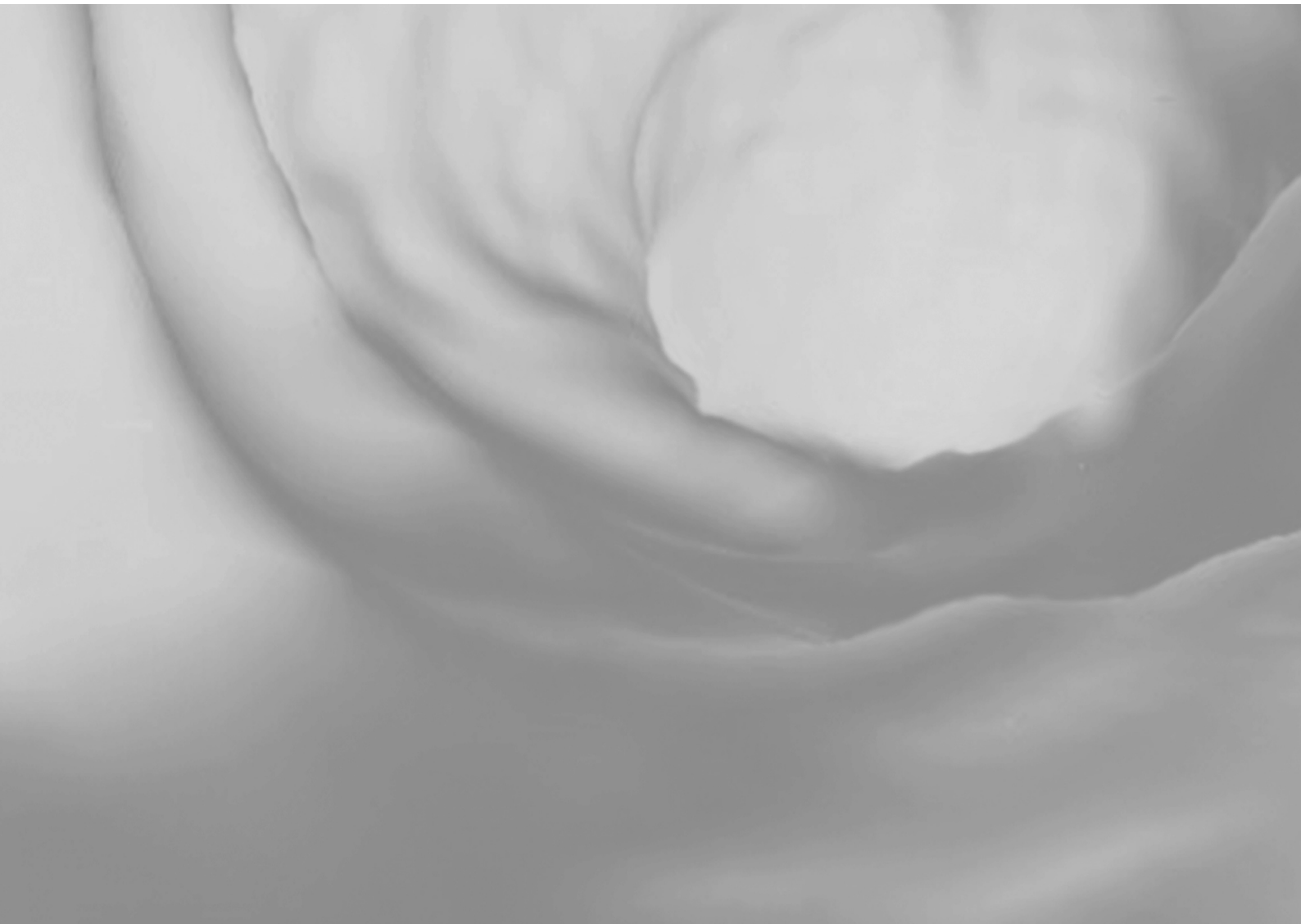
# Chapter 1

## INTRODUCTION



**During the past years, folate has emerged as an important factor in the prevention of a whole array of diseases: anemia, heart disease, stroke, neural tube defects, mental health and cancer.[1] Furthermore, not only folate, but also other B-vitamins are essential in disease prevention. This thesis describes studies that have been conducted to clarify the role of folate and related B-vitamins in colorectal carcinogenesis. The introduction starts with explaining the possible mechanism by which folate and related B-vitamins may exert their protective role in colorectal carcinogenesis, followed by a description of the literature (human observational studies and human intervention studies), and ending with the rationale and the outline of the thesis.**

# THE POTENTIAL MECHANISM

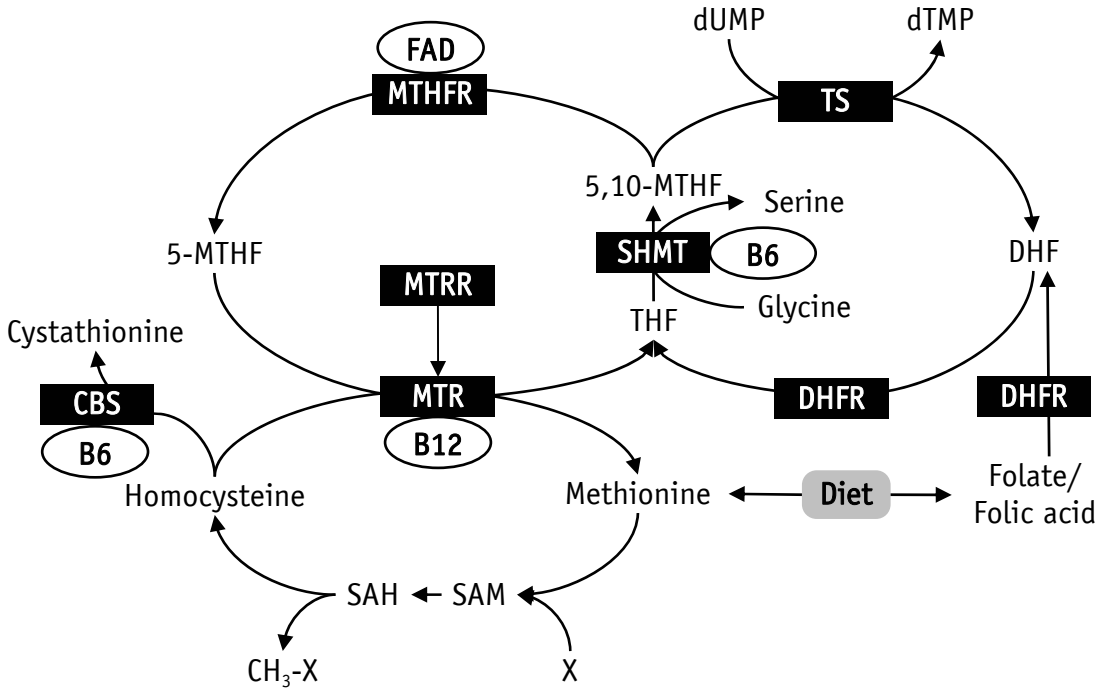
## DNA methylation and DNA synthesis: folate and the one-carbon metabolism

Folate is a B-vitamin that is essential in DNA metabolism. Two mechanisms have been proposed by which folate status or intake might modulate colorectal cancer risk: folate deficiency can affect DNA methylation[2] or incorporation of uracil instead of thymidine in DNA, leading to defective DNA synthesis.[3]

Folate is responsible for mediating the transfer of one-carbon moieties, such as methyl groups (see figure 1.1). As 5-methyltetrahydrofolate (5-methylTHF), folate provides methyl groups for S-adenosyl methionine, which serves as a methyl donor for over 100 biochemical reactions, including the methylation of DNA.[2] DNA methylation is an important event in gene regulation: it is involved in gene expression, chromatin configuration and structural stability of DNA, binding of transcriptional factors and other proteins, mutations and imprinting (reviewed in [4]). Colorectal neoplasms, both carcinomas and adenomas, show a decreased global DNA methylation level compared to normal tissue.[5,6] Conversely, other studies have shown methylation of the promoter region of specific tumor suppressor genes in colorectal tumors,[7] which is increasingly recognized to play an important role in cancer development through silencing of gene transcription.[8]

Furthermore, deficiency of folate affects purine and pyrimidine synthesis.[3,9] 5,10-Methylenetetrahydrofolate (5,10-methyleneTHF) acts as a cofactor in the conversion of deoxyuridylate (dUMP) to thymidylate (dTMP). This is the only *de novo* source of thymidylate, an essential precursor of DNA biosynthesis,[10] which is required for DNA replication and repair. Low cytosolic levels of 5,10-methyleneTHF decrease synthesis of dTMP, increasing the cellular dUMP/dTMP ratio and uracil (dUTP) misincorporation into DNA.[11] Uracil is excised from DNA, generating transient single-strand breaks that could result in more hazardous double-strand breaks if two opposing strand breaks are formed.[12] The induction of strand breaks has been associated with chromosomal aberrations, which in turn have been established to be a risk factor for cancer.[13]

***Figure 1.1*** *The one-carbon metabolism*




*Abbreviations: FAD: flavin adenine dinucleotide; MTHFR: methylenetetrahydrofolate reductase; 5-MTHF: 5-methyltetrahydrofolate; 5,10-MTHF: 5,10-methylenetetrahydrofolate; MTR: methionine synthase; MTRR: methionine synthase reductase; THF: tetrahydrofolate; SHMT: serine hydroxymethyltransferase; TS: thymidylate synthase; dUMP: deoxyuridylate; dTMP: thymidylate; DHF: dihyfrofolate; DHFR: dihydrofolate reductase; SAM: S-adenosyl methionine; SAH: S-adenosyl homocysteine; CBS: cystathionine-β-synthase*

## Enzymes in the one-carbon metabolism

Many enzymes play important roles in the one-carbon metabolism. Folic acid has to be fully reduced to tetrahydrofolate (THF) before it can carry a one-carbon unit. This is carried out by dihydrofolate reductase (DHFR), which reduces folate to dihydrofolate (DHF) and also reduces DHF to THF.[14] Methylenetetrahydrofolate reductase (MTHFR) catalyzes the irreversible conversion of 5,10-methyleneTHF to 5-methylTHF.[2] Thymidylate synthase (TS) catalyzes the conversion of dUMP to dTMP, using 5,10-methyleneTHF as a cofactor. Serine hydroxymethyltransferase (SHMT) catalyzes the reversible conversion of serine and THF to glycine and 5,10-methyleneTHF. These products are both involved in purine and pyrimidine nucleotide synthesis.[15] In line with this role of providing precursors for DNA synthesis, elevated levels of SHMT activity are found in rapidly proliferating cells,[16] particularly tumor cells.[17] Human cells contain both cytosolic (cSHMT or SHMT1) and mitochondrial (mSHMT or SHMT2) forms of SHMT. Cystathionine-β-synthase (CBS) catalyzes the conversion of homocysteine to cystathionine, a pathway which competes with the remethylation of homocysteine to methionine by methionine synthase (5-methyltetrahydrofolate-homocysteine S-methyltransferase; MTR). MTR is maintained in its active form by methionine synthase reductase (MTRR).[18]

## Other B-vitamins in the one-carbon metabolism

Other B-vitamins play a role in the one-carbon metabolism as co-factors for the metabolizing enzymes. Flavin adenine dinucleotide (FAD), a metabolite of vitamin B2 (riboflavin), serves as a cofactor for MTHFR.[19-21] FAD was found to modify MTHFR activity in healthy subjects.[22] Vitamin B6 (pyridoxine) is a cofactor for SHMT.[23] Moreover, vitamin B6 works as a cofactor for CBS. Vitamin B12 (cobalamin) is a cofactor for MTR.[23]

## Genetic variation in the one-carbon metabolism

Functional polymorphisms exist in most of the genes encoding the enzymes that play a role in the one-carbon metabolism. Thus, genetic variation in the one-carbon metabolism might influence DNA methylation and synthesis processes.

A common C-to-T substitution in the *MTHFR* gene at nucleotide 677 converts an alanine to valine and produces diminished enzyme activity *in vivo*.[24] The prevalence of *TT* individuals in Europe ranges from 8% in Germany to 18% in Italy.[25] Another polymorphism in *MTHFR*, *A1298C*, occurs at approximately the same prevalence.[26] These two polymorphisms seem to be linked: individuals with a *677TT* genotype almost always have a *1298AA* genotype and vice versa.[26,27]

The *TS* gene has a 28-bp tandem repeat sequence in the 5'-untranslated region that has been shown to be polymorphic, containing most frequently two or three repeats.[28] The number of tandem repeats affects TS activity levels, probably mediated through effects of the repeats on translation efficiency. Kawakami *et al.* reported no association between *TS* genotype and mRNA levels, but *TS 3R/3R* genotype was associated with higher TS protein level.[29] The *TS* tandem repeat genotype was significantly different in Chinese and Caucasian subjects in a study by Marsh *et al.*: 67% of Chinese were homozygous for the triple repeat compared with 38% of Caucasians.[30] Rare alleles containing more repeats have also been found.[31,32]

In 2000, Ulrich *et al.* identified a second common polymorphism in the *TS* gene: a 6bp-deletion at bp 1494 in the 3'-untranslated region (*TS 1494del6*). The prevalence of the homozygous 6bp-deletion was 7% in a Caucasian population (n=95).[33] The function of this polymorphism has not yet been reported.

Heil *et al.* identified the *SHMT1 C1420T* polymorphism and reported that *CC* individuals had lower erythrocyte and plasma folate levels. The prevalence of the *TT* genotype was 9%.[34]

A large number of mutations and several polymorphisms in the *CBS* gene have been found in patients with homocystinuria, which is a metabolic disorder due to CBS deficiency.[35] Most mutations are rare; one of the most abundant polymorphisms in *CBS*, an insertion of 68 bp in exon 8 (*844ins68*), occurs in about 5% of Caucasian alleles.[35]

In 1997, the *MTR A2756G* polymorphism was identified, which converts an aspartic acid into a glycine.[36] Frequency of the *GG* genotype ranges between 1% and 11%. Most studies suggest that plasma homocysteine levels are lower in those with the *G* allele.[37]

The *MTRR A66G* polymorphism results in the substitution of isoleucine with methionine at codon 22. In some studies, the *AA* genotype was associated with elevated homocysteine levels, although not all. The prevalence of the *GG* genotype ranges from 8% in Hawaiians to 50% in Hispanics, and varied between 19 and 29% in Caucasians.[37]

A 19bp-deletion polymorphism in intron I of *DHFR* was identified in 2004. Because this deletion removes a SP1 transcription factor binding site, it is possible that this polymorphism acts to decrease *DHFR* transcription. 17% of control individuals (mainly Caucasian) was homozygote for the deletion.[38]

### Polymorphism in *MTHFR* and DNA synthesis and DNA methylation

Some studies indicate that the *MTHFR 677 TT* genotype is associated with global DNA hypomethylation in peripheral blood cells,[39-41] probably restricted to those with a low folate intake,[40] although one study could not demonstrate an association.[42] Studies examining the association between *MTHFR* genotype and promoter methylation also show contradictory results.[43-45] To our knowledge, only one study examined the relationship between *MTHFR* genotype and uracil misincorporation in human lymphocyte DNA, and in this study the uracil content was similar for all *MTHFR* variants.[42] From a Japanese study it was suggested that the haplotype with low enzymatic activity of MTHFR, that consisted of *MTHFR 1298 CC*, *677 TT*, and the combination of *1298 AC* and *677 CT* genotypes, is linked with promoter hypermethylation in proximal colon cancer.[43]

*In summary, via the one-carbon metabolism, folate and vitamins B2, B6 and B12 are needed for DNA methylation and DNA synthesis. Defects in these two processes have been linked to colorectal carcinogenesis. Furthermore, many enzymes, like MTHFR, TS, and MTR, are needed for the conversions in the one-carbon metabolism. The working of some of these enzymes (MTHFR, SHMT, MTR, CBS) depends on B-vitamins. Genetic variation in these enzymes may lead to alterations in DNA methylation and DNA synthesis.*

## EPIDEMIOLOGICAL EVIDENCE

### Human observational studies

#### B-vitamins and colorectal cancer

Lashner[46] was the first to publish a human observational study on folate intake and colorectal neoplasia: a case-control study among patients with ulcerative colitis. Patients

with ulcerative colitis commonly have decreased folate levels, partially due to use of the drug sulfasalazine, a competitive inhibitor of folate absorption.[47] Furthermore, patients with ulcerative colitis are at greater risk of developing colorectal cancer than the general population.[48] In the study by Lashner *et al.*, the use of folate supplements was associated with a 62% lower incidence of neoplasia compared with individuals not using folate supplements, although not statistically significant, possibly due to lack of power (odds ratio (OR) 0.38, 95% confidence interval (CI) 0.12;1.20).[46]

After this study, many other human observational studies on folate and colorectal cancer risk have been published. In 2005, Sanjoaquin *et al.*[49] published a meta-analysis on folate and colorectal cancer risk, including 7 cohort and 9 case-control studies. In cohort studies, the association between folate consumption and colorectal cancer risk was stronger for dietary folate (folate from foods alone; relative risk (RR) for high *vs.* low intake 0.75, 95% CI 0.64;0.89) than for total folate (including folate from supplements; RR for high *vs.* low intake 0.95, 95% CI 0.81;1.11). In case-control studies, the summary estimate for dietary folate was 0.76 (0.60;0.96) and for total folate the RR was 0.81 (0.62;1.05). These results offer support for the hypothesis that folate has a small protective effect against colorectal cancer.[49]

Results for other B-vitamins have been less often investigated. Studies investigating the association between vitamin B2[50,51] or vitamin B12 intake[51-53] and colorectal cancer risk report null associations. Studies examining the association between vitamin B6 intake and colorectal cancer risk show either no association[50-52] or an inverse association.[53-57]

### B-vitamins and colorectal adenomas

Adenomas, or adenomatous polyps, are benign neoplasms of glandular epithelium in which there is atypia of varying degrees. They are found throughout the large bowel. Colorectal adenomas are accepted widely as precursors of colorectal cancer in humans, a progression that has been termed the **adenoma-carcinoma sequence**, as a result of the accumulation of genetic and/or epigenetic alterations in genes involved in the regulation of key cellular processes, such as cell proliferation, apoptosis and DNA repair.[58] Although there is no direct proof of the transformation of adenomas to cancer, evidence from autopsy, clinical, epidemiological, and molecular genetic studies has contributed to the development of this theory. Autopsy studies have shown that populations at high risk for colorectal cancer also have a high prevalence of adenomas compared with populations at low risk for colon cancer.[59] Furthermore, removal of adenomas leads to a reduction in the subsequent risk of cancer.[60]

Colorectal adenomas themselves have been the endpoint in a number of studies since 1986, when Hoff *et al.* published on risk factors thought to be important in the etiology of colon cancer.[61] The first observational study on folate and colorectal adenomas was

published in 1993.[62] Since then, many other studies have been published.[63-74] Most epidemiologic studies examining the association between folate intake or status and colorectal adenoma risk observed an inverse association,[62-72] which was statistically significant in some studies,[62-66] whereas two studies did not find an association.[73,74]

The association between adenoma risk and other B-vitamins has also been investigated in observational studies. Three studies examined vitamin B2 intake, of which two did not find an association,[63,75] and the third reported a weak inverse association, that did not reach statistical significance.[72] Results concerning vitamin B6 intake are rather consistent: this was examined in four observational studies, which all showed statistically significant inverse associations.[63,66,72,75]. Furthermore, there was a suggestive inverse association between colorectal adenoma risk and plasma concentrations of pyridoxal 5'-phosphate, the main active form of vitamin B6, in the Nurses' Health Study.[57] Vitamin B12 results are not clear yet. Two studies reported null results for vitamin B12;[63,72] a third study reported a statistically non-significant inverse association between vitamin B12 intake and colorectal adenomas.[66]

*Based on results of human observational studies, folate seems to have a small protective effect against colorectal cancer. Most, but not all, human observational studies conducted on intake of folate or vitamin B6 and colorectal adenomas observed an inverse association. Vitamins B2 and B12 have been studied less often and results are more ambiguous.*

### Genetic variation in the one-carbon metabolism and colorectal cancer

In 2004, a review on polymorphisms in genes involved in the one-carbon metabolism and colorectal cancer risk was published.[37] In seven out of ten studies described in this review, there was a moderately reduced colorectal cancer risk in subjects with the *MTHFR 677 TT* genotype compared with the *CC* genotype, with relative risks ranging from 0.45 to 0.9, although most did not reach statistical significance. Two studies were null and one small study showed an increased risk that was not statistically significant.[37] Two studies that were not included in this review also reported not statistically significant increased risks.[74,76] In four studies reporting on *MTHFR A1298C* genotype and colorectal cancer, risk was modestly reduced in *CC* compared with *AA* genotypes, with relative risks in the range of 0.6-0.8, mostly not statistically significant (reviewed in [37]). However, two studies that were not included in this review showed increased risks.[74,76]

As far as we know, two studies have been published on the *TS* tandem repeat polymorphism and colorectal cancer risk.[77,78] In a nested case-control study within the Physicians' Health Study, the *TS 2R/2R* genotype was associated with decreased colorectal cancer risk and better survival of colorectal cancer,[77] but in a Hungarian case-control study, colorectal cancer risk was lowest for heterozygotes (*2R/3R*).[78]

In the Physicians' Health Study, *SHMT1* genotype was not associated with colorectal cancer risk or with plasma folate and plasma homocysteine.[79]

The *CBS 844ins68* genotype has been published in relation to colorectal cancer risk in three studies. In one study, the frequency of *844ins68* heterozygotes was borderline statistically significantly lower in colorectal cancer patients compared with controls (9.7% in controls and 5% in colorectal cancer cases; p=0.05). Homozygosity for *844ins68* was not detected.[80] In line with this, the second study reported a weak inverse association that did not reach statistical significance,[51] but the third study showed a slightly increased risk (OR 1.10, 95% CI 0.27;4.53).[74]

Three studies on the *MTR A2756G* genotype and colorectal cancer risk reported a reduced risk in *GG* compared with *AA* genotype,[74,81,82] which was statistically significant in one study,[82] but a fourth study reported no association.[51] Prevalences of the *GG* genotype were ~ 3-4% in colorectal cancer patients and ranged from 3% to 11% in control subjects.

As far as we know, only one study reported results on *MTRR A66G* genotype and colorectal cancer risk, reporting an increased risk for *GG* compared with *AA* genotype (OR 1.4, 95% CI 0.9;2.0). The prevalence of the *GG* genotype was 15% among colorectal cancer cases and 12% among control subjects.[51]

### Genetic variation in the one-carbon metabolism and colorectal adenomas

*MTHFR C677T* genotype does not seem to be associated with adenoma risk, with statistically non-significant relative risks ranging from 0.35 to 2.41.[37,74] Two studies reported results on *MTHFR A1298C* and colorectal adenoma risk, one showing an increased risk in people with the *CC* genotype,[74] and the other showing a slightly decreased, not statistically significant, risk in people with the *CC* genotype.[71]

As far as we know, two studies have been published on the *TS* tandem repeat polymorphism and colorectal adenoma risk. These studies could not show an association between *TS* tandem repeat polymorphism and colorectal adenoma risk.[83,84] The *TS 1494del6* polymorphism has been published once in relation to colorectal adenoma risk, and in this study it was not associated significantly with risk of colorectal adenomas.[84]

The *CBS 844ins68* genotype has been published in relation to colorectal adenoma risk in one study and found a statistically non-significant inverse association.[74]

Two studies on *MTR A2756G* genotype and colorectal adenomas showed a statistically non-significant reduced risk for *GG* genotype,[69,74] and one study reported a statistically non-significant increased risk.[85]

*In summary, colorectal cancer risk seems to be decreased by MTHFR 677 TT and MTHFR 1298 CC genotypes, although results are not entirely consistent. For colorectal adenoma risk, the results regarding*

*both MTHFR genotypes are inconsistent. Other polymorphisms have been studied less extensively and show heterogeneous results.*

### Gene-vitamin interactions in colorectal cancer risk

The risk reduction of the *MTHFR 677 TT* genotype with regard to colorectal cancer risk seems to be greatest in those with high intake of folate[86-88] or vitamins B2,[51] B6,[51,88] or B12.[51,88] Most interactions were not statistically significant. Two studies did not observe a clear pattern for the interplay between folate intake and *MTHFR C677T* genotype,[51,89] and one study reported no interaction between vitamin B12 intake and *MTHFR C677T* genotype.[87]

One study reported a greater reduced risk for colorectal cancer in white people with the *MTHFR 1298 CC* genotype in combination with a low folate intake.[89] However, another study reported no significant interactions between *MTHFR A1298C* genotype and intake of folate and vitamins B2, B6 and B12. Furthermore, this same study reported no interactions between intake of folate and vitamins B2, B6 and B12 and *CBS 844ins68*, *MTR A2756G*, and *MTRR A66G* genotypes.[51] One study suggested a statistically non-significant interaction between folate intake and *MTR A2756G* genotype: for those with high folate intake, there was a risk reduction for the *GG* genotype compared with the *AA* and *AG* genotypes, but this risk reduction was not found for those with low folate intake. There was no interaction between vitamin B12 intake and *MTR A2756G* genotype.[81]

### Gene-vitamin interactions in colorectal adenoma risk

Although the *MTHFR C677T* genotype alone may not be associated with adenoma risk, this genotype may modify the association between intake of B-vitamins and colorectal adenomas. Like in colorectal cancer risk, the risk of colorectal adenomas seems to be reduced most in those with *MTHFR TT* genotype and high folate intake,[65,70,90,91] although some studies do not show an interaction between folate and *MTHFR C677T* genotype.[69,71]

Only two studies examined the joint effect of *MTHFR C677T* genotype and vitamin B12 intake with respect to colorectal adenoma risk. Levine *et al.*[91] did not find an interaction, but Ulrich *et al.*[90] showed a similar interaction as observed for folate and *MTHFR C677T* genotype. The latter study is the only one that investigated the interaction between vitamin B6 intake and *MTHFR C677T* genotype, again finding a similar interaction. As far as we know, the interaction between vitamin B2 intake and *MTHFR C677T* genotype has not been published.

Ulrich *et al.* found an interaction between folate intake and *TS* tandem repeat genotype in a case-control study on colorectal adenoma occurrence: among *3R/3R* individuals, high folate intake was associated with a 2-fold decreased risk of colorectal adenomas, but among *2R/2R* individuals, high folate intake was associated with a 1.5-fold increased risk.

The interaction between vitamin B12 and *TS* genotype showed a similar trend.[84] In a nested case-control study within the Health Professionals Follow-up Study, the combination of folate and *TS* tandem repeat polymorphism was not related to colorectal adenomas.[83]

The only study that reported on the interplay between folate, vitamin B6, vitamin B12 and *MTR A2756G* genotype with regard to colorectal adenoma risk did not show any significant interactions.[85]

*In summary, the interactions between folate intake and MTHFR C677T genotype are rather consistent in that the greatest risk reduction for colorectal cancer or adenomas by intake of folate and maybe other B-vitamins can be found in those with the TT genotype. Interactions between other polymorphisms in genes involved in the one-carbon metabolism and B-vitamins have not been studied often with respect to colorectal neoplasia risk, and in different combinations, so it is not possible to draw firm conclusions from these results.*

## Human intervention studies: folic acid and markers for colorectal carcinogenesis

### Intervention studies on folic acid and colorectal adenoma recurrence

The presence of adenomas is presently considered to be the only intermediary biomarker of colorectal cancer for which firm validation data exist.[60] Unfortunately, a very limited amount of information is available from folic acid intervention trials that have used adenoma recurrence in colorectal adenoma patients as the primary endpoint.[92,93] Paspatis *et al.* indicated that folic acid supplementation (1 mg/day) might reduce the recurrence of colorectal adenomas in a randomized controlled trial: after one year of supplementation, the percentages of adenoma recurrence were 23% and 38% in the folate and placebo groups, respectively, and after two years of intervention these percentages were 13% and 28%. These differences did not reach statistical significance.[92] Results from the second study have only been published as an abstract.[93] Preliminary results show that daily intervention with 1 mg folic acid did not reduce the incidence of colorectal adenomas: the RR for the folic acid group compared with the placebo group was 1.04 (95% CI 0.90;1.20) for one or more adenomas and 1.31 (95% CI 0.90;1.89) for advanced lesions, after three years of intervention. Furthermore, there was weak evidence that folic acid increases the risk of multiple adenomas (RR 1.44, 95% CI 1.03;2.02).[93]

### Intervention studies on folic acid and molecular biomarkers for colorectal cancer

Earlier intervention studies have generally observed that folic acid supplementation alters presumed biomarkers of colon cancer in a favorable manner.[94-99] In these studies, effects of a three,[94,95,97] six,[96] or twelve-month[98,99] intervention with a daily dose of 2 mg,[97] 5 mg,[94,95,98,99] or 10 mg[96] folic acid was investigated. Favorable effects were found on DNA

hypomethylation,[95,96] colonic mucosal cell proliferation,[97,99] loss of heterozygosity of the tumor suppressor gene *DCC* (deleted in colorectal cancer),[99] or activity of the proto-oncogene ornithine decarboxylase.[94] In a study by Kim *et al.*, six months of intervention increased genomic DNA methylation and decreased *p53* strand breaks, although after twelve months the effects were the same in the placebo group as in the intervention group.[98] Recently, an intervention study utilizing a more physiological dose of folic acid (400 µg/day) over ten weeks also observed a significant increase in genomic DNA methylation in the rectal mucosa of subjects with colorectal adenomas.[100] The numbers in these studies are relatively small, and in most no distinction is made between people with different *MTHFR* genotypes.

Until now, uracil misincorporation and gene promoter methylation in rectal mucosa DNA have not been used as endpoints in folic acid intervention studies. Only the uracil content of peripheral blood mononuclear cell DNA has been previously shown to revert to lower, more normal, levels in folate-deficient people by daily treatment with 5 mg folate over eight weeks.[3] As far as we know, human intervention studies with other B-vitamins have not been published related to colorectal cancer.

*In summary, human folic acid intervention studies in colorectal adenoma patients, using presumed molecular intermediate biomarkers for colorectal cancer as endpoints, mainly show favorable effects of folic acid intervention. Effects on colorectal adenoma recurrence are not clear yet. Intervention studies with B-vitamins other than folic acid have not been published.*

## RATIONALE FOR THE STUDIES IN THIS THESIS

As shown in this chapter, folate and vitamins B2, B6 and B12, as well as genetic variation in the one-carbon metabolism, may have a role in colorectal carcinogenesis through DNA methylation and DNA synthesis processes.

Human observational studies suggest that folate and vitamin B6 might have a small protective effect against colorectal cancer and maybe also colorectal adenomas. Colorectal cancer risk seems to be decreased by *MTHFR C677T* and *A1298C* genotypes, although the role of these polymorphisms in colorectal adenoma risk is unclear. The interactions between intake of B-vitamins and *MTHFR C677T* genotype are rather consistent in that the greatest risk reduction for colorectal cancer or adenomas by intake of folate or other B-vitamins can be found in those with the *TT* genotype.

Human intervention studies in colorectal adenoma patients show that folic acid supplementation may have favorable effects on global DNA methylation, cell proliferation and *p53* strand breaks, but effects on colorectal adenoma recurrence are not clear yet.

Although it appears from observational studies that folate is inversely associated with colorectal adenoma risk, this effect has not yet been quantified to a sufficient extent. Moreover, it is unclear what the exact role of the *MTHFR C677T* genotype in this association is. The association between vitamins B2, B6 and B12 and colorectal adenomas has also not been clarified yet, just like interactions between those vitamins and genetic variance in one-carbon metabolism-related genes other than *MTHFR*.

Human intervention studies that have been carried out to date included only small numbers of participants, so it was not possible to differentiate between different *MTHFR* genotypes. Furthermore, the effects that have been studied mostly concern DNA methylation and not DNA synthesis.

In the Netherlands, the mean folate intake is about 200 μg/day [101-103], which is below the Dutch RDI of 300 μg. Enrichment of foods with folic acid and supplement use are not common in the Netherlands. Thus, a sizeable portion of the population may be ingesting insufficient quantities of folate to sustain normal DNA metabolism. Intake of other B-vitamins may also be lower than in for example the United States. This makes it interesting to investigate if the associations between B-vitamin intake and colorectal neoplasia as reflected above do exist in a Dutch population.

With this in mind, we wanted to answer the following research questions:

*What is the association between intake of B-vitamins, polymorphisms in one-carbon metabolism-related genes, and colorectal adenoma risk?*
*Does an interaction exist between B-vitamins and these polymorphisms in colorectal adenoma risk?*

To answer these questions, we executed a systematic literature research with a meta-analysis. Furthermore, we investigated these research questions in a Dutch case-control study on colorectal adenoma risk. We focused on *MTHFR C677T*, *TS* tandem repeat, and *SHMT1 C1420T* polymorphisms. We did not take into account the other polymorphisms that are mentioned in this chapter, as *MTHFR A1298C* is linked with *C677T*, and the prevalence of most of the other polymorphisms is too low to examine in the studies that we conducted.

We also investigated the association between folate intake, *MTHFR C677T* genotype, and promoter methylation (gene-specific DNA methylation) in cases from this case-control study.

*Does supplementation with folic acid and vitamin B12 alter DNA methylation and DNA synthesis processes?*
*Does a dependency on the MTHFR C677T genotype exist?*

To answer these two questions, we carried out an intervention study with a high dosage of folic acid (5 mg/day) and vitamin B12 (1.25 mg/day). A placebo group was included to compare the results with. Assignment of the intervention or placebo capsules took place at random, to ensure comparability of the two groups. Randomization was stratified according to MTHFR C677T genotype. We focused on two markers for colorectal carcinogenesis that have not been investigated in human intervention studies before: promoter methylation (gene-specific DNA methylation) and uracil misincorporation in DNA (DNA synthesis).

*Which mechanisms are involved?*

As indicated above, in this thesis we focused on genetic polymorphisms and DNA methylation and DNA synthesis, in molecular epidemiological studies. These studies are part of a multidisciplinary project. Other studies from this project will be published in a related thesis by Linette Pellis. Several *in vitro* studies were conducted in different human colon epithelial cell lines. The effects of long-term exposure to different concentrations (10, 20, 50, 100, and 200 ng/mL) and forms of folate (synthetic folic acid vs. 5-methylTHF) on cell growth and intracellular levels of THF, 5-methylTHF, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), and iron were examined. Furthermore, the effects on gene expression were investigated, to determine the underlying mechanism using cDNA microarray technology.

## Outline of the thesis

In this thesis, we present the associations between folate/folic acid and other B-vitamins that are involved in the one-carbon metabolism, on the one hand, and risk of colorectal adenomas or other potential intermediate biomarkers for colon cancer, namely incorporation of uracil in DNA and promoter methylation of tumor suppressor and DNA repair genes, on the other hand. We studied these associations in human observational studies and a human intervention study. The main findings from these studies are summarized and discussed in the general discussion (**chapter 7**).

### Human observational studies

In **chapter 2** we present a systematic literature review of human observational studies that have assessed the association between intake of folate and risk of colorectal adenomas, including a meta-analysis to quantify this association.

In **chapters 3** and **4**, we evaluate the associations between intake of folate and other B-vitamins and colorectal adenomas in a Dutch case-control study. The study population consists of 768 cases with a history of colorectal adenomas and 709 endoscopy-based

controls. In **chapter 3**, emphasis is put on the relationship with the *MTHFR C677T* genotype, and the possible interplay between folate and vitamin B2 intake. **Chapter 4** takes the *TS* tandem repeat and *SHMT1 C1420T* genotypes into account.

**Chapter 5** uses data from cases from the same case-control study. We selected cases with the *MTHFR 677 CC* or *TT* genotype in the highest or lowest tertile of folate intake. In this chapter, we relate folate intake to methylation status of the promoter of six tumor suppressor and DNA repair genes in DNA derived from paraffin-embedded adenoma tissue blocks.

### Human intervention study

**Chapter 6** describes the results of a randomized, placebo-controlled intervention study with a high dose of folic acid and vitamin B12 in patients with a history of colorectal adenomas. Randomization was stratified according to the *MTHFR C677T* genotype of the participants. The end-points of the study are uracil content of rectal mucosa DNA and promoter methylation of tumor suppressor and DNA repair genes.

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# Chapter 2

## THE ASSOCIATION BETWEEN FOLATE INTAKE AND COLORECTAL ADENOMA RISK: A SYSTEMATIC LITERATURE REVIEW AND META-ANALYSIS

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**Folate intake or status may be inversely associated with colorectal adenoma risk. This study aims at providing an overall estimate on the association between folate intake or status and colorectal adenoma risk.**

**A systematic literature search was carried out for epidemiologic studies that reported on folate intake (both from diet and supplements) or folate status in plasma or erythrocytes, and colorectal adenomas. Meta-analyses were conducted of risk estimates for highest exposure category and of risk estimates per unit exposure, including data from 4 cohort studies and 10 case-control studies. To account for potential sources of between-study heterogeneity, random effects models were used.**

**In the 14 observational studies, relative risks (RRs) for highest *vs.* lowest intake of folate ranged from 0.43 to 1.47. The pooled RRs (95% confidence interval (CI)) for highest *vs.* lowest exposure category were 0.85 (0.71;1.01) for folate intake from the diet, 0.75 (0.61-0.93) for total folate intake (including supplements), and 0.75 (0.60;0.94) for plasma folate. Meta-analyses considering a continuous increase in folate intake or plasma folate level show comparable results. The associations seemed slightly stronger for men. Other stratifications did not lead to remarkable differences in pooled estimates. Two intervention studies that were not peer-reviewed showed contradictory results.**

**Observational epidemiological studies show that folate intake or status is inversely associated with colorectal adenomas. We do not expect that these results have been influenced by small study bias. It is not possible to draw conclusions from human intervention studies.**

## INTRODUCTION

Colorectal cancer is the third most common cancer worldwide.[1] Evidence from experimental and observational studies suggests that folate deficiency may play a role in colorectal cancer development.[2] A meta-analysis, using data from 7 cohort and 9 case-control studies that examined the association between folate intake and colorectal cancer risk, suggested that folate may indeed have a small protective effect against colorectal cancer. This effect was stronger for dietary folate intake than for total folate intake (including supplements). Summary estimates from cohort studies and case-control studies were similar: relative risks (RRs) (95% confidence interval (CI)) for dietary folate were 0.75 (0.64;0.89) and 0.76 (0.60;0.96), respectively.[3]

Colorectal adenomas are widely accepted as precursors of colorectal cancer in humans,[4] a progression that has been termed the **adenoma-carcinoma sequence**. Although there is no direct proof of the transformation of adenomas to cancer, evidence from autopsy, clinical, epidemiological, and molecular genetic studies has contributed to the development of this theory. Autopsy studies have shown that populations at high risk for colorectal cancer also have a high prevalence of adenomas compared with populations at low risk for colon cancer,[5] and removal of adenomas leads to a reduction in the subsequent risk of cancer.[6] Adenomas are highly prevalent in the general population.[5] Therefore, adenomas

are frequently studied epidemiologically, as studies on adenomas might indicate the risk factors that are important in the early stages of carcinogenesis. Many observational studies have been published on the association between folate intake and colorectal adenoma risk, but it is unsure if these indeed show the same results as the studies conducted on colorectal cancer. In the current study, we evaluated the evidence from all published human observational and intervention studies on this topic in a systematic literature review and a meta-analysis.

The aim of the meta-analysis was to provide pooled estimates on the association between folate intake (both from foods and supplements) or folate status (for example, erythrocyte folate or plasma folate levels), and colorectal adenoma risk, based on estimates from cohort studies and case-control studies. Stratified analyses were carried out for different study designs, geographic region, sex, publication year, potential confounders adjusted for, and *MTHFR C677T* genotype.

## METHODS

In 1997, the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) published the expert report *Food, Nutrition and the Prevention of Cancer: a global perspective*.[7] WCRF International is currently working on the production of a second report, to be published in 2007. This systematic literature review on colorectal adenomas was carried out within the framework of that project, according to a specification manual that can be found on the internet (http://www.wcrf.org/research/research_pdfs/slr_manual_15.doc).

### Databases

To ensure comprehensive retrieval of relevant papers, we searched the following electronic databases: PubMed (searched for studies from 1946), Embase (from 1973), ISI Web of Science (from 1945), Biological Abstracts (from 1969), Latin American and Caribbean Center on Health Sciences Information (LILACS; from 1982), Cochrane Library (from 1968), Current Contents (from 1996), and CAB Abstracts (from 1972). We searched for all papers that were published until February 2005. The searches were carried out by an information specialist.

### Search strategy

A search strategy was constructed to identify human studies reporting on the association between food, nutrition and physical state and the risk of colon and rectal adenomas. Folate-related search terms in this search strategy included 'folate*' and 'folic acid'. The

exposure search strategy was combined with the following search terms regarding outcome: ('colorectal neoplasms [MeSH]' or 'intestinal polyps [MeSH]') or (('neoplasm*' or 'tumor*' or 'tumour*' or 'polyp' or 'polyps' or 'polypectomy' or 'polyposis' or 'polypoid' or 'adenoma*' or 'benign*') and ('colon' or 'rectum' or 'rectal' or 'colorectum' or 'colorectal' or 'large bowel' or 'large intestine' or 'gut')). This search strategy was adapted for use in the different databases by the information specialist.

## Selection of relevant papers

After searching the databases, we followed a study selection procedure of references. The first selection step was based on title and keywords. When these suggested that the reference concerned cancer and any food, nutrition or physical state related exposure, the reference was selected for the next step. This second step was based on the contents of the abstract. In case selected citations did not have an abstract, the decision of in- or exclusion of this citation was taken in the third selection step. For this third step, the remaining articles were read in full paper to see if they were human studies concerning colorectal adenomas and food, nutrition or physical state. If the papers were considered to be eligible, they were included for the WCRF International systematic literature search. Additionally, an extra selection step was carried out, and consisted of scanning all included full papers for exposures related to folate. Furthermore, we undertook hand searches to identify references that were not detected in the database search.

## Inclusion criteria

We included all cohort studies (including nested case-control studies and case-cohort studies) and case-control studies that examined the association between folate intake or status and colorectal adenoma risk for meta-analysis. Studies had to be peer-reviewed and published as full paper. We included only human studies, and papers written in all languages. Studies other than cohort or case-control studies were also included, but only for the qualitative part of this review.

## Data extraction

We extracted data from all identified observational studies by use of a standardized Access database, developed by WCRF International. Extracted data included study characteristics and results of individual studies. Study characteristics included study design, type of analysis, study population, recruitment procedure, response rates, and assessment method of dietary data and laboratory measurements. Examples of results are exposure, range of intake, number of exposure categories, numbers of cases and non-cases per exposure category, type of outcome (e.g., risk ratio, odds ratio), adjustments,

unadjusted and adjusted outcomes and 95% confidence intervals. A random 30% of extracted papers was checked by a second reviewer.

## Selection of estimates for meta-analysis

When data-extraction was completed, it was decided which studies could be used for meta-analysis, based on availability of data.

When multiple publications existed from one study, we included the most recent results. When results from different independent populations within one study were reported, for example, men and women, both were included. When multiple results were reported for a study population, the best estimate was selected for inclusion. E.g., when several estimates were reported that differed in the number of adjustments, we considered the maximally adjusted estimate as the best estimate, except when it was explicitly stated in the paper that a less adjusted one was considered the most valid estimate.

## Data analysis

We performed two kinds of meta-analysis. The first is a meta-analysis of risk estimates comparing the highest category of folate intake or blood level with the lowest category. The second is a 'per unit' meta-analysis of risk estimates for a continuous increase in folate intake or blood level. When possible, we calculated the RRs per unit of 400 µg/day of folate intake, 10 ng/mL of plasma or serum folate, and 100 ng/mL of erythrocyte folate.[8,9] Meta-analyses were performed using random effects models to account for heterogeneity, taking into account between-study variation originating from for example design, population, exposure level and data-analysis. We quantified the extent of heterogeneity using $I^2$, the percentage of total variation across studies that is attributable to heterogeneity between studies rather than sampling variation within studies.[10]

When estimates from at least two studies were provided for different subgroups, stratified analyses were carried out for such subgroups, e.g., different study designs (cohort or case-control), geographic region (USA, Europe, or other), sex (male, female), site of adenomas (left- or right-sided), size of adenomas (large or small), publication year (before or after 2000; cut-off point chosen to get an equal numbers of studies in each subgroup), potential confounders adjusted for (for example, adjusted for fiber or not), and *MTHFR C677T* genotype (*CC / CC* and *CT* or *TT*).

We created Begg's and Egger's funnel plots to detect small study bias. All statistical analyses were carried out in Stata 8.0 (Stata Corp., College Station, TX).

# RESULTS

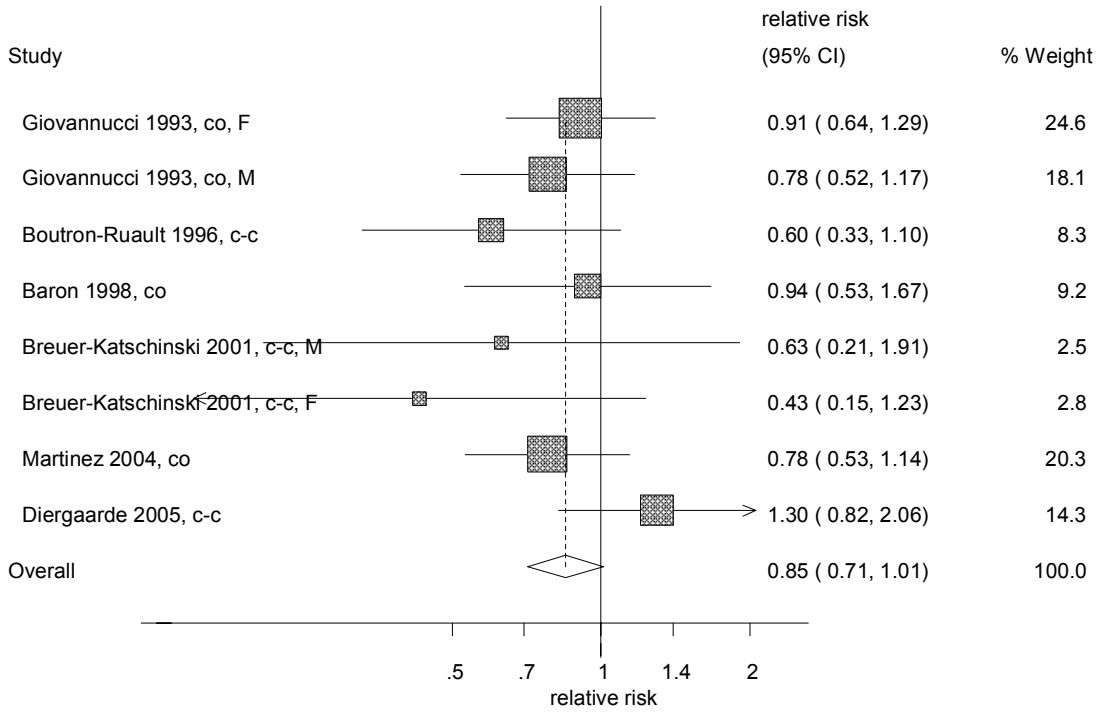
## Included studies

We identified 27 papers describing observational studies that presented data on folate intake or blood folate levels and colorectal adenoma risk.[11-37] Of these papers, one was excluded for meta-analysis because it was a cross-sectional study,[11] four were excluded because they did not provide the data required to calculate RRs for meta-analysis,[12-15] and two were excluded because they only provided risk estimates for the association between folate intake and colorectal adenomas in subgroups of people with different polymorphisms in genes that play a role in folate metabolism, and not an overall estimate.[16,17] Of the remaining 20 papers, seven described four cohort studies[18-24] (table 2.1.a) and thirteen described ten case-control studies[25-37] (table 2.1.b).

## Meta-analyses

Enough estimates were available to perform the high-versus-low meta-analyses for dietary folate intake, total folate intake (including supplements), and plasma folate. There was only one study that examined erythrocyte folate.[26] Furthermore, a sufficient number of estimates was available to conduct meta-analyses for continuous exposure to dietary

***Figure 2.1*** *Forest plot for risk of colorectal adenomas in individuals with high dietary folate intake versus low dietary folate intake, considering 8 estimates from 4 cohort studies and 3 case-control studies*




*Abbreviations: co: cohort study; c-c: case-control study; F: women; M: men*

***Table 2.1.a*** *Overview of cohort studies included in the meta-analyses, all conducted in the USA*

| Reference | Study | Sex | Number of cases | Measure of exposure | Range of exposure | Categories | Adjustments |
|---|---|---|---|---|---|---|---|
| [18] | Giovannucci 1993* | Women | 564 | Dietary folate | Not reported | 5 | Age, BMI, alcohol, parental history of colorectal cancer, saturated fat, fiber, indication for colonoscopy, history of endoscopy |
| [18] | Giovannucci 1993† | Men | 331 | Dietary folate | Not reported | 5 | Age, BMI, alcohol, parental history of colorectal cancer, saturated fat, fiber, indication for colonoscopy, history of endoscopy |
| | | | 331 | Total folate | Median: 847 vs. 241‡ | 5 | |
| [19] | Baron 1998 | Mixed | 260 | Dietary folate | Median: 388 vs. 214‡ | 4 | Age, sex, energy intake, fat, fiber, clinical center, colonoscopy interval |
| | | Mixed | 259 | Total folate | Median: 391 vs. 243‡ | 4 | |
| [20] | Chen 1998* | Women | 237<br>209 | Total folate<br>(also *MTHFR CC/CT*) | ≥508 vs. ≤310‡ | 3 | Age |
| | | Women | 237<br>209 | Total folate<br>(also *MTHFR CC/CT*) | Continuous | - | Age |
| [21] | Platz 2000* | Women | 326 | Total folate | Continuous | - | - |
| [22] | Giovannucci 2003† | Men | 325 | Total folate, *MTHFR CC/CT* | ≥497 vs. <338‡ | 3 | Age, previous endoscopy, year of endoscopy, smoking, aspirin, BMI, family history of colon cancer, energy intake, methionine, red meat, alcohol |
| | | | 325 | Total folate, *MTHFR CC/CT* | Continuous | - | |
| [23] | Martinez 2004 | Mixed | 495 | Dietary folate | >399 vs. <229‡ | 4 | Age, (sex,) number of colonoscopies, history of polyps |
| | | Women | Not reported | Total folate | >664 vs. <275‡ | 4 | ″ |
| | | Men | Not reported | Total folate | >664 vs. <275‡ | 4 | ″ |
| | | Women | Not reported | Plasma folate | >5.63 vs <2.22§ | 4 | ″ |
| | | Men | Not reported | Plasma folate | >5.63 vs <2.22§ | 4 | ″ |
| | | Mixed | 495 | Total folate | Continuous | - | ″ |
| | | Mixed | 495 | Dietary folate | Continuous | - | ″ |
| | | Mixed | 495 | Plasma folate | Continuous | - | ″ |
| [24] | Chen 2004† | Men | 377 | Total folate | Continuous | - | Age, previous endoscopy, year of endoscopy, family history of colon cancer, smoking, aspirin, BMI, physical activity, red meat, methionine |

* *Nurses' Health Study*
† *Health Professionals Follow-up Study*
‡ *µg/day*
§ *ng/mL*

***Table 2.1.b*** *Overview of case-control studies included in the meta-analyses*

| Reference | Study | Country | Sex | N cases/ N controls | Measure of exposure | Range of exposure | Categories | Adjustments |
|---|---|---|---|---|---|---|---|---|
| [25] | Paspatis 1995 | Greece | Mixed | 62/50 | Serum folate | Continuous | - | - |
| [26] | Bird 1995* | USA | Women | 152/161 | Total folate | >576 vs. <242‡ | 4 | Age, date of sigmoidoscopy, center, energy intake, smoking, alcohol, fat, fiber |
| | | | Men | 180/189 | Total folate | >576 vs. <242‡ | 4 | |
| | | | Women | 152/161 | Plasma folate | Median: 16.9 vs. 3§ | 4 | Age, date of sigmoidoscopy, center, energy intake, smoking, alcohol, fat, fiber |
| | | | Men | 180/189 | Plasma folate | Median: 16.9 vs. 3§ | 4 | |
| [27] | Boutron-Ruault 1996 | France | Mixed | 362/426 | Dietary folate | >402.5(m)/371.8(f) vs. <243.6(m)/218.5(f) ‡ | 5 | Age, sex, energy intake |
| [28,29] | Enger 1996* / Witte 1996* | USA | Mixed | 488/488 | Dietary folate | Continuous | - | - |
| [30] | Tseng 1996 | USA | Women | 131/244 | Total folate | >319.9 vs. <172.3‡ | 4 | Age, BMI, energy intake, smoking, supplements, family history of colon cancer, fat, fiber, alcohol |
| | | | Men | 105/165 | Total folate | >386.1 vs. <212.4‡ | 4 | |
| [31] | Ulrich 1999 | USA | Mixed | 247/297 | Total folate, *MTHFR CC* | >434 vs. <268‡ | 3 | Age, sex, BMI, HRT, fat, fiber, B12, B6, methionine, alcohol |
| | | | Mixed | 527/645 | Total folate | Continuous | - | - |
| | | | Mixed | 527/645 | Dietary folate | Continuous | - | - |
| [32] | Levine 2000* | USA | Mixed | 518/554 | Total folate | Continuous | - | - |
| | | | Mixed | 336/355 | Plasma folate | Continuous | - | - |
| | | | | 296/318 | (also *MTHFR CC/CT*) | | | |
| [33] | Breuer-Katschinski 2001 | Germany | Women | 88/90 | Dietary folate | Not reported | 5 | Energy intake, BMI, social class |
| | | | Men | 94/88 | Dietary folate | Not reported | 5 | ″ |
| | | | Mixed | 182/178 | Dietary folate | Continuous | - | - |
| [34] | Pufulete 2003 | United Kingdom | Mixed | 35/76 | Dietary folate | Continuous | - | - |
| | | | | 35/76 | Serum folate | Continuous | - | - |
| [35] | Marugame 2003 | Japan | Men | 177/192 | Plasma folate | >5.5 vs. ≤5.5§ | 2 | Hospital, rank in the Self Defence Forces, alcohol, smoking, BMI. |
| | | | Men | 177/192 | Plasma folate | Continuous | - | |
| | | | | 151/171 | (also *MTHFR CC*) | | | |
| [36] | Boyapati 2004 | USA | Mixed | 176/228 | Total folate | >473 (m)/537(f) vs. <306(m)/247(f) ‡ | 3 | Age, sex, energy intake |
| | | | Mixed | 88/119 | (also *MTHFR CC*) | | 3 | ″ |
| | | | Mixed | 176/228 | Total folate | Continuous | - | ″ |
| | | | Mixed | 177/228 | Dietary folate | Continuous | - | ″ |
| [37] | Diergaarde 2005 | The Netherlands | Mixed | 278/414 | Dietary folate | ≥205.1 vs. ≤174.3‡ | 3 | Age, sex, energy intake |
| | | | Mixed | 278/414 | Dietary folate | Continuous | - | ″ |

* *Kaiser Permanente case-control study*
‡ *µg/day*
§ *ng/mL*

folate intake, total folate intake, and plasma or serum folate. Three studies reported on erythrocyte folate,[25,32,34] but there was significant heterogeneity between these studies, so no pooled estimate is presented for this exposure. In none of the overall pooled analyses, there was evidence of between-study heterogeneity.

In the meta-analysis of risk estimates for the highest compared with the lowest dietary folate intake, we observed an overall pooled RR (95%CI) of 0.85 (0.71;1.01). The reported RRs ranged from 0.43[33] to 1.30[37] (figure 2.1).

Results from stratified analyses are shown in table 2.2. Case-control studies (all conducted in Europe) yielded slightly stronger pooled RRs than cohort studies (all conducted in the USA), although there was more between-study heterogeneity in case-control studies. Furthermore, there were slight differences in stratified pooled estimates according to adjustment for fiber.

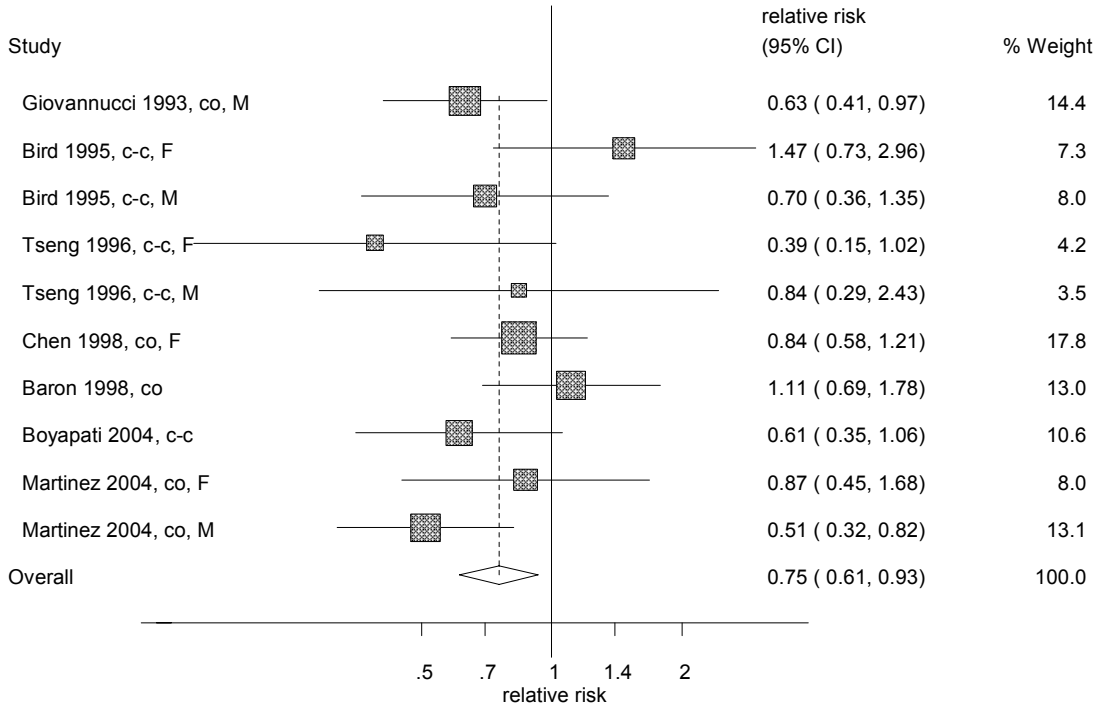
***Table 2.2*** *Stratified pooled relative risks for colorectal adenomas, highest versus lowest level of dietary folate intake*

| | Number of studies | Number of estimates | RR (95%CI) | P for heterogeneity | $I^2$ |
|---|---|---|---|---|---|
| **All studies** | 7 | 8 | 0.85 (0.71;1.01) | 0.42 | 1.6% |
| **Study type (geographic area)** | | | | | |
| Cohort (USA) | 4 | 4 | 0.84 (0.69;1.03) | 0.89 | 0.0% |
| Case-control (Europe) | 3 | 4 | 0.76 (0.44;1.30) | 0.09 | 53.6% |
| **Sex** | | | | | |
| Women | 2 | 2 | 0.74 (0.39;1.43) | 0.19 | 43.2% |
| Men | 2 | 2 | 0.76 (0.52;1.11) | 0.72 | 0.0% |
| **Publication year** | | | | | |
| Before 2000 | 4 | 4 | 0.82 (0.66;1.03) | 0.65 | 0.0% |
| From 2000 onwards | 3 | 4 | 0.85 (0.56;1.29) | 0.15 | 43.3% |
| **Adjustments** | | | | | |
| Adjusted for fiber | 3 | 3 | 0.87 (0.68;1.10) | 0.81 | 0.0% |
| Not adjusted for fiber | 4 | 5 | 0.79 (0.56;1.13) | 0.16 | 39.8% |

In the meta-analysis of risk estimates for a continuous measure of association of dietary folate intake, including 7 estimates from 7 studies,[23,28,31,33,34,36,37] an overall pooled RR (95% CI) of 0.83 (0.66;1.04) per 400 µg/day was observed. Reported RRs ranged from 0.40[34] to 1.96[37]. There was only one cohort study,[23] but results did not change when this study was excluded from the analyses (data not shown). After stratification for geographic area, results from studies from the USA were similar to overall results: pooled RR 0.83, 95% CI 0.67;1.02. Stratification for publication year did not influence the results much, although the studies published before 2000 showed more between-study heterogeneity compared with those published after 2000 (data not shown). The association seemed slightly stronger for pooled unadjusted estimates (RR 0.75, 95% CI 0.53;1.06) compared with pooled adjusted estimates (RR 0.93, 95% CI 0.63;1.38).

The forest plot of the meta-analysis for the highest versus the lowest category of total folate intake is shown in figure 2.2. The overall pooled RR (95% CI) was 0.75 (0.61;0.93). The reported RRs ranged from 0.39[30] to 1.47[26].

***Figure 2.2*** *Forest plot for risk of colorectal adenomas in individuals with high total folate intake versus low total folate intake, considering 10 estimates from 4 cohort studies and 3 case-control studies*




*Abbreviations: co: cohort study; c-c: case-control study; F: women; M: men*

Results from stratified analyses are shown in table 2.3. All studies were conducted in the USA. Again, the pooled estimates derived from cohort studies was comparable to that derived from case-control studies, and the association seemed a bit stronger for men than for women. The two studies that were published after 2000 showed a stronger association and less between-study heterogeneity that those published before 2000. The pooled estimate from studies that were not adjusted for fiber showed a slightly stronger association than those that were adjusted for fiber. The association for *MTHFR 677 CC* or *CC* and *CT* genotypes combined was slightly weaker than the overall association. Moreover, it was not possible to calculate pooled RRs for other *MTHFR* genotypes, as only the *CC* or *CC* and *CT* genotypes were taken as the reference category in most of the studies.

The RR (95% CI) per increment of 400 µg/day consumption of total folate was 0.91 (0.83-1.00). This pooled RR was based on three cohort studies[21,23,24] and three case-control studies.[31,32,36] All studies were conducted in the USA. Reported RRs varied between 0.77[23] and 1.02[24]. The inverse association seemed slightly stronger in cohort studies (pooled RR

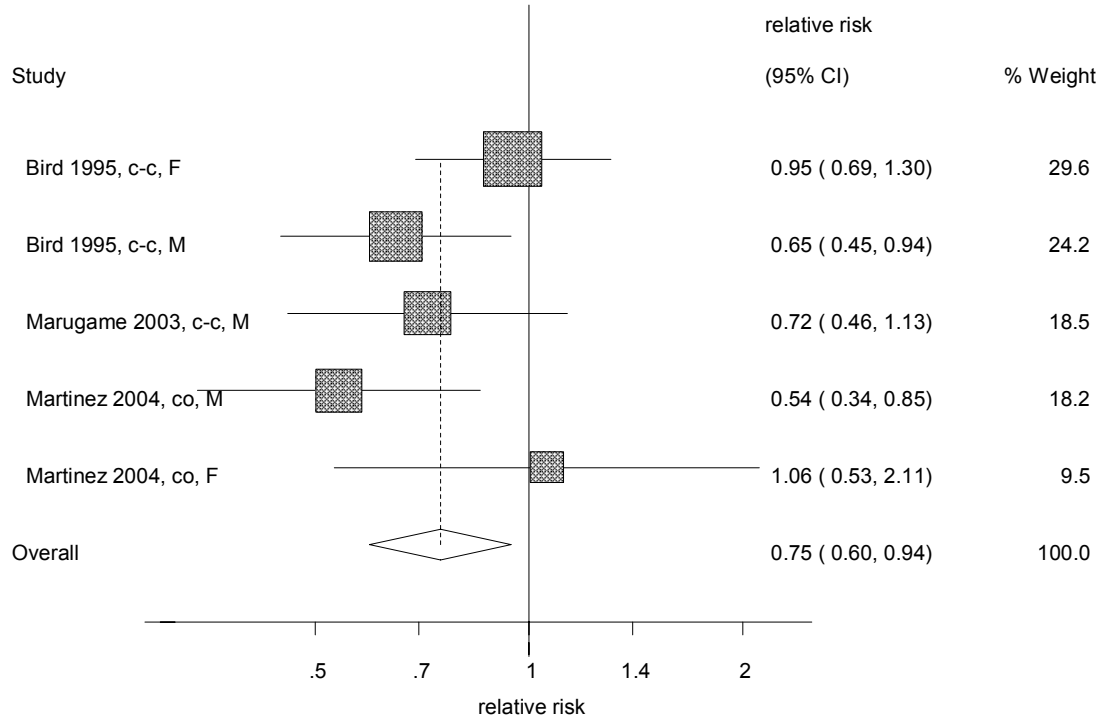
0.88, 95% CI 0.74;1.04) and for adjusted estimates (pooled RR 0.86, 95% CI 0.76;1.01). The pooled estimate for the *MTHFR 677 CC* and *CT* genotype was comparable to the overall estimate (data not shown).

***Table 2.3*** *Stratified pooled relative risks for colorectal adenomas, highest versus lowest level of total folate intake*

| | Number of studies | Number of estimates | RR (95%CI) | P for heterogeneity | $I^2$ |
|---|---|---|---|---|---|
| **All studies** | 7 | 10 | 0.75 (0.61;0.93) | 0.19 | 27.2% |
| **Study type (Geographic area)** | | | | | |
| Cohort (all USA) | 4 | 5 | 0.76 (0.58;0.99) | 0.17 | 37.3% |
| Case-control (all USA) | 3 | 5 | 0.75 (0.50;1.12) | 0.20 | 33.0% |
| **Sex** | | | | | |
| Women | 4 | 4 | 0.86 (0.58;1.28) | 0.18 | 38.4% |
| Men | 4 | 4 | 0.61 (0.46;0.80) | 0.78 | 0.0% |
| **Publication year** | | | | | |
| Before 2000 | 5 | 7 | 0.83 (0.64;1.07) | 0.23 | 26.7% |
| From 2000 onwards | 2 | 3 | 0.61 (0.45;0.84) | 0.43 | 0.0% |
| **Adjustments** | | | | | |
| Adjusted for fiber | 4 | 6 | 0.82 (0.58;1.15) | 0.15 | 38.8% |
| Not adjusted for fiber | 3 | 4 | 0.70 (0.54;0.90) | 0.34 | 10.9% |
| ***MTHFR C677T* genotype** | | | | | |
| *CC* or *CC+CT* | 4 | 4 | 0.90 (0.72;1.13) | 0.50 | 0.0% |

The overall pooled RR (95%CI) for the highest compared with the lowest plasma folate level was 0.75 (0.60;0.94). Reported estimates ranged from 0.54[23] to 1.06[23]. In figure 2.3, the corresponding forest plot is shown.

***Figure 2.3*** *Forest plot for risk of colorectal adenomas in individuals with high plasma folate versus low plasma folate, considering 5 estimates from 1 cohort study and 2 case-control studies*




*Abbreviations: co: cohort study; c-c: case-control study; F: women; M: men*

Results from stratified analyses are reported in table 2.4. It was not possible to stratify according to study type, geographic area, publication year or adjustments for confounding factors, as there were too few studies. We did stratify according to sex: the pooled association seemed stronger for men. Furthermore, the pooled estimate for the *MTHFR 677 CC* and *CT* genotypes was comparable to the overall pooled estimate.

***Table 2.4*** *Stratified pooled relative risks for colorectal adenomas, highest versus lowest level of plasma folate*

| | Number of studies | Number of estimates | RR (95%CI) | P for heterogeneity | $I^2$ |
|---|---|---|---|---|---|
| **All studies** | 3 | 5 | 0.75 (0.60;0.94) | 0.23 | 29.4% |
| **Sex** | | | | | |
| Women | 2 | 2 | 0.97 (0.73;1.29) | 0.78 | 0.0% |
| Men | 3 | 3 | 0.64 (0.50;0.81) | 0.67 | 0.0% |
| ***MTHFR C677T* genotype** | | | | | |
| *CC+CT* | 2 | 2 | 0.79 (0.56;1.10) | 0.64 | 0.0% |

The meta-analysis for plasma or serum folate (per increment of 10 ng/mL) and colorectal adenoma risk is based on one cohort study[23] and four case-control studies.[25,32,34,35] The overall pooled RR (95% CI) was 0.91 (0.85;0.97). There were too few studies to perform most stratified analyses, and the few stratifications that were performed did not reveal any differences between strata (data not shown).

We made funnel plots and performed Begg's and Egger's tests to assess whether small study bias was present in these meta-analyses. However, none of the tests were statistically significant and we did not suspect small study bias based on funnel plots (data not shown).

## Studies not included for meta-analysis

Of the observational studies that were excluded in this meta-analysis, four describe results from studies using the same population as studies that were included.[14-17] The other studies are a cross-sectional study from Norway[11] and two case-control studies that did not provide enough information for meta-analysis. These two studies are conducted in Spain, Majorca,[12] and the USA, New York.[13] The cross-sectional study only investigated erythrocyte folate and reported a statistically significant inverse association between erythrocyte folate levels and risk of high-risk adenomas.[11] In the study from Benito *et al.*, a statistically significant inverse association between dietary folate intake and colorectal adenoma risk was reported: the OR for the highest quartile of intake compared to the lowest quartile was 0.27, p for trend=0.0004.[12] Nair *et al.* reported a slightly lower serum folate level in adenoma patients compared with controls, but this difference was far from statistically significant.[13] A paper that was not included in our meta-analysis because it

was not peer-reviewed reported preliminary results from a Brazilian case-control study. In that study, a statistically non-significant positive association between total folate intake and colorectal adenoma risk was reported.[38]

Four studies presented results on the association between erythrocyte folate levels and colorectal adenoma risk.[25,26,32,34] All four studies reported a higher erythrocyte folate level in controls compared to colorectal adenoma patients, which was not statistically significant. Furthermore, Levine *et al.* reported a statistically non-significant inverse association between erythrocyte folate level and colorectal adenoma risk, but investigated subgroups with either the *MTHFR 677 CC* or *CT* genotype, or the *MTHFR 677 TT* genotype.[32] Bird *et al.* reported an inverse association between erythrocyte folate level and colorectal adenomas; when the analyses were stratified according to sex, the inverse association was statistically significant for men and null for women.[26]

Besides these observational studies, two intervention studies have been published that used recurrence of colorectal adenomas as endpoint.[39,40] However, these studies were not (yet) peer-reviewed. The first was a pilot study that was published as a letter to the editor, and reported results from a two-year randomized intervention study with 1 mg of folic acid per day or placebo. After one year, the percentages of adenoma recurrence were 23% and 38% in the folic acid and placebo groups, respectively; after two years, these percentages were 13% and 28%. The differences did not reach statistical significance.[39] The second study reported results from a six-year randomized intervention study with 1 mg of folic acid per day or placebo and was published as an abstract.[40] After three years, recurrence of adenomas was reported in 42% in the placebo group and in 44% in the folic acid supplementation group (RR 1.04, 95% CI 0.90;1.20). The incidence of advanced adenomas was modestly higher in the folic acid group (RR 1.31, 95% CI 0.90;1.89). After six years, these RRs were similar, but subjects in the folic acid group tended to have greater adenoma multiplicity: the mean (standard deviation) number of adenomas per subject was 0.55 (0.89) in the placebo group and 0.79 (1.40) in the folic acid group (rate ratio 1.44, 95% CI 1.03;2.02).

## DISCUSSION

The results from these meta-analyses suggest an inverse association between folate intake or status and colorectal adenoma risk. The risk of colorectal adenomas is 25% lower among those in the highest category of plasma folate or total folate intake compared with those in the lowest category, with no evidence of heterogeneity between the study estimates. These associations are statistically significant. For dietary folate, there was a borderline statistically significant 15% lower risk of colorectal adenomas for those in the highest category of intake compared with those in the lowest category. Meta-analyses

considering a continuous increase in folate intake or plasma folate level showed comparable results.

Folate intake might be correlated with other dietary factors. The associations between folate intake and colorectal adenoma risk might therefore be confounded by other dietary factors that are associated with colorectal adenomas, for example fiber intake. We were able to stratify the analyses of highest *vs.* lowest intake category of folate intake according to adjustment for fiber intake. Indeed, the associations seemed slightly stronger for studies that did not adjust for fiber intake, especially for total folate intake: the pooled RR for studies adjusted for fiber was 0.82, while the pooled RR for studies that were not adjusted for fiber was 0.70. This might indicate that fiber is a confounding factor in the association between folate intake and colorectal adenomas, and that the estimates that were not adjusted for fiber are overestimated to some extent. However, the associations for studies that did adjust for fiber were still inverse. Firm conclusions from these findings cannot be drawn, as these are based on a few observations. Furthermore, when we stratified the per-unit analyses for estimates that were either adjusted for any factor or unadjusted, results were not consistently affected.

Stratification for sex indicated that the associations might be stronger for men than for women, although this difference was not statistically significant. There are some suggestions that the etiology of colorectal carcinogenesis may be different for men and women, maybe due to physiological factors such as hormones and bile metabolism.[41] Other potential explanations for the difference between men and women may be related to differences in reporting bias, or chance.[42]

Where possible, we stratified the results for different *MTHFR* genotypes. However, only a few studies considered this genotype. Moreover, it was only possible to pool the estimates for the *MTHFR 677 CC* or *CC* and *CT* genotype, as this genotype was taken as the reference category in most of the studies. The results indicate that the associations are weaker for *MTHFR CC* or *CC* and *CT* genotype, which is compatible with observations that the greatest risk reduction for colorectal cancer can be found in those with the *MTHFR 677 TT* genotype.[43]

Stratification for other factors, including study type (cohort studies *vs.* case-control studies), or publication year (before 2000 or from 2000) did not influence the results in a consistent way.

The associations were stronger for total folate intake than for dietary folate intake. Probably this is caused by the difference in range of intake, which is wider for total folate intake than for dietary folate intake. Accordingly, both the within and the between subject variance in intake is larger for total folate intake. Another reason for the differences between dietary and total folate intake may be the fact that total folate intake can be established with more precision than dietary folate intake. In addition, folic

acid from supplements is more stable and has a higher bioavailability than folate from foods, and folate from foods is subject to differences in food processing,[44] unlike folic acid supplements. Validation studies show that total folate intake assessment correlates better with blood folate than dietary folate intake assessment.[18,45,46]

The finding that plasma folate was inversely associated with colorectal adenomas, to the same extent as total folate intake, strengthens the conclusions. Plasma folate is not subject to measurement errors due to inaccuracies in dietary intake assessment and differences in bioavailability. However, plasma folate levels might be influenced by the disease: tissues with rapidly replicating cells are dependent on folate, which is needed for normal DNA synthesis. In neoplastic cells, like in adenomas, cell division and thus DNA replication also occur at an accelerated rate. Thus, these tissues also need folate, and hence, plasma folate levels might be decreased in adenoma cases, although there is no empirical evidence for this assumption yet. As plasma levels in case-control studies are assessed after adenomas have developed, lower plasma levels might be the result of the disease instead of being the cause. This problem does not relate to cohort studies. However, since only two case-control studies and one cohort study assessed plasma folate, it was not possible to perform stratified meta-analyses for study type. The effects seemed similar for case-control studies and cohort studies, but we are not able to draw conclusions.

A concern about combining results from different studies in a meta-analysis is heterogeneity between studies. This might be caused by among others differences in study design, population, exposure level and data-analysis. We tested for heterogeneity in the meta-analyses. Furthermore, we calculated $I^2$, the percentage of total variation across studies that is attributable to heterogeneity rather than chance. In most of the meta-analyses of this study, no heterogeneity was indicated. However, in two stratified meta-analyses, substantial heterogeneity was present, though not statistically significant. Both concerned the per-unit meta-analysis on dietary folate intake: European studies (p=0.09, $I^2$=65.7%) and studies published before 2000 (p=0.07, $I^2$=69.3%). In both cases, only two studies were included. To meet the possibility of heterogeneity, all analyses were carried out using random effects models, which also include a term that represents between-study variation.

We tried to avoid publication bias as much as possible by searching many databases and by including papers in all languages. However, publication bias cannot be ruled out. Small study bias is a type of publication bias that might be caused by either journals being reluctant to publish results from small studies, or authors being hesitant to submit small studies, especially when results are unexpected. Based on funnel plots and Begg's and Egger's tests that we performed, we do not suspect small study bias.

We excluded two studies because these studies did not provide enough information in order to be used for the meta-analyses.[12,13] However, the results from these studies fit into the overall estimates that we found in the meta-analyses. A case-control study from Brazil that was excluded because it was not peer-reviewed showed a positive association between total folate intake and colorectal adenoma risk. However, this was a small study and hence had very broad confidence intervals,[38] so it is not likely that inclusion of this study would have influenced our results importantly.

Two intervention studies were excluded because these were not peer-reviewed.[39,40] The first reported results from a two-year intervention with 1 mg of folic acid per day or placebo. The results suggest a decrease in adenoma occurrence in the folic acid supplementation group, although the difference from the placebo group was not statistically significant.[39] The second study was published as an abstract, and also reported results from an intervention study with 1 mg of folic acid per day or placebo. Recurrence of adenomas did not seem to be influenced by folic acid intervention. However, the incidence of advanced adenomas was modestly higher in the folic acid group, and subjects in the folic acid group tended to have greater adenoma multiplicity.[40]

In conclusion, results from this systematic literature review indicate that folate might have a protective role in colorectal adenoma development. Our results correspond with results from a meta-analysis on the association between folate and colorectal cancer.[3] Stratification did not reveal important sources of variation between study results, except that associations appeared to be stronger in men. Until now, no peer-reviewed intervention studies have been published that investigate the effect of folic acid supplementation on adenoma recurrence. Two publications that were not peer-reviewed report contradictory results. Hence, it is not possible to draw conclusions from intervention studies.

## ACKNOWLEDGEMENTS

We would like to thank all members of the Dutch SLR team for their help with the conduct of this study: Evert-Jan Bakker, Marian Bos, Akke Botma, Simone Croezen, Anouk Geelen, Claudia Kamphuis, Mirjam Meltzer, Marianne Renkema, Fleur Schouten, and Evelien Smit.

This work was funded by WCRF International. It forms part of the WCRF International project preparing the report: *Food, Nutrition, Physical Activity and the Prevention of Cancer: a Global Perspective*. It does not necessarily represent the views of WCRF International; an independent panel will assess all the information from a series of systematic reviews of all cancer sites, and will draw conclusions and make recommendations based on these reviews, and on other relevant evidence.

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# Chapter 3

## DIETARY INTAKE OF FOLATE AND RIBOFLAVIN, *MTHFR C677T* GENOTYPE, AND COLORECTAL ADENOMA RISK: A DUTCH CASE-CONTROL STUDY

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**We investigated the associations between dietary intake of folate and vitamin B2, *MTHFR C677T* genotype, and colorectal adenomas in a Dutch case-control study.**

**Data of cases with at least one histologically confirmed colorectal adenoma (n=768) and controls with no history of any type of colorectal polyp (n=709) were included. Dietary intake was assessed using a food-frequency questionnaire. Multivariable models included age and, if appropriate, dietary folate and calcium intake.**

**The adjusted odds ratio (OR) and 95% confidence interval (CI) for the highest compared with the lowest sex-specific tertile of intake were 1.32 (95% CI 1.01;1.73) for folate and 0.51 (95% CI 0.36;0.73) for vitamin B2. Folate seemed to be a risk factor, especially when vitamin B2 intake was low; vitamin B2 was inversely associated with adenomas, especially with relatively high folate intake. No association was observed between *MTHFR C677T* genotype and colorectal adenomas. The inverse association between vitamin B2 intake and colorectal adenoma risk seemed to be more pronounced among those with the *MTHFR TT* genotype.**

**We conclude that this study does not provide evidence for a decreased colorectal adenoma risk for subjects with high dietary intake of folate. It suggests, however, an inverse association between vitamin B2 and colorectal adenomas, which may be more relevant for those with the *MTHFR TT* genotype.**

## INTRODUCTION

Colorectal adenomas are highly prevalent in the Western world. Among asymptomatic, average-risk patients, prevalence of adenomas is ~ 25% in colonoscopy studies.[1] As certain colorectal adenomas are considered precursors of colorectal cancer,[2,3] prevention of colorectal adenomas may decrease the occurrence of colorectal cancer.

Folate is hypothesized to have a beneficial effect on the development of colorectal adenomas and carcinomas. Folate is essential in DNA metabolism; deficiency affects DNA methylation and purine and pyrimidine synthesis.[4] Vitamin B2 (riboflavin) plays a prominent role in folate metabolism. Flavin adenine dinucleotide, a metabolite of vitamin B2, serves as a cofactor for methylenetetrahydrofolate reductase (MTHFR[5-7]). MTHFR is an important enzyme in folate metabolism; it catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate.[8] Flavin adenine dinucleotide was found to modify MTHFR activity in healthy subjects.[9]

Most epidemiologic studies examining the association between folate intake or status and colorectal adenoma risk observed an inverse association,[10-20] which was statistically significant in some studies,[10-14] whereas two studies did not find an association.[21,22] We know of only two observational studies on vitamin B2 intake and colorectal adenoma risk: in one study no association was found,[11] whereas the other study found a nonsignificant

inverse association (odds ratio (OR) for highest versus lowest tertile of intake 0.67, 95% confidence interval (CI) 0.39;1.17[20]).

A common C-to-T substitution in the *MTHFR* gene at nucleotide 677 converts an alanine to valine and is associated with decreased enzyme activity.[23] Studies investigating the association of *MTHFR C677T* genotype and colorectal adenoma risk show nonsignificant relative risks ranging from 0.35 to 2.41.[13,17,19,20,22,24-26] However, *MTHFR C677T* genotype may modify the association between intake of B-vitamins and colorectal adenomas; several studies indicate that the *MTHFR TT* genotype in combination with a low folate status may be a risk factor for colorectal adenomas,[13,18,24,26] although some studies do not show an interaction.[17,19] As far as we know, only one published study evaluated the interaction between vitamin B2 intake and *MTHFR C677T* genotype, in which there was no evidence of an interaction.[20]

Most studies on folate and colorectal adenoma or cancer risk are conducted in the United States. Intake of folate and vitamin B2 in the United States is high compared with the Netherlands, where supplements are not regularly used and foods are not enriched with folate and only recently with vitamin B2. Therefore, we evaluated the associations between intake of folate and vitamin B2 and colorectal adenoma risk in a Dutch case-control study, taking into account potential confounding or effect modifying variables, such as alcohol consumption, smoking, and intake of other B-vitamins. In addition, we examined whether these associations are modified by *MTHFR C677T* genotype.

# MATERIALS AND METHODS

## Study population

The POLIEP-study is a case-control study conducted in the Netherlands to investigate gene-environment interactions and risk of colorectal adenomas. Participants were recruited among those undergoing endoscopy in ten outpatient clinics between June 1997 and October 2002. The study design has been previously described.[27]

We defined cases as those with at least one histologically confirmed colorectal adenoma ever in their life. Controls had no history of any type of polyps, proven by complete visualization of the colon (i.e., full colonoscopy or sigmoidoscopy combined with X-ray). Eligibility criteria were Dutch speaking, of European origin, of ages 18 to 75 years at time of endoscopy, no hereditary colorectal cancer syndromes (i.e., familial adenomatous polyposis or hereditary nonpolyposis colorectal cancer), no chronic inflammatory bowel disease, no history of colorectal cancer, and no (partial) bowel resection. Response rates varied from 35% to 91% in different outpatient clinics; overall response was 54%.

Of 1,526 eligible participants, we excluded 49 subjects with insufficient dietary data. Thus, the analyses included 1,477 participants: 768 cases and 709 controls. Of 24

participants, no DNA sample was available for *MTHFR* genotyping and the *MTHFR* gene could not be amplified in one DNA sample; therefore, analyses using data on *MTHFR* genotype included 1,452 participants: 751 cases and 701 controls.

## Questionnaires

Participants filled out self-administered questionnaires on diet, medical history, and several lifestyle factors, according to their habits in the year previous to their colonoscopy or complaints. Dietary intake was assessed with a standardized and validated semiquantitative food-frequency questionnaire that was originally developed for the Dutch cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC[28]). Subsequently, intake of energy and nutrients was calculated using the Dutch food composition table. Folate intake was calculated using recently updated information of folate content in Dutch foods from Konings *et al.*[29] Vegetables, bread, and meat contributed more than 50% of folate intake. The reproducibility of these products as assessed with the questionnaire was for men 0.76, 0.86, and 0.68, respectively, and for women 0.65, 0.78, and 0.80, whereas the relative validities compared with 24-hour recalls were 0.31, 0.76, and 0.47 for men and 0.38, 0.78, and 0.70 for women.[28] The main sources of vitamin B2 were milk and milk products, accounting for about 50% of intake. The reproducibility for milk and milk products was 0.71 for men and 0.79 for women; the relative validity was 0.73 for men and 0.78 for women.[28] The Dutch EPIC food-frequency questionnaire has not yet been validated for folate intake.

## *MTHFR* genotyping

To determine the *MTHFR C677T* polymorphism, we used the PCR-RFLP method described in detail by Frosst *et al.*[23] in DNA isolated from whole blood. PCR was done with internal negative controls. Laboratory staff was blinded to case-control status. To study reproducibility, 20% of the samples were analyzed in duplicate and yielded the same result. In addition, we participated in an external quality control program. Results showed a 100% match with expected genotype.

## Statistical analysis

To investigate the association between nutrient intake and colorectal adenomas, we used logistic regression models. Intakes of nutrients were adjusted for total energy intake using the linear residual regression method of Willett and Stampfer.[30] Sex-specific tertiles of intake were calculated based on the distribution among controls. We calculated odds ratios (ORs) and 95% confidence intervals (CIs) to estimate the relative risk of developing colorectal adenomas. Reference groups were those with the lowest nutrient intake. To

examine the association between *MTHFR C677T* genotype and colorectal adenomas, we used individuals with the *MTHFR CC* genotype as reference.

We examined whether the associations with vitamin intake were modified by sex, alcohol intake, or smoking habits, but no differences were observed between strata. We examined if potential confounding factors (i.e., age, body mass index, physical activity, educational level, smoking, use of non-steroid anti-inflammatory drugs, indication for colonoscopy, family history of colorectal cancer, use of contraceptives, hormone replacement therapy, outpatient clinic, and intake of fat, fiber, alcohol, folate, vitamin B2, vitamin B6, vitamin B12, calcium, total meat, organ meat, fruits and vegetables) were associated both with colorectal adenomas and vitamin intake, and changed the crude estimates by more than 10% when added to the logistic regression models. The final logistic regression models included the covariate age; the vitamin B2 models also included dietary intake of folate and calcium.

Interaction between *MTHFR C677T* genotype and intake of vitamins was studied by stratification to genotype, using those categorized in the lowest tertile of intake and with the *MTHFR CC* genotype as reference, and by testing for different slopes associated with nutrient intake across genotype.

To test for linear trend, we modeled the tertile of nutrient intake as a continuous variable in the logistic regression model, in which each tertile was assigned its median value.

All tests of statistical significance were two sided and the significance level was set at 5%. We used Statistical Analysis Software (SAS version 8, SAS Institute, Cary, NC) for all analyses.

## RESULTS

In table 3.1, characteristics of the study population are shown. Compared with controls, cases were more likely to be male, older, have a slightly higher body mass index, and smoke more. Furthermore, cases usually had a colonoscopy for screening rather than because of complaints, used less contraceptives and hormone replacement therapy, and had a higher intake of total energy, vegetables, fat, alcohol, vitamin B6, and folate. Most of these differences remained after standardization for age and sex.

Table 3.2 shows the associations between dietary intake of folate and vitamin B2 and colorectal adenoma risk for the whole study population and stratified by *MTHFR C677T* genotype. A high dietary folate intake was positively associated with risk of colorectal adenomas, with a borderline significant test for trend (p=0.054). A high intake of vitamin B2 was inversely associated with colorectal adenoma risk, with a statistically significant test for trend. No association was observed between intake of vitamins B6 and B12 and

colorectal adenomas (data not shown). ORs did not change substantially when we excluded those using multivitamin supplements (data not shown).

***Table 3.1*** *Characteristics of the study population*

| | Cases (n=768) | Controls (n=709) | P |
|---|---|---|---|
| Female (%) | 46.5 | 61.6 | <0.01 |
| Age (years)* | 59.1±10.1 | 51.5±13.6 | <0.01 |
| BMI (kg/m$^2$)* | 26.1±3.8 | 25.5±4.1 | <0.01 |
| Smoking status (% ever) | 67.0 | 55.3 | <0.01 |
| Physical activity (% high) | 29.8 | 33.5 | 0.13 |
| Educational level (% low) | 35.7 | 33.0 | 0.30 |
| Family history of colorectal cancer (% yes) | 23.5 | 20.1 | 0.12 |
| Regular NSAID use (≥12 times/year; % yes) | 25.1 | 28.5 | 0.14 |
| Indication for colonoscopy (%) | | | |
| Complaints | 47.7 | 76.7 | |
| Screening | 44.7 | 11.3 | |
| Other/unknown | 7.6 | 12.0 | <0.01 |
| Contraceptive use (% ever)† | 65.2 | 77.6 | <0.01 |
| Hormone replacement therapy (% ever)‡ | 21.1 | 29.6 | 0.03 |
| *MTHFR C677T* genotype (%) | | | |
| CC | 44.7 | 45.8 | |
| CT | 45.0 | 43.1 | |
| TT | 10.3 | 11.1 | 0.72 |
| Dietary intake | | | |
| Energy (kJ/day)* | 8703±2484 | 8434±2498 | 0.04 |
| Total vegetables (g/day)* | 129±53 | 121±46 | <0.01 |
| Total fruits (g/day)* | 192±138 | 185±130 | 0.30 |
| Fat (g/day)* | 83±30 | 79±29 | 0.03 |
| Alcohol (g/day)§ | 9.5 (1.0;24.1) | 4.2 (0.3;15.5) | <0.01 |
| Vitamin B2 (mg/day)* | 1.62±0.57 | 1.59±0.56 | 0.27 |
| Vitamin B6 (mg/day)* | 1.65±0.48 | 1.59±0.45 | 0.02 |
| Folate (ug/day)* | 200±60 | 190±53 | <0.01 |
| Vitamin B12 (ug/day)* | 4.87±2.63 | 4.52±2.00 | <0.01 |
| Calcium (mg/day)* | 1095±433 | 1083±403 | 0.57 |
| Fibre (mg/day)* | 23.6±6.7 | 23.1±6.7 | 0.12 |
| Supplementary multivitamin use (% yes) | 17.5 | 17.9 | 0.82 |
| Supplementary B vitamin use (% yes) | 6.9 | 6.5 | 0.75 |

* *mean ± SD*
† *among women only*
‡ *among postmenopausal women only*
§ *median (25$^{th}$ percentile;75$^{th}$ percentile)*

Compared with individuals with the *MTHFR CC* genotype, *MTHFR CT* and *TT* genotypes were not related to colorectal adenoma risk. The age and sex adjusted ORs (95% CIs) were 1.06 (0.84;1.33) and 0.96 (0.66;1.39), respectively (data not shown). For intake of folate (table 3.2), vitamin B6, and vitamin B12 (data not shown), no effect modification by *MTHFR C677T* genotype was observed. The inverse association for vitamin B2 intake was present in all *MTHFR* genotypes, but seemed more pronounced among those with the *MTHFR TT* genotype. The interaction term did not reach statistical significance (table 3.2).

***Table 3.2*** *Association between dietary intake of folate[*] and vitamin B2[*] and colorectal adenomas, stratified by MTHFR C677T genotype*

| | | Dietary intake (tertiles)[†] | | | | |
|---|---|---|---|---|---|---|
| | *MTHFR* genotype | Low | Medium | High | P trend | |
| Folate[‡] | All | | | | | |
| | N cases/controls | 197/236 | 276/237 | 295/236 | | |
| | OR (95% CI) | 1 (ref.) | 1.29 (0.98;1.69) | 1.32 (1.01;1.73) | 0.054 | |
| | *CC* | | | | | |
| | N cases/controls | 78/119 | 123/104 | 135/98 | | |
| | OR (95% CI) | 1 (ref.) | 1.67 (1.12;2.51) | 1.77 (1.18;2.65) | 0.01 | |
| | *CT* | | | | | |
| | N cases/controls | 88/84 | 124/106 | 126/112 | | |
| | OR (95% CI) | 1.55 (1.00;2.39) | 1.59 (1.06;2.39) | 1.52 (1.02;2.27) | 0.88 | |
| | *TT* | | | | | |
| | N cases/controls | 25/30 | 25/23 | 27/25 | | |
| | OR (95% CI) | 1.22 (0.65;2.30) | 1.41 (0.73;2.74) | 1.43 (0.75;2.73) | 0.69 | |
| P interaction gene-nutrient | | | | | | 0.34 |
| Vitamin B2[§] | All | | | | | |
| | N cases/controls | 250/237 | 288/235 | 230/237 | | |
| | OR (95% CI[c]) | 1 (ref.) | 0.84 (0.64;1.10) | 0.51 (0.36;0.73) | 0.0002 | |
| | *CC* | | | | | |
| | N cases/controls | 102/114 | 134/102 | 100/105 | | |
| | OR (95% CI) | 1 (ref.) | 1.04 (0.69;1.55) | 0.57 (0.36;0.90) | 0.01 | |
| | *CT* | | | | | |
| | N cases/controls | 115/99 | 115/105 | 108/98 | | |
| | OR (95% CI) | 1.21 (0.81;1.81) | 0.84 (0.56;1.27) | 0.68 (0.43;1.08) | 0.09 | |
| | *TT* | | | | | |
| | N cases/controls | 26/22 | 34/24 | 17/32 | | |
| | OR (95% CI) | 1.22 (0.63;2.38) | 1.19 (0.64;2.22) | 0.32 (0.16;0.67) | 0.02 | |
| P interaction gene-nutrient | | | | | | 0.13 |

[*] *Adjusted for total energy intake, according to Willett and Stampfer [30]*
[†] *Cut points for tertiles of daily dietary intake: women: vitamin B2, 1.27mg/1.65mg; folate, 160µg/190µg; men: vitamin B2, 1.51mg/1.92mg; folate, 191µg/220µg*
[‡] *Adjusted for age*
[§] *Adjusted for age and dietary folate and calcium intake*

Folate seemed to be a risk factor with low or medium vitamin B2 intake (table 3.3). Moreover, the inverse association between vitamin B2 and colorectal adenomas was particularly seen when folate intake was relatively high. Statistically, the interaction was not significant (p=0.64).

***Table 3.3*** *Interaction between dietary intake[*, †] of folate and vitamin B2 in colorectal adenoma risk*

| Dietary vitamin B2 intake (tertiles) | Dietary folate intake (tertiles) | | | P trend | P interaction |
|---|---|---|---|---|---|
| | Low | Medium | High | | |
| Low | | | | | |
| N cases/controls | 97/110 | 89/78 | 64/49 | | |
| OR (95% CI) | 1 (ref.) | 1.26 (0.82;1.94) | 1.34 (0.82;2.18) | 0.22 | |
| Medium | | | | | |
| N cases/controls | 67/78 | 111/90 | 110/67 | | |
| OR (95% CI) | 0.73 (0.46;1.15) | 1.04 (0.68;1.58) | 1.42 (0.92;2.19) | 0.01 | |
| High | | | | | |
| N cases/controls | 33/48 | 76/69 | 121/120 | | |
| OR (95% CI) | 0.56 (0.31;1.01) | 0.74 (0.45;2.22) | 0.69 (0.44;1.07) | 0.56 | |
| P trend | 0.31 | 0.01 | 0.02 | | |
| P interaction | | | | | 0.64 |

[*] *Adjusted for total energy intake, according to Willett and Stampfer[30]*
[†] *Adjusted for age and dietary calcium intake*

## DISCUSSION

In this endoscopy-based case-control study, we observed a slightly positive association between dietary folate intake and colorectal adenoma risk, which was especially apparent for those with low vitamin B2 intakes. *MTHFR* genotype did not modify this association. An inverse association between dietary intake of vitamin B2 and colorectal adenoma risk was found, which may especially be important among those with relatively high folate intake or those with the *MTHFR TT* genotype.

As far as we know, one other publication exists on the interaction between vitamin B2 and folate in colorectal carcinogenesis, in which there was a pattern of decreased adenoma risk among those with high intakes of folate and vitamin B2 compared with those with low intakes of folate and vitamin B2. However, in that study the intake of vitamin B2 was substantially higher than in our study.[20]

In a rat model, it was shown that MTHFR was affected by riboflavin deficiency.[5] However, this does not explain why folate intake increases colorectal adenoma risk in those with a low vitamin B2 status. We speculate that folate will become protective in colorectal carcinogenesis only when vitamin B2 intake is high enough. One *in vitro* study showed that at low riboflavin level, nuclear buds decreased significantly with increasing folic acid level, whereas at high riboflavin level, nuclear buds decreased even more with increasing folic acid level. In that study, the interaction between folic acid and riboflavin was statistically significant.[31] These observations might explain why we cannot reproduce most American results, suggesting a protective role of folate intake in colorectal carcinogenesis; cereals in the United States have been enriched with vitamin B2 since 1943,[32] and mean vitamin B2 intake is about 28% higher than in the Netherlands.[33,34]

The positive, but statistically nonsignificant association between dietary folate intake and colorectal adenoma risk conflicts with results from other studies showing an inverse association[10-20] or no association at all.[21,22] Our study population had a low folate intake and the range of intake was narrow compared with that in other studies. Supplement use is not common in the Netherlands; in the Dutch Food Consumption Survey 1998, using a 2-day dietary record, 9.5% of participants reported having used multivitamin supplements, including B-vitamin supplements, on one or both days.[34] Furthermore, as vitamin intake from supplements was not calculated in our study, we focused on dietary intake. In some studies, the inverse association between folate intake and colorectal adenoma risk weakened when the analyses were restricted to dietary folate.[10,24] A limitation of the present study and many other epidemiologic studies to date, is the use of a food-frequency questionnaire for the assessment of dietary folate intake. This EPIC questionnaire showed a positive, but poor correlation between plasma folate and dietary folate intake.[35] However, most food-frequency questionnaires that were validated for folate intake show poor correlation between erythrocyte folate and dietary folate intake.[15,36,37] Validations for total folate intake show higher correlations.[10]

Furthermore, the methods of assessment of folate contents from foods may differ between studies. In our study, we used data of folate contents in foods assessed using a high-performance liquid chromatography-based method, which overall leads to about 25% lower estimates of folate contents than data based on microbiological assays.[29] However, in the Netherlands Cohort Study on Diet and Cancer, which used the same method of folate measurement, an inverse, although not statistically significant, association between folate and colon cancer in men and women andbetween folate and rectal cancer in men was suggested. The intake of folate was about 210 µg/d, which is somewhat higher than in our study.[38]

An alternative explanation for the positive association between folate intake and colorectal adenomas as found in our study might have been consumption of liver. Liver contains not only a high level of folate but also of carcinogens and thus may drive up the ORs for folate. Consumption of liver was not specifically assessed in our study. However, total organ meat was assessed and did not confound the results. Therefore, we assume that the influence of liver consumption, if present at all, will be small.

We found an inverse relationship between vitamin B2 intake and colorectal adenoma risk. An explanation may be that vitamin B2 deficiency reduces MTHFR activity.[5] An inverse association was also found in one other study,[20] whereas another study did not find an association.[11]

We did not find an indication that *MTHFR C677T* genotype modifies the association between intake of folate and colorectal adenoma risk, which is in line with some studies,[17,19] but not with others,[13,18,24,26] not depending on study size. Our data suggest a

nonsignificant interaction between vitamin B2 intake and *MTHFR C677T* genotype in colorectal adenoma occurrence; the inverse association between vitamin B2 intake and colorectal adenomas seemed more pronounced among *MTHFR TT* individuals. In a study examining the structure and properties of MTHFR from *Escherichia coli*, it was found that the *E. coli MTHFR Ala177Val* polymorphism, corresponding to the human *C677T* polymorphism, increases the tendency for MTHFR to lose its flavin adenine dinucleotide cofactor.[6] This finding was also observed in recombinant human *MTHFR*, although more subtle,[7] which may explain our finding of an interaction between vitamin B2 intake and *MTHFR* genotype. In human studies, it was observed that the homocysteine-lowering effect of vitamin B2 was essentially confined to subjects carrying the *MTHFR TT* genotype,[9,39] or even to subjects who carry the *MTHFR TT* genotype and have low folate status.[40]

Case-control studies may be limited by selection and information bias. As screening for colorectal cancer is not common in the Netherlands, most endoscopies are conducted for bowel complaints, which may influence dietary patterns and introduce information bias. However, when we excluded people who had changed their dietary habits because of these complaints (166 cases and 232 controls), it did not affect the results. Information bias might also be caused by the fact that we included prevalent cases, but when we excluded prevalent cases (n=363), results were essentially the same.

In summary, the results from this study do not provide evidence for a decreased colorectal adenoma risk for subjects with high levels of folate intake. Folate seems to be a risk factor for colorectal adenomas, especially when vitamin B2 intake is low. This may particularly be relevant for populations where products are not (yet) or only recently enriched with B-vitamins, such as the Netherlands.

Additionally, the study indicates an inverse association between vitamin B2 intake and colorectal adenoma risk, especially with higher folate intake. This study indicates that there may be an interplay between *MTHFR C677T* genotype and intake of vitamin B2, but not folate, in association with colorectal adenoma risk. Although the study does not provide enough evidence to draw firm conclusions, it brings an interesting speculation about the interactive roles of folate and vitamin B2 in colorectal carcinogenesis, which should be further elucidated.

## ACKNOWLEDGEMENTS

We are grateful to all the people who kindly participated in this study. The participants were recruited with help from the endoscopy staff of the following hospitals in the Netherlands: Slingeland Ziekenhuis (Doetinchem), Ziekenhuis Gelderse Vallei (Ede), Radboud University Nijmegen Medical Centre (Nijmegen), Antonius Ziekenhuis (Nieuwegein), Meander Medisch Centrum (Amersfoort), Ziekenhuis

Rijnstate (Arnhem), Ziekenhuis Rivierenland (Tiel), Slotervaart Ziekenhuis (Amsterdam), Jeroen Bosch Ziekenhuis ('s Hertogenbosch), and Canisius-Wilhelmina Ziekenhuis (Nijmegen). We thank Maria van Vugt and Elly Monster (Division of Human Nutrition, Wageningen University, the Netherlands) for collecting blood samples and assistance with the conduct of this study.

This study was funded by the Netherlands Organization for Health Research and Development (ZonMW), grant 980-10-020, and the Netherlands Foundation for Digestive Diseases (MLDS), grant WS 99-72.

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# Chapter 4

## DIETARY INTAKE OF B-VITAMINS, POLYMORPHISMS IN THYMIDYLATE SYNTHASE AND SERINE HYDROXY-METHYLTRANSFERASE 1, AND COLORECTAL ADENOMA RISK: A DUTCH CASE-CONTROL STUDY

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**Thymidylate synthase and serine hydroxymethyltransferase are two enzymes that are involved in folate metabolism. Both have a functional genetic polymorphism.**

**In a Dutch case-control study, including 768 cases with at least one histologically confirmed colorectal adenoma and 709 polyp-free controls, we investigated the associations between colorectal adenomas and *TS* tandem repeat and *SHMT1 C1420T* polymorphisms, and the interplay between those polymorphisms and dietary intake of B-vitamins in colorectal adenoma occurrence. We also examined the interplay between *TS* and *MTHFR C677T* polymorphisms, since these two enzymes compete for the availability of 5,10-methylenetetrahydrofolate.**

***TS* and *SHMT1* polymorphisms were not associated with adenomas. There was no interaction between the polymorphisms and vitamin B2, folate, or vitamin B12, or between *SHMT1* genotype and vitamin B6, but there was a borderline statistically significant interaction (p=0.054) between *TS* genotype and vitamin B6: the association between vitamin B6 and adenomas seemed positive in those with *TS 3R/3R* genotype, but inverse in those with *TS 2R/2R* genotype. There was no interaction between *TS* and *MTHFR* genotypes.**

**This study does not provide evidence for a role of *SHMT1* genotype in colorectal adenoma occurrence. However, future research has to indicate whether the interplay between vitamin B6 and *TS* genotype is a real effect or a chance finding.**

## INTRODUCTION

Evidence from numerous studies suggests that folate intake and functional polymorphisms in folate metabolism play a role in colorectal carcinogenesis.[1,2] The Thymidylate Synthase gene (*TS*) encodes a key enzyme in folate metabolism that catalyzes the conversion of deoxyuridylate to thymidylate, using 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) as a cofactor. This is the only *de novo* source of thymidylate, an essential precursor of DNA biosynthesis,[3] which is required for DNA replication and repair. *TS* has a 28-bp tandem repeat sequence in the 5' untranslated region that has been shown to be polymorphic, containing most frequently two or three repeats.[4] The number of tandem repeats affects TS activity levels, probably mediated through effects of the repeats on translation efficiency. Kawakami *et al.* reported no association between *TS* genotype and mRNA levels, but *TS 3R/3R* genotype was associated with higher TS protein level.[5] This enhanced protein level may increase the conversion of dUMP to dTMP, reducing the level of uracil that might otherwise be erroneously incorporated into DNA.[6] TS competes with methylenetetrahydrofolate reductase (MTHFR) for the availability of 5,10-methyleneTHF.[7]

As far as we know, two studies have been published on the *TS* tandem repeat polymorphism and colorectal adenoma risk[8,9] and two on colorectal cancer risk.[10,11] Ulrich *et al.* did not find an association between *TS* genotype and colorectal adenoma risk in a case-control study. However, they did find an interaction between folate intake and *TS*

genotype: among *3R/3R* individuals, high folate intake was associated with a 2-fold decreased risk of colorectal adenomas, but among *2R/2R* individuals, high folate intake was associated with a 1.5-fold increased risk. The interaction between vitamin B12 and *TS* genotype showed a similar trend.[8] Chen *et al.* did also not find an overall association between *TS* polymorphism and colorectal adenoma risk in a nested case-control study within the Health Professionals Follow-up Study. Furthermore, total folate or the combination of folate and *TS* polymorphism was not related to colorectal adenomas. However, there was an interaction between *TS* and *MTHFR* genotypes.[9] In a nested case-control study within the Physicians' Health Study, the *TS 2R/2R* genotype was associated with decreased colorectal cancer risk and better survival of colorectal cancer,[10] but in a Hungarian case-control study, colorectal cancer risk was lowest for heterozygotes (*2R/3R*).[11]

Serine hydroxymethyltransferase (SHMT), a vitamin B6-dependent enzyme, catalyzes the reversible conversion of serine and tetrahydrofolate to glycine and 5,10-methyleneTHF. These products are both involved in purine and pyrimidine nucleotide synthesis.[12] In line with this role of providing precursors for DNA synthesis, elevated levels of SHMT activity are found in rapidly proliferating cells,[13] particularly tumor cells.[14] Human cells contain both cytosolic (cSHMT or SHMT1) and mitochondrial (mSHMT or SHMT2) forms of SHMT. Heil *et al.*[15] identified the *SHMT1 C1420T* polymorphism and reported that *CC* individuals had lower erythrocyte and plasma folate levels. However, in the Physicians' Health Study, *SHMT1* genotype was not associated with colorectal cancer risk or with plasma folate and plasma homocysteine.[16] To our knowledge, this polymorphism has not been studied in relation to colorectal adenoma risk.

We previously reported results from a Dutch case-control study, in which folate intake was positively associated with colorectal adenomas, especially when vitamin B2 intake was low. Vitamin B2 intake was inversely associated with colorectal adenomas, especially with relatively high folate intake or among those with *MTHFR 677 TT* genotype, and there was no association between intake of vitamin B6 or vitamin B12 and colorectal adenomas.[17] In the same case-control study, we now examined the associations between *TS* and *SHMT1* genotypes and colorectal adenoma occurrence, and interactions between these genotypes and dietary intake of folate and vitamins B2, B6 and B12. Furthermore, we investigated the interaction between *MTHFR C677T* and *TS* polymorphisms, as these two enzymes compete for the availability of 5,10-methyleneTHF.

## MATERIALS AND METHODS

### Study population

The POLIEP-study is a case-control study conducted in the Netherlands to investigate gene-environment interactions and risk of colorectal adenomas. Participants were recruited among those undergoing endoscopy in ten outpatient clinics in the Netherlands between June 1997 and October 2002. The study design has been described previously.[18]

We defined cases as those with at least one histologically confirmed colorectal adenoma ever in their life. Controls had no history of any type of polyps, proven by complete visualisation of the colon (i.e., full colonoscopy, or sigmoidoscopy combined with X-ray). Eligibility criteria were: Dutch speaking, of European origin, of ages 18 to 75 years at time of endoscopy, no hereditary colorectal cancer syndromes (i.e., familial adenomatous polyposis or hereditary non-polyposis colorectal cancer), no chronic inflammatory bowel disease, no history of colorectal cancer and no (partial) bowel resection. Response rates varied from 35% to 91% in different outpatient clinics; overall response was 54%.

Of 1526 eligible participants, we excluded 49 subjects because they filled out the questionnaires insufficiently. The total study population consisted of 1477 participants: 768 cases and 709 controls.

### Questionnaires

Participants filled out self-administered questionnaires on diet, medical history, and several lifestyle factors, according to their habits in the year prior to their colonoscopy or complaints. To assess dietary intake, we used a standardized and validated semi-quantitative food-frequency questionnaire that was originally developed for the Dutch cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC).[19] Subsequently, intake of energy and nutrients was calculated using the Dutch food composition table.

### Genotyping

DNA was isolated from EDTA treated whole blood with the QIAamp 96 spin blood kit (Qiagen) and stored at 4°C in random order in 8*12 array banks. Samples were interspersed with water controls to check for cross-contamination. Laboratory staff was blinded for case-control status. Of 24 participants, no DNA sample was available for genotyping.

To determine the *TS* tandem repeat polymorphism, we adapted the method described by Horie *et al.*.[4] Each 25 µl PCR reaction contained: 400 nM forward primer (5' GTGGCTCCTGCGTTTCCCCC 3'; Invitrogen) and 400 nM reverse primer (5'

GCTCCGAGCCGGCCACAGGCATGGCGCGG 3'; Invitrogen), 200 μM dNTPs, 2 mM $MgCl_2$, 1x Platinum Taq PCR buffer, 0.5 U Platinum Taq DNA polymerase (Invitrogen), 10 % (v/v) DMSO, and ~ 4 ng/μl genomic DNA. PCR cycling was performed in a PTC-100 thermal cycler (MJ Research) with one cycle of 94°C for 5 min, 35 cycles of 94°C for 30 sec, 61°C for 30 sec and 72°C for 30 sec, and one cycle of 72°C for 5 min. Amplification products were visualized on 2% agarose gel containing ethidium bromide. All water controls were negative. Duplicate analyses were performed only in case of negative or questionable results; no differences were found. The *TS* tandem repeat genotype could not be determined in ten DNA samples, and in ten samples there were more than three or less than two repeats; therefore, analyses using data on *TS* tandem repeat genotype include 1433 participants: 737 cases and 696 controls.

To determine the *SHMT1 C1420T* polymorphism we adapted the method for allelic discrimination using fluorogenic 3' minor groove binding (MGB) probes described by Skibola *et al.*[6] Each 25 μl PCR reaction contained: 300 nM foreward primer (5' CAGAGCCACCCTGAAAGAGTTC 3'; Applied Biosystems) and 300 nM reverse primer (5' AGTGGGCCCGCTCCTTTA 3'; Applied Biosystems), 200 nM of each fluorescently labeled probe (wild type: 5' FAM-CGCCTCTCTCTTC-MGB 3', variant: 5' VIC-CGCCTCTTTCTTC-MGB 3'; Applied Biosystems), 200 μM dNTPs, 3 mM $MgCl_2$, 1x Platinum Taq PCR buffer, 0.5 U Platinum Taq DNA polymerase (Invitrogen), and ~ 4 ng/μl genomic DNA. PCR cycling was performed in an iCycler iQ multiple-color real-time PCR detection system (Bio-Rad), with one cycle of 95°C for 5 min, 45 cycles of 94°C for 30 sec and 62°C for 1 min, and one cycle of 72°C for 5 min. The allelic discrimination option of the iCycler iQ optical system software, version 3.1 (Bio-Rad) was used to determine genotypes. All water controls were negative. Eight percent of all samples were analyzed in duplo; no differences were found. The *SHMT1 C1420T* genotype could not be determined in 11 samples; therefore, analyses using *SHMT1* genotype include 1442 participants: 743 cases and 699 controls.

## Statistical analysis

We computed descriptive statistics for selected characteristics. To investigate the association between nutrient intake, genotypes and colorectal adenomas, we used logistic regression models. Intakes of nutrients were adjusted for total energy intake using the linear residual regression method of Willett and Stampfer [20]. Sex-specific tertiles of nutrient intake were calculated based on the distribution among controls. We calculated odds ratios (ORs) and 95% confidence intervals (CIs) to estimate the relative risk of developing colorectal adenomas. Reference groups were those with the lowest nutrient intake, and/or those with the *TS 3R/3R* or *SHMT1 CC* genotype.

The logistic regression models were adjusted for potential confounding factors (i.e. sex, age, body mass index, physical activity, smoking, use of NSAIDs, indication for

colonoscopy, family history of colorectal cancer, use of contraceptives, hormone replacement therapy, education level and intake of fat, fibre, alcohol, folate, vitamin B2, vitamin B6, vitamin B12, calcium, fruits, and vegetables) that changed the crude estimates by more than 10%. The final models included the covariates age and, if applicable, dietary folate intake; the vitamin B2 models also included dietary calcium intake.

To test for linear trend, we modeled the tertile of nutrient intake as a continuous variable in the logistic regression model, in which each tertile was assigned its median value. Significance of interactions was assessed with likelihood ratio testing of models with relevant multiplicative interaction terms. All tests of statistical significance were two-sided and the significance level was set at 5%. We used Statistical Analysis Software (SAS version 9.1, SAS Institute, Cary, NC) for all analyses.

## RESULTS

Selected characteristics of the study population, stratified for sex and case-control status, are shown in table 4.1. Cases are older than controls, and fewer cases are female compared with controls. Therefore, we used sex-specific tertiles for logistic regression analysis, and we adjusted all models for age.

***Table 4.1*** *Selected characteristics of the study population*

| | Women (n=794) | | Men (n=683) | |
|---|---|---|---|---|
| | Cases (n=357) | Controls (n=437) | Cases (n=411) | Controls (n=272) |
| Age (years)* | 59.0±10.3 | 50.3±14.5 | 59.2±10.0 | 53.6±11.9 |
| Intake of nutrients | | | | |
| Vitamin B2 (mg/d)* | 1.55±0.52 | 1.48±0.54 | 1.68±0.60 | 1.76±0.56 |
| Vitamin B6 (mg/d)* | 1.50±0.41 | 1.44±0.36 | 1.78±0.51 | 1.84±0.47 |
| Folate (µg/d)* | 187±48 | 176±46 | 212±67 | 212±55 |
| Vitamin B12 (µg/d)* | 4.25±1.64 | 4.01±1.74 | 5.40±3.16 | 5.33±2.12 |
| *TS* tandem repeat polymorphism (%) | (n=344) | (n=429) | (n=393) | (n=267) |
| *3R/3R* | 23.6 | 25.2 | 25.7 | 25.5 |
| *2R/3R* | 53.2 | 53.4 | 51.4 | 50.6 |
| *2R/2R* | 23.3 | 21.5 | 22.9 | 24.0 |
| *SHMT1* polymorphism (%) | (n=346) | (n=431) | (n=397) | (n=268) |
| *CC* | 50.3 | 47.1 | 47.1 | 49.6 |
| *CT* | 40.8 | 42.5 | 44.6 | 41.0 |
| *TT* | 9.0 | 10.4 | 8.3 | 9.3 |

* *Mean ± standard deviation*

Both *TS* tandem repeat and *SHMT1 C1420T* genotypes were in Hardy-Weinberg Equilibrium in controls (p *TS*=0.68, p *SHMT1*=0.94). The *TS* and the *SHMT1* polymorphisms were not associated with colorectal adenomas. Compared with the *TS 3R/3R* genotype, the OR (95% CI) for the *2R/3R* genotype was 1.02 (0.80;1.32) and the OR (95% CI) for the *2R/2R*

genotype was 1.05 (0.78;1.42). The OR (95% CI) for the *SHMT1 CT* genotype was 1.01 (0.81;1.26) and for the *TT* genotype 0.85 (0.59;1.23), compared with the *CC* genotype.

***Table 4.2*** *Association between dietary intake of B-vitamins and colorectal adenomas, stratified by TS tandem repeat genotype and SHMT1 genotype*

| Dietary intake (tertiles) | | *TS* genotype | | | P int | *SHMT1* genotype | | | P int |
|---|---|---|---|---|---|---|---|---|---|
| | | *3R/3R* | *2R/3R* | *2R/2R* | | *CC* | *CT* | *TT* | |
| Vitamin B2[*†] | Low | | | | | | | | |
| | N cases/controls | 58/64 | 121/111 | 60/61 | | 118/117 | 106/92 | 15/23 | |
| | OR (95% CI) | 1 (ref.) | 1.2 (0.8;1.9) | 1.1 (0.6;1.8) | | 1 (ref.) | 1.2 (0.8;1.8) | 0.7 (0.3;1.4) | |
| | Medium | | | | | | | | |
| | N cases/controls | 60/56 | 149/129 | 70/44 | | 142/112 | 110/95 | 28/24 | |
| | OR (95% CI) | 0.8 (0.4;1.3) | 0.9 (0.6;1.5) | 1.4 (0.8;2.4) | | 1.0 (0.7;1.4) | 0.8 (0.6;1.3) | 0.9 (0.5;1.7) | |
| | High | | | | | | | | |
| | N cases/controls | 64/56 | 115/124 | 40/51 | | 101/107 | 102/106 | 21/21 | |
| | OR (95% CI) | 0.7 (0.4;1.3) | 0.6 (0.3;0.9) | 0.5 (0.2;0.9) | | 0.5 (0.3;0.8) | 0.6 (0.4;0.9) | 0.5 (0.2;1.0) | |
| | P trend | 0.46 | 0.002 | 0.05 | | 0.14 | 0.014 | 0.25 | |
| | P interaction | | | | 0.11 | | | | 0.64 |
| Vitamin B6[*‡] | Low | | | | | | | | |
| | N cases/controls | 54/66 | 131/125 | 66/40 | | 125/111 | 107/87 | 20/31 | |
| | OR (95% CI) | 1 (ref.) | 1.3 (0.8;2.1) | 2.2 (1.2;3.8) | | 1 (ref.) | 1.2 (0.8;1.8) | 0.6 (0.3;1.2) | |
| | Medium | | | | | | | | |
| | N cases/controls | 57/57 | 125/116 | 45/59 | | 107/117 | 106/96 | 17/22 | |
| | OR (95% CI) | 1.2 (0.7;2.0) | 1.2 (0.8;2.0) | 0.9 (0.5;1.6) | | 0.8 (0.5;1.1) | 1.0 (0.7;1.5) | 0.6 (0.3;1.2) | |
| | High | | | | | | | | |
| | N cases/controls | 71/53 | 129/123 | 59/57 | | 129/108 | 105/110 | 27/17 | |
| | OR (95% CI) | 1.4 (0.8;2.5) | 1.2 (0.7;1.8) | 1.2 (0.7;2.0) | | 1.0 (0.7;1.5) | 0.7 (0.5;1.1) | 1.1 (0.6;2.2) | |
| | P trend | 0.26 | 0.58 | 0.07 | | 0.54 | 0.012 | 0.24 | |
| | P interaction | | | | 0.054 | | | | 0.12 |
| Folate[*§] | Low | | | | | | | | |
| | N cases/controls | 43/60 | 102/113 | 43/56 | | 95/110 | 81/93 | 13/28 | |
| | OR (95% CI) | 1 (ref.) | 1.2 (0.7;2.0) | 1.1 (0.6;2.0) | | 1 (ref.) | 1.1 (0.7;1.6) | 0.6 (0.3;1.2) | |
| | Medium | | | | | | | | |
| | N cases/controls | 62/62 | 143/130 | 61/41 | | 131/114 | 114/97 | 23/22 | |
| | OR (95% CI) | 1.2 (0.7;2.1) | 1.4 (0.9;2.3) | 1.9 (1.1;3.4) | | 1.3 (0.9;1.9) | 1.3 (0.9;2.0) | 1.0 (0.5;1.9) | |
| | High | | | | | | | | |
| | N cases/controls | 77/54 | 140/121 | 66/59 | | 135/112 | 123/103 | 28/20 | |
| | OR (95% CI) | 1.7 (1.0;2.9) | 1.4 (0.9;2.2) | 1.4 (0.8;2.5) | | 1.3 (0.9;1.9) | 1.2 (0.8;1.8) | 1.4 (0.7;2.8) | |
| | P trend | 0.05 | 0.60 | 0.50 | | 0.27 | 0.49 | 0.05 | |
| | P interaction | | | | 0.47 | | | | 0.67 |
| Vitamin B12[*‡] | Low | | | | | | | | |
| | N cases/controls | 47/59 | 115/113 | 60/62 | | 109/116 | 99/91 | 15/26 | |
| | OR (95% CI) | 1 (ref.) | 1.4 (0.8;2.3) | 1.3 (0.8;2.3) | | 1 (ref.) | 1.2 (0.8;1.9) | 0.8 (0.4;1.6) | |
| | Medium | | | | | | | | |
| | N cases/controls | 74/59 | 142/121 | 61/52 | | 128/107 | 122/102 | 27/24 | |
| | OR (95% CI) | 1.5 (0.8;2.5) | 1.3 (0.8;2.0) | 1.4 (0.8;2.5) | | 1.1 (0.7;1.6) | 1.1 (0.8;1.7) | 1.0 (0.5;1.8) | |
| | High | | | | | | | | |
| | N cases/controls | 61/58 | 128/130 | 49/42 | | 124/113 | 97/100 | 22/20 | |
| | OR (95% CI) | 0.9 (0.5;1.6) | 0.9 (0.6;1.5) | 1.0 (0.5;1.8) | | 0.8 (0.6;1.3) | 0.7 (0.5;1.1) | 0.7 (0.3;1.4) | |
| | P trend | 0.54 | 0.047 | 0.36 | | 0.50 | 0.01 | 0.54 | |
| | P interaction | | | | 0.73 | | | | 0.76 |

[*] *Cut points for tertiles of daily dietary intake: Women: vitamin B2: 1.27/1.65mg; vitamin B6: 1.35/1.53mg; folate: 160/190µg; vitamin B12: 3.33/4.47µg; Men: vitamin B2, 1.51/1.92mg; vitamin B6: 1.69/1.95mg; folate, 191µg/220µg; vitamin B12: 4.20/5.80µg*

[†] *Adjusted for age, dietary folate intake and dietary calcium intake*

[‡] *Adjusted for age and dietary folate intake*

[§] *Adjusted for age*

In table 4.2, the interactions between intake of B-vitamins and *TS* and *SHMT1* genotypes are shown. Intake of vitamin B2 was inversely, and intake of folate was positively associated with colorectal adenoma risk in all strata of *TS* and *SHMT1* genotypes. For vitamin B12 intake, no trend was visible, neither within *TS* nor within *SMHT1* genotypes. The combination of vitamin B6 intake and *SHMT1* genotype also showed no specific trend. However, there was a borderline statistically significant interaction between vitamin B6 intake and *TS* in colorectal adenoma occurrence (p interaction=0.054): within those with *TS 3R/3R* genotype, there seemed to be a positive association between vitamin B6 intake and adenomas, but within those with *TS 2R/2R* genotype, there was an inverse association between vitamin B6 intake and adenomas. We also repeated the analyses without 166 cases and 232 controls who had changed their dietary habits because of bowel complaints, and without 363 prevalent cases. The results of these repeated analyses were not materially different (data not shown).

Table 4.3 shows the interaction between *TS* and *MTHFR C677T* genotypes in colorectal adenoma occurrence. Individuals who were homozygous for *MTHFR TT* and *TS 3R/3R* had the lowest risk compared with other combinations of genotypes. However, no statistical interaction was observed.

***Table 4.3*** *Interaction between TS tandem repeat and MTHFR C677T genotypes in colorectal adenoma risk*

| | *TS* genotype | | | P trend | P interaction |
|---|---|---|---|---|---|
| *MTHFR* genotype | *3R/3R* | *2R/3R* | *2R/2R* | | |
| *CC* | | | | | |
| N cases/controls | 74/86 | 180/159 | 74/71 | | |
| OR (95% CI) | 1 (ref.) | 1.32 (0.90;1.92) | 1.21 (0.77;1.90) | 0.38 | |
| *CT* | | | | | |
| N cases/controls | 94/68 | 167/170 | 71/63 | | |
| OR (95% CI) | 1.61 (1.03;2.50) | 1.14 (0.78;1.67) | 1.31 (0.83;2.08) | 0.33 | |
| *TT* | | | | | |
| N cases/controls | 14/22 | 38/35 | 25/21 | | |
| OR (95% CI) | 0.74 (0.35;1.55) | 1.26 (0.73;2.20) | 1.38 (0.72;2.67) | 0.18 | |
| P trend | 0.62 | 0.55 | 0.66 | | |
| P interaction | | | | | 0.12 |

## DISCUSSION

In this case-control study, we could not demonstrate an effect of *TS* tandem repeat polymorphism or *SHMT1 C1420T* polymorphism in colorectal adenoma occurrence. There was also no interaction between any one of the genotypes and intake of vitamin B2, folate, or vitamin B12, and no interaction between *SHMT1* genotype and vitamin B6 intake in colorectal adenoma occurrence. However, there was a suggestion of an interaction between *TS* genotype and vitamin B6 intake: vitamin B6 intake was positively

associated with adenomas within those with *3R/3R* genotype, but negatively within those with *2R/2R* genotype. When combining the *TS* and *MTHFR C677T* genotypes, individuals who were homozygous for *MTHFR TT* and *TS 3R/3R* had the lowest risk of adenomas, but no clear interaction between these genotypes was visible.

Our study is a hospital-based case-control study. All participants underwent endoscopy, which means that the results may not be extrapolated to the general population. An advantage of this approach is that we were able to make a clear distinction between patients and controls. This may be a problem in population-based case-control studies, as colorectal adenomas are common in the general population and often do not give complaints.[21] A disadvantage of this approach is that most endoscopies were conducted for bowel complaints, as screening for colorectal cancer is not common in the Netherlands. Bowel complaints may influence dietary patterns, which may introduce information bias. However, when we excluded 166 cases and 232 controls, who reported having changed their dietary habits because of bowel complaints, results did not change. Another source of information bias is the fact that we included prevalent cases. However, excluding 363 prevalent cases from the analyses also did not affect the results, so we think the effect of information bias will be small.

A limitation of our study is the use of a food-frequency questionnaire, which may not be the most accurate way to assess intake of B-vitamins. Some food-frequency questionnaires that were validated for folate intake show poor correlations between blood folates and dietary folate intake.[22] The EPIC-questionnaire that we used showed a positive, but poor correlation between plasma folate and dietary folate intake.[23] Validations for total folate intake show higher correlations,[24] but supplementary vitamin use was not assessed in our study. Since supplement use is not common in the Netherlands, we focused on dietary intake. Adjusting the logistic regression models for use of multivitamin or B-vitamin supplements did not change the results.

Other observational studies also show no association between *TS* genotype and colorectal adenoma risk.[8,9] For colorectal cancer, Chen *et al.* report a lower risk for *TS 2R/2R* individuals compared with *TS 3R/3R* individuals (relative risk 0.59, 95% CI 0.36;0.98) and a better survival from colorectal cancer for *TS 2R/2R* individuals compared with *TS 2R/3R* and *TS 3R/3R* individuals (hazard ratio 0.57, 95% CI 0.30;1.07). The study did not take intake of nutrients into account.[10] The absence of an effect of genetic polymorphisms on colorectal adenomas[9] that was present for colorectal carcinomas[10] was also reported for the *MTHFR C677T* genotype.[9] Adleff *et al.* reported a lower colorectal cancer risk for *TS 2R/3R* individuals in Hungary.[11]

Chen *et al.* did not report an interaction between folate and *TS* with regard to colorectal adenoma risk.[9] However, Ulrich *et al.* reported that among individuals with the *3R/3R* genotype, folate intake in the highest tertile *vs.* the lowest tertile was associated with a

two-fold decreased risk of colorectal adenomas, whereas among individuals with the *2R/2R* genotype, a 1.5-fold increased risk associated with high folate intake was observed. A similar interaction between *TS* genotype and vitamin B12 intake was found.[8] We also found an interaction between *TS* genotype and vitamin B6 intake. However, the interaction that we found was in the other direction. Ulrich *et al.* did not include vitamin B6 intake in their report.[8] The results of Ulrich *et al.* and our results suggest that intake of vitamin B12 and vitamin B6 is somehow interconnected with *TS* genotype. These vitamins are not directly involved in thymidylate synthesis, but they both are cofactors in key enzymes in the methylation pathway. This could imply that the methylation pathway somehow influences thymidylate synthesis. Clearly, more research is needed to unravel such an influence.

Both Chen *et al.* and Ulrich *et al.* investigated the interplay between *MTHFR C677T* and *TS* genotypes in colorectal adenoma occurrence. Chen *et al.* reported that individuals with combined *TS 2R/2R* and *MTHFR CC* genotypes had the lowest risk of colorectal adenomas compared with individuals with other combinations of genotypes, whereas individuals with combined *TS 2R/2R* and *MTHFR TT* genotype had the highest risk.[9] In contrast, in the study by Ulrich *et al.*, the combination of *TS 2R/2R* and *MTHFR TT* genotypes was the lowest risk group,[8] whereas in our study the combination of *TS 3R/3R* and *MTHFR TT* genotype carried the lowest risk. These differences may be due to study and population characteristics, e.g. different background intake of nutrients, or simply to chance.

In our study, there were nine participants (0.6%) with more than three repeats in the *TS* enhancer region, and one participant with less than two repeats (0.07%). We did not include these participants in the analyses, in accordance with Kawakami *et al.*, who reported more than three repeats in 3 of 133 (2.3%) colorectal cancer cases,[5] and Ulrich *et al.*, who reported more than three repeats in 0.3% of controls.[8]

To our knowledge, studies investigating the association between *SHMT1* genotype and colorectal adenomas have not been published. Chen *et al.* reported no association between *SHMT1* genotype and colorectal cancer risk in the Physicians' Health Study,[16] which is in accordance with our findings. This might indicate that the *SHMT1 C1420T* polymorphism does not have a role in colorectal carcinogenesis.

In conclusion, our results add to the evidence that *SHMT1 C1420T* genotype is not involved in colorectal carcinogenesis. We did find a suggestion of an interaction between *TS* 5' untranslated region tandem repeat polymorphism and vitamin B6 intake in colorectal adenoma occurrence: there was a positive association between vitamin B6 intake and colorectal adenomas among those with the *TS 3R/3R* genotype, but an inverse association among those with the *TS 2R/2R* genotype. Future research has to indicate whether this is a real effect or a chance finding.

## ACKNOWLEDGEMENTS

We are grateful to all the participants in this study and to the endoscopy staff in the following hospitals in the Netherlands: Slingeland Ziekenhuis (Doetinchem), Ziekenhuis Gelderse Vallei (Ede), Radboud University Nijmegen Medical Centre (Nijmegen), Antonius Ziekenhuis (Nieuwegein), Meander Medisch Centrum (Amersfoort), Ziekenhuis Rijnstate (Arnhem), Ziekenhuis Rivierenland (Tiel), Slotervaart Ziekenhuis (Amsterdam), Jeroen Bosch Ziekenhuis ('s-Hertogenbosch), and Canisius-Wilhelmina Ziekenhuis (Nijmegen). We thank Dorien Voskuil, Edine Tiemersma, Maria van Vugt, Elly Monster, Brenda Diergaarde, Petra Wark and Mariken Tijhuis for their roles in the conduct of the case-control study, and Marga Ocké from the National Institute for Public Health and the Environment (RIVM, Bilthoven) for micronutrient calculations.

This work was supported by the Netherlands Organization for Health Research and Development (ZonMw), grant 980-10-020, and by the Netherlands Foundation for Digestive Diseases (MLDS), grant WS 99-72.

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# Chapter 5

## DIETARY FOLATE INTAKE IN COMBINATION WITH *MTHFR 677 TT* GENOTYPE AND PROMOTER METHYLATION OF TUMOR SUPPRESSOR AND DNA REPAIR GENES IN SPORADIC COLORECTAL ADENOMAS

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**Methylation of the promoter region of tumor suppressor genes is increasingly recognized to play a role in cancer development through silencing of gene transcription. We examined the associations between dietary folate intake, *MTHFR C677T* genotype, and promoter methylation of six tumor suppressor and DNA repair genes.**

**Colorectal adenoma patients (n=149) with folate intake in the upper or lower tertile with the *CC* or *TT* genotype were selected from a case-control study. Methylation-specific PCRs were conducted on colorectal adenoma specimens.**

**Overall, the observed percentages of promoter methylation ranged from 15.7% to 64.2%. Folate intake seemed inversely associated with promoter methylation, especially among those with the *TT* genotype. *MTHFR* genotype was not associated with promoter methylation. The interaction between folate intake and *MTHFR* genotype was most pronounced for *$O^6$-MGMT* and *RASSF1A.* Compared with patients with a low folate intake and the *CC* genotype, the adjusted odds ratios (95% confidence interval) of having a methylated *$O^6$-MGMT* or *RASSF1A* promoter were 3.39 (0.82;13.93) and 3.53 (1.08;11.50), respectively, for those with a low folate intake and the *TT* genotype, and 0.37 (0.11;1.29) and 0.78 (0.22;2.76), respectively, for those with a high folate intake and the *TT* genotype; p interaction were 0.02 and 0.06 respectively.**

**In this study, folate intake seems inversely associated with promoter methylation in colorectal adenomas, which may be especially so for those with the *TT* genotype.**

## INTRODUCTION

DNA methylation is an important event in gene regulation: it is involved in gene expression, chromatin configuration and structural stability of DNA, binding of transcriptional factors and other proteins, mutations and imprinting (reviewed in [1]). Colorectal neoplasms, both carcinomas and adenomas, show a decreased global DNA methylation level compared to normal tissue.[2,3] Conversely, other studies have shown methylation of the promoter region of specific tumor suppressor genes in colorectal tumors,[4] which is increasingly recognized to play an important role in cancer development through silencing of gene transcription.[5] Evidence from recent studies suggests that DNA hypomethylation and hypermethylation are independent processes and contribute separately to the process of carcinogenesis.[6,7]

Folate is a vitamin that is essential in DNA metabolism. Deficiency of folate affects purine and pyrimidine synthesis and DNA methylation.[8] As 5-methyltetrahydrofolate (5-methylTHF), folate provides methyl groups for S-adenosyl methionine, which is a universal methyl donor in a large number of biological reactions, including the methylation of DNA.[9] In the Netherlands Cohort Study on Diet and Cancer, that examined the associations between folate and alcohol intake and promoter methylation of genes involved in colorectal carcinogenesis, it was suggested that the prevalence of promoter

hypermethylation was higher in carcinomas from patients with a low folate/high alcohol intake when compared with carcinomas from patients with a high folate/low alcohol intake.[10] Kawakami *et al.* showed that colorectal carcinomas with frequent promoter hypermethylation, derived from Australian patients, have high levels of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) and tetrahydrofolate (THF).[11]

An important enzyme in folate metabolism, methylenetetrahydrofolate reductase (MTHFR), catalyzes the irreversible conversion of 5,10-methyleneTHF to 5-methylTHF.[9] A common C-to-T substitution in the *MTHFR* gene at nucleotide 677 converts an alanine to valine and is associated with decreased enzyme activity.[12] Studies investigating the association between *MTHFR C677T* genotype and colorectal adenoma risk show non-significant relative risks ranging from 0.35 to 2.41.[13-21] However, *MTHFR C677T* genotype may modify the association between intake of folate and colorectal adenomas: several studies indicate that the *MTHFR TT* genotype in combination with a low folate status may be a risk factor for colorectal adenomas,[14,16,17,22] although some studies do not show an interaction.[13,19,21] From a Japanese study it was suggested that the haplotype with low enzymatic activity of MTHFR, that consisted of *MTHFR 1298 CC*, *677 TT*, and the combination of *1298 AC* and *677 CT* genotypes, is linked with promoter hypermethylation in proximal colon cancer.[23]

In this study, we examined the association between dietary folate intake, *MTHFR C677T* genotype, their possible interaction, and promoter methylation of six tumor suppressor and DNA repair genes in sporadic colorectal adenomas. The genes that we analyzed are the tumor suppressor genes Adenomatous Polyposis Coli (*APC-1A*), $p14^{ARF}$, $p16^{INK4A}$, Ras Association Domain Family Protein 1A (*RASSF1A*) and the DNA repair genes human MutL Homolog 1 (*hMLH1*) and $O^6$-methylguanine-DNA methyltransferase ($O^6$-*MGMT*). The reason for choosing these genes is that five of these genes (*APC-1A*, $p14^{ARF}$, $p16^{INK4A}$, *hMLH1*, and $O^6$-*MGMT*) were markedly methylated in colon cancer in a study examining methylation profiles using a series of 12 genes in 15 major human tumor types,[24] and *RASSF1A* has been shown to be frequently methylated in colorectal cancer in the previously mentioned Dutch cohort study.[25]

# MATERIALS AND METHODS

## Study population

The POLIEP-study is a case-control study conducted in the Netherlands to investigate gene-environment interactions and risk of colorectal adenomas. Participants were recruited among those undergoing endoscopy in ten outpatient clinics between June 1997 and October 2002. The study design has been described previously.[21]

Eligibility criteria were: Dutch speaking, of European origin, aged 18 to 75 years at time of endoscopy, no hereditary colorectal cancer syndromes (i.e., familial adenomatous

polyposis or hereditary non-polyposis colorectal cancer), no chronic inflammatory bowel disease, no history of colorectal cancer, and no (partial) bowel resection. Response rates varied from 35% to 91% in different outpatient clinics; overall response was 54%. The total study population consisted of 768 cases, defined as those with at least one histologically confirmed colorectal adenoma ever in their life, and 709 controls, defined as those without any colorectal polyp. In these analyses, only the cases of this case-control study are included.

Formalin-fixed, paraffin embedded adenoma tissue was available from 575 cases. From these cases we selected 164 people in the upper (>212 μg/day) or lower (<183 μg/day) tertile of folate intake with the *MTHFR 677 CC* or *TT* genotype. Of these cases, DNA yield of 15 (mainly tubular) adenomas was too low for MSP, so we included 149 cases in the analyses (19 low folate and *TT* genotype; 17 high folate and *TT* genotype; 57 low folate and *CC* genotype; 56 high folate and *CC* genotype).

## Questionnaires

Participants were asked to fill out self-administered questionnaires according to their habits in the year previous to their colonoscopy or complaints. Dietary intake was assessed with a standardized and validated semi-quantitative food-frequency questionnaire that was originally developed for the Dutch cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC).[26] Subsequently, intake of energy and nutrients was calculated using the Dutch food composition table. Vegetables, bread and meat contributed for more than 50% of folate intake. The reproducibility of these products, as assessed with the questionnaire and expressed as Spearman correlation coefficients, was for men respectively 0.76, 0.86, and 0.68, and for women 0.65, 0.78, and 0.80. The relative validities compared to 24-hour recalls were respectively 0.31, 0.76, and 0.47 for men and 0.38, 0.78, and 0.70 for women.[26]

To be able to adjust for possibly confounding factors, the participants filled out a general questionnaire on medical history and several lifestyle factors, e.g. smoking, physical activity, NSAIDs use.

## *MTHFR* genotyping

Blood was drawn by venipuncture and collected in 9 mL EDTA vacutainers (Greiner Bio-One, Kremsmuenster, Austria) and subsequently stored at –20 °C until DNA extraction. DNA was isolated from 200 μL whole blood using the QIAamp blood kit (QIAGEN, Hilden, Germany), subsequently diluted to a concentration of approximately 20 ng/μL, and stored at 4 °C until analysis.

To determine the *MTHFR C677T* polymorphism, we used the PCR/RFLP method described in detail by Frosst *et al.*[12] PCR was performed with internal negative controls. In cases of

questionable genotypes through weak visualization of fragments, genotype was re-assessed. To test reproducibility, 20% of the samples were analyzed in duplicate and yielded the same result (100% reproducibility). In addition, we participated in an external quality control program. Results showed a 100% match with expected *MTHFR C677T* genotype.

## DNA extraction from adenoma tissue

DNA was extracted from formalin-fixed, paraffin-embedded adenoma tissue (ten to twelve 10 µm thick sections) using the Puregene™ DNA isolation kit (Gentra Systems, Minneapolis, MN). Micro dissection was performed, guided by a hematoxylin and eosin stained 4 µm section, and only those areas containing >60% tumor cells were used. Isolated tissue was incubated overnight at 55 °C in 500 µl cell lysis solution containing 0.5 mg/ml proteinase K (Roche Diagnostics, Mannheim, Germany), followed by 72 hours at 37 °C. Proteins were removed with the protein precipitation solution according to the manufacturer's protocol. DNA was precipitated with 500 µl 100% isopropanol at 4 °C for 30 minutes. The pellet was washed with 500 µl 70% ethanol, air-dried, and subsequently the DNA was rehydrated in 30 µl DNA hydration solution.

## Promoter Methylation Analysis

We determined DNA methylation in the CpG islands of the *APC-1A*, $p14^{ARF}$, $p16^{INK4A}$, *hMLH1*, $O^6$*-MGMT*, and *RASSF1A* gene promoters by chemical modification of genomic DNA with sodium bisulfite and subsequent methylation-specific PCR (MSP).[10,27] Sodium bisulfite treatment of 250 ng of DNA was done according to Millar *et al.*,[28] with glycogen used as carrier. In this reaction, all non-methylated cytosines are converted to uracil, but methylated cytosines (5-methylcytosine) remain as cytosine.

After sodium bisulfite treatment of DNA, we performed methylation-specific PCR (MSP) as described in detail elsewhere.[10,27] All PCRs were performed with controls for unmethylated alleles (DNA from normal lymfocytes), methylated alleles (normal lymfocyte DNA treated *in vitro* with *SssI* methyltransferase, New England Biolabs, Beverly, MA), and a negative PCR control without DNA. Primer sequences and PCR conditions are listed elsewhere.[10] Where more primers are listed for one gene, we used the short primers. Ten µl of each MSP reaction was directly loaded onto 6% denaturating polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination. To study the reproducibility of the nested MSP approach, 35% of specimens were also analyzed after bisulfite modification according to the method of Herman *et al.*[27] Agreement between the two methods was 87%. Bisulfite-treated DNA could not be amplified for *APC-1A* in 2 samples (1.3%), for $p14^{ARF}$ in 8 samples (5.4%), for $p16^{INK4A}$ in 18 samples (12.1%), for *hMLH1* in 9 samples (6.0%), and for both $O^6$*-MGMT* and *RASSF1A* in 12 samples (8.1%).

## Statistical analyses

Descriptive statistics of intake of relevant nutrients and other characteristics were computed, according to methylation status (≥3 gene promoters methylated compared to <3 gene promoters methylated).

We used logistic regression models, which allow correction for possibly confounding factors, to calculate odds ratios (ORs) and 95% confidence intervals (CIs) estimating the relative risk of a methylated promoter. We also examined concurrent methylation in ≥3 genes compared to <3 genes. We selected this cut-off point because the median number of methylated genes in our study was three, similar to Bai *et al.*[29] Those with a low folate intake and *MTHFR CC* genotype were reference.

Logistic regression models were adjusted for age and sex. Furthermore, we examined if potential confounding factors (i.e. body mass index, physical activity, educational level, smoking, use of NSAIDs, use of multivitamin or B-vitamin supplements, indication for colonoscopy, family history of sporadic colorectal cancer, and dietary intake of fat, fiber, alcohol, vitamin B2, vitamin B6, vitamin B12, calcium, iron, fruits, and vegetables) were associated both with promoter methylation of any of the six genes and folate intake, and changed the crude estimates by more than 10% when added to the logistic regression models. The final logistic regression models included the covariates age, sex, body mass index, indication for colonoscopy, and dietary intake of vitamins B2 and B6.

All tests of statistical significance were two-sided and the significance level was set at 5%. We used Statistical Analysis Software (SAS version 8, SAS Institute, Cary, NC) for all analyses.

# RESULTS

The observed percentages of promoter methylation were 51.0% (75/147) for *APC-1A*, 61.7% (87/141) for $p14^{ARF}$, 54.2% (71/131) for $p16^{INK4A}$, 15.7% (22/140) for *hMLH1*, 64.2% (88/137) for $O^6$*-MGMT*, and 40.9% (56/137) for *RASSF1A*. For analyses examining methylation in ≥3 genes compared to <3 genes, we left out eleven cases that we could not classify in either group because the DNA could not be amplified for some of the genes. 58.0% (80/138) Of patients had ≥3 methylated gene promoters.

In table 5.1, characteristics of the study population are shown according to methylation status (≥3 gene promoters methylated compared to <3 gene promoters methylated). The most striking difference observed was a higher percentage of (tubulo)villous adenomas among those with three or more methylated gene promoters compared to those with less than three methylated gene promoters. Remarkably, no differences were observed in age and sex.

***Table 5.1*** *Characteristics of the study population according to methylation status*

| | ≥3 genes methylated (n=80) | <3 genes methylated (n=58) |
|---|---|---|
| Female (%) | 45.0 | 46.6 |
| Age (years)* | 59.2 ± 9.7 | 58.9 ± 9.6 |
| Family history of colorectal cancer (% yes) | 26.3 | 31.0 |
| Indication for colonoscopy (% screening) | 33.8 | 27.6 |
| *MTHFR C677T* genotype (% *TT*) | 23.8 | 25.9 |
| Histopathology (% (tubulo)villous) | 35.0 | 20.7 |
| Dietary intake | | |
| Energy (kJ/day)* | 8948 ± 2228 | 8582 ± 2220 |
| Alcohol (g/day)† | 9.8 (1.5; 24.3) | 8.9 (0.3; 26.3) |
| Vitamin B2 (mg/day)* | 1.67 ± 0.47 | 1.57 ± 0.55 |
| Vitamin B6 (mg/day)* | 1.71 ± 0.44 | 1.61 ± 0.41 |
| Folate (µg/day)* | 205 ± 57 | 204 ± 61 |
| Vitamin B12 (µg/day)* | 5.04 ± 2.34 | 4.83 ± 2.29 |
| Supplementary multivitamin use (% yes) | 16.3 | 19.0 |
| Supplementary B vitamin use (% yes) | 6.3 | 6.9 |

* *mean ± SD*
† *median (25th percentile; 75th percentile)*

Odds ratios for a methylated gene promoter for those with a high compared with a low folate intake and for those with *TT* compared with *CC* genotype, adjusted for confounding factors, are shown in table 5.2. For all genes, there was an inverse association between folate intake and promoter hypermethylation, although none of the associations reached statistical significance. No clear pattern was visible in the associations between *MTHFR*

***Table 5.2*** *Association between dietary intake of folate or MTHFR C677T genotype and gene promoter methylation**

| | Dietary folate intake† | | *MTHFR C677T* genotype | |
|---|---|---|---|---|
| Promoter methylation | <183 µg/day | >212 µg/day | *CC* | TT |
| *APC-1A* | | | | |
| N methylated/not methylated | 40/35 | 35/37 | 61/51 | 14/21 |
| OR (95% CI) | 1 (ref.) | 0.73 (0.33;1.62) | 1 (ref.) | 0.56 (0.25;1.25) |
| *p14ARF* | | | | |
| N methylated/not methylated | 44/29 | 43/25 | 68/40 | 19/14 |
| OR (95% CI) | 1 (ref.) | 0.86 (0.37;1.97) | 1 (ref.) | 0.87 (0.38;1.96) |
| *p16INK4A* | | | | |
| N methylated/not methylated | 36/30 | 35/30 | 55/45 | 16/15 |
| OR (95% CI) | 1 (ref.) | 0.54 (0.22;1.32) | 1 (ref.) | 1.08 (0.46;2.52) |
| *hMLH1* | | | | |
| N methylated/not methylated | 11/59 | 11/59 | 18/87 | 4/31 |
| OR (95% CI) | 1 (ref.) | 0.79 (0.25;2.45) | 1 (ref.) | 0.64 (0.20;2.11) |
| *O6-MGMT* | | | | |
| N methylated/not methylated | 47/22 | 41/27 | 66/37 | 22/12 |
| OR (95% CI) | 1 (ref.) | 0.62 (0.26;1.49) | 1 (ref.) | 1.00 (0.44;2.30) |
| *RASSF1A* | | | | |
| N methylated/not methylated | 27/42 | 29/39 | 40/62 | 16/19 |
| OR (95% CI) | 1 (ref.) | 0.81 (0.35;1.91) | 1 (ref.) | 1.55 (0.69;3.50) |
| At least three genes methylated | | | | |
| N ≥3 /<3 methylated | 40/29 | 40/29 | 61/43 | 19/15 |
| OR (95% CI) | 1 (ref.) | 0.66 (0.29;1.54) | 1 (ref.) | 1.00 (0.44;2.24) |

* *Adjusted for age, sex, BMI, indication for colonoscopy, and dietary intake of vitamin B2 and vitamin B6*
† *Adjusted for total energy intake, according to Willett and Stampfer[30]*

*C677T* genotype and promoter hypermethylation for the individual genes, and the associations were not statistically significant. Analyses examining methylation in ≥3 genes compared with <3 genes led to similar results as those for all genes separately (table 5.2): folate intake was inversely related to promoter methylation, although not statistically significant, and *MTHFR* genotype was not related to promoter methylation.

Table 5.3 shows the interplay between folate and *MTHFR C677T* genotype in gene promoter methylation. Among the different genes, a consistent pattern was noticeable: within those carrying the *CC* genotype, there was no clear association between folate intake and promoter hypermethylation, but within those carrying the *TT* genotype, the association between folate intake and promoter hypermethylation was inverse, although not statistically significant. This association was most obvious for *$O^6$-MGMT*, where the interaction was statistically significant (p=0.02), and for *RASSF1A*, with a borderline statistically significant interaction (p=0.06). Again, the results were the same when analyzing concurrent methylation in ≥3 genes compared to <3 genes (p interaction=0.07). The interactions seemed more pronounced among male participants; however, the study did not have enough power for subgroup analyses according to sex (data not shown).

***Table 5.3*** *Interplay between dietary folate intake and MTHFR C677T genotype in gene promoter methylation*[*]

| | *MTHFR CC* genotype | | *MTHFR TT* genotype | | |
|---|---|---|---|---|---|
| Promoter methylation | Folate intake[†] <183 µg/day | Folate intake[†] >212 µg/day | Folate intake[†] <183 µg/day | Folate intake[†] >212 µg/day | P interaction |
| *APC-1A* | | | | | |
| N methylated/not methylated | 30/26 | 31/25 | 10/9 | 4/12 | |
| OR (95% CI) | 1 (ref) | 0.94 (0.39;2.27) | 0.88 (0.29-2.64) | 0.30 (0.08;1.12) | 0.25 |
| *$p14^{ARF}$* | | | | | |
| N methylated/not methylated | 32/23 | 36/17 | 12/6 | 7/8 | |
| OR (95% CI) | 1 (ref) | 1.20 (0.48;3.00) | 1.70 (0.53-5.48) | 0.49 (0.14;1.68) | 0.10 |
| *$p16^{INK4A}$* | | | | | |
| N methylated/not methylated | 27/21 | 28/24 | 9/9 | 7/6 | |
| OR (95% CI) | 1 (ref) | 0.48 (0.18;1.28) | 0.88 (0.28-2.76) | 0.72 (0.19;2.84) | 0.55 |
| *hMLH1* | | | | | |
| N methylated/not methylated | 8/44 | 10/43 | 3/15 | 1/16 | |
| OR (95% CI) | 1 (ref) | 1.13 (0.33;3.89) | 1.32 (0.29-5.96) | 0.26 (0.03;2.52) | 0.19 |
| *$O^6$-MGMT* | | | | | |
| N methylated/not methylated | 32/19 | 34/18 | 15/3 | 7/9 | |
| OR (95% CI) | 1 (ref) | 1.00 (0.38;2.63) | 3.39 (0.82-13.93) | 0.37 (0.11;1.29) | 0.02 |
| *RASSF1A* | | | | | |
| N methylated/not methylated | 17/34 | 23/28 | 10/8 | 6/11 | |
| OR (95% CI) | 1 (ref) | 1.15 (0.44;3.03) | 3.53 (1.08-11.50) | 0.78 (0.22;2.76) | 0.06 |
| At least three genes methylated | | | | | |
| N ≥3 /<3 methylated | 28/23 | 33/20 | 12/6 | 7/9 | |
| OR (95% CI) | 1 (ref.) | 0.95 (0.37;2.42) | 2.17 (0.66-7.11) | 0.43 (0.13;1.49) | 0.07 |

[*] *Adjusted for age, sex, BMI, indication for colonoscopy, and dietary intake of vitamin B2 and vitamin B6*
[†] *Adjusted for total energy intake, according to Willett and Stampfer*[30]

## DISCUSSION

In this observational study, we examined whether dietary folate intake and *MTHFR C677T* genotype are associated with promoter methylation of tumor suppressor genes and DNA repair genes in colorectal adenoma specimens. Nonsignificant inverse associations were observed between dietary folate intake and promoter methylation in the genes under study, especially among those carrying the *MTHFR TT* genotype. The interaction between folate intake and *MTHFR C677T* genotype was most pronounced for *$O^6$-MGMT* and *RASSF1A*. The *MTHFR C677T* genotype alone was not associated with promoter methylation.

Most other studies examining promoter methylation in sporadic adenomas found frequencies of methylation of individual genes that were either lower[6,29,31-35] or in the same range as in our study.[6,29,36] Only Bai *et al.* found a higher percentage of promoter methylation of *hMLH1*[29]. However, comparing our results with other studies is difficult for several reasons: different studies examine different genes, and background exposures and characteristics of adenomas (for example tubular/villous) may vary between studies. We selected participants from a Dutch case-control study, in which folate intake was positively associated with adenoma occurrence.[21] Dietary folate intake in this case-control study was relatively low (mean: 195 μg/day). This may partly account for the relatively high percentages of promoter methylation in our study. Differences in adenoma characteristics may explain a further part of the relatively high methylation frequencies that we found. In our study, villous adenomas show higher frequencies of promoter methylation than tubular adenomas. Furthermore, 15 of 164 adenomas (9.1%), which comprised mainly tubular adenomas, could not be analyzed due to insufficient DNA yield. Two studies that observed lower frequencies of methylation included only adenoma samples that showed low-grade dysplasia,[33] or only tubular adenomas.[34] Conversely, other studies indicate similar methylation patterns in both advanced and non-advanced adenomas.[6,29]

To our knowledge, we are the first to investigate the role of folate, *MTHFR C677T* genotype, and their interplay in promoter methylation in colorectal adenomas. In colorectal cancer, however, a few studies looked at the association between folate or *MTHFR C677T* genotype and promoter methylation. Van Engeland *et al.*[10] studied promoter methylation of the same six genes in the Netherlands Cohort Study on Diet and Cancer. The prevalence of promoter methylation was higher in carcinomas from patients with low folate/high alcohol intake when compared with carcinomas from patients with high folate/low alcohol intake, although the differences were not statistically significant.[10] Although alcohol intake was not taken into account in our study, we state that this is in accordance with our adenoma results: in the case-control study from which our participants are selected, alcohol intake did not modify or confound the association

between folate intake and adenoma occurrence; furthermore, stratification in the Netherlands Cohort Study on Diet and Cancer was done primarily on folate intake and secondarily on alcohol intake.[10] Kawakami *et al.*[11] examined the associations between the folate intermediates 5,10-methyleneTHF and THF in colorectal carcinomas from Australian patients, polymorphisms including *MTHFR C677T*, and methylation of gene promoters. They showed that colorectal carcinomas with frequent promoter methylation have high levels of folate intermediates in tissue. However, like we, they did not find a relationship between *MTHFR C677T* polymorphism and gene promoter methylation.[11] The interplay between folate and *MTHFR C677T* was not studied. In a study by Paz *et al.*[37], *MTHFR C677T* genotype was also not related to frequency of promoter methylation in cancer patients. Folate intake was not taken into account.[37]

It would be interesting to study the influence of polymorphisms in other genes that are involved in the one-carbon metabolism on promoter methylation. However, in the current study, we selected the participants based on the *MTHFR C677T* genotype and folate intake. Therefore, there is not enough power to take other polymorphisms into account. Another interesting biomarker to study would be global DNA hypomethylation. This is not possible within the scope of the present study because of scarcity of adenoma tissue.

The use of a food-frequency questionnaire may not be the most accurate way to assess folate status. Most food-frequency questionnaires that were validated for folate intake show poor correlation between erythrocyte folate and dietary folate intake.[38,39] The EPIC questionnaire that we used showed a positive, although poor correlation between plasma folate and dietary folate intake [40]. We were not able to assess erythrocyte folate or plasma folate in the current study. We focused on dietary folate intake, since supplement use is not common in the Netherlands and supplementary folic acid intake was not assessed in this study. Adjusting the logistic models for supplementary multivitamin or B-vitamin use did not change the results.

Cases were asked to recall their dietary habits from the past, which may have led to misclassification. However, systematic errors in dietary recall are less likely to bias results from case-case comparisons as misclassification will presumably be at random. So if this had an influence on our results, it will have attenuated them.

We chose to examine a set of six genes that are frequently methylated in colorectal cancer.[24,25] Mutations in the *APC* tumor suppressor gene that result in loss of APC function are thought to be a key initiating event in familial and sporadic colorectal cancer.[41] One of the proposed mechanisms is that loss of APC function leads to the accumulation of β-catenin, which in turn results in transcriptional activity of Wnt target genes.[41] The *INK4A/ARF* locus gives rise to two distinct transcripts from different promoters, namely $p16^{INK4A}$ and $p14^{ARF}$. Both $p16^{INK4A}$ and $p14^{ARF}$ induce cell-cycle arrest.[42] Thus, inactivation of $p16^{INK4A}$ or $p14^{ARF}$ contributes to uncontrolled proliferation in human neoplasia.

Transcriptional silencing of the *RASSF1A* gene by promoter methylation has been observed in many cancers. Thus far, it has been shown to play a role in cell cycle regulation, apoptosis, and microtubule instability.[43] The *hMLH1* gene is a DNA mismatch repair gene. Mismatch repair is required for the cell to accurately copy its genome during cellular proliferation. Errors made during DNA duplication, such as deletions, insertions, and mismatched base pairs, are substrates for this system.[44] $O^6$-MGMT is a DNA repair protein that removes mutagenic and cytotoxic adducts from the $O^6$ position of guanine in DNA.[45] Clearly, methylation of all these genes can play an important role in colorectal carcinogenesis. As folate intake, *MTHFR C677T* genotype and the interplay between them may influence the promoter methylation process, this may be of importance in the development of colorectal adenomas or carcinomas.

In summary, this study suggests that folate intake is inversely associated with promoter methylation in colorectal adenomas, especially in those with *TT* genotype. This study is the largest study to date. Despite the numbers being still small, results from separate genes point in the same direction and some interactions between folate intake and *MTHFR C677T* genotype reach statistical significance. Therefore, we believe that our results justify conducting studies with larger sample sizes.

## ACKNOWLEDGEMENTS

We thank Dorien Voskuil, Edine Tiemersma, Maria van Vugt, Elly Monster, Brenda Diergaarde, Petra Wark, and Mariken Tijhuis for their roles in the conduct of the case-control study, as well as Jan Harryvan and Annelies Bunschoten for the genotype assessment, Sandra van den Bosch for her support in the methylation assessment, Goos van Muijen and Hanneke Braam for tissue collection and DNA isolation, Han van Krieken for re-evaluation of polyp histology, and Marga Ocké for micronutrient calculations. We are grateful to all the people who kindly participated in this study. The participants were recruited with help from the endoscopy staff of the following hospitals in the Netherlands: Slingeland Ziekenhuis (Doetinchem), Ziekenhuis Geldere Vallei (Ede), Radboud University Nijmegen Medical Centre (Nijmegen), Antonius Ziekenhuis (Nieuwegein), Meander Medisch Centrum (Amersfoort), Ziekenhuis Rijnstate (Arnhem), Jeroen Bosch Ziekenhuis ('s Hertogenbosch), and Canisius-Wilhelmina Ziekenhuis (Nijmegen).

This study is funded by the Netherlands Organization for Health Research and Development (ZonMW), grant 980-10-020, and the Netherlands Foundation for Digestive Diseases (MLDS), grant WS 99-72.

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# Chapter 6

## THE EFFECT OF FOLIC ACID AND VITAMIN B12 ON PROMOTER METHYLATION AND URACIL INCORPORATION IN RECTAL MUCOSA DNA AMONG *MTHFR C677T* GENOTYPES: A RANDOMIZED, PLACEBO-CONTROLLED INTERVENTION STUDY

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**Adequate folate availability is necessary to sustain normal DNA synthesis and normal patterns of DNA methylation, and these features of DNA can be modified by *MTHFR C677T* genotype. This study investigated the effect of daily supplementation with 5 mg folic acid and 1.25 mg vitamin B12 on uracil misincorporation into DNA and promoter methylation.**

**86 Subjects with a history of colorectal adenomas and *MTHFR CC* or *TT* genotype were randomly assigned to either folic acid and vitamin B12 or placebo for six months. Randomization was stratified for *MTHFR* genotype. At baseline and after six months, uracil incorporation and promoter methylation of six tumor suppressor and DNA repair genes were measured in DNA from rectal biopsies.**

**In the intervention group, the uracil content of DNA increased 0.60 pg/μg, whereas in the placebo group it increased 0.15 pg/μg. The 95% confidence interval (CI) of the difference in response was -0.19;1.09 pg/μg. The probability of promoter methylation increased in the intervention group compared with the placebo group (odds ratio 1.67, 95% CI 0.95;2.95) Both effects were more pronounced in people with the *TT* genotype.**

**This study suggests that supplementation with high doses of folic acid and vitamin B12 can increase uracil incorporation and enhance promoter methylation in colorectal adenoma subjects. Since such alterations might feasibly increase the risk of neoplastic transformation, more research is needed to fully define the consequences of these molecular alterations.**

## INTRODUCTION

Folate is an essential vitamin in DNA synthesis and repair. As 5-methyltetrahydrofolate (5-methylTHF), folate provides methyl groups for S-adenosylmethionine, the universal methyl donor for a large number of biological reactions, including the methylation of DNA.[1] DNA methylation is involved in gene expression, chromatin configuration and structural stability of DNA, binding of transcriptional factors and other proteins, and imprinting.[2] Furthermore, 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) acts as a cofactor in the conversion of deoxyuridylate to thymidylate, and thus is essential for DNA synthesis.

Inadequate folate availability in humans has been shown to increase uracil misincorporation into DNA and decrease global hypomethylation, two factors which may be operative in colorectal carcinogenesis.[1] Methylation of the promoter region of tumor suppressor genes is increasingly recognized to play an important role in cancer development, through silencing of gene transcription.[3]

Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme in folate metabolism that catalyzes the conversion of 5,10-methyleneTHF to 5-methylTHF.[1] A common C-to-T substitution in the *MTHFR* gene at nucleotide 677 converts an alanine to valine and produces diminished enzyme activity *in vivo*.[4] Most studies indicate that the *TT* genotype is associated with global DNA hypomethylation in peripheral blood cells,[5-7]

probably restricted to those with a low folate intake,[6] although one study could not demonstrate an association.[8] Studies examining the association between *MTHFR* genotype and promoter methylation show ambiguous results.[9,10] To our knowledge, one study examined the relationship between *MTHFR* genotype and uracil misincorporation in human lymfocyte DNA, showing a similar uracil content for all *MTHFR* variants.[8]

In the Netherlands, the mean folate intake is about 200 μg/day,[11] which is below the Dutch RDI of 300 μg. Enrichment of foods with folic acid and supplement use are not common in the Netherlands. Thus, part of the population may be ingesting insufficient quantities of folate to sustain normal DNA metabolism.

Previous studies in subjects with colorectal adenomas using global DNA methylation in normal colorectal mucosa as a primary endpoint, indicate that folic acid supplementation (0.4-10 mg/day) can reverse DNA hypomethylation.[12-15] A study examining the effect of a high-dose folic acid intervention in colorectal adenoma patients showed a non-significant reduction in adenoma recurrence.[16] The numbers in these studies are relatively small, and in most no distinction is made between different *MTHFR* genotypes.

To further explore the effects of folate supplementation on relevant molecular events in the colon, we conducted a randomized, placebo-controlled intervention study with a high dose of folic acid and vitamin B12. Vitamin B12 was added to prevent the risk of masking a vitamin B12 deficiency. We examined the effect of a six-month intervention on uracil misincorporation and promoter hypermethylation of six tumor suppressor and DNA repair genes in DNA from rectal mucosa biopsies. The study was conducted among individuals with a history of colorectal adenomas, and the possible modulation of effects by the *MTHFR C677T* genotype was taken into account.

# MATERIALS AND METHODS

## Study population

We recruited participants among patients from a Dutch case-control study[17] who have the *MTHFR 677 CC* or *TT* genotype and a history of sporadic colorectal adenomas. Eligibility criteria were: Dutch speaking; of European origin; aged 18 to 80 years; no hereditary colorectal cancer syndromes; no chronic inflammatory bowel disease; no history of colorectal cancer; no (partial) bowel resection; not pregnant or lactating; no significant liver or renal disease; not using anti-epileptic medication, antifolate medication or supplements containing B-vitamins. The participants were recruited between March 2002 and February 2003. We obtained written informed consent from all participants.

The study was conducted in three hospitals in the Netherlands: the Radboud University Nijmegen Medical Centre in Nijmegen, the Gelderse Vallei Hospital in Ede, and the

Slingeland Hospital in Doetinchem. The study protocol was approved by the Ethical Committees of all participating centers.

## Design

After stratification by *MTHFR C677T* genotype, participants were allocated to vitamin or placebo capsules at entry into the study. Capsules were produced by Dutch BioFarmaceutics (Helmond, The Netherlands). After analysis, vitamin capsules appeared to contain 4.6 mg folic acid (pteroylmonoglutamic acid) and 1.1 mg vitamin B12 (cyanocobalamin), whereas placebo capsules contained <0.04 µg folic acid en <0.002 µg vitamin B12. Within each genotype group, treatment was allocated using random permuted blocks with lengths of four and six. A random number table was used to determine block lengths and allocation within each block. All participants and study personnel were blinded to treatment assignment for the duration of the study. Compliance was judged by pill-return counts and by analyzing plasma homocysteine and erythrocyte folate levels before and after the intervention period.

## Data collection

Dietary intake was assessed with a semi-quantitative food-frequency questionnaire that was originally developed for the Dutch cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC).[18] The participants also completed a general questionnaire on medical history and lifestyle factors. Participants were advised not to alter their diet or lifestyle during the study.

Venous blood samples were obtained before and after the intervention period, to measure plasma homocysteine, serum and erythrocyte folate, and serum vitamin B12 levels. Rectal biopsies of normal appearing mucosa were obtained by flexible sigmoidoscopy without bowel preparation. Biopsies were immediately snap-frozen in liquid nitrogen and stored in liquid nitrogen until extraction of DNA for determination of uracil misincorporation and promoter methylation. DNA was extracted using TRIZOL (Invitrogen, Breda, The Netherlands).

The uracil content of DNA samples was measured using the method of Blount and Ames[19] with modifications.[20,21] A quality control sample was tested in triplicate with each run. The mean intra-run %CV was 10.1% while the between day %CV was 12.1% (over twelve days). All samples were analyzed in duplicate.

The genes analyzed for promoter methylation are the DNA repair genes $O^6$-*MGMT* and *hMLH1* and tumor suppressor genes that affect important cellular processes such as the cell cycle ($p14^{ARF}$, $p16^{INK4A}$, *RASSF1A*) and the Wnt signalling pathway (*APC*). All of these genes are reported to be frequently methylated in colorectal cancer.[22,23] We determined DNA methylation of the CpG islands of the promoters of these genes by chemical

modification of 500 ng of genomic DNA with sodium bisulfite and subsequent methylation-specific PCR (MSP), according to the method described by Herman *et al.*[24], with nested PCR.[23] All PCRs were performed with controls for unmethylated alleles (DNA from normal lymfocytes), methylated alleles (normal lymfocyte DNA treated *in vitro* with *SssI* methyltransferase; New England Biolabs, Beverly, MA), and a control without DNA. Primer sequences and PCR conditions are listed elsewhere.[23] Ten µl of each MSP reaction was loaded onto 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

## Statistical analyses

Descriptive statistics of intake and blood levels of relevant nutrients and other baseline characteristics were computed, according to intervention or placebo group. Because also the non-responders participated in the aforementioned case-control study,[17] we were able to calculate some baseline characteristics of this group.

The primary endpoints were changes in the uracil content and promoter methylation in rectal mucosa DNA. We used paired *t* tests to assess differences in uracil content between baseline and post intervention values in each group. We assessed the differences in response between the intervention and the placebo group with linear regression analysis, to be able to adjust for *MTHFR* genotype. In the total intervention group (n=36), a change in uracil content of 1.3 pg/µg DNA can be detected with a power of 80% and a two-sided confidence interval of 0.95, at a supposed standard deviation of 2 pg uracil/µg DNA.[20] Within the *TT* (n=8) and *CC* (n=28) genotypes, the detectable differences will be 2.8 and 1.5 pg uracil/µg DNA, respectively.

We did not have enough power to assess changes in promoter methylation for all genes separately, as methylation frequencies are too low. Since colorectal carcinogenesis is widely held to be the end result of a multistep process in which the aberrant expression of several genes collectively create a milieu which facilitates the progression of carcinogenesis, we considered it appropriate to assess the methylation of all these genes in a collective manner. Furthermore, folate intake, especially in combination with *MTHFR TT* genotype, was inversely associated with promoter methylation of all six genes in cases of the Dutch case-control study from which our participants were recruited (**chapter 5**). For each participant, we calculated the percentage of gene promoters that were not methylated at baseline, but were methylated after the intervention period (**upmethylated**), and the percentage of gene promoters that were methylated at baseline but not after the intervention period (**downmethylated**). These percentages were converted into logits (ln (p/(1-p))) to calculate odds ratios (ORs) for upmethylation and for downmethylation, taking the placebo group as the reference group. These ORs are an estimate for the probability ratio of either upmethylation or downmethylation of any of

the six genes in the intervention group as compared with the placebo group. We used linear regression analysis for these calculations. The ORs were weighed for the number of genes that were assessed in each participant, to be able to include participants that had missing values for one or more of the six genes.

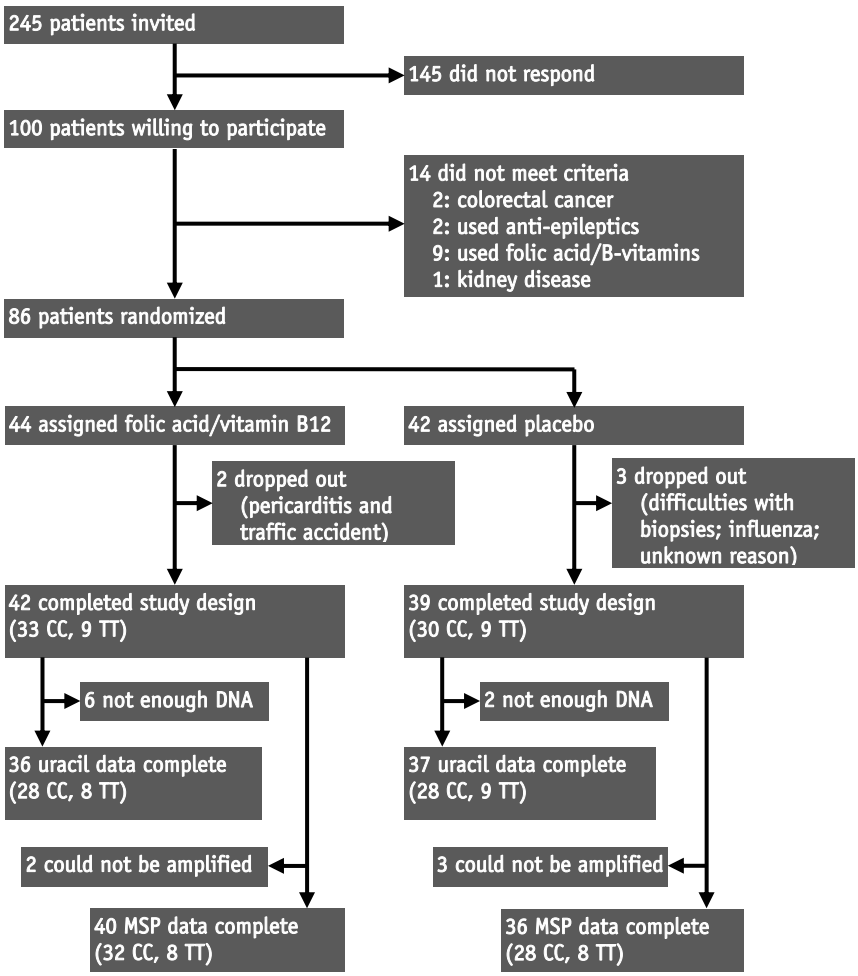
Since individuals with a relatively low folate status might respond differently than those with a relatively high folate status, we conducted subgroup analyses according to baseline serum folate level below or above the median.

We analyzed the data by **intention to treat**, with complete case analyses where data are missing at random. All tests of statistical significance were two-sided and the significance level was set at 5%. We used Statistical Analysis Software (SAS version 9.1, SAS Institute, Cary, NC) for all analyses.

## RESULTS

Figure 6.1 shows the study flowchart. 86 Eligible patients, 67 having the *CC* genotype and 19 the *TT* genotype, were randomly assigned to either the intervention or placebo.

***Figure 6.1*** *Study flowchart*



After randomization, five participants withdraw from the study: four with *CC* genotype (three in the placebo group and one in the intervention group) and one with *TT* genotype, in the intervention group. The reasons for withdrawal were: difficulties with taking rectal mucosa biopsies; traffic accident; influenza; pericarditis; lost to follow-up for unknown reason. Moreover, there were missing data on either the baseline or the end measurement of the primary endpoints due to insufficient quality or quantity of DNA. As we judged these missings to be at random, we did not include these participants in the analyses. One participant (placebo, *CC* genotype) stopped taking the capsules when developing a peptic ulcer, but did not withdraw. This person was included in the analyses.

In table 6.1, baseline characteristics of both study groups and of the non-responders are shown. Although the participants were randomly assigned to the two groups, the proportion of women was somewhat lower in the intervention group (34%) compared with the placebo group (43%). Furthermore, the intervention group tended to have higher levels of serum vitamin B12 and serum and erythrocyte folate, and a lower level of plasma homocysteine, compared with the placebo group; however, none of the differences were statistically significant. When dividing the population in those below and above the median serum folate value at baseline, there were also no statistically significant differences between the intervention and the placebo groups (data not shown).

***Table 6.1*** *Baseline characteristics of the study population*

| | Intervention (n=42) | Placebo (n=39) | Non-responders (n=145) |
|---|---|---|---|
| Sex (% female) | 33 | 46 | 51 |
| Age* | 61.1±8.0 | 61.4±9.9 | 58.5±10.0 |
| Body mass index* | 25.9±3.3 | 27.1±3.8 | 25.7±3.1 |
| Daily intake of food and nutrients: | | | |
| Energy (kJ)* | 8500±2196 | 8281±1964 | 8364±2252 |
| Fat (g)* | 82±26 | 80±21 | 80±27 |
| Vitamin B2 (mg)* | 1.53±0.56 | 1.45±0.52 | 1.52±0.51 |
| Vitamin B6 (mg)* | 1.58±0.41 | 1.51±0.29 | 1.61±0.47 |
| Folate (µg)* | 192±43 | 195±95 | 197±71 |
| Vitamin B12 (µg)* | 4.68±2.18 | 4.84±5.39 | 4.75±3.94 |
| Fibre (mg)* | 23.6±7.0 | 23.5±5.0 | 23.2±6.5 |
| Alcohol (g)† | 8.3 (0.6;22.3) | 10.0 (0.4;23.2) | 6.5 (0.8;21.6) |
| Vegetables (g)* | 124±44 | 130±42 | 127±49 |
| Fruit (g)* | 170±128 | 203±136 | 188±136 |
| Blood levels: | | | |
| Plasma Hcy (µmol/L)* | 11.5±3.0 | 12.7±4.1 | n.a. |
| Serum folate (nmol/L)* | 18.9±9.9 | 14.5±5.9 | n.a. |
| Serum B12 (pmol/L)* | 265±99 | 244±100 | n.a. |
| Erythrocyte folate (nmol/L)* | 750±291 | 641±250 | n.a. |

** Mean±standard deviation*
*† Median (p25;p75)*

We measured erythrocyte folate and plasma homocysteine levels of the participants as measures of compliance (table 6.2). Erythrocyte folate and plasma homocysteine did not change in the placebo group during the study, whereas plasma homocysteine decreased

***Table 6.2*** *Effect of folic acid and vitamin B12 intervention on plasma homocysteine and erythrocyte folate concentrations*

| | Intervention | | | Placebo | | | Difference in response† |
|---|---|---|---|---|---|---|---|
| | Baseline | End | Change* | Baseline | End | Change* | |
| All participants | (n=42) | | | (n=39) | | | |
| Plasma Hcy | 11.5±0.5 | 7.8±0.2 | -3.7 (-4.4;-3.0) | 12.7±0.7 | 13.2±0.7 | 0.5 (-0.1;1.1) | -4.2 (-5.2;-3.2)‡ |
| Ery§ folate | 750±45 | 2556±95 | 1806 (1621;1991) | 641±40 | 601±31 | -40 (-95;16) | 1849 (1651;2048) ‡ |
| *TT* genotype | (n=9) | | | (n=9) | | | |
| Plasma Hcy | 11.5±1.2 | 7.7±0.7 | -3.8 (-5.2;-2.4) | 14.8±1.7 | 14.7±1.9 | -0.1 (-1.1;0.8) | -3.7 (-5.5;-1.9) |
| Ery§ folate | 665±73 | 2895±236 | 2231 (1771;2691) | 579±67 | 506±43 | -73 (-151;4) | 2304 (1799;2808) |
| *CC* genotype | (n=33) | | | (n=30) | | | |
| Plasma Hcy | 11.5±0.5 | 7.8±0.2 | -3.7 (-4.6;-2.8) | 12.1±0.7 | 12.8±0.7 | 0.6 (-0.1;1.4) | -4.3 (-5.5;-3.2) |
| Ery§ folate | 773±53 | 2463±98 | 1690 (1506;1874) | 659±48 | 630±37 | -29 (-98;39) | 1719 (1511;1927) |

** End - baseline (95%CI)*
*† Change intervention – change placebo (95%CI)*
*‡ Adjusted for MTHFR genotype*
*§ Ery: erythrocyte*

significantly in the intervention group and erythrocyte folate increased significantly in the intervention group. Furthermore, we counted returned pills from the participants. Of 81 participants that completed the study, five did not return their remaining pills, one stopped taking the pills, seven took between 90% and 95% of the pills, and 68 took more than 95% of the pills. Based on blood measurements and pill return counts, we judged that compliance was adequate.

***Table 6.3*** *Effect of folic acid and vitamin B12 intervention on uracil levels in rectal mucosa DNA*

| | Intervention | | | Placebo | | | Difference in response† |
|---|---|---|---|---|---|---|---|
| | Baseline | End | Change* | Baseline | End | Change* | |
| All participants | (n=36) 0.36±0.10 | 0.96±0.21 | 0.60 (0.10;1.10) | (n=37) 0.41±0.12 | 0.56±0.15 | 0.15 (-0.22;0.53) | 0.45 (-0.19;1.09) p=0.16‡ |
| *TT* genotype | (n=8) 0.33±0.12 | 1.11±0.49 | 0.79 (-0.27;1.84) | (n=9) 0.24±0.15 | 0.35±0.35 | 0.11 (-0.66;0.88) | 0.68 (-0.72;2.08) p=0.32 |
| *CC* genotype | (n=28) 0.37±0.13 | 0.92±0.24 | 0.55 (-0.03;1.13) | (n=28) 0.46±0.15 | 0.63±0.17 | 0.17 (-0.26;0.60) | 0.38 (-0.36;1.12) p=0.31 |
| Serum folate <14.3 nmol/L | (n=14) 0.65±0.20 | 0.66±0.27 | 0.01 (-0.71;0.73) | (n=22) 0.36±0.11 | 0.39±0.19 | 0.03 (-0.32;0.38) | -0.03 (-0.78;0.71) p=0.93‡ |
| Serum folate ≥14.3 nmol/L | (n=22) 0.17±0.09 | 1.15±0.30 | 0.98 (0.33;1.62) | (n=15) 0.48±0.24 | 0.81±0.25 | 0.33 (-0.44;1.10) | 0.60 (-0.43;1.63) p=0.25‡ |

** End - baseline (95% CI)*
*† Change intervention – change placebo (95% CI)*
*‡ Adjusted for MTHFR genotype*

Table 6.3 shows uracil levels in rectal mucosa DNA before and after the intervention period. Within the total intervention group, the level of uracil increased approximately 2.5-fold during the study (p=0.02), whereas in the placebo group, the small increase in uracil did not achieve statistical significance. The increase in the intervention group was 0.45 pg uracil/μg DNA more compared with the placebo group (p=0.16). When stratifying

the analyses for *MTHFR* genotype, the effects of the intervention were similar in both the *TT* and the *CC* group, but seemed more pronounced in the *TT* group. However, the numbers were too small to demonstrate statistically significant differences between the genotype groups. When stratifying the analyses according to baseline serum folate, the effect of the intervention seemed to be limited to those with baseline serum folate above the median.

***Table 6.4*** *Effect of folic acid and vitamin B12 intervention on promoter methylation in rectal mucosa DNA*

| | | | Upmethylation* | Downmethylation† |
|---|---|---|---|---|
| | N intervention | N placebo | OR (95% CI) | OR (95% CI) |
| All participants | 40 | 36 | 1.67 (0.95-2.95) | 0.85 (0.52-1.34)‡ |
| *TT* genotype | 8 | 8 | 2.36 (0.57-9.79) | 0.39 (0.11-1.40) |
| *CC* genotype | 32 | 28 | 1.53 (0.83-2.84) | 1.03 (0.63-1.67) |
| Serum folate <14.3 nmol/L | 17 | 21 | 1.16 (0.63-2.14) | 1.16 (0.56-2.40)‡ |
| Serum folate ≥14.3 nmol/L | 23 | 15 | 1.91 (0.78-4.66) | 0.61 (0.33-1.12)‡ |

* *Upmethylation: not methylated at baseline, but methylated after the intervention period*
† *Downmethylation: methylated at baseline, but not methylated after the intervention period*
‡ *Adjusted for MTHFR genotype*

The results of the MSP analyses are shown in table 6.4. In the total group, intervention with folic acid and vitamin B12 was positively associated with upmethylation, which means that the probability of upmethylation was higher in the intervention group than in the placebo group. Intervention seemed inversely associated with downmethylation. Both associations did not reach statistical significance. Again, results were similar in both genotype groups, but seemed more pronounced in those with the *TT* genotype. Furthermore, the associations seemed restricted to those with baseline serum folate above the median. When we considered all six genes separately, results pointed in the same direction for all genes except *APC* (data not shown); however, numbers were too small to draw conclusions from these analyses.

## DISCUSSION

In this randomized, placebo-controlled trial, we observed that supplementation with folic acid and vitamin B12 for six months increased the probability of promoter methylation in rectal mucosa DNA of patients with a history of sporadic colorectal adenomas. Moreover, the uracil content in rectal mucosa DNA increased ~ 2.5-fold as a result of intervention, although this increase was not significantly greater than a much more modest increase observed among those who received the placebo. Although the uracil content of peripheral blood mononuclear cell DNA has been previously shown to revert to lower, more normal, levels with the treatment of folate deficiency,[25] our study is the first human folic acid intervention study to our knowledge to examine uracil misincorporation and promoter methylation in rectal mucosa DNA as primary endpoints. These endpoints in the colonic mucosa are of particular relevance since: 1) excess uracil incorporation and

promoter hypermethylation of critical genes are mechanistically implicated in colorectal carcinogenesis,[1,3] 2) the colon appears to be an especially sensitive organ in regard to how folate status might modulate cancer risk,[1] and 3) the biochemical and molecular alterations that arise as a result of changes in folate status are known to be highly tissue-specific.[26] Although we did not have sufficient power to observe significant effects of *MTHFR* genotype, the abovementioned effects seemed more pronounced in people with the *TT* genotype. Moreover, the effects were mainly present in individuals with serum folate levels above the median at baseline.

The results from this study contradict our initial hypotheses. Earlier studies have generally observed that folic acid supplementation alters purported biomarkers of colon cancer in a favorable manner.[12-14,27-29] In these studies, effects of a three,[12,27,28] six,[13] or twelve-month[14,29] intervention with a daily dose of 2 mg,[28] 5 mg,[12,14,27,29] or 10 mg[13] folic acid was investigated. Favorable effects were found on DNA hypomethylation,[12,13] colonic mucosal cell proliferation,[28,29] loss of heterozygosity of the tumor suppressor gene *DCC* (deleted in colorectal cancer),[29] or activity of the proto-oncogene ornithine decarboxylase.[27] In a study by Kim *et al.*, six months of intervention increased genomic DNA methylation and decreased *p53* strand breaks, although after twelve months the effects were the same in the placebo group.[14] Recently, an intervention study utilizing a physiological dose of folic acid (400 µg/day) over ten weeks also observed an increase in genomic DNA methylation in rectal mucosa of colorectal adenoma patients.[15]

The presence of adenomas is presently considered to be the only intermediary biomarker of colorectal cancer for which firm validation data exist, since removal of such lesions leads to a reduction in the subsequent risk of cancer.[30] Unfortunately, a very limited amount of information is available to date from folate intervention trials that have used adenoma recurrence as the primary endpoint. A small study investigating daily supplementation with 1 mg folic acid for two years suggested a reduction in adenoma recurrence,[16] but the preliminary report from a large, placebo-controlled multicenter trial showed no reduction in adenoma recurrence.[31] In summary, human folic acid intervention studies to date generally have shown favorable effects on biochemical, molecular and cytokinetic biomarkers for colorectal cancer whereas the paucity of data available from adenoma recurrence trials precludes any firm conclusions regarding effects on the latter biomarker.

The high dosage of folic acid (and vitamin B12) that was used in our study is feasibly responsible, at least in part, for the counterintuitive nature of our results. In animal models of colon cancer, folate supplementation has been shown to be protective under most conditions, but if it is given in very high doses or at a stage of carcinogenesis where neoplastic transformation has already firmly been established, it instead enhances the

development of neoplasms.[32-34] In these animal studies, daily doses of folic acid exceeding the basal requirements increased rather than decreased the number of neoplasms.

We used the synthetic, fully oxidized form of folate (pteroylmonoglutamic acid), which is normally fully metabolized by the intestine before it is released into the plasma as 5-methylTHF; consequently, the latter form is the sole circulating form of folate under normal conditions. However, studies show that this absorption and biotransformation process is saturated at doses in the region of 400 µg folic acid or less.[35] At higher doses, synthetic folic acid is also transported into the blood and may enter in large quantities. Compelling data about possible antagonistic activities of this fully oxidized form of folate in tissues is lacking, although occasional concern has been voiced about this possibility.[36]

In a human intervention study that was performed to examine the effect of folic acid supplementation on neural tube defects, a higher all-cause mortality and mortality from breast cancer was found in participants in two intervention groups (with 200 µg or 5 mg folic acid daily during pregnancy) compared with the placebo group.[37] As this was not a prespecified hypothesis, these results have to be interpreted cautiously, but still it is something that deserves attention. In a study examining the effects of megadose vitamin supplementation in healthy elderly people, subjects that took megadoses of any of several B-vitamins (B1, B2, niacin, B6 and folate) had lower absolute circulating lymfocyte counts than did subjects not on supplements.[38]

A relative lack in knowledge about the specific pathways by which promoter methylation and uracil incorporation effect a greater risk of cancer might also explain our counterintuitive observations. Although it is presumed that increased promoter methylation and increased uracil content are pro-carcinogenic, there still exist large gaps in the link between these particular molecular alterations and the appearance of cancer. We cannot exclude the possibility that other molecular events that we did not examine were being altered by the folic acid intervention and that they may play a much more important mechanistic role in determining cancer risk. For example, we cannot exclude the possibility that a promoter for a critical proto-oncogene or other pro-carcinogenic element in the genome also became hypermethylated as a result of intervention, thereby diminishing cancer risk.

Folate is not the only vitamin that is needed for DNA synthesis and DNA methylation. Vitamins B2, B6 and B12 also play a prominent role in the one-carbon metabolism. Flavin adenine dinucleotide (FAD), a metabolite of vitamin B2, serves as a cofactor for MTHFR.[39] Vitamin B6 is a cofactor for serine hydroxymethyltransferase, which catalyzes the conversion of tetrahydrofolate to 5,10-methylTHF.[40] Although the mean intakes of vitamin B2 and vitamin B6 in this study are in the range of the Dutch recommended dietary intake, it is possible that some people had a suboptimal intake. Vitamin B6 and especially vitamin B2 intakes are lower in the Netherlands compared to the United States. We

reported that vitamin B2 intake may be of importance in the association between folate intake and colorectal adenoma risk.[17] Unfortunately, we lack statistical power to do subgroup analyses according to intake of vitamin B2 or vitamin B6 in this intervention study. Vitamin B12 is a cofactor for methionine synthase, which is needed for methylation of homocysteine to produce methionine.[40] As vitamin B12 was supplemented in such a high dose that even people that can only passively take up vitamin B12, should take up enough, we do not expect that this influenced our results.

The results of this study suggest that, among individuals with a history of colorectal adenomas, high doses of supplemental folic acid and vitamin B12 can alter promoter methylation of a selected array of genes in the colonic mucosa in a manner that is usually perceived as pro-carcinogenic. Parallel changes in uracil content were also observed although fell short of statistical significance. The cellular consequences of these molecular alterations become better understood by the results of the subgroup analysis. These results revealed that the effects were confined to those with higher baseline folate status, and may provide direction as to which individuals might be the most suitable candidates for folate supplementation. Our results underscore the importance of further studies needed to determine whether or not folate supplementation is an effective cancer chemopreventive agent, and if so, what the appropriate dose, timing, form of folate, and subject may be for such an intervention.

## ACKNOWLEDGEMENTS

We are grateful to the participants of this study, the field workers of the POLIEP-study and the endoscopy staff of the participating hospitals. We thank Jenneke Poortman for her help with the DNA isolation, Sandra van den Bosch for her support in the methylation assessment, and Pieter van 't Veer and Jan Burema for statistical assistance.

This work was supported by the Netherlands Organization for Health Research and Development (ZonMw), grant 980-10-020, and by the Netherlands Foundation for Digestive Diseases (MLDS), grant WS 99-72. J.B.M. and J.W.C. were supported in part by the USA Agricultural Research Service, under agreement no. 581950-9-001 and by NIH grants K05 CA100048 and U54 CA100971. Any opinions, findings, conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the US Department of Agriculture.

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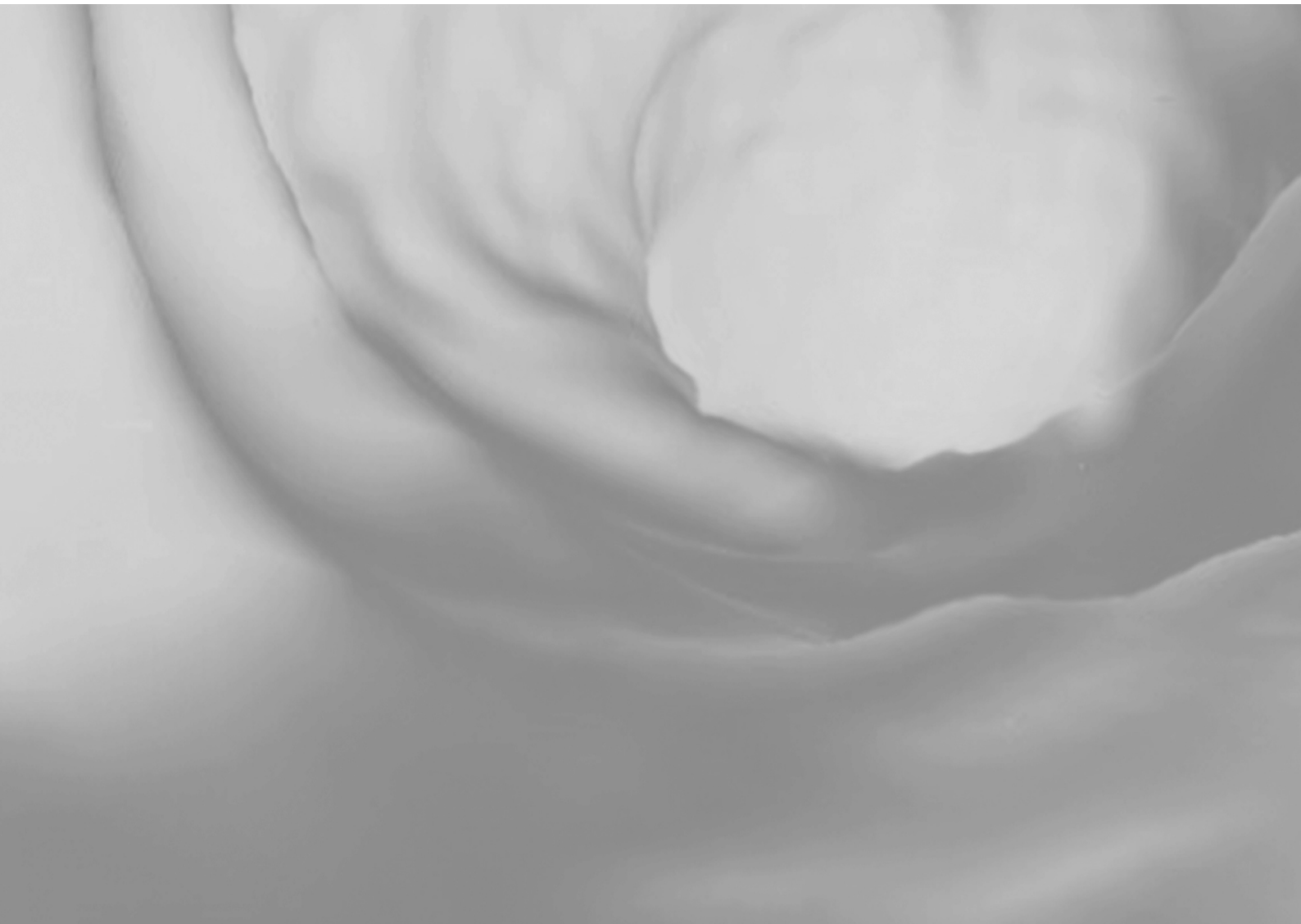
# Chapter 7

## GENERAL DISCUSSION



**In the studies described in this thesis, the associations between B-vitamins and presumed intermediate biomarkers for colorectal cancer were investigated, taking the genetic variation that exists in the one-carbon metabolism into account. This chapter summarizes the results from these studies. After this summary, the findings are discussed, with the emphasis on epidemiologic and molecular considerations. This chapter ends with general conclusions, possible public health implications of our findings, and recommendations for future research.**

## MAIN FINDINGS

The findings of the studies described in this thesis are schematically summarized in table 7.1.

***Table 7.1*** *Overview of the outcomes of studies described in this thesis*

| Study type | Exposure | Outcome | Type of result | Result† | Chapter |
|---|---|---|---|---|---|
| **Observational studies** | | | | | |
| Meta-analysis | Folate | Adenoma risk | RR | - | 2 |
| Case-control study, overall | Dietary folate | Adenoma occurrence | OR | + | 3 |
| Case-control study, *MTHFR TT* | Dietary folate | Adenoma occurrence | OR | 0 | 3 |
| Case-control study, overall | Vitamin B2 | Adenoma occurrence | OR | - | 3 |
| Case-control study, *MTHFR TT* | Vitamin B2 | Adenoma occurrence | OR | - - | 3 |
| Case-case analyses, overall | Dietary folate | Promoter methylation | OR | - | 5 |
| Case-case analyses, *MTHFR TT* | Dietary folate | Promoter methylation | OR | - - | 5 |
| **Intervention study** | | | | | |
| Overall | Folic acid+B12 | Uracil incorporation | $\Delta_{intervention} - \Delta_{placebo}$ | + | 6 |
| *MTHFR TT* | Folic acid+B12 | Uracil incorporation | $\Delta_{intervention} - \Delta_{placebo}$ | ++ | 6 |
| Overall | Folic acid+B12 | Promoter methylation | $OR_{upmethylation}$ | + | 6 |
| *MTHFR TT* | Folic acid+B12 | Promoter methylation | $OR_{upmethylation}$ | ++ | 6 |

† *-: inverse association; --: stronger inverse association; 0: null association; +: positive association; ++: stronger positive association.*

The meta-analysis described in **chapter 2** shows an inverse association between folate intake and colorectal adenoma risk (total folate intake: pooled relative risk (RR) for highest *vs.* lowest category 0.75, 95% confidence interval (CI) 0.61;0.93; pooled RR per 400 µg/day increase in intake 0.91, 95% CI 0.83;1.00). In the case-control study that we conducted, described in **chapters 3** and **4**, a slightly positive association between dietary folate intake and colorectal adenoma risk (odds ratio (OR) highest *vs.* lowest tertile 1.32, 95% CI 1.01;1.73), and an inverse association between vitamin B2 intake and colorectal adenoma risk (OR highest *vs.* lowest tertile 0.51, 95% CI 0.36;0.73) was found, especially among those with *MTHFR 677 TT* genotype. A null association was observed between intake of vitamin B6 or vitamin B12 and colorectal adenomas. Furthermore, the combined intake of these B-vitamins may be important. The polymorphisms in the one-carbon metabolism studied (*MTHFR C677T*, *TS* tandem repeat, and *SHMT1 C1420T*) did not seem to influence colorectal adenoma risk when dietary factors were not taken into account. In **chapter 5**, relatively high folate intake (>212 µg/day) was mildly inversely associated

with promoter methylation of six selected tumor suppressor and DNA repair genes in adenoma tissue as compared with low folate intake (<183 µg/day), with statistically non-significant ORs ranging from 0.54 to 0.86. This effect was mainly restricted to those carrying the *MTHFR 677 TT* genotype. In the intervention study described in **chapter 6**, a high dosage of synthetic folic acid and vitamin B12 seemed to increase uracil incorporation ($\Delta_{intervention}$ - $\Delta_{placebo}$ 0.45, 95% CI -0.19;1.09) and promoter methylation of the same set of genes as studied in **chapter 5** ($OR_{upmethylation}$ 1.67, 95% CI 0.95;2.95), both biomarkers measured in rectal mucosa DNA of patients with a history of colorectal adenomas. Again, the effect seemed more pronounced in those with *MTHFR 677 TT* genotype.

It can be concluded that we almost consistently found unexpected results for folic acid or folate intake: neither in the case-control study nor in the intervention study did we observe a favorable effect on either colorectal adenoma occurrence or presumed intermediate biomarkers for colorectal cancer. In the following section, these results that seem contradictory to the literature are discussed.

## EPIDEMIOLOGICAL CONSIDERATIONS ON OBSERVATIONAL STUDIES

Observational epidemiological studies have methodological limitations that need to be taken into account when appraising their results. Most of the limitations that are relevant to the individual studies have already been addressed in the separate discussions in **chapters 2** through **5**. This section elaborates on these separate methodological discussions. It integrates the issues relevant for the interpretation of the results of the complete thesis.

### Selection of cases and controls

Most case-control studies on colorectal adenomas conducted in the USA used cases and controls enrolled during population screening for colorectal cancer.[1-4] Obviously, this is an advantage. This advantage has to do with selection of cases and controls, which is one of the most critical issues in observational studies, and may be especially important for studies on colorectal adenomas.

The advantages of recruiting participants based on population screening are that most participating people do not have bowel complaints, or at least, this is not the reason for visiting the screening. Furthermore, adenoma cases that are enrolled during population screening are newly diagnosed cases, and controls enrolled during population screening almost certainly do not have colorectal adenomas.

Unfortunately, in the Netherlands, there is no population screening for colorectal cancer. This means that the source from which cases can be selected is confined to people undergoing endoscopy on a clinical indication. This group may comprise more prevalent cases, who might have more difficulty remembering their dietary habits before the development of their colorectal adenomas. Furthermore, endoscopy-based cases probably have more bowel complaints and therefore may have altered their dietary habits.

There are two options for selection of the control participants: either a community-based or an endoscopy-based control group. Choosing a community control group inevitably includes people having adenomas, as colorectal adenomas are highly prevalent in the general population.[5] This will lead to misclassification in the outcome and attenuation of results. The choice of an endoscopy-based control group almost certainly excludes the possibility of control participants having colorectal adenomas. However, this has disadvantages as well: it may harm generalization to the general population, and the controls may be more similar to the cases than community-based controls, which may also attenuate the results. Furthermore, these controls may also have bowel complaints as the reason for the colonoscopy and thus altered dietary habits. Studies that included both a community-based and an endoscopy-based control group show inconsistent results when the two distinct control groups are compared.[6,7] In our study, we chose to include an endoscopy-based control group. Therefore, we might expect that the results are attenuated to some degree, but we do not have reasons to believe that this will invert the results.

## Bias

Another important issue in observational epidemiological studies concerns bias. In our case-control study, most potential sources of bias result from the inability to perform a screening-based study. We might expect misclassification of exposure (information bias) to occur because of altered dietary habits in participants with bowel complaints, or because of the fact that half of the cases were prevalent cases. However, exclusion of those with complaints or those with prevalent adenomas from the analyses did not change the results, so the effect of this type of information bias is likely to be small. Misclassification of exposure could also have occurred as the interval between endoscopy and recruitment for the study could be up to several months. However, there is no reason to believe that this misclassification is other than non-differential, so it could at most attenuate the results. As we do not expect participants to be aware of risk factors for adenomas, we think the effect of differential recall bias for that reason, if present at all, will be small.

## Confounding

Confounding addresses the problem of mistaking potential causes with factors that are associated both with a real causal factor and the disease itself, and is almost inherent to observational research. It is possible to identify confounding in a study by noting a change in the risk ratio after having added a potential confounding factor to the analyses. In our case-control study, we investigated which factors fulfilled these criteria and examined if the odds ratios changed more than 10% by adding these factors to the logistic models. As the odds ratios remained approximately stable after adjusting for possible confounding factors, we conclude that confounding is not a major topic in our study. However, residual confounding (due to unmeasured variables or measurement errors) cannot be ruled out. The few factors that did influence the results, of which age had the greatest impact, were included in the logistic regression models.

## Use of food-frequency questionnaires

The use of self-administered questionnaires to assess habitual food intake as a determinant, is another critical aspect of observational epidemiological research. We examined intake of folate and other B-vitamins by use of a self-administered food-frequency questionnaire.[8] This may not be the most accurate way to assess folate status, but unfortunately, no appropriate blood samples were available to assess plasma or erythrocyte folate levels.

Most food-frequency questionnaires that were validated for folate intake show poor correlation between erythrocyte folate and dietary folate intake,[1,9,10] although validations for total folate intake (including supplements) show higher correlations.[11] The EPIC questionnaire that we used showed a positive, but poor correlation between plasma folate and dietary folate intake.[12] Since supplement use is not common in the Netherlands[13] and supplementary folic acid intake was therefore not assessed quantitatively in our study, we focused on dietary folate intake. Adjusting the logistic regression models for supplementary multivitamin or B-vitamin use did not change the results.

The main sources of folate in our population were vegetables, bread, and meat, accounting for more than 50% of folate intake. The reproducibility of the assessment of these products, as assessed with the questionnaire and expressed as Pearson correlation coefficients, was 0.76, 0.86, and 0.68, respectively, for men, and 0.65, 0.78, and 0.80 for women, while the relative validities compared to 24-hour recalls were 0.31, 0.76, and 0.47 for men and 0.38, 0.78, and 0.70 for women.[8] Especially for vegetables, the validity is rather low, indicating that there might have been errors in the exposure assessment (non-differential misclassification of exposure). This might have influenced the association between folate intake and colorectal adenomas as found in our study.[14] Furthermore, it is difficult to assess actual intake, as folate contents of foods and folate

bioavailability might differ according to, for example, the method of preparation.[15] This also may be a disturbing factor in the identification of associations between food intake and disease risk.[16]

Although food frequency questionnaires have certain disadvantages, using plasma or erythrocyte folate levels in case-control studies is not perfect either. Adenomas contain highly proliferative cells, and thus need folate to grow. Therefore, the presence of adenomas may lead to decreased folate levels, although there is no empirical evidence for this assumption yet. Hypothetically, lower folate levels in plasma or erythrocytes can be a consequence of the disease rather than a cause. As the exposure in cohort studies is presumably assessed before the onset of the disease, this disadvantage of the use plasma or erythrocyte levels of folate is of less significance in prospective studies.

In our study, folate content of foods was determined using data from Koning *et al.*[17], which were assessed with high-performance liquid chromatography. This method is considered to be more accurate than the previously more frequently used microbiological method. These new estimates give ~ 20% lower folate levels compared with the microbiological method. As these differences are about the same for most foods, we expect them not to influence the results much.

The main sources of vitamin B2 in our study were milk and milk products, accounting for about 50% of vitamin B2 intake. The reproducibility for milk and milk products was 0.71 for men and 0.79 for women, and the relative validity was 0.73 for men and 0.78 for women. Potatoes, bread and meat accounted for 50% of vitamin B6 intake, and meat was the main source for vitamin B12 intake, accounting for almost 50% of intake. The reproducibility for potatoes was 0.85 for men and 0.75 for women, and the validity was 0.58 for men and 0.70 for women.[8] Accordingly, there probably is less noise in the assessment of intake of vitamins B2, B6 and B12 than in the assessment of intake of folate. Furthermore, fewer differences in bioavailability and content of vitamins are expected for milk, milk products and bread as a result of food processing, as these products are ready-to-eat.

## Intake of folate and other B-vitamins in the Netherlands

Our study population had a relatively low folate intake. As stated above, we focused on dietary intake. In some studies, the inverse association between folate intake and colorectal adenoma risk weakened when the analyses were restricted to dietary folate,[4,11] which was also apparent from the meta-analyses that we performed (**chapter 2**). However, reversal of the association, only by excluding supplement use, is unlikely.

A more likely explanation for the positive association between folate intake and colorectal adenomas as found in our study is that the background intake in the Netherlands is different from that in other countries, especially the USA, where most of the studies on

folate and colorectal adenomas were carried out. An example of the mentioned differences is the intake of vitamin B2. In the USA, flour has been enriched with vitamin B2 since 1943,[18] and therefore the intake is ~ 28% higher than in the Netherlands.[13,19] From our study there are indications that the association between folate intake and colorectal adenoma risk may depend on vitamin B2 status, so the relatively low intake of vitamin B2 may explain part of the positive association. The intake of fruit juice is another example. Smith-Warner *et al.*[7] found an inverse association between vegetable juices and fruit juices and colorectal adenomas, that was not found between total consumption of vegetables and fruit and colorectal adenomas. In the same population, an inverse association between folate intake and colorectal adenomas was found,[4] which might therefore be due to consumption of fruit juices. Fruit juices are an important source of folate in the USA.[20] In the Netherlands, juice consumption is much lower, but conversely, meat is an important source of folate, accounting for 16% of folate intake in our study. This may partly explain the difference in folate results between studies from the USA and our own.

In the case-case analyses, we did observe a beneficial effect of dietary folate intake on presumed biomarkers for colorectal cancer, although not statistically significant: promoter methylation of tumor suppressor and DNA repair genes decreased. In these analyses, the same questionnaire was used. The advantage of these analyses is that these are not hampered by a control group that may not be optimal. On the other hand, one may doubt if the biomarkers in these analyses show the whole picture. This is discussed in further detail in next section.

## CONSIDERATIONS ON A MOLECULAR LEVEL: INTERMEDIATE MARKERS

Two outcome measures that are related to colorectal cancer risk were used in our studies: gene-specific DNA methylation in both the observational study and the intervention study, and uracil misincorporation as a marker for DNA synthesis in the intervention study.

### Gene-specific DNA methylation

In our study, we examined promoter methylation as a proxy of DNA methylation. When thinking of folate as a methyl donor, it seems logical that promoter methylation increases. However, this reasoning is too simplistic and contradicts thinking of folate as an **anti-neoplastic agent**, as promoter methylation of tumor suppressor genes is associated with neoplasia. The interplay between global hypomethylation and gene-specific hypermethylation is not yet fully established. In carcinogenesis, it is assumed that global DNA hypomethylation is an early event, which is followed by hypermethylation

of the promoter region of certain tumor suppressor genes, which may accelerate the carcinogenic process.[21] There have been some speculations about the mechanism that links DNA hypomethylation to promoter hypermethylation: DNA methyltransferase, the enzyme that is responsible for DNA methylation in mammalian cells[22] increases in response to DNA hypomethylation, and this increase could cause local increases in DNA methylation.[23,24] This mechanism is supported by findings that folate deficient diets may induce both DNA hypomethylation and increased DNA methyltransferase activity in liver samples of rats.[25,26]

Another issue is hypermethylation of other genes than the ones studied. We focused on six tumor suppressor and DNA repair genes that are frequently methylated in colorectal neoplasms. However, it is not known whether promoters of oncogenes become methylated at the same time as tumor suppressor genes, and what the net effect is of these changes in methylation.

## Gene expression

Promoter methylation leads to silencing of gene expression.[27] In that way, promoter methylation of tumor suppressor and DNA repair genes could lead to an increased risk of colorectal cancer. To support our findings, we wanted to look at the gene expression of the six tumor suppressor and DNA repair genes of which we studied promoter methylation, for example with real time reverse transcriptase PCR. However, RNA isolated from the rectal mucosa biopsies from participants to our intervention study was found to be degraded. Therefore, it was not possible to directly link gene expression levels with methylation.

For the same reason, our original plan to perform cDNA microarray analyses on the samples of the intervention study could not be executed. However, even if we had been able to perform this analysis, interpretation would have been difficult. When the array technology came into use, some six years ago, the technique seemed very promising: it would be possible to study the effects of for example nutrients on thousands of genes at the same time, and in this way it would be possible to unravel the underlying mechanism. In practice, it appeared that this was a bit too straightforward. By performing cDNA microarray analyses, gene expression profiles of thousands of genes are generated. Until now, it has not been clear how this huge amount of information should be interpreted. This observation is not unique to cDNA microarray techniques; it is fundamentally attached to the 'omics'-approach: how to deal with the vast amounts of information genomics, proteomics, and metabolomics generate? This issue of multiplicity of outcome measures has been prevalent earlier but never urgent enough for epidemiology to give it much consideration. However, 'genomics thrusts these issues to the forefront', as mentioned by R. Millikan.[28] Besides this, there are more methodological issues regarding

genomics, to the solution of which epidemiology may contribute: when using genomics approaches in observational studies, these studies should incorporate epidemiological principles relevant to observational research as well. This means that bias needs to be avoided, potential confounders need to be measured, and reproducibility has to be ensured.[29] Until now, these issues have not always been adequately dealt with.

## DNA synthesis

We investigated uracil incorporation in our study, as a marker for DNA synthesis. This means that both aspects that are held responsible for the beneficial effect of folic acid in colorectal carcinogenesis, namely DNA synthesis and DNA methylation, are covered in this study. We think this is an advantage of the study, because both mechanisms could be influenced by folic acid intervention, but we do not know to what extent and in what proportion.

We are the first to study uracil misincorporation in rectal mucosa biopsies in a human folic acid intervention study. Therefore, we cannot compare our results to others. Only one other study[30] investigated the effect of folic acid intervention on genomic DNA methylation and *p53* strand breaks, which is also a marker for DNA synthesis. In this study, six months of intervention increased genomic DNA methylation and decreased *p53* strand breaks, so favorable effects of folic acid intervention were seen on both DNA methylation and DNA synthesis. However, the effects were the same in the placebo group after twelve months.[30]

## *MTHFR 677 TT* genotype, DNA methylation and DNA synthesis

We observed stronger associations for those with the *MTHFR 677 TT* genotype, both in the observational and in the intervention study. As MTHFR irreversibly converts 5,10-methyleneTHF to 5-methylTHF, this enzyme controls whether folate is employed for DNA synthesis or DNA methylation.[21] The hypothesis is that impaired MTHFR activity, which may be caused by the *MTHFR C677T* genotype, increases colorectal cancer risk as a result of reduced 5-methylTHF levels and associated DNA hypomethylation. However, it is observed that the *TT* genotype is generally associated with a decrease in colorectal cancer risk[31] in subjects with normal folate status. The reason might be that low activity of MTHFR may increase plasma 5,10-methyleneTHF, which is crucial for DNA synthesis and repair, and thereby may enhance DNA stability by reducing uracil misincorporation and chromosomal strand breaks.[21] A normal or high folate status guarantees sufficient levels of 5-methylTHF, essential for adequate DNA methylation. However, when folate levels are low, this balance might be disrupted: in that case, levels of both 5-methylTHF and 5,10-methyleneTHF might be too low to sustain normal DNA methylation and DNA synthesis. Indeed, in most studies, risk of colorectal cancer and adenomas seems highest in subjects

with the *MTHFR TT* genotype and low folate levels, and these subjects seem to gain most by increased folate levels.[31]

In our intervention study, we could not demonstrate a favorable effect of folic acid intervention. However, the effect was even less favorable in those with the *MTHFR TT* genotype. It seems that the adverse effects of both a low and a very high dosage of folate are the largest in people with the *MTHFR TT* genotype.

### Concluding considerations on intermediate markers

Even if the biomarkers that we selected have been chosen well, they still remain indicators of mechanisms which are believed to contribute to the development of cancer. Although these biomarkers have been demonstrated to arise in colorectal neoplastic tissue, it is unknown if the risk of colorectal cancer will actually be influenced when these markers change.

Recurrence of adenomas is the only intermediate biomarker for colorectal cancer for which firm validation data exist: removal of adenomas leads to a reduction of cancer risk.[32] However, one can even have doubts about this biomarker, as not all colorectal adenomas will develop into cancer.[33,34] Until now, two folic acid intervention studies used recurrence of adenomas as the endpoint.[35,36] Paspatis *et al.* performed a two-year randomized intervention study with 1 mg folic acid per day or placebo, and found a statistically non-significant reduction in adenoma recurrence in the folic acid group compared with the placebo group.[35] The second study was only published as an abstract and reported no differences in adenoma recurrence between the folic acid and placebo group, and suggestions for a modestly higher incidence of advanced adenomas and greater adenoma multiplicity in the folic acid group. This study also used 1 mg of folic acid/day.[36] We will further reflect on folate intervention studies in the next section.

## FOLATE INTERVENTIONS: TIMING, DOSAGE, AND FORM

### Timing of folic acid supplementation

The folic acid intervention study that we performed was explicitly aimed at a population of people with a history of adenomas. The reason was that these people already have a predisposition for neoplastic growth, and thus an effect of intervention might be expressed sooner. However, this may also hold an explanation for the positive associations observed in our intervention study. Folate is necessary for the growth of healthy tissues to sustain normal DNA synthesis, and folate deficiency in tissues with rapidly replicating cells results in the opposite: ineffective DNA synthesis. In neoplastic cells, DNA replication and cell division also occur at an accelerated rate. Inhibition of

folate metabolism in neoplastic cells causes ineffective DNA synthesis, resulting in inhibition of tumor growth.[37] It is imaginable that a high dosage of folate causes the opposite: stimulation of growth of a tumor or proliferated tissue that is already present. This could have happened in our study population, which consisted of patients with a history of colorectal adenomas and hence potentially already proliferated mucosal tissue. There is evidence from animal studies to support this hypothesis. In a study in *Apc+/-Msh2-/-* mice, folic acid supplementation decreased the number of small intestinal adenomas, colonic adenomas and colonic aberrant crypt foci compared with a moderate degree of folate deficiency when administrated before the establishment of neoplastic foci. Conversely, when provided after the establishment of neoplastic foci, folic acid seemed to have an opposite effect.[38] Similar effects were found in $Apc^{Min}$ mice.[39]

However, other folic acid intervention studies that were carried out in patients with colorectal adenomas saw advantageous effects of supplementation on global DNA hypomethylation,[30,40-42] colonic mucosal cell proliferation,[43,44] loss of heterozygosity of the tumor suppressor gene *DCC* (deleted in colorectal cancer),[44] or activity of the proto-oncogene ornithine decarboxylase.[45] This contradicts this hypothesis.

## Dosage and form of folate

In our intervention study, we used a high dosage of synthetic folic acid (pteroylmonoglutamic acid), which is in line with earlier studies. This has to be metabolized in the body into 5-methylTHF, the normal form of folate transported in plasma. However, studies show that this absorption and biotransformation process is already saturated at dosages in the region of 400 µg folic acid or less.[46,47] At higher dosages, synthetic folic acid is also transported into the blood. Although occasional concern has been voiced about possible antagonistic activities of this fully oxidized form of folate in tissues,[48] compelling data to support this hypothesis lack.

In **chapter 1**, we mentioned the *in vitro* studies that will be part of the thesis by Linette Pellis. In these studies, differential effects were found on gene expression in human colon epithelial cell lines in respons to different forms of folate (synthetic folic acid *vs.* 5-methylTHF), which may indicate that the outcome of the study is in part dependent on the form of folate that is supplied (unpublished data).

However, the use of high dosages of synthetic folic acid is not uncommon in other studies investigating the effect of folic acid intervention on presumed biomarkers for colorectal cancer: earlier studies used dosages between 2 mg and 10 mg per day over a period of three to twelve months, and reported favorable effects.[30,40,41,43-45] As the intervention period and the dosage of folic acid in our study fall within the range of these intervention studies, this might not explain our findings.

## Other issues related to the intervention study

Our study included more participants than other folic acid intervention studies, but the number of participants cannot explain the direction of the effect. Moreover, gene-environment interactions also do not seem a sufficient explanation. Our study was the first in which it has been able to look at gene-environment interactions: we executed subgroup analyses according to *MTHFR C677T* genotype, but our overall results point in the same direction as the stratified results and seem even stronger for those with the *MTHFR TT* genotype.

Apart from size, our study essentially differs from the earlier folic acid intervention studies in only two aspects. First, we examined other presumed biomarkers for colorectal cancer risk than the ones used previously; this aspect has been discussed before. Second, our supplements also included a high dosage of vitamin B12. We added vitamin B12 because we wanted to prevent masking a vitamin B12 deficiency as a consequence of folic acid supplementation.[49] The dosage of vitamin B12 that we used in our study was high enough to prevent vitamin B12 deficiency, even if one could only passively absorb vitamin B12. We did not find indications that this might have influenced DNA methylation or DNA synthesis in a disadvantageous manner.

# GENERAL CONCLUSIONS

As stated before, the positive association between dietary folate intake and colorectal adenoma risk as found in our case-control study contradicts results from other observational studies. Part of this positive association may be explained by the fact that also vitamin B2 intake in our study was low, and meat being an important source of folate intake in our study. Vitamin B2 is necessary for MTHFR to work well. Meat, especially organ meat, contains several nutrients that have been associated with an increased risk of (colorectal) cancer.

However, some aspects regarding the conduct of this study cannot be set aside. It is not possible to rule out all sources of confounding and bias, although we tried to correct for possible confounding factors and we performed several subgroup analyses to detect potential sources of bias, without leading to changes in the results. Furthermore, folate intake was estimated by means of a food frequency questionnaire, which might not be the most accurate method to assess folate intake. Additionally, we were not able to perform a screening-based case-control study. Taking all these considerations into account, some doubts about our positive association remain, and we do not dare to draw firm conclusions from this single study.

Although the results from the intervention study correspond to the results from the case-control study, there are essential differences between the two studies. In the intervention

study, a high dosage of synthetic folic acid was supplemented to a group of patients, after colorectal adenomas had been established in these patients. A placebo group was included to compare the results with, and assignment of the intervention or placebo capsules took place at random. Therefore, both study groups are comparable to each other. Maybe the set of biomarkers that we chose is not complete, and does not reflect actual cancer risk, although associations with colorectal cancer are suggested.[50,51] The fact that we included biomarkers that account for both mechanisms via which folate is thought to exert its anti-neoplastic effect strengthens our results. Other folic acid intervention studies also only investigated a limited number of biomarkers that may not reflect actual cancer risk, so the possibility exists that these studies missed disadvantageous effects due to incompleteness of the markers. Furthermore, our results correspond to results from animal studies concerning high dosages of folic acid administration after the establishment of precancerous lesions.

Also from human studies, evidence is accumulating that folate might have adverse effects. In a folic acid intervention study on neural tube defects, a higher all-cause mortality and specifically a higher mortality from breast cancer was found in two intervention groups (200 μg or 5 mg per day during pregnancy) compared with the placebo group.[52] The intervention study by Cole *et al.*[36], that was mentioned before, suggested a higher incidence of advanced adenomas and greater adenoma multiplicity in the folic acid group compared with the placebo group. Recently, a randomized controlled intervention study, the NORVIT trial, was carried out in Norway to assess the effect of 800 μg folic acid and/or 400 μg vitamin B12 per day for 3.5 years on myocardial infarction. 3749 Post-myocardial infarction patients were included. One of the endpoints in this study was newly diagnosed cancer. The RR for cancer for the folic acid/vitamin B12 group was 1.40 (95% CI 0.96-2.03) compared with the group that did not receive folic acid/vitamin B12 (Prof. Dr. K.H. Bønaa, University of Tromsø, Norway, personal communication).

Taking all these arguments together, we conclude that a potential adverse effect of a high dose of folic acid should be considered, especially when administered after colorectal neoplastic lesions have been established.

If the results of this thesis are correct then folic acid shows another, largely unknown and darker face than the brilliant one we have seen before. Have we – until recently – only met Doctor Jekyll without having to face Mister Hyde? As long as we cannot exclude the existence of Mister Hyde, it cannot harm to be cautious.

## PUBLIC HEALTH IMPLICATIONS

The first message from this thesis is that a balanced intake of B-vitamins, and genetic variants in the one-carbon metabolism, may be important. This should be kept in mind when prescribing folic acid, and should be taken into account when thinking of enrichment of food with vitamins.

Another notion that can be deduced from this thesis is to be careful with folic acid, especially when used in high dosages. This might have implications for patient care. A dosage of 5 mg folic acid is prescribed in the Netherlands, for example to prevent a neural tube defect in high-risk pregnancies, in case of macrocytic anemia, or to avoid adverse effects from methotrexate therapy in rheumatoid arthritis. As undiagnosed adenomas are prevalent in the general population, this high dose of folic acid could have unintentional effects on the colorectal mucosa, at least on a molecular level. Although this does not necessarily imply an increase in risk of colorectal cancer or adenomas, it is something to take into account.

## FUTURE RESEARCH

### Folate intervention study in healthy people

To avoid the aspect of timing in the exploration of the effects of folate in colorectal mucosa, it would be interesting to perform a randomized intervention trial in healthy people. However, this will obviously bring about some problems:

- Biomarkers

  Occurrence of colorectal adenomas or cancer is the best outcome. However, in people who do not have preneoplastic lesions, it may take years to develop adenomas or cancer, if these develop at all. Therefore, either very many participants are needed, or the participants should be followed for a very long period of time.

  Choosing for another biomarker will lead to other problems. As discussed before, it is not known what the effect of molecular biomarkers, like changes in DNA methylation or DNA synthesis, will be on the actual risk of cancer. Furthermore, it is not clear if changes in these biomarkers can be demonstrated in healthy people. Therefore, a feasibility study should be conducted to test demonstrability of those biomarkers in colorectal mucosa biopsies, for example in people undergoing colonoscopy who turn out to have healthy colorectal mucosa.

  If effects on these biomarkers could be demonstrated in lymfocytes, conducting this type of study will be more acceptable to participants and therefore more feasible: instead of taking rectal mucosa biopsies, it will be sufficient to take blood samples. An

additional advantage is that this is more economical. This should also be investigated in a feasibility study.

- Recruitment of participants

  It will be difficult to recruit healthy people for such a trial, as they will have to undergo colonoscopy to exclude adenoma patients and take capsules for a long time, without expecting to benefit from it.

- Dosage of folate

  As it would be unethical to supplement people with a potentially harmful dosage or form of folate, pilot studies need to be performed to see if for example low dosages of folic acid do show indications for favorable effects. The same holds for different forms of folate.

## Points of attention

Regardless of what study will be performed, some points need to be addressed:

- When molecular biomarkers will be used, it is important to include all different aspects that might play a role in the one-carbon metabolism. For example, when looking at promoter methylation, also oncogenes should be examined, and preferably a quantitative measurement should be performed. When both genomic DNA methylation and promoter methylation are assessed, it might be possible to link these.
- It would be highly recommended to assess metabolites from the one-carbon metabolism in mucosa biopsies, for example different forms of folate, S-adenosyl methionine and S-adenosyl homocysteine. In that case, the processes in the mucosa can be deduced.
- As effects of folate also depend on other B-vitamins, these should also be taken into account. For example, an intervention group with vitamin B2 might be included. This will open opportunities to study the combined effects of different B-vitamins.

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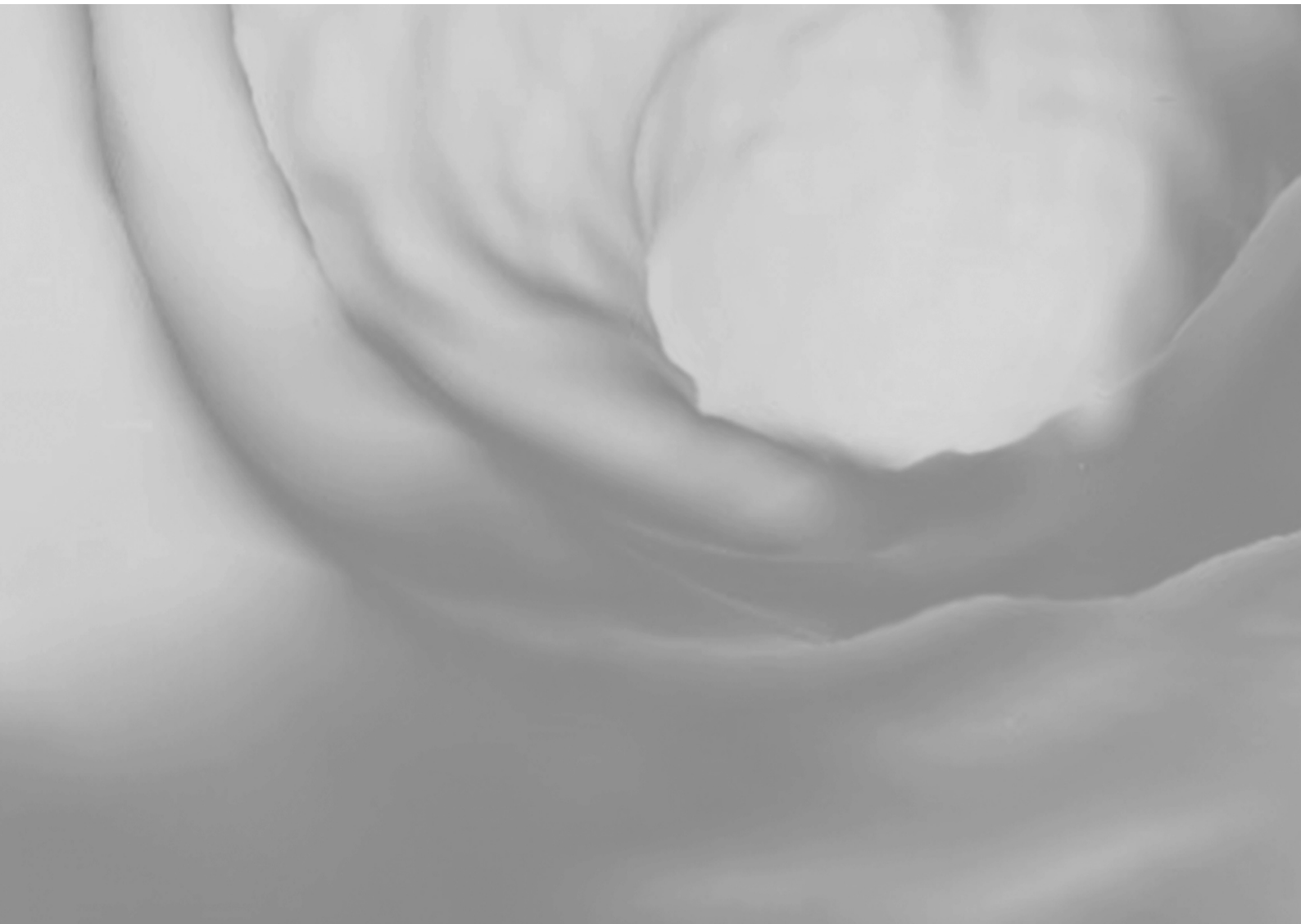
# SUMMARY



**During the past years, folate has emerged as an important factor in the prevention of a whole array of diseases: anemia, heart disease, stroke, neural tube defects, mental health and cancer. Furthermore, not only folate, but also other B-vitamins are essential in disease prevention. This thesis describes studies that have been conducted to clarify the role of folate and related B-vitamins in colorectal carcinogenesis.**

**Chapter 1** describes the proposed mechanism through which these vitamins may exert their protective roles in colorectal carcinogenesis. Folate is responsible for mediating the transfer of methyl groups, via the so-called one-carbon metabolism. Folate deficiency can affect DNA methylation or incorporation of uracil instead of thymidine in DNA, leading to defective DNA synthesis. Both factors may be operative in colorectal carcinogenesis. Many enzymes, like methylenetetrahydrofolate reductase (MTHFR), thymidylate synthase (TS), methionine synthase (MTR), serine hydroxymethyltransferase (SHMT), and dihydrofolate reductase (DHFR), are needed for the conversions in the one-carcon metabolism. This is where other B-vitamins enter: flavin adenine dinucleotide, a metabolite of vitamin B2, acts as a cofactor for MTHFR; vitamin B6 acts as a cofactor for SHMT; and vitamin B12 acts as a cofactor for MTR. Functional polymorphisms exist in most of the genes encoding the enzymes that play a role in the one-carbon metabolism. Therefore, genetic variation in the one-carbon metabolism might influence DNA methylation and synthesis processes.
Human observational studies suggest that folate and vitamin B6 might have a small protective effect against colorectal cancer and maybe also colorectal adenomas. However, the associations between vitamins B2 and B12 and colorectal adenomas are not clear yet. Colorectal cancer risk seems to be decreased among those with *MTHFR C677T* and *A1298C* genotypes, although the role of these polymorphisms in colorectal adenoma risk, and also that of polymorphisms in other genes, is unclear. The interactions between intake of B-vitamins and *MTHFR C677T* genotype are rather consistent in that the greatest risk reduction for colorectal cancer or adenomas by intake of folate or other B-vitamins can be found in those with the *TT* genotype.
Human intervention studies in colorectal adenoma patients show that folic acid supplementation may have favorable effects on global DNA methylation, cell proliferation, and *p53* strand breaks. However, these studies included only small numbers of patients, did not take *MTHFR* genotype into account, and the effects studied mostly do not concern DNA synthesis.

In this thesis, the following research questions are addressed:

- *What is the association between intake of B-vitamins, polymorphisms in one-carbon metabolism-related genes, and colorectal adenoma risk?*
- *Does an interaction exist between B-vitamins and these polymorphisms in colorectal adenoma risk?*

- *Does supplementation with folic acid and vitamin B12 alter DNA methylation and DNA synthesis processes?*
- *Does a dependency on the MTHFR C677T genotype exist?*
- *Which mechanisms are involved?*

To answer these questions, we executed a systematic literature review of human observational studies that have assessed the association between intake of folate and risk of colorectal adenomas, including meta-analyses to quantify this association (**chapter 2**). Meta-analyses were conducted of risk estimates for highest exposure category and of risk estimates per unit exposure, including data from 4 cohort studies and 10 case-control studies, using random effects models. The pooled relative risks (95% confidence interval (CI)) for highest *vs.* lowest exposure category were 0.85 (0.71;1.01) for folate intake from the diet, 0.75 (0.61;0.93) for total folate intake (including supplements), and 0.75 (0.60;0.94) for plasma folate. The associations seemed slightly stronger for men. Other stratifications did not lead to remarkable differences in pooled estimates. We concluded that folate intake or status is inversely associated with colorectal adenoma risk.

We further investigated these research questions in a Dutch case-control study on colorectal adenoma risk (**chapters 3** and **4**). We focused on *MTHFR C677T*, *TS* tandem repeat, and *SHMT1 C1420T* polymorphisms. Data of cases with at least one histologically confirmed colorectal adenoma (n=768) and controls with no history of any type of colorectal polyp (n=709) were included. The adjusted odds ratio (OR) and 95% CI for the highest compared with the lowest sex-specific tertile of intake were 1.32 (95% CI 1.01;1.73) for folate and 0.51 (95% CI 0.36;0.73) for vitamin B2. Folate seemed to be a risk factor, especially when vitamin B2 intake was low; vitamin B2 was inversely associated with adenomas, especially with relatively high folate intake. No association was observed between vitamin B6 or vitamin B12 intake and colorectal adenomas. Furthermore, *MTHFR*, *TS* and *SHMT1* polymorphisms were not associated with adenomas when dietary factors were not taken into account. The inverse association between vitamin B2 intake and colorectal adenoma risk seemed to be more pronounced among those with the *MTHFR TT* genotype. We also found suggestions for an interaction between *TS* genotype and vitamin B6 intake: the association between vitamin B6 and adenomas seemed positive in those with *TS 3R/3R* genotype, but inverse in those with *TS 2R/2R* genotype. We conclude that this study does not provide evidence for a decreased colorectal adenoma risk for subjects with high dietary intake of folate. It suggests, however, an inverse association between vitamin B2 and colorectal adenomas, which may be more relevant for those with the *MTHFR TT* genotype.

We also investigated the association between dietary folate intake, *MTHFR C677T* genotype, and promoter methylation of six tumor suppressor and DNA repair genes in cases from this case-control study (**chapter 5**). Methylation of the promoter region of tumor suppressor genes is increasingly recognized to play a role in cancer development through silencing of gene transcription. Colorectal adenoma patients (n=149) with folate intake in the upper or lower tertile with the *CC* or *TT* genotype were selected. Methylation-specific PCRs were conducted on colorectal adenoma specimens. Overall, the observed percentages of promoter methylation in the evaluated genes (*APC*, $p14^{ARF}$, $p16^{INK4A}$, $O^6$-*MGMT*, *hMLH1*, *RASSF1A*) ranged from 15.7% to 64.2%. Folate intake seemed inversely associated with promoter methylation, especially among those with the *TT* genotype. *MTHFR* genotype was not associated with promoter methylation in itself. The interaction between folate intake and *MTHFR* genotype was most pronounced for $O^6$-*MGMT* and *RASSF1A*. Compared with patients with a low folate intake and the *CC* genotype, the adjusted ORs (95% CI) of having a methylated $O^6$-*MGMT* or *RASSF1A* promoter were 3.39 (0.82;13.93) and 3.53 (1.08;11.50), respectively, for those with a low folate intake and the *TT* genotype, and 0.37 (0.11;1.29) and 0.78 (0.22;2.76), respectively, for those with a high folate intake and the *TT* genotype. We conclude that folate intake seems inversely associated with promoter methylation in colorectal adenomas, which may be especially so for those with the *TT* genotype.

We carried out a randomized controlled intervention study (**chapter 6**), focusing on two markers for colorectal carcinogenesis: promoter methylation (gene-specific DNA methylation) and uracil misincorporation in DNA (DNA synthesis). 86 Subjects with a history of colorectal adenomas and *MTHFR CC* or *TT* genotype were randomly assigned to either folic acid (5 mg/day) and vitamin B12 (1.25 mg/day) or placebo. Randomization was stratified for *MTHFR* genotype. At baseline and after six months, uracil incorporation and promoter methylation of six tumor suppressor and DNA repair genes were measured in DNA from rectal biopsies. In the intervention group, the uracil content of DNA increased 0.45 pg/μg more than in the placebo (95% CI of the difference in response -0.19;1.09 pg/μg). The number of methylated promoters increased in the intervention group compared *w*ith the placebo group (OR 1.67, 95% CI 0.95;2.95). Both effects were more pronounced in people with the *TT* genotype. This study suggests that supplementation with high doses of folic acid and vitamin B12 may increase uracil incorporation and enhance promoter methylation in colorectal adenoma subjects.

As indicated above, in this thesis we focused on genetic polymorphisms and DNA methylation and DNA synthesis, in molecular epidemiological studies. These studies are part of a multidisciplinary project. Other studies from this project will be published in a related thesis by Linette Pellis, including several *in vitro* studies conducted in different

human colon epithelial cell lines. The effects of long-term exposure to different concentrations and forms of folate on cell growth and intracellular levels of folate metabolites and iron, and the effects on gene expression were investigated.

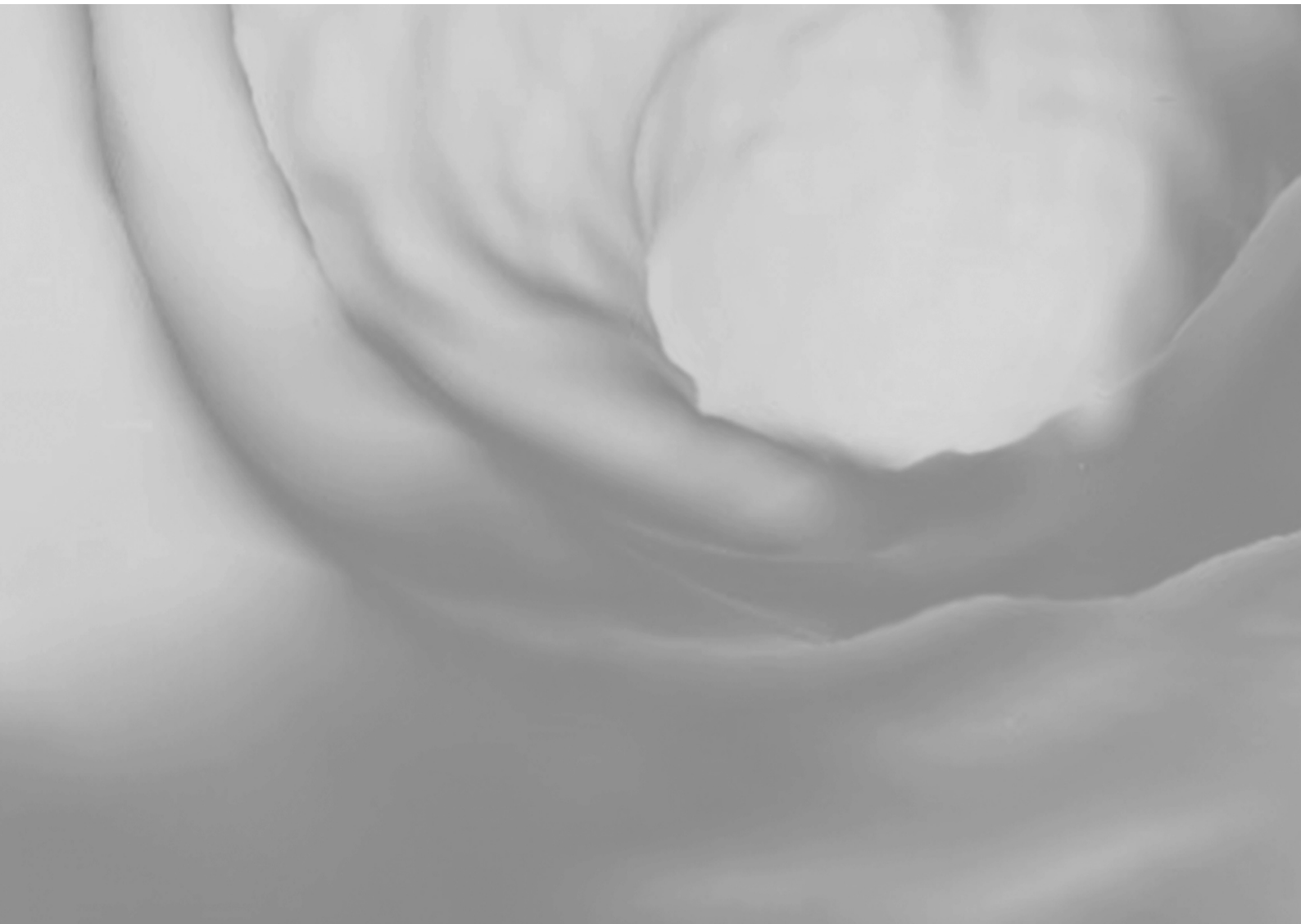
## Discussion

The main findings from these studies are summarized and discussed in **chapter 7**. The positive association between dietary folate intake and colorectal adenoma risk as found in our case-control study contradicts results from other observational studies. Part of this positive association may be explained by the fact that also vitamin B2 intake in our study was low, and meat being an important source of folate intake in our study. However, it is not possible to rule out all sources of confounding and bias, although we tried to correct for possible confounding factors and we performed several subgroup analyses to detect potential sources of bias, without leading to changes in the results. Furthermore, folate intake was estimated by means of a food frequency questionnaire, which might not be the most accurate method to assess folate intake. Taking all these considerations into account, some doubts about our positive association remain, and we do not dare to draw firm conclusions based on this single study.

Although the results from the intervention study correspond to the results from the case-control study, there are essential differences between the two studies. In the intervention study, a high dosage of synthetic folic acid was supplemented to a group of patients, after colorectal adenomas had been established in these patients. A placebo group was included to compare the results with, and assignment of the intervention or placebo capsules took place at random. Maybe the set of biomarkers that we chose is not complete, and does not reflect actual cancer risk, but the fact that we included biomarkers that account for both DNA methylation and DNA synthesis strengthens our results. Other folic acid intervention studies also investigated a limited number of biomarkers that do not reflect actual cancer risk, so the possibility exists that these studies missed disadvantageous effects due to incompleteness of the markers. Furthermore, our results correspond to results from animal studies concerning high dosages of folic acid administration after the establishment of precancerous lesions. Taking all these arguments together, we conclude that a high dose of folic acid may indeed have adverse effects, especially when administered after colorectal neoplastic lesions have been established.

**If the results of this thesis are correct then folic acid shows another, largely unknown and darker face than the brilliant one we have seen before. Have we – until recently – only met Doctor Jekyll without having to face Mister Hyde? As long as we cannot exclude the existence of Mister Hyde, it cannot harm to be cautious.**

# SAMENVATTING



**Gedurende de laatste jaren is van folaat bekend geworden dat het een belangrijke factor in de preventie van een groot aantal ziekten is: bloedarmoede, hart- en vaatziekten, neurale buisdefecten, mentale gezondheid en kanker. Daarnaast zijn ook andere B-vitaminen van belang bij ziektepreventie. Dit proefschrift beschrijft onderzoek dat is uitgevoerd naar de rol van folaat en gerelateerde B-vitaminen bij het ontstaan van dikkedarmkanker.**

**Hoofdstuk 1** beschrijft het mogelijke mechanisme waardoor foliumzuur en andere B-vitaminen hun beschermende rol zouden kunnen uitoefenen bij het ontstaan van dikkedarmkanker. Folaat is verantwoordelijk voor het doorgeven van methylgroepen. Een tekort aan folaat kan de DNA-methylering beïnvloeden, of zorgen voor de inbouw van uracil in plaats van thymidine in DNA, wat kan leiden tot verstoorde DNA-synthese. Beide factoren kunnen van belang zijn bij het ontstaan van dikkedarmkanker. Verscheidene enzymen, zoals methyleentetrahydrofolaatreductase (MTHFR), thymidylaatsynthase (TS), methioninesynthase (MTR), serine hydroxymethyltransferase (SHMT) en dihydrofolaatreductase (DHFR) zijn nodig voor de omzettingen in het folaatmetabolisme. Hier zijn ook andere B-vitaminen bij betrokken: flavine adenine dinucleotide (FAD) - een metaboliet van vitamine B2 - is een cofactor voor MTHFR, vitamine B6 werkt als cofactor voor SHMT1 en vitamine B12 werkt als cofactor voor MTR. In de meeste genen die voor deze enzymen coderen komen functionele polymorfismen voor. Daarom kan genetische variatie in het folaatmetabolisme de DNA-synthese en de DNA-methylering beïnvloeden.

Humane observationele studies suggereren dat folaat en vitamine B6 een klein beschermend effect tegen dikkedarmkanker kunnen hebben, en misschien ook tegen dikkedarmadenomen, een goedaardig voorstadium van dikkedarmkanker. De verbanden tussen de vitaminen B2 en B12 en adenomen zijn echter niet eenduidig. Het risico op dikkedarmkanker lijkt verlaagd te zijn bij mensen met de *MTHFR 677 TT* of *1298 CC* genotypes, maar de rol van deze polymorfismen bij het ontstaan van dikkedarmadenomen, en ook die van polymorfismen in andere genen, is onduidelijk. De interacties tussen de inname van B-vitaminen en *MTHFR C677T* genotype zijn tamelijk consistent: de grootste risicoreductie van dikkedarmkanker of –adenomen door folaat en andere B-vitaminen wordt in mensen met het *TT* genotype gevonden.

Humane interventiestudies met patiënten met dikkedarmadenomen laten zien dat suppletie met foliumzuur gunstige effecten kan hebben op globale DNA-methylering, celproliferatie en DNA-breuken. Deze studies waren echter klein, hielden geen rekening met het *MTHFR C677T* genotype, en er werden geen effecten op de DNA-synthese onderzocht.

In dit proefschrift komen de volgende vraagstellingen aan de orde:

- *Wat is het verband tussen inname van B-vitaminen, polymorfismen in genen gerelateerd aan het folaatmetabolisme, en risico op dikkedarmadenomen?*
- *Bestaat er een interactie tussen B-vitaminen en deze polymorfismen in het risico op dikkedarmadenomen?*
- *Beïnvloedt suppletie met foliumzuur en vitamine B12 de DNA-methylering en DNA-synthese?*
- *Bestaat er een afhankelijkheid van het MTHFR C677T genotype?*
- *Welke mechanismen zijn daarbij betrokken?*

Om deze vragen te beantwoorden, hebben we een systematisch literatuuronderzoek uitgevoerd naar humane observationele studies die het verband tussen folaat en risico op dikkedarmadenomen hebben onderzocht. Daarbij hebben we ook meta-analyses uitgevoerd om dit verband te kwantificeren (**hoofdstuk 2**). We voerden meta-analyses uit van risicoschattingen voor de hoogste blootstellingscategorie ten opzichte van de laagste en van risicoschattingen van blootstelling per eenheid. We includeerden gegevens van vier cohortstudies en tien patiënt-controle onderzoeken, en gebruikten random effect modellen. De gecombineerde (**pooled**) relatieve risico's (RR's) (95% betrouwbaarheidsintervallen (BI's)) voor de hoogste versus de laagste categorie van blootstelling waren 0.85 (0.71;1.01) voor folaatinname uit de voeding, 0.75 (0.61;0.93) voor totale folaatinname (inclusief supplementen), en 0.75 (0.60;0.94) voor plasmafolaat. De verbanden leken wat sterker te zijn voor mannen. Andere stratificaties leidden niet tot opmerkelijke verschillen in pooled schattingen. We concludeerden dat er een omgekeerd verband is tussen folaatinname of –status en risico op dikkedarmadenomen.

Verder hebben we deze onderzoeksvragen onderzocht in een Nederlands patiënt-controle-onderzoek naar het risico op dikkedarmadenomen (**hoofdstukken 3** en **4**). We hebben ons gericht op de polymorfismen *MTHFR C677T*, *TS* tandem repeat, en *SHMT1 C1420T*. Gegevens van patiënten met tenminste één histologisch bevestigd adenoom in de dikke darm (n=768) en controles zonder dikkedarmpoliepen in de geschiedenis (n=709) werden ingesloten. De gecorrigeerde odds ratio (OR) en 95% BI voor het hoogste vergeleken met het laagste geslachtsspecifieke tertiel van inname was 1.32 (1.01;1.73) voor folaat en 0.51 (0.36;0.73) voor vitamine B2. Folaat leek vooral een risicofactor bij lage inname van vitamine B2; de inverse associatie tussen vitamine B2 en adenomen in de dikke darm was met name aanwezig bij een relatief hoge folaatinname. Er werd geen verband gevonden tussen inname van vitamine B6 of B12 en dikkedarmadenomen. Bovendien waren de polymorfismen in *MTHFR*, *TS* en *SHMT1* niet geassocieerd met adenomen als geen rekening werd gehouden met voedingsfactoren. Het inverse verband tussen inname van vitamine

B2 en risico op dikkedarmadenomen leek sterker aanwezig te zijn bij degenen met het *MTHFR 677 TT* genotype. We vonden ook suggesties voor een interactie tussen het *TS* genotype en inname van vitamine B6: er leek een positief verband te zijn tussen vitamine B6 en adenomen bij degenen met het *TS 3R/3R* genotype, maar een inverse verband bij degenen met het *TS 2R/2R* genotype. Onze conclusie is dat deze studie geen aanwijzingen geeft voor een verlaagd risico op colorectale adenomen bij hoge folaatinname via de voeding. Er wordt echter wel een inverse verband gesuggereerd tussen inname van vitamine B2 en dikkedarmadenomen, dat vooral relevant lijkt voor degenen met het *MTHFR 677 TT* genotype.

We hebben ook onderzoek gedaan naar het verband tussen inname van folaat via de voeding, *MTHFR C677T* genotype en promoter methylering van zes tumorsuppressor- en DNA repairgenen in patiënten afkomstig uit het patiënt-controle-onderzoek (**hoofdstuk 5**). Methylering van de promoterregio van tumorsuppressorgenen wordt steeds vaker gezien als een factor in de ontwikkeling van kanker door remming (**silencing**) van de gentranscriptie. Patiënten met dikkedarmadenomen (n=149) met folaatinname in het hoogste of laagste tertiel en het *MTHFR 677 CC* of *TT* genotype werden geselecteerd. Methyleringsspecifieke PCR's werden uitgevoerd op weefselblokjes van dikke-darmadenomen. De geobserveerde percentages van promotermethylering in de onderzochte genen (*APC*, $p14^{ARF}$, $p16^{INK4A}$, $O^6$*-MGMT*, *hMLH1*, *RASSF1A*) varieerden tussen 15.7% en 64.2%. Folaatinname leek inverse geassocieerd met promotermethylering, vooral bij degenen met het *TT* genotype. Het *MTHFR* genotype op zich was niet geassocieerd met promotermethylering. De interactie tussen folaatinname en *MTHFR* genotype was het duidelijkst voor $O^6$*-MGMT* en *RASSF1A*. Vergeleken met patiënten met een lage folaatinname en het *CC* genotype, waren de OR's (95% BI's) voor een gemethyleerde $O^6$*-MGMT* of *RASSF1A* promoter respectievelijk 3.39 (0.82;13.93) en 3.53 (1.08;11.50) voor patiënten met een lage folaatinname en het *TT* genotype, en 0.37 (0.11;1.29) en 0.78 (0.22;2.76) voor patiënten met een hoge folaatinname en het *TT* genotype. Onze conclusie is dat folaatinname inverse geassocieerd lijkt te zijn met promotermethylering in dikkedarmadenomen, en dat dit met name het geval is voor degenen met het *TT* genotype.

We hebben een gerandomiseerde, placebo-gecontroleerde interventiestudie uitgevoerd (**hoofdstuk 6**), waarbij we ons gericht hebben op twee biomarkers die van belang zijn bij het ontstaan van dikkedarmkanker: promotermethylering (genspecifieke DNA-methylering) en uracilmisincorporatie in DNA (DNA-synthese). 86 Deelnemers met eerdere dikkedarmadenomen en het *MTHFR CC* of *TT* genotype werden gerandomiseerd toegewezen aan óf suppletie met foliumzuur (5 mg per dag) en vitamine B12 (1.25 mg per dag), óf

een placebo. De randomisatie was gestratificeerd voor het *MTHFR* genotype. Aan het begin van de studie en na zes maanden werden de uracilincorporatie en promotermethylering van zes tumorsuppressor- en DNA repairgenen gemeten in DNA uit rectale slijmvliesbiopten. In de interventiegroep steeg het uracilgehalte van het DNA 0.45 pg/µg meer dan in de placebogroep (95% BI van het verschil in respons -0.19;1.09 pg/µg). Het aantal gemethyleerde promoters nam toe in de interventiegroep in vergelijking met de placebogroep (OR 1.67, 95% BI 0.95;2.95). Beide effecten waren sterker aanwezig in degenen met het *TT* genotype. Deze studie suggereert dat suppletie met hoge doseringen folaat en vitamine B12 uracilincorporatie en promotermethylering in patiënten met dikkedarmadenomen verhoogt.

Zoals eerder aangegeven, richtten we ons in dit proefschrift op genetische polymorfismen en DNA-methylering en –synthese in moleculair epidemiologische studies. Deze studies zijn onderdeel van een multidisciplinair project. Andere studies uit dit project zullen gepubliceerd worden in een gerelateerd proefschrift van Linette Pellis, waarin verscheidene *in vitro* studies zullen worden beschreven die zijn uitgevoerd in verschillende humane colonepitheel-cellijnen. Hierin werden de effecten van langdurige blootstelling aan verschillende concentraties en vormen van folaat op folaatmetabolieten en ijzer onderzocht. Bovendien werden de effecten op genexpressie onderzocht om andere mechanismen in kaart te brengen.

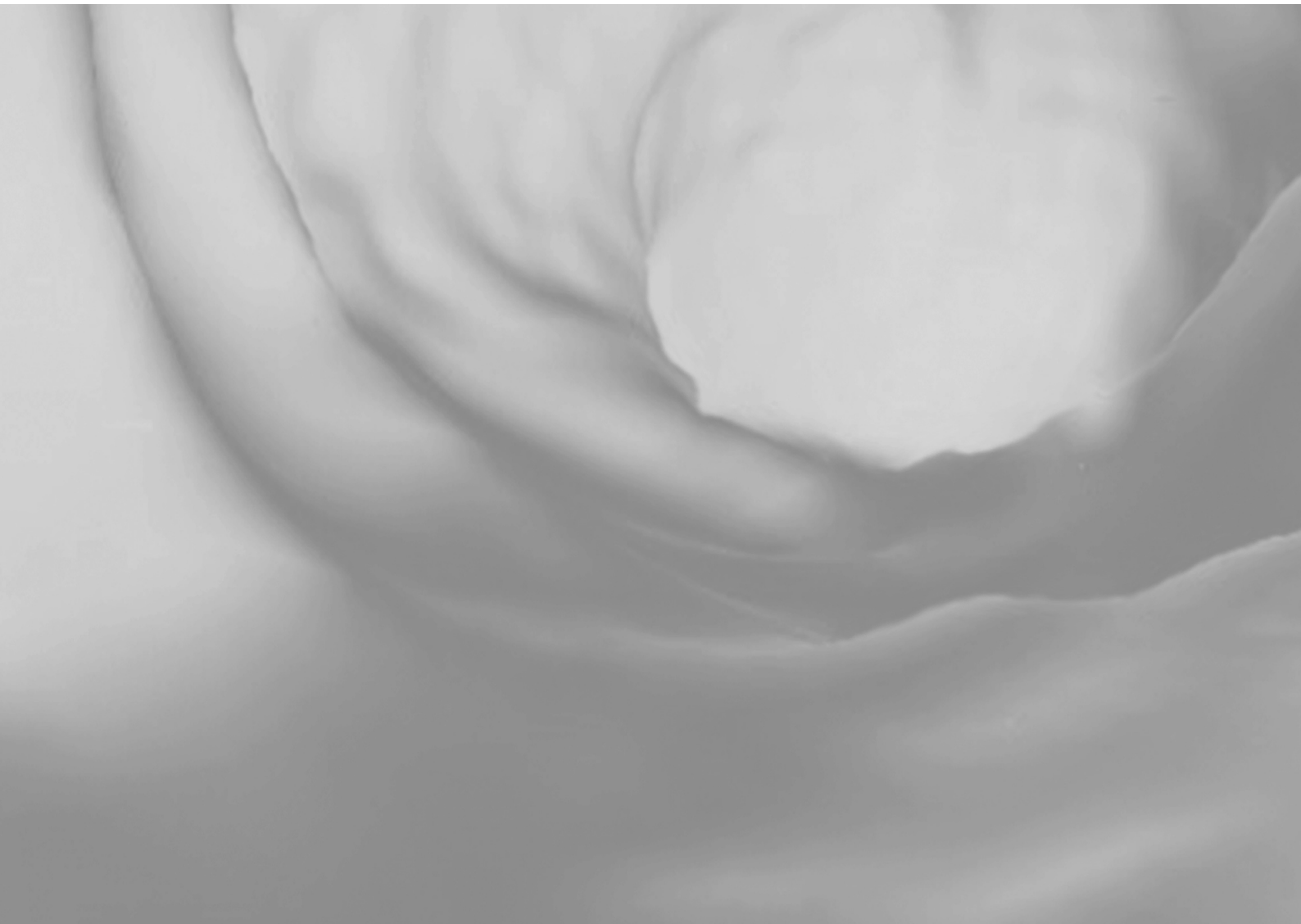
## Discussie

De belangrijkste bevindingen van de onderzoeken worden samengevat en bediscussieerd in **hoofdstuk 7**. Het positieve verband tussen folaatinname via de voeding en het risico op dikkedarmadenomen dat wij in ons patiënt-controleonderzoek vonden is in tegenspraak met resultaten van het meeste eerder uitgevoerde onderzoek. Dit positieve verband kan gedeeltelijk toegeschreven worden aan de relatief lage inname van vitamine B2 in ons onderzoek, en aan het feit dat vlees een belangrijke bron van folaat was in ons onderzoek. Er zijn echter aspecten aan de opzet van deze studie die niet zomaar genegeerd kunnen worden. Zo is het niet mogelijk om alle bronnen van confounding en bias uit te sluiten, hoewel we geprobeerd hebben om te corrigeren voor mogelijke confounders, en verschillende subgroepanalyses hebben uitgevoerd om mogelijke bronnen van bias te detecteren, zonder dat dit leidde tot veranderingen in de resultaten. Bovendien was de folaatinname geschat met behulp van een voedselfrequentievragenlijst, wat misschien niet de meest accurate methode is voor het bepalen van de folaatinname. Als we al deze overwegingen meenemen, blijven er twijfels bestaan over het positieve verband, en we durven dan ook geen conclusies te trekken op basis van deze ene studie.

De resultaten van de interventiestudie komen overeen met die van het patiënt-controle onderzoek. Wel zijn er essentiële verschillen tussen de twee onderzoeken. In de interventiestudie werd een hoge dosering foliumzuur gegeven aan een groep patiënten, nadat bij deze patiënten adenomen in de dikke darm waren gediagnosticeerd. Er was gebruik gemaakt van een placebogroep om de resultaten van de interventie mee te vergelijken, en de toewijzing van de interventie en de placebo gebeurde door middel van loting. Het is mogelijk dat de combinatie van biomarkers die wij gebruikt hadden niet compleet is, of dat deze biomarkers niet het werkelijke kankerrisico weergeven. Het feit dat we biomarkers hebben gebruikt die een maat vormen voor zowel de DNA-methylering als de DNA-synthese versterkt onze resultaten. Andere interventiestudies met foliumzuur onderzochten ook een beperkt aantal biomarkers, dus de mogelijkheid bestaat dat deze studies nadelige effecten van foliumzuur hebben gemist doordat de combinatie van markers niet compleet was. Bovendien komen onze resultaten overeen met resultaten uit dierstudies waarin hoge foliumzuurdoseringen werden toegediend nadat voorstadia van kanker zich ontwikkeld hadden. Als we al deze argumenten samennemen, kunnen we concluderen dat een hoge dosering foliumzuur inderdaad nadelige effecten kan hebben, vooral bij toediening nadat neoplastische laesies in de dikke darm zijn gediagnosticeerd.

**Als de resultaten van dit proefschrift juist zijn, dan laat foliumzuur een ander, vooralsnog onbekend en zorgwekkender gezicht zien dan het stralende gezicht dat we tot nu toe hebben gezien. Hebben we tot voor kort alleen dokter Jekyll ontmoet zonder kennis te hebben gemaakt met meneer Hyde? Zolang we niet kunnen uitsluiten dat meneer Hyde bestaat, kan het geen kwaad om voorzichtig te zijn.**

# DANKWOORD



**In deze laatste pagina's van mijn proefschrift wil ik graag iedereen bedanken die er, bewust of onbewust, voor heeft gezorgd dat het me gelukt is een proefschrift te schrijven. In het bijzonder denk ik hierbij aan:**

Alle mensen, en dit waren er maar liefst 1500, die belangeloos aan het FOCO-onderzoek en de POLIEP-studie hebben deelgenomen: zonder deelnemers is het natuurlijk onmogelijk om humane studies uit te voeren. Dankuwel!

Daarna komt natuurlijk het project-team aan de beurt. Ellen Kampman was mijn co-promotor en dagelijks begeleider. Ellen, ik wil je graag bedanken voor alle steun, en je gekleurde verschijning! Hoewel het gelukkig niet zo heel vaak voor kwam, wist je me als dat nodig was zo weer uit een dipje te halen. Mijn promotor Frans Kok: in het begin was je wat meer zijdelings betrokken bij het project, maar naar het einde toe (en ook in crisissituaties) raakte je er nauwer bij betrokken. Ik heb het ook erg gewaardeerd dat je in de laatste periode zoveel tijd voor overleg vrij wilde maken. Mijn co-promotor Jaap Keijer: hoewel je iets minder intensief betrokken was bij de dagelijkse gang van het project, heb ik veel gehad aan je moleculair-biologische inbreng, en daardoor ook je frisse kijk op de epidemiologie. Verder was ik blij dat ik niet de enige AIO op dit project was, want samen sta je sterker. Mijn mede-AIO Linette Pellis was verantwoordelijk voor het moleculair-biologische gedeelte. Linette, jij hebt me ingewijd in de moleculaire wereld, wat nog heel wat voeten in de aarde had voor een epidemioloog als ik. Ook heb ik veel steun aan je gehad tijdens onze zwangerschap, tot twee maal toe! Dank jullie wel, alle vier.

De andere AIO's die werkzaam waren op de POLIEP-studie, Edine Tiemersma, Brenda Diergaarde, Petra Wark en Mariken Tijhuis, wil ik bedanken voor de fijne samenwerking. Het kost heel wat moeite om zo'n groot patiënt-controle onderzoek draaiende te houden en ervoor te zorgen dat de werving en data-verzameling soepel verlopen! Hierbij was ook een heel belangrijke rol weggelegd voor Maria van Vugt. Dankjewel allemaal! Verder was Petra al die jaren mijn kamergenoot, wat altijd zorgde voor de nodige ontspanning Dankjewel, ook voor alle kopjes thee! Ook Mariken zorgde voor veel afleiding. Fijn dat je paranimf wilt zijn, staan we daar samen met een buikje! Brian Buijsse en Saskia van den Berg hebben als student bijgedragen aan hoofdstuk 3. Hartelijk dank daarvoor.

Als epidemioloog was het een hele omslag om ook laboratoriumwerk te gaan doen. In het DNA-lab van de afdeling heb ik de *MTHFR*-genotyperingen uitgevoerd. Ik wil Annelies Bunschoten bedanken omdat ze mij met veel geduld heeft ingewerkt in de PCR-wereld. Later werd haar rol overgenomen door Jan Harryvan en Marleen Visker, die ook de andere polymorfismen hebben bepaald. Dankjewel!

De methyleringsanalyses heb ik uitgevoerd bij de afdeling Pathologie van de Universiteit Maastricht. Ik wil Manon van Engeland graag bedanken voor het scheppen van de mogelijkheden. Verder wil ik met name Sandra van den Bosch, maar ook de andere Angels, bedanken voor hun uitleg en hulp bij de analyses. Zonder jullie had ik het niet gered! Tijdens dit Maastrichtse uitstapje mocht ik steeds blijven logeren bij Boukje van Dijk, die al tijdens mijn studie in Nijmegen een goede vriendin is geworden. Dankjewel voor de vriendschap en de gastvrijheid! Ik vind het erg fijn dat je straks ook naast me staat als paranimf.

Het FOCO-onderzoek is uitgevoerd in drie ziekenhuizen: het Universitair Medisch Centrum St. Radboud in Nijmegen, Ziekenhuis de Gelderse Vallei in Ede, en het Slingeland Ziekenhuis in Doetinchem. Ik ben veel dank verschuldigd aan de artsen die hun vrije tijd hebben opgeofferd om monsters af te nemen voor de studie: Fokko Nagengast, Pieter Friederich, Ben Witteman, Jeroen van Bergeijk, Jan Uil en Sybrand de Boer. Daarnaast heb ik ook veel hulp gehad van het endoscopiepersoneel, het laboratoriumpersoneel (met name Annie van Schaik en Tamara Lamers), en de secretaresses.

Naast deze drie ziekenhuizen hebben we in zeven andere ziekenhuizen deelnemers geworven voor de POLIEP-studie: het Ziekenhuis Rivierenland in Tiel, het Antonius-ziekenhuis in Nieuwegein, het Rijnstateziekenhuis in Arnhem, Meander Medisch Centrum in Amersfoort, het Slotervaartziekenhuis in Amsterdam, het Canisius-Wilhelmina Ziekenhuis in Nijmegen en het Jeroen Bosch Ziekenhuis in Den Bosch. Ik wil graag alle betrokken gastro-enterologen, endoscopiepersoneel en secretaresses bedanken voor hun hulp bij de werving. Verder dank ik Marga Ocké van het RIVM voor het berekenen van de voedingsinname van alle deelnemers.

Jimmy Crott and Joel Mason from Tufts Medical School: thank you very much for doing the uracil analyses and your very useful comments on the manuscript.

In de loop van mijn AIO-project ben ik ook betrokken geraakt bij het grote WCRF-project waarbinnen meta-analyses worden uitgevoerd naar voeding en kanker. Ik wil met name Marije Schouten, Bianca Stam, Jan Burema en Pieter van 't Veer bedanken voor hun hulp bij het uitvoeren van de meta-analyse over folaat en adenomen. Maar ook de rest van het team: hartelijk bedankt voor de fijne samenwerking, die we nu natuurlijk gaan voortzetten!

Ook de overige collega's van de afdeling Humane Voeding wil ik graag bedanken, vooral de AIO's. Ik ging altijd met plezier naar het werk, en dat is heel wat waard! Ook heb ik

veel gehad aan al het geregel ‘achter de schermen’ van héél veel mensen van binnen en buiten de afdeling. Dank jullie wel voor alles.

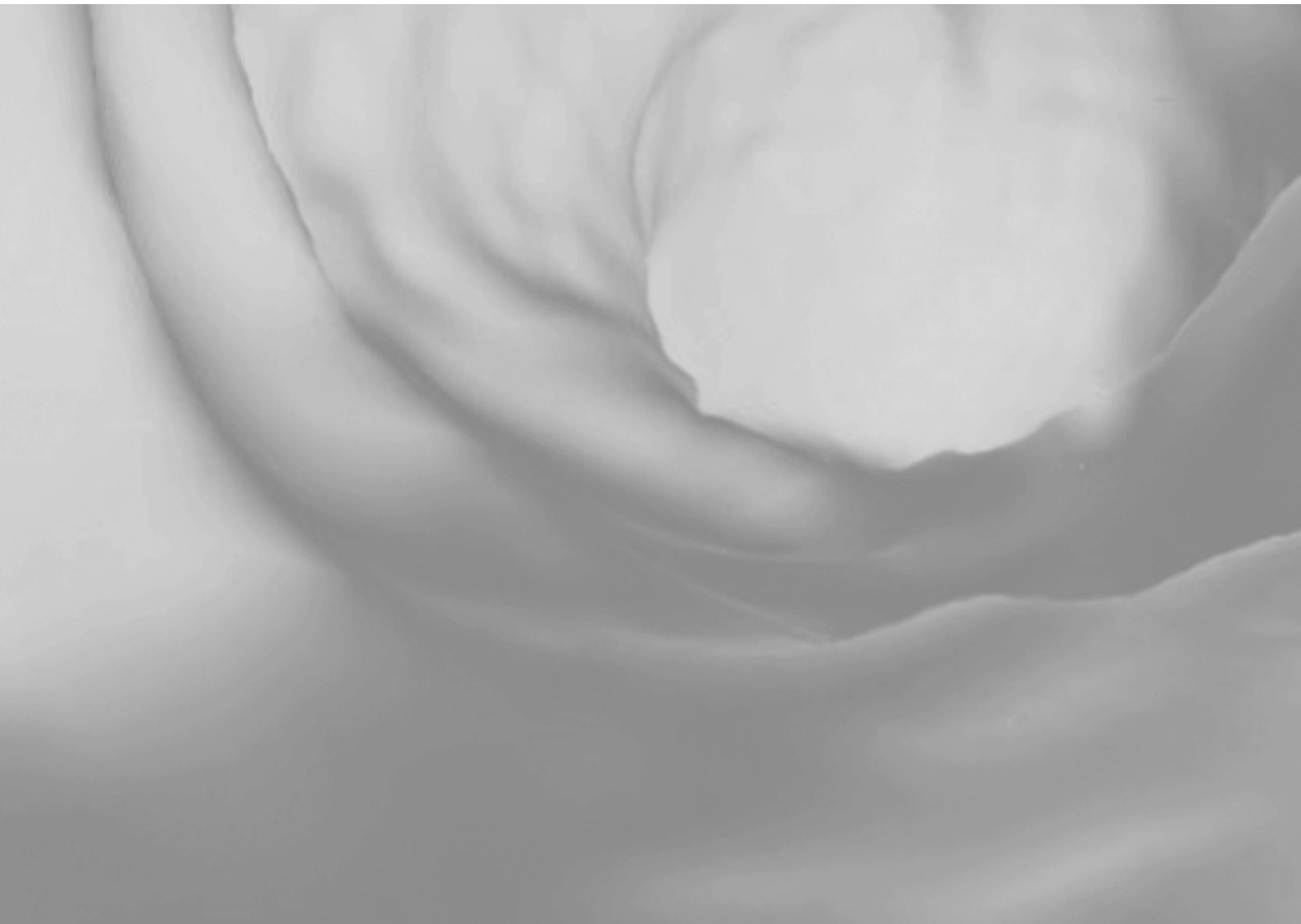
Lian van Druenen wil ik hartelijk danken voor het ontwerpen van de kaft en de hulp bij het lay-outen. Ik vind het echt prachtig geworden, en zo’n mooie knalkleur! En ik vind het erg fijn dat ik je op het laatst voor alle kleine probleempjes mocht bellen, ondanks dat je het zelf zo druk hebt.

Natuurlijk heb ik ook veel steun gehad aan al mijn vrienden uit Boxtel, Nijmegen, Eindhoven en Boekel. Het zijn er teveel om op te noemen, maar voor jullie allemaal geldt: hartelijk dank voor de vriendschap en de nodige ontspanning!

Mijn familie is ook erg belangrijk voor me. Met name papa, mama, Bregje en Kerstin wil ik bedanken voor al hun steun en belangstelling. Ik ben erg blij met zo’n veilige thuishaven, en hoop dat dat altijd zo zal blijven. Ook Bram hoort hier bij. Verder mag ik me gelukkig prijzen met mijn geweldige, lieve oma Alwine. Hoewel het duidelijk is dat je ouder wordt, blijf je altijd met me meeleven, zowel thuis als op het werk.
Lieve Veerle, hoewel je natuurlijk nog niet weet wat het schrijven van zo´n boekje inhoudt en je er misschien ook niet direct aan hebt bijgedragen, heb je er wel voor gezorgd dat het leven nóg leuker is dan het altijd al was!
Lieve René, jij bent mijn grote steun en toeverlaat in alles! Natuurlijk is het praktisch, een epidemioloog-in-spé in huis. Maar ook daarnaast zou ik je niet meer kunnen missen. Ik ben zó blij dat ik je heb ontmoet, en ik ben erg trots op jou, op ons. Laten we nog lang zo verder gaan!

**Iedereen: hartelijk dank voor alles!**

# PUBLICATION LIST AND EDUCATIONAL PROGRAMME



# PUBLICATION LIST

## Peer reviewed papers

Robertson C, Van den Donk M, Primic-Zakelj M, MacFarlane T, Boyle P. The association between induced and spontaneous abortion and risk of breast cancer in Slovenian women aged 25-54. Breast 2001;10(4):291-8.

Pijnappel RM, Peeters PH, Van den Donk M, Holland R, Hendriks JH, Deurloo EE, Mali WP. Diagnostic strategies in non-palpable breast lesions. Eur J Cancer 2002;38(4):550-5.

Pijnappel RM, Van den Donk M, Holland R, Mali WP, Peterse JL, Hendriks JH, Peeters PH. Diagnostic accuracy for different strategies of image-guided breast intervention in cases of nonpalpable breast lesions. Br J Cancer 2004;90(3):595-600.

Van den Donk M, Buijsse B, Van den Berg SW, Ocké MC, Harryvan JL, Nagengast FM, Kok FJ, Kampman E. Dietary intake of folate and riboflavin, MTHFR C677T genotype, and colorectal adenoma risk: a Dutch case-control study. Cancer Epidemiol Biomarkers Prev 2005;14(6), 1562-6.

## Published abstracts

Van den Donk M, Pellis EPM, Keijer J, Kok FJ, Kampman E. The role of folic acid and vitamin B12 in colorectal carcinogenesis in genetically different individuals. Eur J Cancer Prev 2000;9(6). (Poster presentation at '18$^{th}$ Annual symposium of the European Cancer Prevention Organisation (ECP): Precancerous lesions of the digestive tract', Maastricht, the Netherlands, 12-14 October 2000)

Van den Donk M, Pellis EPM, Keijer J, Kok FJ, Nagengast FM, Kampman E. The role of folic acid and vitamin B12 in colorectal carcinogenesis in genetically different individuals--design of a study. IARC Sci Publ. 2002;156:499-500. (Poster presentation at 'European conference on nutrition & cancer', Lyon, France, 21-24 June 2001)

## Other abstracts

Van den Donk M, Pijnappel RM, Peeters PHM, Verkooijen HM, Peterse JL, Holland R, Mali W. De diagnostiek van niet-palpabele borstafwijkingen. (Poster presentation at 25th WEON, Texel, the Netherlands, 7-9 June 2000)

Pellis EPM, van Hal NLW, Van den Donk M, Kampman E, Keijer J. Effects of folic acid on the human colonic cell line HT29. (Poster presentation at 'Homocysteine metabolism congress', Sorrento, Italy, 1-5 July 2001)

Van den Donk M, Buijsse B, Kok FJ, Kampman E. Inname van B-vitamines, *MTHFR C677T* genotype en het risico op colorectale adenomateuze poliepen. (Poster presentation at 27th WEON, Nijmegen, the Netherlands, 6-7 June 2002)

Pellis EPM, Van Hal NLW, Van den Donk M, Kampman E, Keijer J. The role of folic acid and vitamin B12 in colorectal carcinogenesis in genetically different individuals. (Poster presentation at 'FASEB Summer research conference on folic acid, vitamin B12 & one carbon metabolism', Snowmass Village, Colorado, USA, 3-8 August 2002)

Van den Donk M, Pellis EPM, Keijer J, Kok FJ, Nagengast FM, Kampman E. The role of folic acid and vitamin B12 in colorectal carcinogenesis in genetically different individuals--design and preliminary findings. (Poster presentation at 'FASEB Summer research conference on folic acid, vitamin B12 & one carbon metabolism', Snowmass Village, Colorado, USA, 3-8 August 2002)

Pellis L, Van den Donk M, Franssen-van Hal N, Venema D, Baykus H, Kampman E, Keijer J. Molecular and physiological steady state effects of two different folic acid concentrations on human colon epithelial cell lines. (Poster presentation at 'First international conference on folates, analysis, bioavailability and health', Warschau, Poland, 11-14 February 2004)

Van den Donk M, Buijsse B, Van den Berg S, Harryvan J, Nagengast FM, Kok FJ, Kampman E. De inname van folaat, vitamine B2 en andere B-vitamines via de voeding, *MTHFR C677T* genotype, en risico op colorectale adenomen. (Oral presentation at 29th WEON, Leiden, the Netherlands, 10-11 June 2004)

Van den Donk M, Pellis EPM, Van Engeland M, Crott JW, Nagengast FM, Kok FJ, Keijer J, Kampman E. Het effect van foliumzuur en vitamine B12 op DNA-synthese en DNA-methylering in rectaal slijmvlies bij mensen met verschillend MTHFR C677T genotype: een gerandomiseerde, placebo-gecontroleerde interventiestudie. (Oral presentation at 30th WEON, Wageningen, the Netherlands, 2-3 juni 2005)

Van den Donk M, Van Engeland M, Pellis EPM, Witteman BJM, Kok FJ, Keijer J, Kampman E. De inname van folaat via de voeding, *MTHFR C677T* genotype en promoter methylering in sporadische colorectale adenomen. (Poster presentation at 30th WEON, Wageningen, 2-3 juni 2005)

Van den Donk M, Pellis L, Crott JW, Van Engeland M, Friederich P, Nagengast FM, Van Bergeijk JD, De Boer SY, Mason JB, Kok FJ, Keijer J, Kampman E. The effect of folic acid and vitamin B12 on promoter methylation and uracil incorporation in rectal mucosa DNA among *MTHFR C677T* genotypes: a randomized, placebo-controlled intervention trial. (Poster presentation at 'Fourth annual AACR international conference: Frontiers in cancer prevention research', Baltimore, Maryland, USA, 30 October-2 November 2005)

# EDUCATIONAL PROGRAMME

## Discipline specific activities

ECP symposium 'Precancerous lesions of the digestive tract', Maastricht, 2000

IARC course 'Molecular Epidemiology', Lyon, France, 2000

VLAG course 'Ecophysiology of the gastrointestinal tract', Wageningen, 2001

NIHES course 'Clinical trials and drug risk assessment', Utrecht, 2001

IARC conference 'Nutrition and cancer', Lyon, France, 2000

VLAG masterclass 'From nutrigenomics to healthy food', Wageningen, 2001

UMC St Radboud symposium 'Homocysteine, folate and vitamin B12 in cardiovascular and neurological disease', Ravenstein, 2001

VLAG thematic meeting 'The future of molecular epidemiology', Wageningen, 2002

FASEB conference 'Folic acid, vitamin B12 and one-carbon metabolism', Snowmass Village (CO), USA, 2002

Centre for Human Nutrigenomics thematic day 'Intestinal function', Wageningen, 2002

UMC St Radboud symposium 'Micro arrays in biomedical sciences', Nijmegen, 2002

Centre for Human Nutrigenomics / VLAG discussion symposium 'Future perspectives of human health; the role for nutrigenomics', Wageningen, 2003

NIHES symposium 'Patients, populations and the role of scientific research', Rotterdam, 2003

WCFS minisymposium 'Folate metabolism: new approaches and insights', Wageningen, 2003

WUR symposium 'Folate and age-related disease', Wageningen, 2004

AACR conference 'Frontiers in cancer prevention research', Baltimore (MD), USA, 2005

NWO annual meeting of Nutritional Science Community, Papendal, 2000-2003

NWO symposium 'Nutrition and chronic diseases', Utrecht, 2000-2004

WEON annual symposium of the Dutch Epidemiology Society, 2000-2005

## General courses

VLAG PhD week, Nijmegen, 2000

OWU course 'Organizing and supervising thesis work', Wageningen, 2002

CENTA course 'Scientific writing', Wageningen, 2004

WGS course 'Career perspectives', Wageningen, 2005

## Optional courses and activities

Preparation PhD research proposal

WUR-HNE PhD study tour, 2001

WUR-HNE meetings of Homocysteine Club, 2000-2004

WUR-HNE meetings of Journal Club, 2000-2004

The work described in this thesis was funded by the Netherlands Organization for Health Research and Development (ZonMW), grant 980-10-020, and the Netherlands Foundation for Digestive Diseases (MLDS), grant WS 99-72, and was conducted at the Division of Human Nutrition of Wageningen University, the Netherlands.

The financial support of Wageningen University for the printing of this thesis is gratefully acknowledged.

Design cover and lay-out: Van Druenen ontwerp & dtp, Veldhoven, the Netherlands
Printed by: PrintPartners Ipskamp, Enschede, the Netherlands