Dietary folate, genetic variation and DNA methylation in sporadic colorectal cancer

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Voor oom Fred en oma van Keeken

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1

Introduction

Adequate intake of the B-vitamin folate is important for the prevention of neural tube defects during embryonal development (1,2). These observations led to a regulation in the US and Canada of mandatory fortification of flour and grains with folic acid in the year 1998, and several other countries are currently discussing whether or not to introduce nation-wide folic acid fortification. Folic acid supplementation may also prevent stroke (3). In addition, several observational studies suggested that high intake of dietary folate may protect against colorectal cancer, although results have been inconsistent (4,5). One explanation for such inconsistencies may be the recently hypothesized dual role of folate in colorectal carcinogenesis, i.e. it may protect against neoplasia in normal colorectal mucosa whereas folate supplementation might enhance existing pre-malignant lesions (6). Accounting for approximately 13% of total cancer incidence, colorectal cancer is the second most common type of cancer and the second most common form of cancer death (7) in Europe, indicating the importance of studying potential determinants of the disease.

The focus of this thesis was to study associations of dietary folate and other methyl donors with overall colorectal cancer, and specifically colorectal tumors characterized by the presence or absence of gene mutations or gene promoter hypermethylation. We also studied whether genetic variants of folate metabolizing enzymes and epigenetic regulators modify these associations. In addition, we studied the occurrence of, and overlap between specific molecular characteristics in colorectal tumors.

Dietary methyl donors and colorectal cancer

Folate: naturally occurring folates and folic acid

Folate is a B-vitamin mainly present in green leafy vegetables, fruits, dairy products, meat, bread and potatoes (8). The term "folate" is used for a group of compounds with similar chemical and nutritional properties and includes folates that occur naturally in our diet, as well as folic acid which is the chemically stable synthetic form of folate used in dietary supplements and fortified foods. Naturally occurring folates predominantly exist as 5-methyl-tetrahydrofolate (5-methyl-THF), which is also the main circulating form of folate, and typically are a mixture with other tetradydrofolates such as 5,10-methylene-THF, 10-formyl-THF and other less common forms (9,10). They are mostly polyglutamates and may differ in length depending on the number of attached glutamate chains. These polyglutamates first have to be hydrolyzed to monoglutamates in order to enable transport through cell membranes. Folic acid is a monoglutamate and may directly be transported through the intestinal brush border without this enzymatic conversion (6), and the bioavailability of folic acid is therefore higher compared to naturally occurring folates (11).

Folate metabolism

Folate is needed for the biosynthesis of the pyrimidine nucleoside thymidine and of purines, and thereby plays an essential role in DNA synthesis. The folate intermediate 5,10-methylene-THF serves as a methyl donor to convert deoxyuridine monophosphate (dUMP) into deoxythymidine monophosphate (dTMP) (Figure 1, Panel A). Folate deficiency may result in accumulation of dUMP, induce uracil mis-incorporation in DNA

(instead of thymidine) and result in subsequent DNA instability, chromosomal damage and malignant transformation (12).

In addition, folate is a methyl group donor which may influence DNA methylation such as CpG island promoter hypermethylation or global hypomethylation, both of which are often observed in colorectal cancer (13,14). The conversion of homocysteine into methionine, with 5-methyl-THF as a substrate, is important for the synthesis of S-adenosylmethionine, which is the universal methyl group donor needed for methylation processes (15). Since methionine itself is present in our diet, it is considered as a methyl group donor in addition to folate.

Vitamins B2, B6 and B12 are involved in folate metabolism, and may therefore modulate the bioavailability of methyl groups (15). Vitamin B2, or riboflavin, is the cofactor for methylenetetrahydrofolate reductase (MTHFR), the enzyme that reduces 5,10-methylene-THF in 5-methyl-THF. Methionine synthase (MTR) and methionine synthase reductase (MTRR) convert homocysteine into methionine, which is a vitamin B12-dependent reaction. In addition, vitamin B6 is involved in the conversion of tetrahydrofolate into 5,10-methylenetetrahydrofolate, one of the steps of the folate cycle (15).

Adequate intakes of dietary folate, methionine, vitamins B2, B6 and B12 ensure a sufficient supply of methyl groups, and may be hypothesized to prevent DNA instability and aberrant DNA methylation thereby protecting against colorectal cancer.

Associations between folate, other methyl donors and colorectal cancer

The relation between dietary folate and colorectal cancer has previously been studied in many observational studies, but results have been inconsistent. A meta-analysis estimating the aggregate effect of folate, suggested that dietary folate may have a small protective effect against colorectal cancer (an overall risk reduction of approximately 25%), and that this effect was not present for total folate intake (i.e. including folic acid supplements) (5). However, a literature review showed that out of the 23 case-control and cohort studies conducted so far, only 10 studies suggested a protective effect of folate on colorectal cancer (4).

Relatively high methionine intake did not seem to protect against CRC (16-19). In addition, whereas a potential protective effect of vitamin B2 intake against colorectal adenomas was observed in one study (20), it was not associated with colorectal cancer (19,21). Several studies demonstrated an inverse association between vitamin B6 intake or plasma levels of vitamin B6 and CRC (18,22-26), although in one study, a positive association was observed between the vitamin and rectal tumors among women (17).

There may be a number of reasons for inconsistencies between observational studies, for example differences in study design, study populations and endpoints. In this respect, some of the studies had a prospective cohort design (16,17,22-25), whereas others were case-control studies (18-21). Moreover, some studies were conducted among women only (16,17,23-25) or in relation to colorectal adenomas (20), and there are considerable differences in the level of vitamin intake between studies. Also, particularly study populations in the US may be exposed to higher levels of folic acid intake due to the use of dietary supplements (27), and it is currently unknown if folic acid has a similar effect on colorectal carcinogenesis as compared to dietary folate.

We aimed to investigate the associations between dietary folate, methionine, vitamin B2 and vitamin B6 in a prospective setting in the Netherlands Cohort Study on diet and cancer (NLCS) within a large group of incident colorectal cancer patients (chapter 3).

Molecular pathways to colorectal cancer

Hereditary versus sporadic colorectal cancer

Colorectal cancer may either be caused by hereditary factors, or alternatively, develop as sporadic colorectal cancer. Two well-characterized types of hereditary colorectal cancer are familial adenomatous polyposis coli (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome). Individuals with FAP have a mutation in the *Adenomatous Polyposis Coli (APC)* gene and typically develop multiple colorectal adenomatous polyps already in early adulthood, which are likely to result in colorectal cancer (28). FAP accounts for less than 1% of colorectal cancer cases (29). Patients with HNPCC often have germline mutations in the mismatch repair (MMR) genes *MLH1, MSH2* and *MSH6*, which have been observed in 39%, 42% and 18% of MMR mutation carriers among a large group of colorectal cancer patients (30). However, only 1-5% of colorectal cancer cases are estimated to develop as a result of HNPCC (29,31,32). The majority of colorectal cancers occurs sporadically (from here called colorectal cancer) and will be the investigated in this thesis. In the Netherlands, almost 11000 colorectal cancer patients have been identified in the year 2005, and 4562 patients died of the disease (33).

Gene mutations driving colorectal carcinogenesis

In the early 1990s, a genetic model of the carcinogenesis of colorectal cancer was postulated by Fearon and Vogelstein (34). The authors described the sequential transformation from normal colorectal epithelium, through aberrant crypt foci, colorectal adenomas, colorectal carcinomas and finally metastasis, as a process during which accumulation of gene mutations occur which contribute to carcinogenesis. Mutations of the tumor suppressor gene *APC* were observed to be early events in colorectal carcinogenesis. *Kirsten-ras* (*KRAS*) oncogene mutations would be involved in the transformation from early to late adenomas, and mutations in the *P53* tumor suppressor gene in the development of adenomas to carcinomas. This model was used by many scientists in the field of colorectal cancer research in the years thereafter. With new technologies that are available nowadays, it has become possible to perform whole-genome sequence analyses. When screening 18,191 genes for gene mutations, it appeared that less than 15 mutations were likely to be responsible for tumor initiation or progression, and that *APC*, *KRAS* and *P53* are mutated in the majority of colorectal tumors (35).

Microsatellite instability and chromosomal instability

Other mechanisms that may contribute to colorectal carcinogenesis involve either microsatellite instability (MSI), or chromosomal instability (CIN). MSI is a type of genetic instability that is characterized by length alterations within simple repeated

microsatellite sequences (36). MSI occurs in approximately 15% of colorectal cancers and may be caused by the inactivation of mismatch repair (MMR) genes. Most sporadic MSI tumors show epigenetic silencing by promoter hypermethylation, of the MMR gene *MLH1* (37,38). Alternatively, CIN refers to the rate with which whole chromosomes (or large portions thereof) are gained or lost in cancers. CIN tumors therefore contain an abnormal chromosomal content and are thought to represent the remaining 85% of colorectal cancers (39).

CpG island methylator phenotype (CIMP)

In addition to genetic alterations, loss of gene function may occur through epigenetic gene silencing. DNA hypermethylation of CpG islands in gene promoters is a predominant epigenetic alteration that results in inactivation of transcriptional activity (13). It has been estimated that in colorectal cancer, approximately 5% of all known genes shows promoter hypermethylation and that more genes are hypermethylated than mutated, underscoring the importance of epigenetic alterations in colorectal cancer (40).

A distinct subset of colorectal cancers harbors widespread promoter hypermethylation and is referred to as the CpG island methylator phenotype (CIMP) (41,42). Different CIMP subclasses have been proposed based on the number of methylated genes in CIMP marker panels, and their correlation with other molecular alterations in colorectal cancer (43). CIMP-high colorectal cancers are associated with MSI, *BRAF* mutations, wild-type *P53*, older age, female sex and location in the proximal colon (44-46). CIMP-low tumors exhibit less extensive promoter methylation and have been associated with *KRAS* mutations and male sex (47,48). Finally, CIMP-negative tumors have low frequencies of promoter methylation and are associated with CIN, suggesting that CIMP and CIN are two independent distinct molecular mechanisms in colorectal carcinogenesis (49,50). Similar subclasses were suggested by independent research groups (43,51).

However, there may be more than these three CIMP subgroups depending on the presence or absence of MSI, *BRAF* or *KRAS* mutations (43,52), indicating that the classification of CIMP continues to be a subject of debate. Moreover, different panels of methylation markers have been used in the past to define CIMP (53). The clinical outcome for CIMP and related molecular characteristics may also differ. In this respect, CIMP-high and MSI were associated with a reduction of colon cancer-specific mortality, whereas *BRAF* mutations were associated with high mortality (54). Conversely, among patients with microsatellite stable (MSS) tumors, CIMP was associated with a shorter 5-year survival (55). Although CIMP, MSI and *BRAF* mutations strongly correlate, the question therefore remains whether colorectal tumors with these characteristics develop through one distinct molecular pathway.

To validate current hypotheses about molecular correlates in colorectal cancer, we studied the occurrence and overlap of the related characteristics CIMP, *MLH1* hypermethylation, MSI and *BRAF* mutations (chapters 5 and 6). Since some molecular aberrations may be inversely correlated, we also investigated associations between *MLH1* promoter methylation and *APC*, *KRAS* and *BRAF* mutations (chapter 2). In addition, we studied the associations between *MGMT* promoter methylation and G:C>A:T mutations in *KRAS* and *APC*, which may provide more insight in a possible sequence of events during colorectal carcinogenesis (chapter 2).

To date, little is known about the environmental and genetic determinants of CIMP, and this question will be addressed in this thesis.

Dietary methyl donors and molecular phenotypes in colorectal cancer

Folate and tumors characterized by gene mutations

Since folate is important for nucleotide biosynthesis and DNA stability, it may be hypothesized that sufficient folate intake prevents the introduction of gene mutations. The association between dietary folate and the risk of developing tumors with gene mutations has been investigated in a limited number of studies, but with inconsistent results. Individuals with a history of colorectal adenoma and high folate intake were at reduced risk of colorectal adenoma recurrence with *KRAS* mutation (56). However, folate intake was not associated with colorectal cancer, with or without *KRAS* mutations, among individuals in two large prospective cohort studies (57). We previously observed that folate was not associated with *KRAS* mutations in colon cancer, but suggestions for positive and inverse associations were observed with rectal cancer risk depending on the type of *KRAS* mutation (58).

The association between intake of green leafy vegetables, an important source of folate, and *APC* mutation status in colorectal adenomas has previously been investigated (59). However, whether folate intake is associated with *APC* mutations in colorectal cancer has not previously been studied, and was therefore investigated in this thesis (chapter 4).

Dietary methyl donors and promoter hypermethylation in colorectal cancer

High intakes of dietary methyl donors may prevent CpG island promoter hypermethylation in colorectal cancer. A pilot study among 120 colorectal cancer patients previously conducted within the NLCS supported this hypothesis, since low intake of folate and methionine in combination with high alcohol intake was associated with increased promoter hypermethylation (60). However, the association in that study was weak, observed with at least one out of 6 methylated markers as an endpoint, and did not reach statistical significance. High fruit intake, a source of folate, was associated with reduced risk of colon carcinomas harboring MSI and *MLH1* hypermethylation (61). High alcohol intake potentially reduces the bioavailability of folate which may affect DNA methylation in the colonic mucosa (62-64), and was associated with MSI in colorectal cancer (65). However, no associations were observed between dietary folate, vitamins B6 and B12, methionine and alcohol with CIMP in colorectal cancer in a large case-control study (65).

Conversely, it has been observed that high folate and vitamin B12 levels were associated with *increased* tumor methylation among colorectal cancer patients (66). Similarly, two important forms of circulating folate were associated with hypermethylation of genes in colorectal tumors (67). Moreover, in a randomized intervention trial in subjects with a history of colorectal adenoma, folate supplementation resulted in an increase of promoter hypermethylation in rectal mucosa (68).

These observations indicate that the precise effect of dietary methyl donors is unclear and needs further investigation. Moreover, the effect of vitamin B2 on DNA methylation has not previously been investigated. Therefore, we investigate associations between dietary folate, methionine, vitamins B2 and B6 with *MLH1* hypermethylation, MLH1 expression, MSI and *BRAF* mutations (chapter 5) and with CIMP in colorectal cancer (chapter 7).

Polymorphisms in folate metabolizing enzymes and epigenetic regulators

Folate metabolizing enzymes

The bioavailability of methyl groups depends on the activity of folate metabolizing enzymes. Single nucleotide polymorphisms in genes encoding these enzymes have been reported to alter enzymatic activity. For example, the C677T and A1298C polymorphisms in the *MTHFR* gene reduce enzymatic activity of the MTHFR enzyme (69,70). Polymorphisms in *MTR* and *MTRR* were associated with lower plasma homocysteine concentrations (71,72) suggesting that such genetic variants may lower catalytic activity of the MTR and MTRR enzymes.

The *MTHFR* C677T and A1298C polymorphisms were generally inversely associated with overall colorectal cancer in several observational studies (73,74), although positive associations have been reported (75-78). In addition, individuals with these polymorphisms were more likely to develop colorectal tumors with a CpG island hypermethylation phenotype (79,80) or microsatellite instability (MSI) (81). The *MTR* A2756G polymorphism was inversely associated with overall colorectal cancer in two studies (82,83) but was not associated with CIMP (79). Though the association of *MTRR* polymorphisms with promoter hypermethylation has not been investigated to date, it was observed that some *MTRR* polymorphisms may increase the risk of developing colorectal adenomas and carcinomas (19,84,85).

Interaction between methyl donors and folate metabolizing enzymes

Several studies suggested an interaction between methyl donor intake and *MTHFR* genotypes in colorectal cancer. In this respect, an inverse association between intake of methyl donor nutrients and colorectal adenomas or colorectal cancer was more pronounced in subjects with the *MTHFR* 677TT genotype (19,20,75,78,86-88). One study reported that a protective effect of high folate intake against colorectal adenoma was present only among individuals with *MTHFR* 1298AA or AC genotypes (76). However, literature on the other folate metabolizing enzymes and their potential interactions with methyl donor intake is limited. An interaction between folate and vitamin B6 and the *MTRR* A66G polymorphism has been suggested in one study (21).

Interestingly, folate intake or folate status and *MTHFR* C677T polymorphisms may also interact to affect methylation status. It has been observed that the *MTHFR* 677TT variant was associated with hypomethylation in blood cells of subjects with low folate status (89). Moreover, folate intake was inversely associated with *MGMT* hypermethylation (90) or MSI (91) in colorectal cancer among *MTHFR* 677TT

homozygotes, and inversely associated with CIMP among carriers of the rare allele of *MTHFR* A1298C (79).

DNA methyltransferases

Whereas folate metabolizing enzymes are involved in the provision of methyl groups, DNA methyltransferases catalyze the transfer of these methyl groups from S-adenosylmethionine into CpG dinucleotides of DNA (Figure 1, Panel B). The DNA methyltransferase 3b (DNMT3b) is involved in *de novo* methylation (92), and several experimental studies suggested that DNMT3b depletion can reduce promoter CpG island hypermethylation (93-95). Moreover, DNMT3b expression was associated with *p16* and *RASSF1A* promoter methylation in non-small cell lung cancer (96), and with a promoter hypermethylator phenotype in breast cancer (97).

The *DNMT3b* C>T (rs2424913) polymorphism was found to significantly increase enzymatic DNMT3b activity, and was associated with an increased risk of lung cancer (98), prostate cancer (99), colorectal polyps including colorectal adenomas (100) or with prognosis of head and neck cancer (101). However, the association between this polymorphism and overall colorectal cancer, or with a hypermethylation phenotype, has not previously been investigated.

Histone methyltransferases

Methylation of histones plays a critical role in maintaining epigenetic silencing by promoter hypermethylation of genes involved in colorectal cancer (102). It has been hypothesized that DNA methyltransferases such as DNMT3b, may only have an effect on the chromatin if histone tails are first methylated by histone methyltransferases (Figure 1, Panel B), and that DNA methylation may thus depend on the activity of histone methyltransferases (103).

The retinoblastoma protein (Rb) interacting zinc finger gene (*RIZ* or *PRDM2)* is a histone methyltransferase which may act as a tumor suppressor and *PRDM2* frame shift mutations have been observed in colorectal tumors with MSI (104). Polymorphisms in *PRDM2* were inversely associated with lung cancer (105) but associated with increased risk of breast cancer (106). Euchromatin Histone Methyltransferase-1 (*EHMT1*) and -2 (*EHMT2*) are other histone methyltransferases, and genetic variants of these genes were modestly associated with breast cancer risk in a large case-control study (106). However, the potential impact of genetic variants of *PRDM2*, *EHMT1* and *EHMT2* have not previously been studied in relation to colorectal cancer, and is addressed in the present thesis.

The aim in this study was to determine the occurrence of polymorphisms in genes encoding folate metabolizing enzymes (*MTHFR, MTR* and *MTRR*), the DNA methyltransferase *DNMT3b*, and histone methyltransferases (*EHMT1, EHMT2* and *PRDM2*). We estimated associations of these polymorphisms with overall colorectal cancer risk and with tumors with or without CIMP, *MLH1* hypermethylation or MSI (chapter 6). Moreover, we investigated whether the effect of methyl donor intake on these hypermethylation-associated endpoints may be modified by genetic variants of folate metabolizing enzymes, DNA methyltransferase 3b or histone methyltransferases (chapter 7).

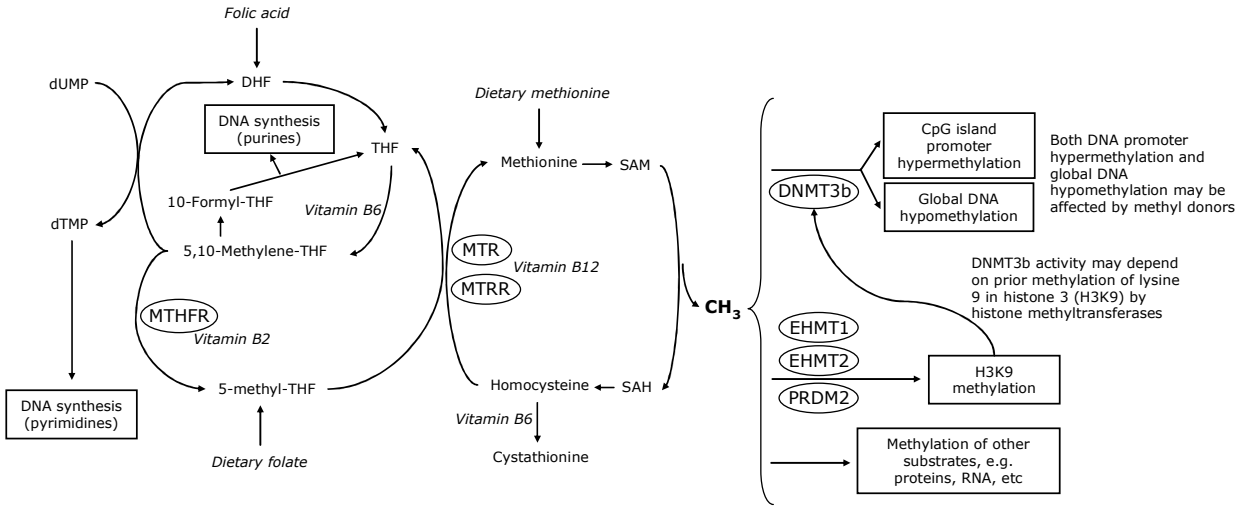

Figure 1. Folate metabolizing enzymes, DNA methyltransferases, histone methyl transferases, DNA synthesis and DNA methylation

Ovals represent the enzymes of which SNPs are investigated in this study. dUMP: deoxyuridine monophosphate, dTMP: deoxythymidine monophosphate, DHF: dihydrofolate, THF: tetrahydrofolate, FAD: flavine adenine dinucleotide, SAM: S-adenosyl methionine, SAH: S-adenosyl homocysteine, MTHFR: methylene tetrahydrofolate reductase, MTR: methionine synthase, MTRR: methionine synthase reductase, DNMT3b: DNA methyltransferase 3b, PRDM2: PR domain 2, EHMT: euchromatine histone methyltransferase, H3K9: lysine 9 of histone 3.

The Netherlands Cohort Study on diet and cancer (NLCS)

All studies described in this thesis were conducted within the NLCS, which is a population-based prospective cohort study, initiated in 1986 (107). The NLCS includes 120,852 men and women who filled out a self-administered food frequency questionnaire at baseline to obtain dietary information and other risk factors for cancer (Figure 2). The cohort was followed-up for cancer occurrence. A subcohort of 5,000 subjects was randomly selected after baseline exposure measurement, to estimate accumulation of person-time in the cohort through biennial follow-up of vital status. Overall colorectal cancer was the endpoint in chapter 3, and could be investigated among 2,349 cases from a follow-up period of 13.3 years after baseline. Subcohort members still alive in the year 2000 were asked to collect mouth swabs, from which DNA was extracted and used for genotyping. Tumor material was collected of colorectal cancer patients identified within the first 7.3 years after baseline. In total, there were 734 patients of whom sufficient DNA could be extracted for genotyping and other molecular analyses.

Outline of this thesis

A priori hypotheses

Two hypotheses play a central role in the work presented here. Since adequate folate status is important for sufficient nucleotide synthesis and DNA stability, it may prevent the introduction of gene mutations and thereby protect against carcinogenesis. Therefore, we hypothesized that high folate intake is inversely associated with colorectal tumors harboring gene mutations.

Secondly, sufficient bioavailability of methyl groups may reduce aberrant CpG island promoter hypermethylation. We therefore hypothesized that high intakes of dietary methyl donors and of other B-vitamins involved in the folate metabolism are inversely associated with CpG island promoter hypermethylation in colorectal cancer.

Both of these hypotheses indicate that adequate intake of folate and other methyl donors may contribute to preventing colorectal cancer.

Overview of research questions

Figure 3 provides an overview of the research questions with references to chapters in which the investigated associations are reported. In chapter 2, we investigated associations between *MLH1* promoter methylation and *APC*, *KRAS* and *BRAF* mutations, and between *MGMT* promoter methylation and G:C>A:T mutations in *KRAS* and *APC*. The associations between dietary folate, methionine, vitamin B2 and vitamin B6 with overall colorectal cancer risk are presented in chapter 3. Whether folate intake is associated with *APC* mutations in colorectal cancer was investigated in chapter 4. In addition, we investigated the associations between dietary folate, methionine, vitamins B2 and B6 with *MLH1* hypermethylation, MLH1 expression, MSI and *BRAF* mutations in chapter 5 and with CIMP in chapter 7. Chapter 6 addresses the associations between genetic variants of folate metabolizing enzymes *MTHFR*, *MTR* and *MTRR*, the DNA methyltransferase 3b and histone methyltransferases *PRDM2*, *EHMT1* and *EHMT2* with

overall colorectal cancer and with tumors with or without CIMP, *MLH1* hypermethylation or MSI. Finally, in chapter 7 we investigated whether the effect of methyl donor intake on overall colorectal cancer and these hypermethylation-associated endpoints, may be modified by the genetic variants.

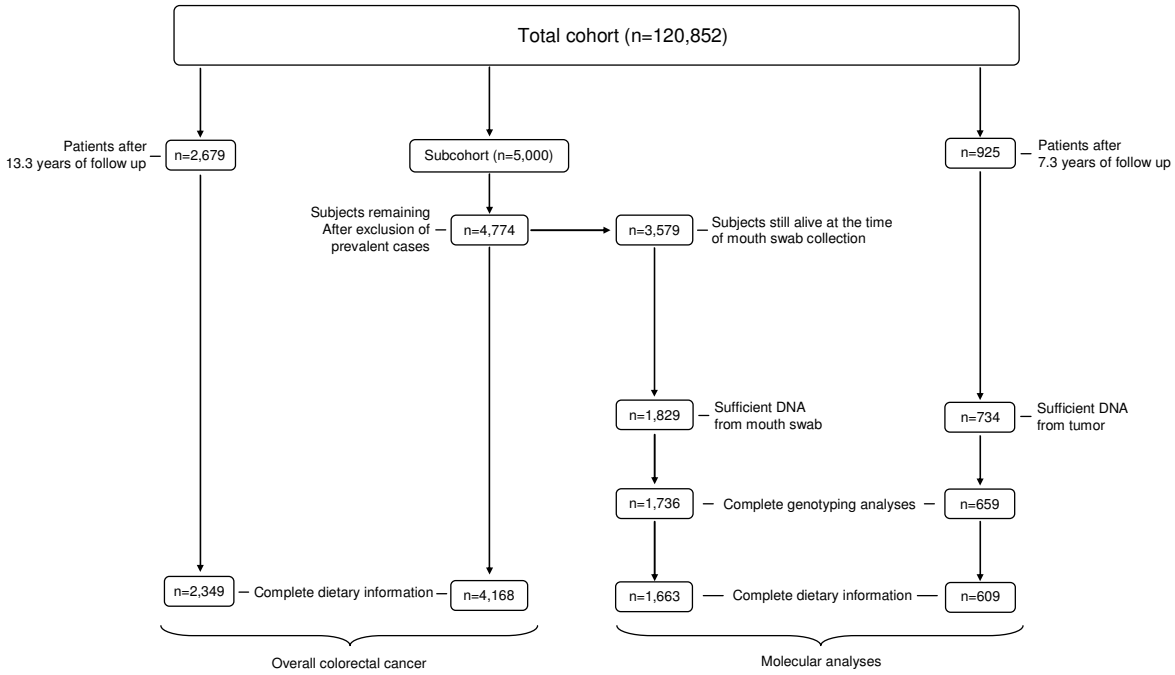

Figure 2. Subcohort members and colorectal cancer patients in the Netherlands Cohort Study on diet and cancer

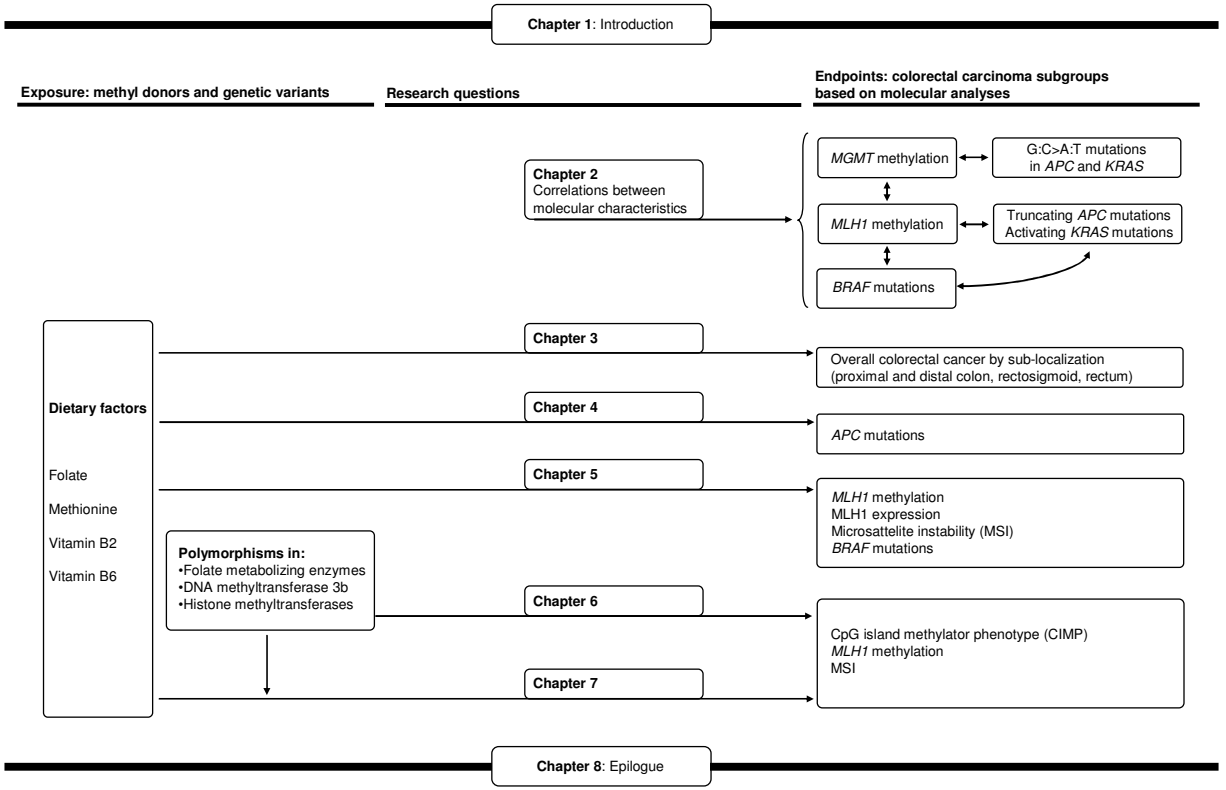

Figure 3 Overview of the research questions in the current thesis

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2

MGMT and *MLH1* promoter methylation versus *APC*, *KRAS* and *BRAF* gene mutations in colorectal cancer: indications for distinct pathways and sequence of events

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Abstract

Background: To study how caretaker gene silencing relates to gatekeeper mutations in CRC, we investigated whether *MGMT* and *MLH1* promoter hypermethylation are associated with *APC, KRAS* and *BRAF* mutations among n=734 CRC patients.

Design: We compared *MGMT* hypermethylation with G:C>A:T mutations in *APC* and *KRAS*, and with the occurrence of such mutations in CpG or non-CpG dinucleotides in *APC*. We also compared *MLH1* hypermethylation with truncating *APC* mutations, and activating *KRAS* and *BRAF* mutations.

Results: Only 10% of the tumors showed both *MGMT* and *MLH1* hypermethylation. *MGMT* hypermethylation occurred more frequently in tumors with G:C>A:T *KRAS* mutations (55%) compared to those without these mutations (38%, $P<0.001$). No such difference was observed for G:C>A:T mutations in *APC*, regardless of whether mutations occurred in CpG or non-CpG dinucleotides. *MLH1* hypermethylation was less common in tumors with *APC* mutations ($P=0.006$) or *KRAS* mutations ($P=0.001$), but was positively associated with *BRAF* mutations ($P<0.001$).

Conclusions: MGMT hypermethylation is associated with G:C>A:T mutations in *KRAS*, but not in *APC*, suggesting that *MGMT* hypermethylation may succeed *APC* mutations but precedes *KRAS* mutations in colorectal carcinogenesis. *MLH1*-hypermethylated tumors harbor fewer *APC* and *KRAS* mutations and more *BRAF* mutations, suggesting that they develop distinctly from an *MGMT*-methylator pathway.

Introduction

In addition to mutations in oncogenes and tumor suppressor genes, epigenetic alterations, including aberrant methylation of DNA repair genes, play an important role in the initiation and development of cancer (1). Although aberrations in these repair genes (also referred to as caretaker genes) do not directly affect cell growth, they can result in a higher rate of mutations in oncogenes and tumor suppressor genes (called gatekeeper genes) (2,3). Promoter hypermethylation inhibits the expression of DNA repair genes, occurs in early stages of cancer development and possibly even precedes somatic mutations in the initiation of colorectal cancer (CRC) (4-6). Hypermethylation of O^6-methylguanine DNA methyltransferase (*MGMT*) for example, has been observed in normal appearing colorectal tissue adjacent to tumor tissue, which suggests that *MGMT* methylation may occur prior to mutations in other key genes early in the multi-step process of colorectal carcinogenesis (7,8). The O^6-MGMT enzyme prevents G:C>A:T point mutations by removing alkyl adducts from the O^6 position of guanine (9), and it was suggested that *MGMT* hypermethylation may lead to G:C>A:T mutations in *KRAS* and *TP53* (10,11) and that loss of MGMT may lead to such mutations in *PIK3CA* (12). Although an association of G:C>A:T *APC* mutations with *APC* hypermethylation and concurrent hypermethylation of *MGMT* was previously suggested (13), the relation between *MGMT* hypermethylation exclusively and G:C>A:T mutations in the *APC* gene has not previously been studied.

Promoter hypermethylation of the mismatch repair gene Human Mut-L Homologue 1 (*MLH1*) is associated with microsatellite instability and *BRAF* mutations in CRC (14-18), and also likely to be an early event in colorectal carcinogenesis (5,19). It was previously observed that microsatellite instable tumors may harbor fewer aberrations in *KRAS*, *TP53* or *APC*, indicating that these tumors develop through a distinct pathway (20-23). In this respect, an interesting recent observation was that *MGMT* and *MLH1* promoter hypermethylation were mutually exclusive (24). These results also indicate the existence of several distinct pathways in CRC, although the sample size used in that study was relatively small.

Because epigenetic silencing of caretaker genes may result in gatekeeper gene mutations, we aimed to investigate whether an association exists between *MGMT* hypermethylation and G:C>A:T *APC* mutations, and to study how this relates to the association between *MGMT* hypermethylation and G:C>A:T *KRAS* mutations. We also assessed whether *MLH1* promoter methylation is associated with mutations in *APC*, *KRAS*, and *BRAF* in a large cohort of incident sporadic CRC patients.

Methods

Study population and tissue samples

Tissue material was obtained from incident CRC patients from the Netherlands Cohort Study on diet and cancer (NLCS), which has been described in detail elsewhere (25). Briefly, this prospective cohort study was initiated in September 1986 and includes 58,279 men and 62,573 women aged 55-69 years and free of disease at baseline, who originated from 204 Dutch municipalities with computerized population registers. The

entire cohort is monitored for cancer occurrence by annual record linkage to the Netherlands Cancer Registry (NCR, comprising nine cancer registries in The Netherlands) and to PALGA (Pathologisch Anatomisch Landelijk Geautomatiseerd Archief), a nationwide network and registry of histopathology and cytopathology reports (26,27).

The PALGA database was used to identify and locate tumor tissue in Dutch pathology laboratories. CRC was classified according to disease site as follows: colon, i.e. proximal and distal colon, rectosigmoid and rectum. At baseline, all cohort members filled out a self-administered food-frequency questionnaire through which information about age, sex and family history of colorectal cancer was also obtained.

Tumor material of the CRC patients was collected after approval by the ethical review boards of Maastricht University, the NCR and PALGA. In addition, all 54 pathology laboratories in the Netherlands agreed to make relevant tissue samples available from PALGA upon request. These tissue samples were retrieved between August 1999 and December 2001, as described previously (28).

In total, 734 incident CRC patients were identified from a follow-up period of 7.3 years after baseline, of whom a PALGA report of the lesion as well as sufficient DNA was available (28).

Promoter methylation analyses

DNA methylation in the CpG islands of the *MGMT* and *MLH1* gene promoters was determined by chemical modification of genomic DNA with sodium bisulfite and subsequent Methylation Specific PCR (MSP) as described in detail elsewhere (29). In brief, 500 ng of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega), again treated with NaOH, precipitated with ethanol and resuspended in H_2O.

To facilitate MSP analysis on DNA retrieved from formalin-fixed, paraffin-embedded tissue, DNA was first amplified with flanking PCR primers that amplify bisulfite modified DNA but do not preferentially amplify methylated or unmethylated DNA. The resulting fragment was used as a template for the MSP-reaction (30,31).

All PCRs were performed with controls for unmethylated alleles (DNA from normal lymphocytes), methylated alleles (normal lymphocyte DNA treated in vitro with SssI methyltransferase (New England Biolabs)) and a control without DNA. Ten µl of each MSP reaction was directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide and visualized under UV illumination. MSP analyses were successfully performed for 693 patients for the *MGMT* promoter and for 686 patients for the *MLH1* promoter.

Gene mutation analyses

Mutation analyses of the mutation cluster region (MCR) in *APC* (codons 1286-1520), was performed as described previously (32). Briefly, nested PCR was used to amplify the MCR in four overlapping DNA fragments and the purified fragments were sequenced. Two observers independently evaluated the sequence patterns and data entry. One or more fragments of the MCR could not be analyzed completely from 72 CRC patients, leaving 662 patients with complete information on *APC* mutation status for data analysis. Mutations that lead to the introduction of a stop codon (truncating mutations) subsequently lead to functional loss of the *APC* protein. Such functional *APC*

mutations were observed in 37% of CRCs (32) and are considered in the current study, as well as frameshift mutations in *APC*. Exon 1 (codons 12 and 13) of the *KRAS* oncogene was analyzed successfully for all 734 patients using nested PCR, followed by direct sequencing of purified fragments, as previously described (28).

G:C>A:T mutations (being G>A and/or C>T mutations) in either of the two genes were also considered, irrespective of the consequence of the mutation. It is important to note that in *APC*, these mutations may occur via two different chemical transformations depending on the orientation of the mutated cytosine or guanine. Methylated cytosines in the context of a CpG dinucleotide are prone to spontaneous deamination (33), whereas non-CpG cytosines are more likely to mutate by alkyl adduct formation (Figure 1).

We also included mutations of the *BRAF* gene in our study, and for this purpose, the common V600E *BRAF* mutation was analyzed as previously described in detail (34).

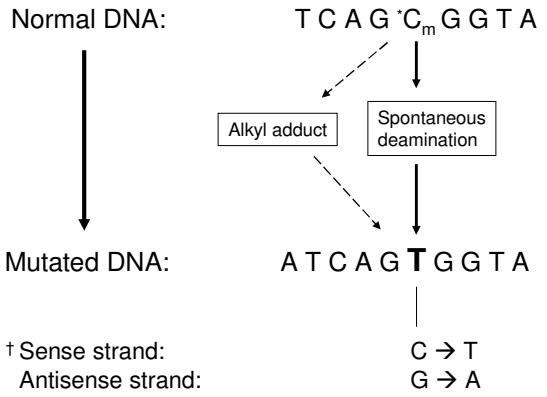

Figure 1. G:C>A:T transitions caused by deamination and possibly alkylation in CpG dinucleotides

* Methyl cytosine with neighboring guanine that mutates to thymine primarily by spontaneous deamination and possibly by alkyl adduct formation.

† C>T mutations with corresponding G>A mutations on the other DNA strand

Statistical analyses

Data analyses were conducted separately for CRCs with or without promoter hypermethylation of *MGMT* and *MLH1*. First, we evaluated the association between *MGMT* and *MLH1* hypermethylation. Then, we compared the prevalence of *MGMT* hypermethylation with G:C>A:T point mutations in *KRAS*, or in *APC* (irrespective of leading to a truncation or not), and with the occurrence of *BRAF* mutations. We also determined whether the percentage of *MGMT* hypermethylation differed between G:C>A:T mutations in *APC* that occur in either CpG or non-CpG dinucleotides.

The prevalence of *MLH1* hypermethylation was compared with mutations that lead to functional alterations of *APC*, *KRAS* and *BRAF*, i.e. truncating and frameshift *APC* mutations, activating *KRAS* mutations and V600E *BRAF* mutations. In addition, we assessed the relation between *BRAF* mutations and truncating *APC* mutations and

activating *KRAS* mutations. Potential differences in prevalence or differences in patient characteristics were tested by Chi-square tests or t-tests where appropriate.

All statistical analyses were performed with the STATA statistical software package (intercooled STATA, version 9.1).

Results

Overall, hypermethylation of *MGMT* and *MLH1* was observed in 41% and 22% of the patients respectively (Table 1). We observed that hypermethylation of both *MGMT* and *MLH1* occurred in only 66 (10%) of the patients with available MSP analyses. Women more often showed *MLH1* hypermethylation (26%) compared to men (19%, P=0.03), whereas no sex-difference existed for *MGMT* hypermethylation (Table 1). In addition, patients less frequently had a positive family history of colorectal cancer when they showed hypermethylation in *MGMT* or *MLH1*, although these differences were only borderline statistically significant. However, this difference was significant when considering patients with hypermethylation in either of the genes, since a family history of colorectal cancer was observed in 8% of the patients with *MGMT* and/or *MLH1* methylation compared to 13% among patients showing no methylation (P=0.03, data not shown). Whereas *MGMT* hypermethylation was independent of tumor sub-localization, we observed that *MLH1* hypermethylation occurred more often in proximal colon tumors (35%) compared to distal colon (16%), rectosigmoid (16%) and rectal tumors (13%, p<0.001). All other characteristics presented in Table 1 did not substantially differ between cases with or without *MGMT* and *MLH1* hypermethylation.

The relation between *MGMT* hypermethylation and the occurrence of G:C>A:T mutations in *KRAS* and *APC* is shown in Table 2. Tumors with G:C>A:T *KRAS* mutations showed a higher percentage of *MGMT* hypermethylation (55%) compared to those without G:C>A:T point mutations (38%, P<0.001). The percentages of *MGMT* hypermethylation in tumors with or without G:C>A:T mutations in *APC* did not differ significantly. Since G:C>A:T mutations in *APC* may also occur via spontaneous deamination when occurring in CpG dinucleotides, we stratified *MGMT* hypermethylation according to the orientation of these mutations. However, we then observed that the frequency of *MGMT* hypermethylation was similar in tumors with G:C>A:T *APC* mutations in either CpG or non-CpG dinucleotides.

Table 1. Comparison of colorectal cancer patients with or without *MGMT* and *MLH1* promoter hypermethylation

	Promoter hypermethylation *							
	MGMT				*MLH1*			
	Methylated		Unmethylated		Methylated		Unmethylated	
Number of patients (%)	287	(41%)	406	(59%)	152	(22%)	534	(78%)
Age at diagnosis (yr, mean (SD))	68.0	(4.3)	67.9	(4.3)	68.0	(4.4)	68.0	(4.2)
P-value ‡			*0.64*				*0.76*	
Sex								
Men	156	(41%)	229	(59%)	73	(19%)	309	(81%)
Women	131	(43%)	177	(57%)	79	(26%)	225	(74%)
P-value			*0.59*				*0.03*	
Family history of colorectal cancer (% yes) †	22	(8%)	47	(12%)	10	(7%)	62	(12%)
P-value			*0.10*				*0.08*	
Tumor sub-localization †								
Proximal colon	95	(41%)	137	(59%)	80	(35%)	150	(65%)
Distal colon	82	(38%)	132	(62%)	35	(16%)	178	(84%)
Rectosigmoid	37	(46%)	43	(54%)	12	(16%)	64	(84%)
Rectum	67	(42%)	92	(58%)	21	(13%)	138	(87%)
P-value			*0.65*				*<0.001*	
Dukes' stage †								
A	84	(48%)	91	(52%)	36	(21%)	139	(79%)
B	87	(39%)	138	(61%)	52	(23%)	172	(77%)
C	72	(41%)	105	(59%)	44	(25%)	133	(75%)
D	32	(40%)	48	(60%)	14	(18%)	62	(82%)
P-value			*0.28*				*0.63*	
Differentiation †								
Good	27	(40%)	40	(60%)	12	(18%)	55	(82%)
Moderate	174	(40%)	257	(60%)	86	(20%)	340	(80%)
Poor	41	(41%)	58	(59%)	31	(32%)	67	(68%)
Undifferentiated	2	(29%)	5	(71%)	2	(29%)	5	(71%)
P-value			*0.93*				*0.07*	

* Complete data on *MGMT* and *MLH1* hypermethylation were available for 693 and 686 out of 734 patients, respectively. 66 patients had hypermethylation of both *MGMT* and *MLH1*

† Data on family history of colorectal cancer, tumor sub-localization, Dukes' stage and differentiation were unavailable for 3, 9, 38 and 93 patients respectively

‡ P-value for the difference between the Methylated and Unmethylated groups

Table 2. *MGMT* Promoter hypermethylation versus G:C>A:T mutations in *KRAS* and *APC*

		MGMT promoter hypermethylation *			
		Methylated		Unmethylated	
KRAS †	G:C>A:T mutation	79	(55%)	64	(45%)
	No G:C>A:T mutation	208	(38%)	342	(62%)
	P-value			*<0.001*	
APC ‡	G:C>A:T mutation §	135	(41%)	198	(59%)
	No G:C>A:T mutation	127	(42%)	177	(58%)
	P-value			*0.75*	
APC	G:C>A:T mutation – CpG ¶, ††	17	(44%)	22	(56%)
	G:C>A:T mutation – non-CpG **, ††	88	(39%)	135	(61%)
	P-value			*0.63*	

* Complete data on *MGMT* promoter hypermethylation were available for 693 out of 734 patients

† *KRAS* mutation analysis succeeded for all 734 patients

‡ *APC* mutation analyses were available for 662 out of 734 patients

§ G>A with matching C>T point mutation at similar loci in both DNA strands

¶ G:C>A:T mutation in a CpG dinucleotide: the cytosine or guanine that mutated had a neighbouring guanine or cytosine, respectively

** G:C>A:T mutation in non-CpG dinucleotide: the cytosine or guanine that mutated did not have a neighbouring guanine or cytosine, respectively

†† 79 (21%) of 379 patients with G:C>A:T *APC* mutations had more than one such a mutation that did not consistently occur in CpG or non-CpG dinucleotides

We also assessed the relation between *MLH1* hypermethylation and the occurrence of truncating or frameshift *APC* mutations, or activating *KRAS* or *BRAF* mutations. We observed less *MLH1* hypermethylation in patients with a truncating *APC* mutation (17%) compared to patients without such a mutation (26%, *P*=0.006, Table 3). A similar contrast could be observed for *APC* frameshift mutations, since *MLH1* methylation occurred in 15% of the tumors with an *APC* frameshift mutation, versus 25% in tumors without frameshift mutation in *APC* (*P*=0.01). Less *MLH1* hypermethylation was also observed in patients with activating *KRAS* mutations compared to cases without these mutations (15% and 26% respectively, *P*=0.001). Conversely, a strong positive association was present between *MLH1* hypermethylation and activating *BRAF* mutations, since 47% of the tumors with *BRAF* mutations showed *MLH1* hypermethylation compared to 17% of *MLH1* hypermethylation in tumors harboring wild type *BRAF* (*P*<0.001).

Table 3. Promoter hypermethylation of *MLH1* versus mutations in *APC*, *KRAS* or *BRAF*

		***MLH1* promoter hypermethylation** *			
		Methylated		**Unmethylated**	
APC (truncation) †	Mutation	40	(17%)	195	(83%)
	No mutation	106	(26%)	295	(74%)
	P-value		*0.006*		
APC (frameshift mutation)	Mutation	19	(15%)	110	(85%)
	No mutation	127	(25%)	375	(75%)
	P-value		*0.01*		
KRAS (activating mutation) ‡	Mutation	40	(15%)	218	(85%)
	No mutation	112	(26%)	316	(74%)
	P-value		*0.001*		
BRAF (activating mutation) §	Mutation	51	(47%)	58	(53%)
	No mutation	97	(17%)	462	(83%)
	P-value		*<0.001*		

* Complete data on *MLH1* promoter hypermethylation was available for 686 out of 734 patients
† *APC* mutation analyses were available for 662 out of 734 patients
‡ *KRAS* mutation analysis succeeded for all 734 patients
§ *BRAF* mutation analysis succeeded for 697 out of 734 patients

Finally, we observed that *BRAF* mutations were more common in tumors without *APC* mutations, or without *KRAS* mutations (*P*<0.001 for both associations respectively, Table 4). No association was observed between *BRAF* mutations and *MGMT* promoter methylation (data not shown).

Table 4. Comparison of *BRAF* mutations with mutations in *APC* and *KRAS*

		V600E *BRAF* mutation *			
		Mutation		**No mutation**	
APC (truncation) ‡	Mutation	21	(8%)	227	(92%)
	No mutation	91	(20%)	358	(80%)
	P-value		*<0.001*		
KRAS (activating mutation) §	Mutation	13	(5%)	244	(95%)
	No mutation	99	(23%)	341	(77%)
	P-value		*<0.001*		

* *BRAF* mutation analyses were available for 697 out of 734 patients
† Complete data on *MLH1* and *MGMT* promoter hypermethylation were available for 693 and 686 out of 734 patients, respectively
‡ *APC* mutation analyses were available for 662 out of 734 patients
§ *KRAS* mutation analysis succeeded for all 734 patients

Discussion

Here we studied the prevalence of promoter hypermethylation of the DNA repair genes *MGMT* and *MLH1* in relation to somatic mutations in *APC*, *KRAS* and *BRAF* in colorectal carcinogenesis, in a large group (n=734) of unselected, incident colorectal cancer patients. *MGMT* hypermethylation was associated with G:C>A:T *KRAS* mutations. However, there was no association between *MGMT* hypermethylation and G:C>A:T mutations in *APC*, irrespective of the occurrence of these mutations in CpG or non-CpG dinucleotides. This indicates that G:C>A:T *APC* mutations are independent of, and possibly occur prior to *MGMT* hypermethylation. Whereas there was a positive association between *MLH1* hypermethylation and *BRAF* mutations, it was inversely associated with *APC* mutations and *KRAS* mutations.

These results support the hypothesis that *MGMT* hypermethylation may lead to somatic mutations in *KRAS*, and that functional loss of *MGMT* may occur early in colorectal carcinogenesis (8). This is the first study in which the association between *MGMT* methylation and G:C>A:T *APC* mutations was investigated. A possible explanation for the lack of association between these two events may be the orientation of cytosines or guanines, which may "mask" a potential association between *MGMT* methylation and G:C>A:T mutations. If these mutations occur in CpG dinucleotides, they may also result from spontaneous deamination (33), whereas alkyl adduct formation is more likely the primary cause in non-CpG dinucleotides. *MGMT* removes alkyl adducts from the O^6 position of guanine, and it was previously observed that G:C>A:T mutations are repaired less effectively via *MGMT* when they occur in CpG dinucleotides (35). Moreover, it was also observed that *MGMT* hypermethylation occurred less frequently in tumors with G:C>A:T *P53* mutations in CpG dinucleotides compared to such mutations in non-CpG dinucleotides (10). In order to investigate whether this difference could explain the lack of association with G:C>A:T *APC* mutations in our study, we stratified the analyses for those mutations occurring in CpG or non-CpG dinucleotides. However, stratification did not reveal a difference in *MGMT* methylation between these two types of G:C>A:T *APC* mutations, and this therefore suggests that the association does not exist. *MGMT* hypermethylation in combination with hypermethylation of *APC* or *MLH1* may be associated with G:C>A:T *APC* mutations (13). However, in that study the association was not studied for *MGMT* hypermethylation exclusively, and the conclusion was based on rather small numbers of cases. Moreover, Halford et al previously investigated the association between O^6-MGMT protein expression and G:C>A:T *APC* mutations (36). Although the investigators demonstrated a weak association with G:C>A:T mutations in the combination of *APC*, beta-catenin, *KRAS* and *P53*, no association was observed with G:C>A:T mutations in *APC* exclusively.

In view of these results, a plausible explanation is to assume that in colorectal carcinogenesis, truncating *APC* mutations may occur prior to epigenetic silencing of *MGMT* by promoter hypermethylation. Mutations in *KRAS* are viewed as occurring in a later stage of colorectal carcinogenesis than *APC* mutations, and we observed an association between *MGMT* hypermethylation and *KRAS* mutations. Esteller et al previously observed a similar association between *MGMT* hypermethylation and G:C>A:T mutations in *KRAS* (11). Moreover, an association of *MGMT* hypermethylation with these mutations in the *P53* gene, which usually occur in a late stage of colorectal

carcinogenesis (10), was observed. We propose that the sequence of events in the multi-step model of colorectal carcinogenesis as proposed by Fearon and Vogelstein (37), may include *MGMT* methylation as depicted in Figure 2. It should be noted, however, that the molecular endpoints studied in tumor tissue are cross-sectional data, and can therefore only provide indirect evidence for a potential sequence of events. Nevertheless, such data provide more insight in colorectal carcinogenesis, and may be an important source for new hypotheses about the possible timing of molecular events.

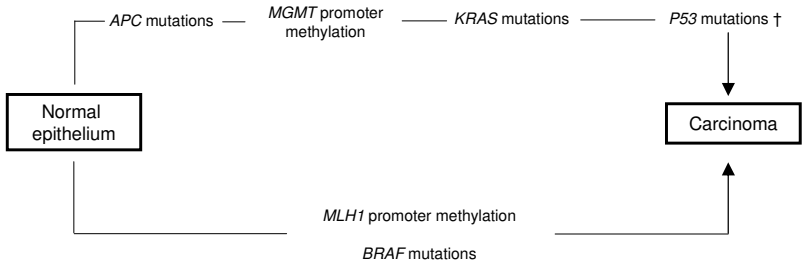

Figure 2. *MGMT* and *MLH1* promoter methylation and mutations in *APC*, *KRAS* and *BRAF* in the carcinogenesis of sporadic colorectal cancer *

* Adapted from Fearon and Vogelstein, Cell 1990. Adenomas were not analyzed in the study and were therefore omitted from this model
† P53 mutation status was not analyzed in this study

A second observation in the current study was that *MLH1* hypermethylation was related to lower frequencies of somatic mutations in *APC* and *KRAS*, which suggests that such tumors develop through a distinct genetic pathway. This is in line with the observed high correlation between *MLH1* methylation and microsatellite instability in the colorectum (15-17) and its inverse relationship with mutations in other key genes involved in colorectal carcinogenesis (20-23). In addition, we have previously observed a strong positive association between *MLH1* methylation and MSI, and that *APC* and *KRAS* mutations are rare in MLH1 deficient tumors among CRC patients included in the current study (18,38). Moreover, strong associations between *MLH1* hypermethylation, MSI and *BRAF* mutations were previously observed (14). In this respect, we demonstrated that *BRAF* mutated tumors were strongly associated with *MLH1* hypermethylation, and moreover, that these tumors also harbored less mutations in *APC* and *KRAS*. These observations confirm previous results showing that *BRAF* mutations were inversely associated with *KRAS* mutations and with *APC* hypermethylation (39,40). On the other hand, *MGMT* promoter hypermethylation was not associated with *BRAF* mutations and our results as well as observations of a smaller study (24) show that concurrent promoter hypermethylation of *MLH1* and *MGMT* is rare. We therefore suggest that these are additional indications showing that tumors with epigenetic silenced *MGMT* may arise through a distinct hypermethylation - associated pathway.

Patients with *MGMT* and/or *MLH1* hypermethylation less often had a positive family history of colorectal cancer but there were no age differences between patients with, or without hypermethylation. This suggests that in these tumors, exogenous factors may

have played a larger role in colorectal carcinogenesis compared to tumors that do not develop through a hypermethylated pathway.

We conclude that *MGMT* inactivation by hypermethylation may cause somatic mutations in *KRAS*, but not in *APC*. This suggests that in the process of colorectal carcinogenesis, *MGMT* hypermethylation may succeed the introduction of *APC* mutations, but precedes *KRAS* mutations. Tumors with *MLH1* inactivation by hypermethylation seem to develop through a distinct pathway associated with fewer *APC* and *KRAS* mutations, and this pathway is possibly distinct from an *MGMT*-methylator pathway. Colorectal carcinogenesis may be more susceptible to the influence of exogenous factors when hypermethylation plays an important role.

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3

Dietary folate, methionine, riboflavin and vitamin B6 and risk of sporadic colorectal cancer

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Abstract

Adequate intake of folate, methionine, riboflavin and vitamin B-6 may prevent aberrant DNA methylation and thereby protect against colorectal cancer (CRC). However, previous epidemiological studies investigating associations between dietary intakes of these nutrients and CRC have been inconsistent. We investigated the associations between intakes of folate, methionine, riboflavin and vitamin B-6 and CRC risk, accounting for the sub-localization of the tumor.

Within the Netherlands Cohort Study on diet and cancer (n=120,852), 2,349 cases and 4,168 subcohort members were available for data analyses from a follow-up period of 13.3 y after baseline. Gender-specific adjusted incidence rate ratios (RR) were calculated over quintiles of dietary intake in case-cohort analyses.

Folate intake was not associated with CRC risk in either men or women. However, methionine was associated with decreased risk of proximal colon cancer among men (RR=0.57 for highest versus lowest quintile of intake, P_{trend}=0.03) and rectal cancer among women (highest vs. lowest quintile: RR=0.45, P_{trend}=0.05). Riboflavin was non-significantly associated with decreased proximal colon cancer risk among women (RR=0.61, P_{trend}=0.07). Conversely, there was a strong positive association between vitamin B-6 and rectal cancer among women (RR=3.57, P_{trend}=0.01).

Our findings suggest that relatively high methionine intake may protect against proximal colon cancer in men and rectal cancer in women, but that folate may not have a protective effect. This is the second prospective cohort study in which vitamin B-6 intake was associated with increased risk of rectal tumors in women, which might suggest that this vitamin enhances rectal cancer in women.

Introduction

It has been proposed that folate may affect colorectal carcinogenesis, because of its role in the synthesis of nucleic acid and DNA methylation (1). Folate deficiency may result in uracil mis-incorporation in DNA which possibly leads to DNA instability and gene alterations. It may also cause aberrant DNA methylation such as CpG promoter hypermethylation or global hypomethylation and which in turn may contribute to colorectal carcinogenesis. For example, hypermethylation of gene promoters is known to result in silencing of tumor suppressor genes and DNA repair genes, which in turn may enhance carcinogenesis (2). Therefore, it may be hypothesized that adequate folate levels possibly protect against CRC. However, epidemiological studies on the relation between dietary folate intake and colorectal cancer risk have not consistently shown a protective effect of high folate intake (3,4). Also, it has been suggested that the effect of folate supplementation may depend on the stage of colorectal carcinogenesis, i.e. it would protect against carcinogenesis in normal colorectal tissue, but might enhance already existing lesions (5,6). In this respect, the relation between dietary folate and the etiology of colorectal cancer continues to be the subject of debate.

Like folate, methionine is required for the synthesis of S-adenosylmethionine, which is the universal methyl group donor needed for methylation processes (7). Adequate intakes of folate or methionine through the diet ensure a sufficient supply of methyl groups, and it may be hypothesized that this also prevents aberrant DNA methylation. Other B-vitamins such as riboflavin and vitamin B-6 are involved in the folate-mediated 1-carbon metabolism, and may therefore modulate the bioavailability of methyl groups. Riboflavin, as flavin adenine dinucleotide (FAD), is the cofactor for methylenetetrahydrofolate reductase (MTHFR), the enzyme that influences homocysteine (tHcy) remethylation and DNA methylation. Low riboflavin status was previously observed to be associated with increased tHcy concentration, which possibly results in lower availability of methyl groups (8). In addition, vitamin B-6 is involved in the conversion of tetrahydrofolate into 5,10-methylenetetrahydrofolate, one of the steps of the folate cycle (7). On the other hand, high alcohol intake potentially reduces the bioavailability of folate and such a disruption of the one-carbon metabolism may affect DNA methylation in for example the colonic mucosa (9-11).

The associations between dietary intakes of riboflavin, vitamin B-6 and methionine have been investigated in a number of studies. However, whereas a potential protective effect of riboflavin intake on colorectal adenomas was observed in one study (12) others have not demonstrated an association with CRC at all (13,14). Similarly, relatively high methionine intake did not seem to protect against CRC (13,15-17). Several studies demonstrated an inverse association between vitamin B-6 intake and CRC (17-21), although in one study, a positive association was observed between the vitamin and rectal tumors among women (16). There may be a number of reasons for these inconsistencies between studies, such as the danger of selection bias and possibly recall bias that could have played a larger role in case-control studies. In this respect, some of the studies had a prospective cohort design (15,16,18-21) whereas others were case-control studies (12-14,17). Moreover, some studies were conducted among women only (15,16,19-21) or in relation to colorectal adenomas (12), and there are considerable differences in the level of vitamin intake between studies.

The relation between folate and colorectal cancer is still controversial and previous studies on associations between folate, B-vitamins and methionine with CRC show inconsistencies. This prompted us to investigate the combination of folate, riboflavin, vitamin B-6 and methionine in a prospective setting. The study was conducted among incident CRC patients and subcohort members of the Netherlands Cohort Study on diet and cancer (NLCS), which is a large population-based study with a prospective case-cohort design.

Subjects and methods

Study population

The participants in this study are incident colon and rectal cancer patients and subcohort members from the NLCS, which has been described in detail elsewhere (22). Briefly, the study was initiated in September 1986 and includes 58,279 men and 62,573 women aged 55-69 y at baseline, who originated from 204 Dutch municipalities with computerized population registers. At baseline, participants completed a self-administered food-frequency questionnaire that also provided information about age, sex and other risk factors for cancer. The entire cohort is being monitored for cancer occurrence by annual record linkage to the Netherlands Cancer Registry (NCR, nine cancer registries in The Netherlands). Accumulation of person-time in the cohort has been estimated through biennial follow-up of vital status in a subcohort of 5,000 men and women who were randomly selected after baseline exposure measurement. Cases with prevalent cancer other than non-melanoma skin cancer were excluded from this subcohort, which left 4,774 men and women eligible for analysis. CRC was classified according to disease site as follows: colon, i.e. proximal colon (ICD-O-1 codes 153.0, 153.1, 153.4, 153.5, 153.6) and distal colon (153.2, 153.3, 153.7), rectosigmoid (154.0), rectum (154.1), or ICD-O-1 codes 153.8 and 153.9 if information of the disease site was not available. Follow-up information was available from a follow-up period up to 13.3 y after baseline, i.e. from September 1986 until January 2000. Within this period, 2,679 CRC patients were identified.

Food frequency questionnaire

The self-administered questionnaire was a 150-item semi-quantitative food frequency questionnaire (FFQ), which concentrated on habitual consumption of food and beverages during the year preceding the start of the study, and also contained questions about body weight and -length, smoking status, physical activity, and family history of colorectal cancer. Daily mean nutrient intakes were calculated as the cumulated product of the frequencies and portion sizes of all food items and their tabulated nutrient contents from the Dutch Food Composition Table (NEVO table, 1986) (23). The questionnaire was validated through comparison with a 9-day diet record (24), and the reproducibility was determined (25). Questionnaire data were key-entered twice for all incident cases in the cohort and for all subcohort members in a blinded manner with respect to case/subcohort status. This was done in order to minimize observer bias in coding and interpretation of the data.

Folate data were derived from a validated liquid chromatography trienzyme method (26) used to analyze the 125 most important Dutch foods contributing to folate intake (27). Mean daily intakes of all other relevant nutrients were calculated using the computerized Dutch Food Composition Table (23). Dietary supplement data were also obtained via the food frequency questionnaire. However, the use of B-vitamin supplements was low (7%) and folic acid was generally not included in these supplements in the Netherlands in the late 1980s. Therefore, folic acid supplement use most likely plays a very minor role in our study population, and supplement use was not further accounted for in the analyses.

Statistical analyses

Analyses were performed for all CRC cases combined and for each sub-localization, i.e. proximal colon, distal colon, rectosigmoid and rectum. Dietary factors and other baseline characteristics were evaluated for men and women separately, for subcohort members and CRC cases by calculating means and standard deviations for continuous variables and distributions of the categorical variables.

Cox proportional hazards regression models were used to estimate gender-specific multivariate-adjusted incidence rate ratios (RR) and corresponding 95% confidence intervals (CI) over quintiles of folate intake, methionine, riboflavin and B-6, using the lowest quintiles as reference. Standard errors of the RR were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling from the cohort (28). Tests for dose response trends over the quintiles of intake were estimated by fitting the ordinal exposure variables as continuous variables and evaluated using the Wald test. To account for potentially non-linear effects due to skewed distributions of folate, methionine, riboflavin and vitamin B-6, we also estimated the associations between the quintile variables and CRC by replacing the quintiles with the median intakes within each quintile and compare these RR with the main analyses. The proportional hazards assumption was tested using the scaled Schoenfeld residuals (29), and by fitting the main determinants as time-dependent variables. Analyses were additionally stratified for the first and second half of the follow up time. Because undetected pre-clinical disease may have affected exposure status, we excluded the first year of follow-up in additional sub-analyses and compared these results with the analyses performed over the full 13.3 y of follow-up. In addition to gender-specific analyses, we also conducted overall analyses for men and women combined.

The dietary variables were adjusted for total energy intake by calculating nutrient residuals from the regression of nutrient intake on total energy intake, as described by Willett et al (30). Such nutrient residuals are uncorrelated with total energy intake, and the effect of the variation in nutrient intake can subsequently be estimated independently of a potential effect of energy intake. Tests for heterogeneity were performed to evaluate differences between sub-localizations of tumors, using the competing risks procedure in Stata. However, the SE for the difference of the logHR from this procedure assumes independence of both estimated HR, which would underestimate that SE and thus overestimate the p-values for their difference. Therefore, these p-values and the associated confidence intervals were estimated based on a bootstrapping method that was developed for the case-cohort design (31). For each bootstrap sample, X subcohort members were randomly drawn from the

subcohort of X subjects and Y cases from the total of Y cases outside the subcohort, both with replacement, out of the dataset of X + Y observations. The logHR were obtained from this sample using Stata's competing risks procedure and recalculated for each bootstrap-replication. The confidence interval and p-value of the differences in hazard ratio of the subtypes were then calculated from the replicated statistics. Each bootstrap analysis was based on 1,000 replications.

First, we estimated the individual associations of dietary folate, methionine, riboflavin and vitamin B-6 with CRC in separate models. However, since the bioavailability of methyl groups donated by folate and methionine may be influenced by riboflavin and vitamin B-6, the effects of these nutrients may not be independent and should then be mutually adjusted for. There were no major differences between the associations of these individual models compared to the multivariate analysis including folate, methionine, riboflavin and vitamin B-6 simultaneously, and it was therefore decided to include these main determinants simultaneously in all further analyses.

The covariates considered as potential confounders were suggested risk factors of CRC and those commonly included as confounders in observational studies on the associations of B-vitamins and CRC (12-21). The covariates included were those to influence the RR of any of the main determinants considerably (by more than 5% when including the variables Body Mass Index (BMI), smoking status, energy, calcium and meat, or even more than 10% for alcohol, iron, fat, fibre). In addition, age and having a family history of CRC may be strong determinants for CRC and were therefore included as well.

We also conducted analyses using models only including the four main determinants and the variable age, and compared the results of these crude results to the multivariate-adjusted analyses. After excluding subjects with missing information on these covariates or subjects who only partly filled out the questionnaire, 4,168 subcohort members and 2,349 colorectal cancer cases remained for statistical analyses.

We determined possible interactions between dietary intakes of folate, methionine, riboflavin, vitamin B-6 and alcohol intake. This was done by first testing, in separate models, the gender specific interaction terms between folate on the one hand, with methionine, riboflavin, vitamin B-6 and alcohol on the other, for all CRC tumors combined and for each of the four sub-localizations. The Cox proportional hazard analyses without the interaction terms were subsequently stratified by low or high intake of folate, methionine, riboflavin and vitamin B-6 using the median intakes as cut-off values to define both strata within each variable. The strata used for alcohol intake were (1) abstainers, (2) subjects with intake <30 g/day, and (3) subjects who consumed ≥30 g/day. All statistical analyses were performed with the Stata statistical software package (version 9.1).

Results

During a period of 13.3 y of follow up, 1,389 men and 960 women with CRC were identified who also had complete information on the co-variates used in the adjusted regression analyses. The mean intakes of the main determinants folate, methionine, riboflavin and vitamin B-6 were generally similar between subcohort members and cases in most of the subgroups among both men and women (Table 1).

Table 1 Baseline dietary intake and other characteristics of subcohort members and cancer cases from the Netherlands Cohort Study on diet and cancer [1]

	MEN						WOMEN					
	Subcohort	All tumors	Proximal colon	Distal colon	Recto-sigmoid	Rectum	Subcohort	All tumors	Proximal colon	Distal colon	Recto-sigmoid	Rectum
Patient characteristics												
n [2]	2090	1389	382	467	141	360	2078	960	386	296	78	176
Age(y)	61.3 ± 4.2	62.0 ± 4.1	62.5 ± 4.0	62.0 ± 4.2	62.0 ± 3.9	61.6 ± 4.0	61.4 ± 4.3	62.6 ± 4.0	62.9 ± 4.0	62.1 ± 4.1	62.8 ± 3.6	62.4 ± 3.8
Family History of CRC (% yes)	5.4	9.3	10.0	9.4	10.6	8.1	6.0	9.8	10.9	9.1	7.7	9.1
BMI (kg/m^2)	24.9 ± 2.6	25.2 ± 2.6	25.1 ± 2.6	25.4 ± 2.8	25.6 ± 2.6	25.1 ± 2.5	25.0 ± 3.5	25.1 ± 3.5	25.3 ± 3.5	25.0 ± 3.4	25.1 ± 3.2	25.0 ± 3.6
Smoking Status (%)												
Never	12.8	11.0	11.5	12.0	9.9	9.4	57.6	59.8	61.4	62.1	51.3	58.5
Ex Smoker	52.6	59.9	57.3	64.5	58.2	57.5	20.8	21.2	20.7	21.0	28.2	19.3
Current Smoker	35.6	29.1	31.1	23.5	31.9	33.1	21.6	19.0	17.9	16.9	20.5	22.2
Dietary factors												
Folate (µg/day)	224.6 ± 66.6	222.1 ± 65.0	222.0 ± 68.1	217.4 ± 54.1	227.7 ± 80.5	225.9 ± 65.7	198.5 ± 61.1	199.1 ± 61.3	196.4 ± 58.2	199.4 ± 62.4	196.5 ± 51.7	204.8 ± 67.7
Methionine (mg/d)	1713 ± 293	1701 ± 277	1696 ± 280	1697 ± 2.70	1714 ± 287	1710 ± 2.71	1489 ± 276	1482 ± 278	1474 ± 2.98	1483 ± 251	1450 ± 250	1512 ± 279
Riboflavin (mg/d)	1.58 ± 0.37	1.55 ± 0.34	1.56 ± 0.35	1.54 ± 0.32	1.56 ± 0.34	1.55 ± 0.34	1.45 ± 0.34	1.43 ± 0.35	1.43 ± 0.35	1.46 ± 0.34	1.33 ± 0.30	1.48 ± 0.37
Vitamin B-6 (mg/d)	1.54 ± 0.27	1.55 ± 0.27	1.54 ± 0.27	1.53 ± 0.27	1.55 ± 0.25	1.57 ± 0.29	1.33 ± 0.24	1.33 ± 0.24	1.33 ± 0.25	1.32 ± 0.23	1.33 ± 0.21	1.36 ± 0.25
Alcohol (%)												
0 g/d	14.6	12.4	12.0	14.5	9.9	11.1	32.3	33.8	34.7	33.8	29.5	34.1
< 30 g/d	70.6	69.7	74.6	67.5	73.8	67.2	64.1	61.4	59.8	61.8	61.1	63.1
≥ 30 g/d	14.8	17.9	13.4	18.0	16.3	21.7	3.6	4.8	5.5	4.4	6.4	2.8
Energy (kjoules/d)	9080 ± 2134	8949 ± 2059	8923 ± 2006	8846 ± 2135	8841 ± 1946	9149 ± 2008	7062 ± 1659	6980 ± 1608	6994 ± 1698	6933 ± 1541	7071 ± 1944	6991 ± 1332
Meat (g/d)	137 ± 52	136 ± 49.7	134 ± 49	136 ± 49	137 ± 53	137 ± 50	116 ± 46	115 ± 45	112 ± 46	115 ± 42	123 ± 40	117 ± 47
Fat (g/d)	93.9 ± 14.2	94.0 ± 13.6	95.0 ± 12.4	94.8 ± 14.0	93.7 ± 14.3	92.2 ± 14.1	74.0 ± 10.3	74.1 ± 10.3	73.6 ± 10.8	74.5 ± 10.0	75.4 ± 8.6	74.0 ± 10.3
Fiber (g/d)	28.7 ± 7.3	28.8 ± 7.0	28.6 ± 7.1	28.4 ± 6.6	28.9 ± 6.9	29.4 ± 7.1	25.3 ± 5.8	25.1 ± 5.9	25.2 ± 6.2	24.9 ± 5.5	24.8 ± 6.0	25.4 ± 5.8
Calcium (mg/d)	948 ± 295	932 ± 281	947 ± 286	919 ± 267	932 ± 2.96	944 ± 287	901 ± 268	896 ± 274	898 ± 2.93	909 ± 2.54	804 ± 218	913 ± 2.81
Iron (mg/d)	13.3 ± 2.4	13.3 ± 2.4	13.2 ± 2.26	13.3 ± 2.3	13.5 ± 2.5	13.5 ± 2.4	11.7 ± 2.0	11.6 ± 1.9	11.6 ± 2.0	11.5 ± 1.9	11.6 ± 1.8	11.8 ± 1.9
Vitamin C (mg/d)	98.6 ± 41.6	99.7 ± 40.6	98.8 ± 40.5	96.7 ± 38.6	99.5 ± 40.3	103.1 ± 42.6	108.4 ± 42.7	109.3 ± 45.5	108.0 ± 46.1	109.9 ± 44.4	110.4 ± 36.5	109.5 ± 48.4
Fruits (g/d)	155 ± 114	160 ±112	158 ± 107	158 ± 107	161 ± 121	166 ± 120	197 ± 121	195 ± 83	194 ± 134	195 ± 117	201 ± 98	191 ± 123
Vegetables (g/d)	191 ± 85	192 ± 86	191 ± 91	185 ± 76	192 ± 73	205 ± 95	196 ± 82	194 ± 83	193 ± 83	191 ± 77	206 ± 85	200 ± 88

[1] Values are mean ± SD or percentages if indicated otherwise

[2] Numbers of subcohort members and patients are based on complete availability of the variables presented in this table. Information about the tumor localization was unavailable for 39 men and 22 women

Only the intakes of folate among men with a distal colon tumor and of riboflavin among women with a tumor in the rectosigmoid appeared slightly lower compared to subcohort members. The percentages of subjects with a family history of CRC were generally higher in cases than in subcohort members. There were no striking differences in any of the other variables between subcohort members and CRC patients.

We subsequently estimated multivariate adjusted incident rate ratios over quintiles of folate intake, methionine, riboflavin and vitamin B-6 for men and women.

After 13.3 y of follow-up, folate intake was not significantly associated with risk of CRC at any of the sub sites in neither men (Table 2) nor women (Table 3). Among men, methionine was associated with a decreased risk of proximal colon cancer (RR=0.57, CI=0.28-1.18, P_{trend}=0.03), whereas among women, it was found to be inversely associated with rectal cancer (RR=0.45, CI=0.17-1.20, P_{trend}=0.05). Among women, riboflavin tended to be inversely associated with proximal colon cancer (RR for the highest vs. the lowest quintile of intake=0.61, CI=0.35-1.06, P_{trend}=0.07). Conversely, there was a strong positive association between vitamin B-6 and rectal cancer risk in women (RR=3.57, CI=1.56-8.17, P_{trend}=0.01). An increased RR for vitamin B-6 could also be observed for all CRC tumors combined among women, although this risk was less high and not statistically significant (RR=1.39, CI=0.92-2.08, P_{trend}=0.09). When performing the analyses for men and women together, the highest quintile of vitamin B-6 intake was also positively associated with overall CRC (RR=1.34, CI=1.03-1.74, P_{trend}=0.02), with proximal colon cancer (RR=1.37, CI=0.93-2.01, P_{trend}=0.05) and with rectal cancer (RR=1.86, CI=1.17-2.95, P_{trend}=0.02, data not shown).

Adjustment for potential confounders only slightly aggravated the estimated associations compared to age-adjusted analyses, but did not result in different conclusions (Tables 2 and 3). There were no significant interactions between folate, methionine, riboflavin or vitamin B-6 and alcohol intake for overall CRC or for the individual sub-localizations. In addition, we did not observe consistent differences when comparing the RR between strata of low or high intakes of folate, methionine, riboflavin and vitamin B-6 or alcohol (data not shown).

Although the adjusted models did not meet the proportional hazards assumption according to the Schoenfeld residuals, fitting the main determinants as time-dependent variables showed that there was no interaction with time for these variables. Furthermore, when stratifying the analysis based on follow-up time, e.g. on the first and second half of the follow-up period, we observed no consistent differences compared to analyses on the total follow-up period. This also applied to the results when excluding the first year of follow-up. Estimating the relative risks with the median intake within quintiles only slightly changed the *P*-values for dose-response trends, and did not result in different conclusions. The tests for heterogeneity for the effects over the four subgroups of tumors were not statistically significant.

Table 2 Associations between dietary folate, methionine, riboflavin and vitamin B-6 with colorectal cancer risk in men

		All tumors			Proximal colon		Distal colon		Rectosigmoid		Rectum	
Quintile [1]	**PY** [2]	***n*** [3]	**RR (95% CI)** [4]	**RR (95% CI)** [5]	***n***	**RR (95% CI)** [5]	***n***	**RR (95% CI)** [5]	***n***	**RR (95% CI)** [5]	***n***	**RR (95% CI)** [5]
Folate												
(µg/d)												
1 (160.8)	4840	292	1.00	1.00	77	1.00	111	1.00	29	1.00	65	1.00
2 (189.5)	4971	289	0.93 (0.74-1.17)	0.90 (0.71-1.13)	94	1.14 (0.80-1.61)	88	0.69 (0.50-0.97)	22	0.67 (0.37-1.22)	76	1.05 (0.72-1.55)
3 (212.1)	4848	271	0.88 (0.70-1.13)	0.85 (0.66-1.09)	61	0.77 (0.51-1.16)	94	0.72 (0.50-1.04)	37	1.18 (0.67-2.08)	74	1.03 (0.68-1.55)
4 (240.6)	4822	270	0.89 (0.69-1.14)	0.86 (0.66-1.11)	80	1.02 (0.68-1.51)	88	0.69 (0.47-1.01)	25	0.78 (0.40-1.50)	70	1.00 (0.64-1.56)
5 (297.2)	4829	267	0.93 (0.71-1.20)	0.87 (0.65-1.15)	70	0.97 (0.62-1.52)	86	0.71 (0.46-1.08)	28	0.83 (0.41-1.66)	75	1.01 (0.64-1.60)
P_{trend} [6]			*0.43*	*0.29*		*0.65*		*0.16*		*0.72*		*0.92*
Methionine												
(mg/d)												
1 (1366)	4960	296	1.00	1.00	77	1.00	104	1.00	31	1.00	72	1.00
2 (1555)	4805	284	1.01 (0.80-1.28)	0.96 (0.74-1.26)	91	1.07 (0.70-1.64)	86	0.91 (0.62-1.33)	26	0.83 (0.43-1.58)	71	1.03 (0.66-1.60)
3 (1698)	4971	277	0.96 (0.75-1.22)	0.87 (0.64-1.17)	83	0.87 (0.54-1.41)	91	0.89 (0.58-1.37)	30	0.81 (0.37-1.74)	68	0.98 (0.60-1.61)
4 (1843)	4803	282	0.98 (0.77-1.27)	0.90 (0.63-1.26)	65	0.65 (0.37-1.14)	105	1.13 (0.69-1.87)	26	0.65 (0.28-1.50)	79	1.21 (0.69-2.11)
5 (2093)	4772	250	0.91 (0.68-1.22)	0.79 (0.50-1.25)	66	0.57 (0.28-1.18)	81	1.03 (0.52-2.04)	28	0.65 (0.22-1.93)	70	1.09 (0.53-2.24)
P_{trend}			*0.47*	*0.36*		*0.03*		*0.58*		*0.34*		*0.53*
Riboflavin												
(mg/d)												
1 (1.17)	4944	315	1.00	1.00	82	1.00	93	1.00	35	1.00	89	1.00
2 (1.38)	4968	265	0.82 (0.66-1.03)	0.83 (0.65-1.05)	72	0.82 (0.56-1.20)	94	1.13 (0.80-1.60)	27	0.68 (0.38-1.20)	64	0.67 (0.45-0.99)
3 (1.53)	4815	284	0.91 (0.72-1.15)	0.92 (0.71-1.19)	78	0.91 (0.61-1.35)	108	1.44 (0.98-2.11)	22	0.56 (0.29-1.08)	71	0.73 (0.48-1.11)
4 (1.71)	4681	270	0.90 (0.70-1.16)	0.93 (0.69-1.24)	75	0.96 (0.60-1.52)	98	1.47 (0.95-2.29)	28	0.74 (0.38-1.43)	64	0.65 (0.40-1.03)
5 (2.03)	4903	255	0.83 (0.63-1.08)	0.86 (0.60-1.22)	75	0.91 (0.53-1.58)	74	1.22 (0.71-2.08)	29	0.72 (0.30-1.74)	72	0.66 (0.38-1.14)
P_{trend}			*0.37*	*0.72*		*0.92*		*0.19*		*0.52*		*0.16*
Vitamin B-6												
(mg/d)												
1 (1.22)	4838	257	1.00	1.00	70	1.00	94	1.00	25	1.00	59	1.00
2 (1.40)	4766	272	1.12 (0.89-1.40)	1.12 (0.87-1.43)	77	1.17 (0.80-1.71)	93	1.01 (0.71-1.45)	26	1.09 (0.59-2.04)	69	1.22 (0.81-1.82)
3 (1.53)	4959	283	1.14 (0.89-1.45)	1.13 (0.86-1.49)	82	1.23 (0.81-1.87)	93	0.98 (0.66-1.45)	26	1.09 (0.55-2.18)	78	1.32 (0.84-2.07)
4 (1.67)	4884	302	1.27 (0.99-1.63)	1.30 (0.97-1.75)	76	1.29 (0.81-2.06)	103	1.17 (0.76-1.79)	37	1.62 (0.78-3.36)	76	1.31 (0.81-2.12)
5 (1.88)	4863	275	1.18 (0.90-1.56)	1.29 (0.90-1.84)	77	1.50 (0.86-2.62)	84	1.03 (0.60-1.76)	27	1.28 (0.51-3.24)	78	1.35 (0.76-2.41)
P_{trend}			*0.14*	*0.12*		*0.14*		*0.87*		*0.32*		*0.31*

[1] Quintile (median intake within quintile)

[2] Number of accumulated Person Years (PY) within quintiles of dietary intake

[3] Number of cases

[4] Unadjusted Incidence Rate Ratio (RR): from a regression model including the variables folate, methionine, riboflavin, vitamin B-6 and age.

[5] Adjusted RR: from a regression model including the variables folate, methionine, riboflavin, vitamin B-6, age, family history of colorectal cancer, BMI, smoking status and the intakes of alcohol, energy, meat, fat, fiber, calcium and iron

[6] *P*-value for linear trend over quintiles of intake

Table 3 Associations between dietary folate, methionine, riboflavin and vitamin B-6 with colorectal cancer risk in women

		All tumors			Proximal colon		Distal colon		Rectosigmoid		Rectum	
Quintile [1]	**PY** [2]	***n*** [3]	**RR (95% CI)** [4]	**RR (95% CI)** [5]	***n***	**RR (95% CI)** [5]	***n***	**RR (95% CI)** [5]	***n***	**RR (95% CI)** [5]	***n***	**RR (95% CI)** [5]
Folate (µg/d)												
1 (139.0)	5097	198	1.00	1.00	89	1.00	56	1.00	16	1.00	32	1.00
2 (165.6)	5121	185	0.96 (0.75-1.25)	0.98 (0.75-1.28)	66	0.79 (0.54-1.15)	65	1.19 (0.79-1.80)	15	1.13 (0.48-2.66)	34	1.03 (0.60-1.77)
3 (187.5)	5313	180	0.90 (0.69-1.18)	0.95 (0.71-1.27)	71	0.83 (0.55-1.24)	59	1.11 (0.70-1.75)	14	1.06 (0.42-2.66)	31	0.90 (0.49-1.65)
4 (212.9)	5156	200	1.11 (0.84-1.46)	1.17 (0.87-1.58)	79	1.07 (0.71-1.63)	59	1.21 (0.76-1.94)	19	1.62 (0.67-3.94)	40	1.18 (0.65-2.14)
5 (267.3)	5119	197	1.12 (0.82-1.52)	1.25 (0.89-1.76)	81	1.24 (0.76-2.02)	57	1.34 (0.81-2.22)	14	1.38 (0.51-3.75)	39	1.06 (0.53-2.11)
P_{trend} [6]			*0.31*	*0.10*		*0.17*		*0.37*		*0.28*		*0.73*
Methionine (mg/d)												
1 (1154)	5183	206	1.00	1.00	86	1.00	59	1.00	17	1.00	38	1.00
2 (1351)	5130	214	1.07 (0.84-1.38)	1.02 (0.76-1.36)	87	1.20 (0.80-1.82)	67	0.92 (0.59-1.45)	18	1.38 (0.61-3.15)	36	0.77 (0.43-1.38)
3 (1476)	5081	164	0.83 (0.63-1.10)	0.78 (0.56-1.10)	68	1.01 (0.62-1.65)	53	0.72 (0.42-1.22)	13	1.18 (0.43-3.24)	29	0.54 (0.27-1.05)
4 (1617)	5257	194	0.96 (0.73-1.28)	0.87 (0.59-1.29)	74	1.09 (0.61-1.92)	66	0.80 (0.44-1.45)	17	1.77 (0.56-5.56)	33	0.51 (0.24-1.08)
5 (1841)	5155	182	0.88 (0.65-1.21)	0.76 (0.46-1.26)	71	1.07 (0.51-2.22)	51	0.53 (0.24-1.19)	13	1.91 (0.52-6.92)	40	0.45 (0.17-1.20)
P_{trend}			*0.30*	*0.18*		*0.86*		*0.16*		*0.31*		*0.05*
Riboflavin (mg/d)												
1 (1.04)	5124	211	1.00	1.00	99	1.00	51	1.00	25	1.00	33	1.00
2 (1.26)	5067	194	0.91 (0.71-1.17)	0.89 (0.68-1.17)	79	0.74 (0.51-1.06)	57	1.12 (0.71-1.77)	17	0.81 (0.40-1.65)	35	0.99 (0.57-1.72)
3 (1.42)	5130	188	0.92 (0.71-1.19)	0.92 (0.69-1.23)	62	0.62 (0.41-0.93)	73	1.53 (0.95-2.46)	12	0.65 (0.28-1.51)	34	0.99 (0.55-1.79)
4 (1.59)	5294	187	0.85 (0.65-1.11)	0.84 (0.60-1.16)	71	0.61 (0.38-0.96)	60	1.24 (0.72-2.12)	16	0.86 (0.35-2.07)	34	0.91 (0.47-1.74)
5 (1.89)	5190	180	0.85 (0.63-1.14)	0.79 (0.53-1.18)	75	0.61 (0.35-1.06)	55	1.22 (0.62-2.40)	8	0.55 (0.15-2.03)	40	0.92 (0.41-2.04)
P_{trend}			*0.26*	*0.30*		*0.07*		*0.40*		*0.47*		*0.76*
Vitamin B-6 (mg/d)												
1 (1.05)	5018	198	1.00	1.00	82	1.00	65	1.00	17	1.00	27	1.00
2 (1.21)	5278	189	0.98 (0.76-1.26)	1.04 (0.78-1.37)	75	1.02 (0.69-1.51)	61	0.90 (0.58-1.38)	10	0.67 (0.26-1.73)	38	1.73 (0.97-3.08)
3 (1.32)	5187	178	1.01 (0.75-1.34)	1.11 (0.80-1.52)	73	1.17 (0.75-1.82)	53	0.84 (0.50-1.39)	16	1.16 (0.43-3.11)	32	1.79 (0.91-3.54)
4 (1.44)	5324	194	1.08 (0.81-1.44)	1.23 (0.88-1.73)	85	1.35 (0.84-2.15)	60	0.97 (0.57-1.65)	18	1.31 (0.48-3.54)	28	1.73 (0.83-3.58)
5 (1.63)	4998	201	1.12 (0.81-1.55)	1.39 (0.92-2.08)	71	1.15 (0.65-2.04)	57	1.06 (0.56-2.03)	17	1.34 (0.38-4.64)	51	3.57 (1.56-8.17)
P_{trend}			*0.42*	*0.09*		*0.33*		*0.86*		*0.36*		*0.01*

[1] Quintile (median intake within quintile)
[2] Number of accumulated Person Years (PY) within quintiles of dietary intake
[3] Number of cases
[4] Unadjusted Incidence Rate Ratio (RR): from a regression model including the variables folate, methionine, riboflavin, vitamin B-6 and age.
[5] Adjusted RR: from a regression model including the variables folate, methionine, riboflavin, vitamin B-6, age, family history of colorectal cancer, BMI, smoking status and the intakes of alcohol, energy, meat, fat, fiber, calcium and iron
[6] *P*-value for linear trend over quintiles of intake

Discussion

Here, we investigated the associations between intakes of folate, methionine, riboflavin and vitamin B-6 with CRC risk. Our results do not suggest an association between dietary folate and CRC. However, we observed inverse associations between relatively high methionine intake with proximal colon cancer in men and rectal cancer in women. Also, a modest non-significant inverse association was observed between riboflavin intake and tumors of the proximal colon in women. Conversely, a positive association between vitamin B-6 intake was observed for overall CRC, which was particularly strong for rectal cancer among women.

Previous observational studies did not consistently point to a clear inverse association between folate intake and CRC risk. Next to different sample sizes in those studies, this inconsistency may also have been caused by possible selection bias that is likely to occur in case-control studies but not in cohort studies, potential differences in the level of over- or under-estimation of dietary intake using food-frequency questionnaires, and different adjustments for confounding factors between studies (3). In addition, the inconsistencies may be due to differences between study populations and inherent variation in levels of folate intake. The intake in our study was relatively low, with the median folate intake within quintiles ranging from 160 to 299 µg/day among men and 137 to 270 µg/day among women. However, among subjects with considerably higher levels of intake studied in prospective cohort settings, an inverse association was observed only twice (32,33), whereas three other studies did not suggest this (15,16,34). Moreover, a significant inverse association was observed in a cohort study among men with even lower intakes compared to men in our study (compared highest and lowest category >249 vs. <103 µg/day) (35), indicating that high ranges of intake do not necessarily lead to reduced CRC risk. Nevertheless, in several studies the protective effect of folate was confined to either men or women, or limited to a specific study population or sub site of colorectal cancer, which makes it difficult to generalize the results and hence to be conclusive about whether dietary folate has a chemo preventive effect on CRC.

Despite the current view of the importance of folate in cancer prevention, its role in colorectal carcinogenesis is still a subject of debate. Interestingly, it was recently observed that high plasma folate levels may be associated with increased CRC risk (36), and possibly with breast cancer (37). An alternative hypothesis in this regard, is that although folate supplementation may protect against the occurrence of neoplasia in normal colorectal epithelium it might promote already existing early lesions to cancer, suggesting that the effect depends on the timing of intervention (5). It was also reported that folic acid supplementation was associated with increased risk of advanced lesions or recurrence of multiple adenomas after a follow-up of 3 to 5 y (38). Interestingly, the participants in that intervention study were people with a recent history of colorectal adenomas, and possibly, undetected neoplasms were present in these subjects which may have had a growth advantage in the presence of high concentrations of folic acid. Furthermore, after the introduction of nationwide fortification of cereals with folic acid in the United States and Canada, colorectal cancer incidence increased in these countries which may possibly be due to increased intakes folic acid (39). A potentially harmful effect of folate may also be confined to specific subgroups of cancers based on their molecular characteristics. In this respect, we

previously observed positive associations between folate intake and colorectal carcinomas harboring mutations in the key genes *APC* or *BRAF* among men (40,41), indicating that folate may result in a growth advantage of such tumors.

Regarding methionine, we observed that relatively high intake may be inversely associated with proximal colon cancer in men and rectal cancer in women. Previous studies examining the role of methionine intake did not demonstrate an individual inverse association with colorectal cancer risk (13,15-17), but one study showed that low methionine in combination with low folate and high alcohol intake may lead to increased CRC risk (34). Although we did not observe an interaction between folate and methionine, our findings suggest that methionine, as a methyl donor, may be more effective in preventing CRC than folate in some of the colorectal sub-localizations. We also found a weak inverse association between riboflavin and proximal colon cancer only in women, whereas in men riboflavin was unrelated to colorectal cancer. Riboflavin is a co-factor for MTHFR, and a low intake of the vitamin may reduce the metabolic activity of this enzyme and thereby contribute to colorectal carcinogenesis (8). In addition, the 677C>T polymorphism in *MTHFR* may also affect enzymatic activity, and the occurrence of this polymorphism was associated with reduced CRC risk among subjects with adequate folate status (42). It is worthwhile to further explore potential effect modification by such polymorphisms, however, as of today this information is not yet available for our cohort members.

The reason why an effect of methionine or riboflavin would be site or gender-specific remains unclear. Possibly, the development of tumors harboring promoter hypermethylation, which were observed more often in the proximal colon and among women (41,43,44), is more sensitive to these nutrients. We do not have information on global hypomethylation status; however, it is unknown whether the level of global DNA hypomethylation may differ between tumors in different sub localisations of the colorectum or between men and women.

A remarkable finding in our study was the strong positive association between vitamin B-6 intake and rectal cancer in women, with the RR strongly increased in the highest quintile of intake. Also, when analyzing men and women and all colorectal tumors combined, we observed a modest positive association with the RR increasing evenly over quintiles of intake, suggesting a linear dose-response effect. Meat intake appeared to be the main contributor of vitamin B-6 intake (men 23.8%, women 24.4%) and could therefore have accounted for the positive association of vitamin B6, because meat may also be a risk factor for CRC. However, all analyses were adjusted for meat intake. Interestingly, a positive association with rectal cancer in women has previously been observed in the IOWA women's health population based cohort study (IWHS) (16), while the range of intake among women in that study was considerably higher compared to our study. Notwithstanding this difference, these observations suggest that vitamin B-6 may indeed be associated with increased CRC risk. Although these are observations based on subgroups of patients, and thus caution should be taken in interpreting such findings, these are two prospective cohort studies showing a positive association between vitamin B-6 and rectal cancer. Moreover, we have previously observed that a positive association between vitamin B-6 intake and CRC was most pronounced among individuals with tumors harboring human mut-L homologue 1 (*MLH1*) promoter hypermethylation (41). In view of this finding, we hypothesize that relatively high vitamin B-6 intake may increase promoter

hypermethylation and thereby enhances the development of tumors with a methylation-associated phenotype. However, because an inverse association between the vitamin and CRC has been suggested in a number of previous prospective cohort studies (18-21), the role of vitamin B-6 clearly needs further attention. In future research, the role of vitamin B-6 in a hypermethylation-associated pathway should be studied and preferably be investigated in even larger cohort studies.

The current study was a population-based prospective cohort design with a long follow-up period. The large number of incident CRC patients is likely to have minimized the probability of reporting results based on chance alone. It is also unlikely that selection bias has occurred, because the follow up of cases and subcohort members was almost complete. Recall bias was probably low, certainly if compared with retrospective case-control designs, although measurement error is unavoidable with self administered food frequency questionnaires and may still have biased results to some extent. After a validation study however, it was concluded that the questionnaire is sufficiently able to rank subjects according to dietary intake (24) suggesting reasonably adequate validity of the single baseline measurement. Furthermore, potential pre-clinical disease is not likely to have affected exposure status, since the results did not substantially change after excluding the first year of follow-up.

This study could not account for potential changes in dietary habits over time, since there have been no follow-up measurements of dietary intake. We do nonetheless have information on the reproducibility of the baseline FFQ, which was determined from five annually repeated measurements in independent random samples from the cohort. The average decline of the correlation between follow up measures was only minimal indicating that the ability of the baseline FFQ to rank subjects according to dietary intake is maintained relatively well over a period of at least five years (25). Moreover, subjects aged 55-69 years at baseline and these elderly people tend to have a more stable dietary habits compared to younger individuals (22). Although follow up was longer than five years, for these reasons, we consider that potential changes in dietary intake over time have presumably not influenced the estimated associations to a great extent.

In conclusion, the current large prospective cohort study suggests that dietary folate does not protect against CRC in this Dutch population, and that methionine may reduce colorectal cancer risk of the proximal colon in men and rectum among women. Riboflavin also tended to reduce the risk of proximal colon tumors in women. Our study is the second cohort study to show a positive association between vitamin B-6 and rectal cancer among women, which is interesting considering a possible similarity with the recent insight in a potentially harmful effect of high folate levels in colorectal carcinogenesis. However, we underscore the need for larger studies or meta-analyses and to further investigate the role of these nutrients in colorectal cancer development.

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Dietary folate and *APC* mutations in sporadic colorectal cancer

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Abstract

Folate deficiency has been associated with colorectal cancer risk, and may be involved in colorectal carcinogenesis through increased chromosome instability, gene mutations and aberrant DNA methylation. Within the Netherlands Cohort Study on diet and cancer, we investigated associations between dietary folate intake and colorectal cancer risk with (*APC*$^+$) and without (*APC*$^-$) truncating *APC* mutations, accounting for hMLH1 expression and *K-ras* mutations. In total, 528 cases and 4,200 subcohort members were available for data analyses out of the study cohort (n=120,852) from a follow-up period between 2.3 and 7.3 years after baseline. Adjusted gender-specific incidence rate ratios (RR) over tertiles of folate intake were calculated in case-cohort analyses for colon and rectal cancer. Although relatively high folate intake was not associated with overall colorectal cancer risk, it reduced the risk of *APC*$^-$ colon tumors in men (RR=0.58, 95% CI=0.32-1.05, P_{trend}=0.06 for the highest versus the lowest tertile of folate intake). In contrast, it was positively associated with *APC*$^+$ colon tumors in men (highest vs. lowest tertile: RR=2.77, CI=1.29-5.95, P_{trend}=0.008), and even stronger when additional lack of hMLH1 expression and *K-ras* mutations were excluded (RR=3.99, CI=1.43-11.14, P_{trend}=0.007). Such positive associations were not observed among women, neither was folate intake associated with rectal cancer when *APC* mutation status was accounted for. Relatively high folate consumption reduced the risk of *APC*$^-$ colon tumors, but folate intake was positively associated with *APC*$^+$ colon tumors among men. These opposite results may indicate that folate enhances colorectal carcinogenesis through a distinct *APC* mutated pathway.

Introduction

Folate deficiency is hypothesized to increase the risk of colorectal carcinogenesis through various mechanisms (1). It can cause uracil misincorporation in DNA, which in turn may lead to DNA strand breaks and chromosome instability. In addition, a low folate status can cause global DNA hypomethylation and hypermethylation of promoter regions, which may impair the expression of tumor suppressor genes and DNA repair genes (1). Considering these mechanisms it could be expected that sufficient intake of dietary folate protects against colorectal cancer. Epidemiological studies on the relation between dietary folate intake and colorectal cancer risk however, have not consistently shown a protective effect of high folate intake (2,3). This inconsistency may partly be explained by the possible dual role of folate in carcinogenesis, that is, folate would prevent carcinogenesis in normal healthy tissue but may promote growth of existing tumors (4). In addition, accounting for molecular events underlying the carcinogenic process may further clarify the effect of folate intake on colorectal carcinogenesis, but this was not addressed in most of these epidemiological studies.

A well-characterized molecular alteration in colorectal carcinogenesis is the occurrence of mutations in the adenomatous polyposis coli (*APC*) gene. Somatic mutations in this tumor suppressor gene were found mainly in its mutation cluster region in a significant proportion of sporadic colorectal carcinomas, varying from 34% to more than 80% (5-8). This underscores the importance of *APC* gene mutations in sporadic colorectal carcinogenesis. In an earlier study in colorectal cancer patients (8) we found that 37% of the patients had tumors harboring an *APC* mutation resulting in a stop codon, which leads to a truncated and therefore inactive *APC* protein. In addition, Diergaarde *et al* (9) found an indication for a positive association between folate intake and colorectal adenomas, but the associations did not differ between adenomas with or without truncating *APC* mutations. In another study, Diergaarde observed a differential association between consumption of green leafy vegetables, an important source of dietary folate, and the risk of carcinomas with or without truncating *APC* mutations (10).

The exact mechanism through which folate may alter *APC* functionality remains unclear however. Possibly, there is an indirect effect of decreased activity of the DNA repair gene O^6-methylguanine DNA methyltransferase (*O^6-MGMT*) which was found to be associated with hypermethylation of its promoter region (11). Consequently, this can lead to an increased risk of tumors with G:C>A:T mutations in other key-genes, like *APC*, involved in colorectal cancer (12,13).

Several studies have suggested that colorectal tumors may arise through distinct pathways based on specific molecular alterations. In this respect, it has been reported that mutations in *APC* and *K-ras* occur less often in tumors with microsatellite instability (MSI) compared to microsatellite stable ones (14-17). MSI is known to be strongly associated with the lack of expression of the DNA mismatch repair gene *hMLH1* (18,19). Tumors harboring this type of instability occur predominantly in the proximal colon, are often poorly differentiated, and are more frequently present in women at older age (14,20). Furthermore, exogenous factors such as smoking behavior (21,22) and dietary factors (7,23-25) may be differentially associated with the risk of cancer development in MSI and non-MSI tumors. In addition, differential associations were also observed between folate intake and mutations in *K-ras* (26-29).

Therefore, it is important to account for potential aberrations in *K-ras* and *hMLH1* and for differences in tumor location and sex when assessing the potential effect of dietary folate on colorectal cancer risk and *APC* mutations.

In view of these findings, this study aimed to investigate the associations between folate intake and the risk of colon and rectal cancer, with and without truncating *APC* mutations. It was also assessed whether these associations were independent of *K-ras* mutations and hMLH1 expression. The study was conducted within the framework of the Netherlands Cohort Study on diet and cancer (NLCS), of which the study population consumed relatively low levels of dietary folate compared to some other Western countries, since folic acid fortification is not allowed in the Netherlands.

Subjects and methods

Study population

The participants in this study are incident colon and rectal cancer patients and subcohort members from the Netherlands Cohort Study (NLCS), which has been described in detail elsewhere (30). Briefly, the study was initiated in 1986 and includes 58,279 men and 62,573 women aged 55-69 years at baseline, who originated from 204 Dutch municipalities with computerized population registers. Numbers of subcohort members and cases are displayed in a flow chart (Figure 1). At baseline, participants completed a self-administered food-frequency questionnaire that also contained questions about other risk factors for cancer. The entire cohort is being monitored for cancer occurrence by annual record linkage to the Netherlands Cancer Registry (NCR, nine cancer registries in The Netherlands) and to PALGA, a nationwide network and registry of histopathology and cytopathology reports (31). Accumulation of person-time in the cohort has been estimated through biennial vital status follow-up of a subcohort of 5,000 men and women who were randomly selected after baseline exposure measurement. Cases with prevalent cancer other than non-melanoma skin cancer were excluded from this subcohort, which left 4,774 men and women eligible for analysis.

The PALGA registry was not yet implemented in some of the municipalities included in the study in 1986, but reached full coverage by the end of 1988. Incomplete coverage may introduce selection bias, and also possible preclinical disease may have affected exposure status. For these reasons we excluded the first 2.3 years of follow-up from the analyses. 101 subcohort members were either deceased or diagnosed with cancer other than non-melanoma skin cancer within this period, leaving 4,673 men and women for analysis. From 1989 to 1994, 925 incident cases were identified with histologically confirmed colorectal cancer of whom 815 could also be linked to a PALGA report of the lesion. The PALGA database was used to identify and locate tumor tissue in Dutch pathology laboratories. Colorectal cancer was classified according to disease site as follows: colon, i.e. proximal colon (ICD-O-1 codes 153.0, 153.1, 153.4, 153.5, 153.6) and distal colon (153.2, 153.3, 153.7, 153.8 153.9), rectosigmoid (ICD-O-1 code 154.0) and rectum (ICD-O-1 code 154.1). Information about age at baseline, sex and family history of colorectal cancer (at baseline) was retrieved from the NLCS database.

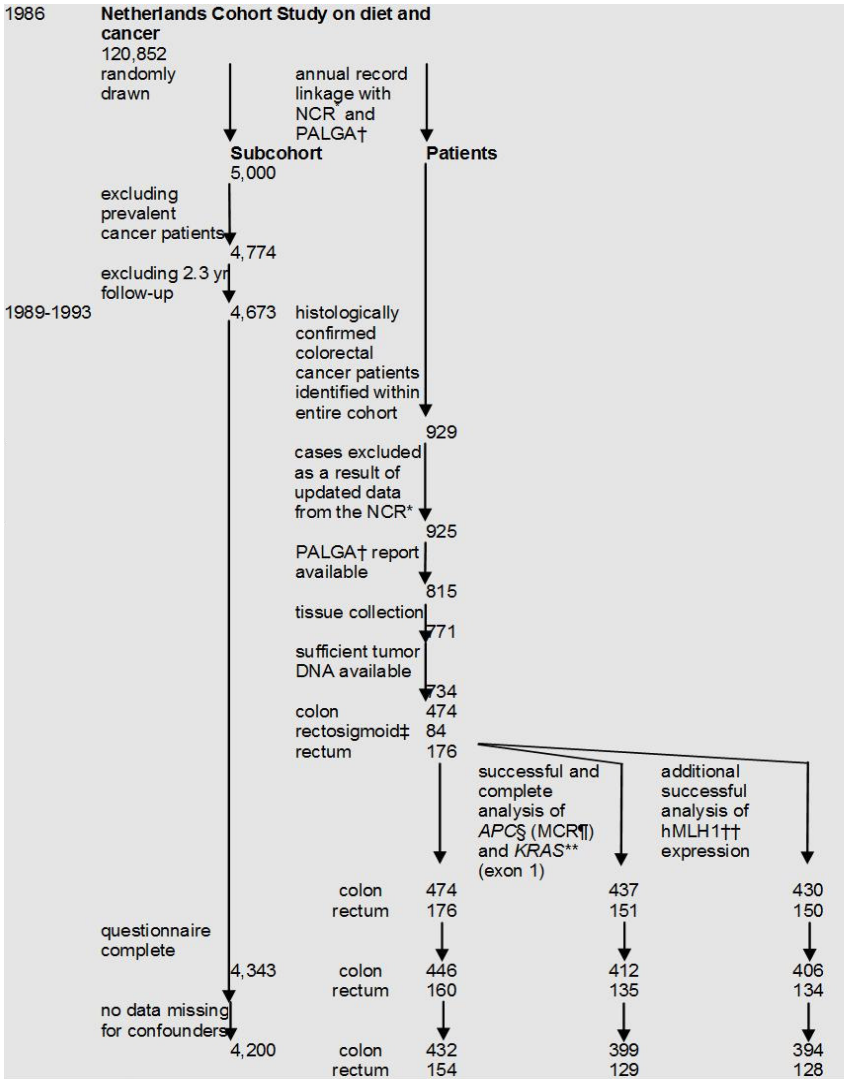

Figure 1. Flow diagram of subjects on whom the analyses were based

* Netherlands Cancer Registry
† Pathologisch Anatomisch Landelijk Geautomatiseerd Archief
‡ Patients with rectosigmoid tumors were not included in the analyses
§ Adenomatous polyposis coli
¶ Mutation cluster region
** Kirsten ras
†† human mut-L homologue 1

Tissue samples

Tumor material of the colorectal cancer patients was collected after approval by the ethical review boards of Maastricht University, the NCR and PALGA (32). In addition, all pathology laboratories in the Netherlands agreed to make relevant tissue samples

available from PALGA upon request. From the 815 tissue samples that were scattered over 54 pathology laboratories in the Netherlands, 734 samples (90%) could be traced and were retrieved between August 1999 and December 2001.

Gene mutation analyses

Genomic DNA was isolated from the paraffin sections after macrodissection of tumor cells as described previously (32). Gene mutation analyses of the mutation cluster region in *APC* (codons 1286-1520), was performed as previously described (8). Briefly, nested PCR was used to amplify the mutation cluster region in four overlapping DNA fragments and the purified fragments were sequenced. Two observers independently evaluated the sequence patterns and data entry. From 72 colorectal cancer patients, one or more fragments of the *APC* gene could not be analyzed completely, leaving 662 patients for data analysis.

The exon 1 fragment of the *K-ras* oncogene, spanning codons 8-29, was analyzed successfully of all 734 patients using nested PCR, followed by direct sequencing of purified fragments as described (32).

hMLH1 expression analysis

Immunohistochemical analyses were performed and scored on 4 µm sections of formalin-fixed, paraffin-embedded cancer tissue and adjacent normal tissue using a monoclonal antibody against hMLH1, as previously described (7). Two investigators reviewed the immunohistochemical staining profiles independently and discrepancies were re-examined and discussed with a pathologist until consensus was reached. hMLH1 expression status was determined successfully in 721 (98%) of the 734 patients.

Food frequency questionnaire

The self-administered questionnaire was a 150-item semi-quantitative food frequency questionnaire, which concentrated on habitual consumption of food and beverages during the year preceding the start of the study, and also contained questions about body weight and -length, smoking status, physical activity, and family history of colorectal cancer. Daily mean nutrient intakes were calculated as the cumulated product of the frequencies and portion sizes of all food items and their tabulated nutrient contents from the Dutch Food Composition Table (NEVO table, 1986) (33). The questionnaire was validated through comparison with a 9-day diet record (34). Questionnaire data were key-entered twice for all incident cases in the cohort and for all subcohort members in a blinded manner with respect to case/subcohort status. This was done in order to minimize observer bias in coding and interpretation of the data.

Folate data were derived from a validated liquid chromatography trienzyme method (35) used to analyze the 125 most important Dutch foods contributing to folate intake (36). Mean daily intakes of all other relevant nutrients were calculated using the computerized Dutch Food Composition Table (33). Dietary supplement data were also obtained via the food frequency questionnaire. However, the use of B-vitamins and/or multivitamin supplements was low (7% and 4% respectively), and folic acid was generally not included in supplements in the Netherlands in the late 1980s. Therefore, folic acid supplement use probably plays a very minor role in our study population, and

dietary supplement use was not further accounted for in the analyses. Cases and subcohort members with incomplete or inconsistent dietary data were excluded from analyses (27,34). Hence, 446 colon cancer cases, 160 rectal cancer cases, 76 rectosigmoid cancer cases, and 4,343 subcohort members remained.

Statistical analyses

Data analyses were conducted overall, as well as stratified for men and women, and for colon and rectal tumors with and without truncating *APC* mutations, described here as APC^+ tumors and APC^- tumors respectively. The group of cases without truncating *APC* mutations consists of cases with tumors containing a wild-type *APC* gene (28%), a missense mutation (29%), or a silent mutation (6%). Since the number of patients with a rectosigmoid tumor was too small for adequate stratified analyses, we included these patients only when assessing associations for all colorectal tumors combined. Furthermore, the rectosigmoid is regarded as a clinically applied term rather than an anatomically defined transitional zone between the colon and rectum (32).

The analyses were repeated for tumors with *APC* mutations, with exclusion of those tumors that also contained activating *K-ras* mutations and were hMLH1 deficient. Out of 125 truncating *APC* colon tumors, 64 (49%) exclusively contained a truncating *APC* mutation. In order to investigate the effect of folate on specific point mutations, analyses were conducted for tumors with G:C>A:T mutations in *APC*, irrespective of the presence of truncating *APC* mutations or other gene defects.

Intake of dietary folate and other baseline characteristics were evaluated for subcohort members and colon and rectal cancer cases with or without *APC* mutations, by calculating mean and standard deviation of the continuous variables folate intake (µg/day), age (years), body mass index (BMI, kg/m^2), energy (kJ/day), alcohol (g/day), total fat (g/day), fiber (g/day), vitamin C (mg/day), vitamin B-2 (mg/day), vitamin B-6 (mg/day), iron (mg/day) and methionine (mg/day), as well as distributions of the variables family history of colorectal cancer (yes/no), smoking status (never/ex/current smoker), and physical activity in leisure time (<30, 30-60, 60-90, >90 min/day). Differences in mean values or distributions of variables between groups of cases with and without truncating *APC* mutations were tested with the Student t-test, the Mann-Whitney-U-test, or the Chi-square test where appropriate.

Cox proportional hazards regression models were used to estimate age-adjusted and multivariate-adjusted gender specific incidence rate ratios (RR) and corresponding 95% confidence intervals (CI) of colon and rectal cancer for tertiles of folate intake. In addition, associations were estimated for tumors with or without truncating *APC* mutations. Standard errors of the RRs were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling from the cohort (37). The proportional hazards assumption was tested using the scaled Schoenfeld residuals (38). Tests for dose response trends over the different tertiles of folate intake were estimated by fitting the ordinal exposure variables as continuous variables and evaluated using the Wald test.

The covariates included in the multivariate analyses were those found to significantly contribute to the multivariate model ($P \leq 0.05$) for colon and/or rectal cancer, or to influence the RR by more than 10 percent. This applied for the variables age, family history of colorectal cancer, BMI, energy, fiber, vitamin C, vitamin B-2, vitamin B-6, iron and methionine. After excluding subjects with missing information on

one or more of these covariates 4,200 subcohort members remained for statistical analyses, as well as 399 colon- and 129 rectal cancer cases with complete *APC* and *K-ras* mutation analyses, and 394 colon- and 128 rectal cancer cases with additional complete hMLH1 expression analyses.

Several factors were previously found to be associated with colorectal cancer risk, or capable to modify the association between dietary folate and colorectal cancer risk, i.e. smoking (22,39,40), alcohol (41,42), vitamins B-2 and B-6 (43,44), and iron intake (45). We therefore determined possible effect modification of these factors, as well as for sex, by testing for interaction and by stratified analyses.

All statistical analyses were performed with the STATA statistical software package (intercooled STATA, version 9.1).

Results

We first explored mean dietary intakes and other characteristics measured at baseline for cancer cases and subcohort members. Folate intake among males with APC^+ colon tumors was higher compared to males with APC^- colon tumors (Table 1). Furthermore, we observed that intakes of vitamin B-2 and iron were significantly higher among men with APC^+ colon tumors compared to men with APC^- colon tumors. All other characteristics presented in Table 1 did not differ substantially between tumors with and without truncating *APC* mutations.

In order to further investigate the associations between folate intake, colon cancer risk and *APC* mutation status, we have calculated relative risks for men and women (Table 2). Folate intake was not associated with overall colon cancer risk in either men or women. However, it reduced the risk of APC^- colon tumors in men (RR 0.58, CI 0.32-1.05 for the highest versus the lowest tertile of folate intake; P_{trend} = 0.06). In contrast, there was a strong positive association between dietary folate and APC^+ colon tumors in men (RR 2.77, CI 1.29-5.95, P_{trend} = 0.008), whereas no association could be observed in women with this type of colon tumors. When calculating associations between folate intake and cancer risk for APC^+ colon tumors without an additional mutated *K-ras* gene and with hMLH1 expression, the positive association appeared even stronger in men (RR 3.99, CI 1.43-11.14, P_{trend} = 0.007) but not in women. Although not statistically significant, high folate intake reduced the risk of colon tumors in women harboring G:C>A:T point mutations in the *APC* gene (RR 0.45, CI 0.18-1.14; P_{trend} = 0.10). This was not observed in men.

Table 1 Baseline dietary intake and other characteristics of cancer cases and subcohort members from the Netherlands Cohort Study on diet and cancer in 1986 [1]

Characteristic	Men					Women				
	Subcohort	Colon		Rectum		Subcohort	Colon		Rectum	
		APC- [2]	*APC*+ [3]	*APC*-	*APC*+		*APC*-	*APC*+	*APC*-	*APC*+
N	2040	143	70	46	38	2136	131	55	26	19
Folate(µg/day)	224.9 ± 75.2	215.8 ± 65.6	247.9 ± 67.9 [6]	221.6 ± 52.1	218.1 ± 69.8	198.7 ± 67.2	186.7 ± 69.2	195.2 ± 56.9	218.5 ± 94.8	211.0 ± 56.9
Age(y)	61.3 ± 4.2	63.0 ± 4.3	62.7 ± 3.9	62.2 ± 4.7	62.4 ± 3.6	61.4 ± 4.3	63.3 ± 3.9	62.7 ± 4.2	62.2 ± 4.0	62.9 ± 3.3
Family History (% yes)	5.4	11.9	8.6	6.5	15.8	5.7	9.9	12.7	15.4	0
BMI (kg/m^2)	25.0 ± 2.6	25.5 ± 2.9	25.7 ± 2.9	25.2 ± 2.6	24.8 ± 2.7	25.1 ± 3.56	25.4 ± 3.7	25.9 ± 3.2	25.4 ± 3.9	25.7 ± 3.0
Smoking Status (%)										
Never	12.8	11.9	8.6	8.7	13.2	58.2	61.8	74.6	57.7	73.7
Ex Smoker	51.7	65.7	64.3	56.5	52.6	20.6	24.4	16.4	30.8	10.5
Current Smoker	35.5	22.4	27.1	34.8	34.2	21.2	13.7	9.1	11.5	15.8
Physical Activity (%) [4]										
<30 min/day	17.7	9.9	20.3	13.3	15.8	24.3	28.7	33.3	23.1	47.4
30-60 min/day	30.5	35.5	30.4	20.0	36.8	31.3	31.0	27.8	42.3	21.1
60-90 min/day	18.8	22.0	14.5	22.2	13.2	22.7	21.7	20.4	23.1	26.3
>90 min/day	32.0	32.6	34.8	44.4	34.2	21.7	18.6	18.5	11.5	5.3
Dietary Factors										
Energy (kjoules/day)	9084 ± 2134	8815 ± 1698	9183 ± 2166	9204 ± 1769	8875 ± 1604	7044 ± 1656	6785 ± 1492	7239 ± 2003	7071 ± 1415	7592 ± 1296
Alcohol (g/day) [5]	14.9 ± 16.8	15.3 ± 15.9	16.8 ± 18.8	15.2 ± 17.1	18.6 ± 19.4	5.8 ± 9.5	6.2 ± 12.2	3.9 ± 9.2	6.8 ± 7.9	5.8 ± 11.2
Fat (g/day)	94.3 ± 28.4	92.0 ± 23.5	94.1 ± 28.8	93.0 ± 24.5	89.7 ± 25.4	73.9 ± 22.7	71.5 ± 21.9	77.1 ± 25.8	74.3 ± 22.2	83.5 ± 18.8
Fiber (g/day)	28.7 ± 8.7	28.9 ± 7.7	30.3 ± 7.9	30.0 ± 7.7	29.1 ± 10.2	25.3 ± 7.0	24.2 ± 7.5	24.8 ± 7.2	24.6 ± 4.0	26.3 ± 5.3
Vitamin C (mg/day)	98.8 ± 42.9	102.3 ± 42.9	111.6 ± 42.9	113.9 ± 51.9	98.7 ± 48.0	108.6 ± 44.5	101.9 ± 48.8	105.6 ± 43.1	114.8 ± 34.0	117.5 ± 52.2
Vitamin B-2 (mg/day)	1.58 ± 0.45	1.53 ± 0.39	1.65 ± 0.44 [6]	1.53 ± 0.35	1.49 ± 0.34	1.45 ± 0.41	1.37 ± 0.34	1.46 ± 0.44	1.53 ± 0.48	1.53 ± 0.29
Vitamin B-6 (mg/day)	1.54 ± 0.38	1.54 ± 0.35	1.62 ± 0.37	1.58 ± 0.35	1.46 ± 0.40	1.33 ± 0.32	1.28 ± 0.30	1.37 ± 0.37	1.34 ± 0.27	1.45 ± 0.32
Iron (mg/day)	13.2 ± 3.3	13.3 ± 3.0	14.5 ± 2.8 [6]	13.6 ± 2.6	13.5 ± 3.3	11.7 ± 2.7	11.3 ± 2.4	11.3 ± 2.5	11.5 ± 2.1	12.1 ± 2.1
Methionine (mg/day)	1716 ± 417	1694 ± 366	1736 ± 387	1658 ± 298	1598 ± 408	1492 ± 366	1442 ± 304	1537 ± 447	1140 ± 337	1613 ± 290

[1] Values are means ± SD, except for Family History (% yes), Smoking Status (%) and Physical Activity (%)
[2] Tumors without a truncating *APC* mutation
[3] Tumors with a truncating *APC* mutation
[4] Information on physical activity was not available for 43 subcohort members, for 6 colon cancer cases and for 1 rectal cancer case.
[5] Information on alcohol intake was not available for 117 subcohort members, for 4 colon cancer cases and for 4 rectal cancer cases.
[6] *P*-Value < 0.05 for the difference between cases in the *APC*- and *APC*+ group.

Table 2 Incidence Rate Ratios (RR) and 95% CI for colon cancer patients according to tertiles of folate intake

	MEN			WOMEN		
Tertiles of folate intake[1]	**N [2]**	**RR[3]**	**95% CI**	**N[2]**	**RR[3]**	**95% CI**
Colon carcinoma, all tumors						
1	74	1.00		74	1.00	
2	56	0.69	0.46-1.02	64	0.94	0.61-1.44
3	83	0.96	0.61-1.54	48	0.82	0.45-1.49
P-value for linear trend		*0.84*			*0.53*	
Colon carcinoma, APC- [4]						
1	60	1.00		56	1.00	
2	39	0.56	0.35-0.88	42	0.85	0.52-1.39
3	44	0.58	0.32-1.05	33	0.79	0.40-1.54
P-value for linear trend		*0.06*			*0.47*	
Colon carcinoma, APC+ [5]						
1	14	1.00		18	1.00	
2	17	1.20	0.56-2.55	22	1.21	0.54-2.70
3	39	2.77	1.29-5.95	15	0.91	0.27-3.06
P-value for linear trend		*0.008*			*0.91*	
Colon carcinoma, APC+, K-ras -, hMLH1+ [6]						
1	7	1.00		11	1.00	
2	10	1.58	0.57-4.35	7	0.67	0.18-2.48
3	22	3.99	1.43-11.14	7	0.77	0.13-4.57
P-value for linear trend		*0.007*			*0.75*	
Colon carcinoma, G:C>A:T point mutations in APC						
1	40	1.00		39	1.00	
2	32	0.66	0.39-1.12	34	0.78	0.41-1.49
3	48	0.86	0.46-1.61	20	0.45	0.18-1.14
P-value for linear trend		*0.63*			*0.10*	

[1] Median intakes of folate within tertiles of folate are 162.7, 211.4, 279.9 µg/day for men, and 142.5, 186.8, 248.0 µg/day for women

[2] Number of person years among subcohort members for the first, second and third tertile of folate intake is 3243, 3282 and 3293 for men, and 3457, 3512 and 3526 for women respectively

[3] RR adjusted for age, family history, BMI, iron, fiber, energy, vitamin B-2, vitamin B-6, vitamin C and methionine

[4] Colon tumors without a truncating *APC* mutation.

[5] Colon tumors with a truncating *APC* mutation.

[6] Colon tumors with a truncating *APC* mutation, no *K-ras* mutation and with hMLH1 expression.

Relative risks were also calculated for tumor sub-localizations within the colon. However, it appeared that folate intake was neither associated with proximal and distal colon cancer risk, nor did these relative risks substantially differ from overall colon cancer risk. Accounting for *APC* mutations in these subgroups did not reveal any differential results (data not shown).

Regarding rectal cancer, we observed that intake of dietary folate was neither associated with overall cancer risk, nor with rectal cancer with or without *APC* truncating mutations in either men or women (see Table 3). However, when conducting the analyses for *APC*$^{+}$ tumors without a *K-ras* mutation and with hMHL1 expression, folate was again positively associated with rectal cancer risk among men (RR 3.69, CI 0.97-13.98; P_{trend} = 0.06).

Table 3 Incidence Rate Ratios (RR) and 95% CI for rectal cancer patients according to tertiles of folate intake

	MEN			WOMEN		
Tertiles of folate intake[1]	**N[2]**	**RR[3]**	**95% CI**	**N[2]**	**RR[3]**	**95% CI**
Rectal carcinoma, all tumors						
1	26	1.00		10	1.00	
2	32	1.11	0.63-1.97	18	1.71	0.72-4.04
3	26	0.91	0.41-2.01	17	1.54	0.55-4.33
P value for linear trend		*0.82*			*0.42*	
Rectal carcinoma, APC- [4]						
1	12	1.00		6	1.00	
2	19	1.31	0.62-2.77	10	1.78	0.58-5.49
3	15	0.93	0.31-2.72	10	1.80	0.46-6.98
P value for linear trend		*0.89*			*0.39*	
Rectal carcinoma, APC+ [5]						
1	14	1.00		4	1.00	
2	13	0.95	0.39-2.31	8	1.60	0.41-6.29
3	11	0.92	0.29-2.99	7	1.25	0.25-6.34
P value for linear trend		*0.89*			*0.82*	
Rectal carcinoma, APC+, K-ras-, hMLH1+ [6]						
1	5	1.00		1	1.00	
2	7	1.98	0.63-6.21	4	3.42	0.30-38.89
3	9	3.69	0.97-13.98	3	2.56	0.16-41.13
P value for linear trend		*0.06*			*0.52*	
Rectal carcinoma, G:C>A:T point mutations in APC						
1	12	1.00		7	1.00	
2	18	1.34	0.61-2.95	8	1.25	0.37-4.22
3	16	1.15	0.39-3.35	9	1.62	0.39-6.64
P value for linear trend		*0.80*			*0.50*	

1 Median intakes of folate within tertiles of folate are 162.7, 211.4, 279.9 µg/day for men, and 142.5, 186.8, 248.0 µg/day for women
2 Number of person years among subcohort members for the first, second and third tertile of folate intake is 3243, 3282 and 3293 for men, and 3457, 3512 and 3526 for women respectively
3 RR adjusted for age, family history, BMI, iron, fiber, energy, vitamin B-2, vitamin B-6, vitamin C and methionine
4 Rectal tumors without a truncating *APC* mutation.
5 Rectal tumors with a truncating *APC* mutation.
6 Rectal tumors with a truncating *APC* mutation, no *K-ras* mutation and with hMLH1 expression.

In addition, we assessed possible associations for all colorectal cancer cases combined, for each of the endpoints considered in this study. Folate intake was also positively associated with *APC*$^+$ colorectal tumors without a *K-ras* mutation and with hMHL1 expression (RR 1.86, CI 0.97-3.55), again being most pronounced among men (RR 3.40, CI 1.54-7.50), although the interaction with sex was not statistically significant. Significant interactions were observed between folate intake and sex in the associations with overall colon cancer risk (P = 0.03), and *APC*$^+$ colon tumors (P = 0.01). None of the other interactions tested were statistically significant, nor did the remaining stratified analyses reveal any different relative risks compared to the overall groups.

Discussion

In this study we investigated the associations between folate intake and colorectal carcinoma risk with and without truncating *APC* mutations. We observed that folate intake in the second and highest tertile reduced the risk of *APC*$^-$ colon tumors among men, while folate intake was positively associated with *APC*$^+$ colon tumors in men. It is striking that this positive association among men was even stronger for *APC*$^+$ colon tumors, and appeared in rectal tumors, when tumors with additional aberrations in *K-ras* and with hMLH1 expression were excluded.

The relation between folate and *APC* mutations has been investigated only once before in colorectal adenomas by Diergaarde et al (9), but in contrast to our study, no differences were observed between the *APC*$^-$ and *APC*$^+$ endpoints. Reasons for this discrepancy might be that adenomas were studied instead of carcinomas, and that a case-control design was used with selected cases versus a case-cohort design with incident cases in our study. Furthermore, in the study of Diergaarde et al (9), the analyses were not stratified for gender, which may have attenuated potential associations in men. On the other hand, the ranges of dietary folate intake within the tertiles of intake were comparable to those in our study population. In another study by Diergaarde et al (10), intake of green leafy vegetables - an important source of dietary folate - was inversely associated with *APC*$^-$ colon carcinomas. Although the association with folate intake was not calculated separately in that study, the results suggest a similarity with the inverse association between folate and *APC*$^-$ colon carcinomas in our study. However, the reason why the protective influence of folate is confined to these tumors remains unclear and needs further investigation.

After determining potential interactions it was found that sex significantly modified the effect of folate intake on overall colon cancer risk, as well as on *APC*$^+$ colon tumors. For this reason, we presented the data for men and women separately in spite of the drawbacks of thereby creating smaller subgroups and less precise estimations. In order to minimize the potential danger of reporting chance findings however, we also conducted analyses for all colorectal tumors combined with and without stratifying for sex. We then observed that folate intake was again positively associated with *APC*$^+$ colorectal tumors without a *K-ras* mutation and with hMHL1 expression, and that this effect was only present among men.

Although one could argue that the number of cases in some subgroups should preferably have been higher, we emphasize that the selection of patients in this study was based on the availability of a tissue sample with sufficient extracted DNA as well as the completeness of analyses of the *K-ras* and *APC* genes and hMLH1 expression. This led to a reduction of the number of cases that could be included in the analyses. However, a tissue sample as well as a sufficient amount of DNA was available for no less than 90% of the patients. Moreover, the overlap of available molecular analyses was high (89%). Finally, it is important to realize that the characteristics of these patients with regard to age, sex, family history of colorectal cancer, smoking behavior and dietary factors neither differed from the 815 patients initially identified, nor from the 734 patients with sufficient tumor DNA. It is therefore unlikely that bias has occurred due to selection of patients.

High folate intake resulted in an increased risk of *APC*$^+$ colon tumors among men. However, this was not expected given the hypothesis that folate prevents DNA

damage. Since it is reasonable to assume that aberrations in other key genes in colorectal carcinogenesis may also be associated with folate intake, we included *K-ras* mutation status and hMLH1 expression in our analyses in order to exclude their potential underlying influence. The observations that *APC* and *K-ras* mutations may be inversely associated with microsatellite instability (14-17), and with hMLH1 expression (18,19), are an important reasons to do so. *APC* mutated colon tumors without additional *K-ras* mutation and with hMLH1 expression were even more strongly positively associated with folate intake in men, and the positive association also appeared for this type of rectal tumors among men. Interestingly, it has been suggested that folic acid supplementation might have a cancer-promoting effect in people with already existing, undiagnosed pre-malignant or malignant lesions (4,46). *APC* mutations are known to play an important role in both tumor initiation as well as in later stages of colorectal cancer development (47). The development of lesions containing early *APC* mutations is possibly more sensitive to a high folate intake, that is, the protective influence of dietary folate on cancer progression might be decreased after an *APC* mutation has occurred compared to tumors that were initiated otherwise.

Other recent studies confirm that multiple alternative genetic pathways to colorectal cancer may exist, since it was found that only a very small number of colorectal tumors (5 to 6%) harbored mutations in all three key genes *K-ras, APC* and *TP53* (48,49). We observed strong positive associations for tumors containing exclusively *APC* mutations in men only. This may indicate that folate potentially mediates an *APC* mutated pathway in the colon and rectum in men, but that its effect differs between men and women. However, the reasons for these differences are unknown, and clearly other studies are warranted to confirm or reject this.

The relative risks of tumors harboring G:C>A:T point mutations in *APC* were slightly decreased among women with colon tumors. Decreased activity of the DNA repair gene *O^6-MGMT* by epigenetic hypermethylation might be an indirect mechanism through which folate intake modifies colorectal cancer risk (11). The O^6-MGMT protein removes adducts from the O^6 position of guanine in DNA (12), which in turn prevents G:C>A:T point mutations. Previously, we reported that *O^6-MGMT* promoter hypermethylation may partly be due to a low folate status (50). It has also been reported that that *O^6-MGMT* hypermethylation is associated with G:C>A:T mutations in *K-ras* and *TP53* (12,13,27). However, others did not observe an association between *O^6-MGMT* expression and G:C>A:T mutations in the *APC* gene alone (51). Further research is therefore needed to investigate whether *APC* mutations might occur via decreased *O^6-MGMT* functionality.

A potential protective effect of folate intake on colorectal cancer risk has been demonstrated only in 10 out of 23 epidemiological studies conducted to investigate this association (2). In addition, we have observed higher risks of colorectal cancer in some subgroups, even though intakes of dietary folate in our study population were relatively low compared to some other Western countries. This raises the question whether high folate intake should be a reason for concern in terms of colorectal cancer risk, particularly in view of differences in recommended dietary intakes between countries and the mandatory fortification of cereals with folic acid in the United States and Canada. It is therefore of crucial importance to gain more insight in the role that dietary folate plays in colorectal carcinogenesis with genetic aberrations like truncating *APC* mutations in future studies.

In conclusion, the results of this study indicate that an inverse association between relatively high folate consumption and carcinogenesis is limited to *APC*$^{-}$ colon tumors, especially in men. Combined with the higher relative risks of *APC*$^{+}$ colon and rectal tumors without additional aberrations in two other key genes involved in colorectal cancer in men, these findings may indicate that folate intake exerts an effect on specific genetic pathways, i.e. an *APC* mutated pathway. Tumors that arise through distinct genetic pathways on the basis of specific genetic aberrations, such as truncating *APC* mutations, possibly have a unique etiology and folate may have a different effect on these pathways in men and women.

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5

Associations of dietary methyl donor intake with *MLH1* promoter hypermethylation and related molecular phenotypes in sporadic colorectal cancer

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Abstract

Intake of dietary factors that serve as methyl group donors may influence promoter hypermethylation in colorectal carcinogenesis. We investigated whether dietary folate, vitamins B2 and B6, methionine and alcohol were associated with *MLH1* hypermethylation, and the related molecular phenotypes of MLH1 protein expression, microsatellite instability (MSI) and *BRAF* mutations in patients with colorectal carcinomas.

Within the Netherlands Cohort Study on diet and cancer (n=120,852), 648 cases (367 men and 281 women) and 4,059 subcohort members were available for data analyses from a follow-up period between 2.3 and 7.3 years after baseline. Gender-specific adjusted incidence rate ratios (RR) were calculated over categories of dietary intake in case-cohort analyses.

The intakes of folate, vitamin B2, methionine and alcohol were not associated with risk of tumors showing *MLH1* hypermethylation, those lacking MLH1 protein expression, or with MSI. Among men, we observed strong positive associations between folate and *BRAF*-mutated tumors (RR=3.04, for the highest versus lowest tertile of intake, P_{trend}=0.03), and between vitamin B6 and tumors showing *MLH1* hypermethylation (highest vs. lowest tertile: RR=3.23, P_{trend}=0.03). Among women, the relative risks of tumors with *BRAF* mutations or *MLH1* hypermethylation were also increased in the highest tertiles of folate and vitamin B6 intake respectively, but these did not reach statistical significance.

The positive associations between folate intake and tumors harboring *BRAF* mutations and between vitamin B6 intake and those showing *MLH1* hypermethylation were most pronounced among men, and may suggest that these vitamins enhance CRC risk through genetic as well as epigenetic aberrations.

Introduction

It has been hypothesized that two forms of genetic instability may contribute to the carcinogenesis of sporadic colorectal cancer (CRC). While microsatellite instability (MSI) occurs in approximately 15% of the sporadic CRCs, chromosomal instability (CIN) would account for the remaining 85% (1). Sporadic tumors with MSI present a distinct molecular phenotype and may develop predominantly as a consequence of hypermethylation of the mismatch repair gene Mut-L Homologue 1 (*MLH1*) (2-4). Furthermore, mutations in the *BRAF* oncogene are strongly associated with *MLH1* promoter methylation and MSI (5), and are an additional type of molecular alteration characterizing this phenotype.

Dietary factors that serve as methyl group donors, such as folate and methionine, potentially have an effect at the level of DNA methylation. A low folate status may decrease genomic DNA methylation, which in turn presumably contributes to the process of carcinogenesis (6). A specific form of aberrant methylation that is frequently observed in carcinogenesis involves CpG island promoter hypermethylation of for example DNA repair genes (7). It may be hypothesized that deficient status or low intake of methyl donors could also lead to increased frequencies of this type of aberrant DNA methylation. In this respect, in a pilot study (n=122 patients), we previously observed a weak association between relatively low folate intake in combination with high alcohol consumption, and increased promoter hypermethylation of at least one out of six of the studied colorectal cancer genes (8). Conversely, it was recently suggested that folate supplementation of 4.6 mg/day in combination with vitamin B12 during 6 months may be associated with increased levels of promoter hypermethylation in colorectal mucosa, although this association was borderline significant (9). These results seem contradictive and it is obviously important to further study the potential effect of relatively high methyl donor intake on the occurrence of promoter hypermethylation.

Other dietary factors, such as vitamins B2 and B6, are also involved in the folate-mediated one-carbon metabolism (Figure 1). These vitamins may therefore modulate the bioavailability of methyl groups and thereby DNA methylation as well (10). Flavin adenine dinucleotide (FAD), a metabolite of vitamin B2, is the cofactor for methylene-tetrahydrofolate reductase (MTHFR), the enzyme that converts 5,10-methylene-tetrahydrofolate into 5-methyl-tetrahydrofolate and thereby enhances DNA methylation. Low vitamin B2 status was previously observed to be associated with increased tHcy concentration, which possibly results in lower availability of methyl groups needed for DNA methylation (11). Interestingly, it was suggested that patients with the *MTHFR* C677T and A1298C polymorphisms, that are associated with lower activity of this enzyme, tended to have a higher level of *MLH1* promoter methylation in colon carcinomas (12). Subjects with the homozygous *MTHFR* 677TT variant also had lower serum folate levels (13), and may be at increased risk of developing tumors harboring MSI (13,14). Vitamin B6 is involved in the conversion of tetrahydrofolate into 5,10-methylenetetrahydrofolate, which is one of the steps of the folate cycle and therefore essential for the subsequent supply of methyl groups.

Conversely, high alcohol intake reduces the bioavailability of folate and such a disruption of the one-carbon metabolism may affect DNA methylation in for example

the colonic mucosa (15,16). Long term alcohol consumption may also result in an increased risk of tumors harboring MSI (17,18).

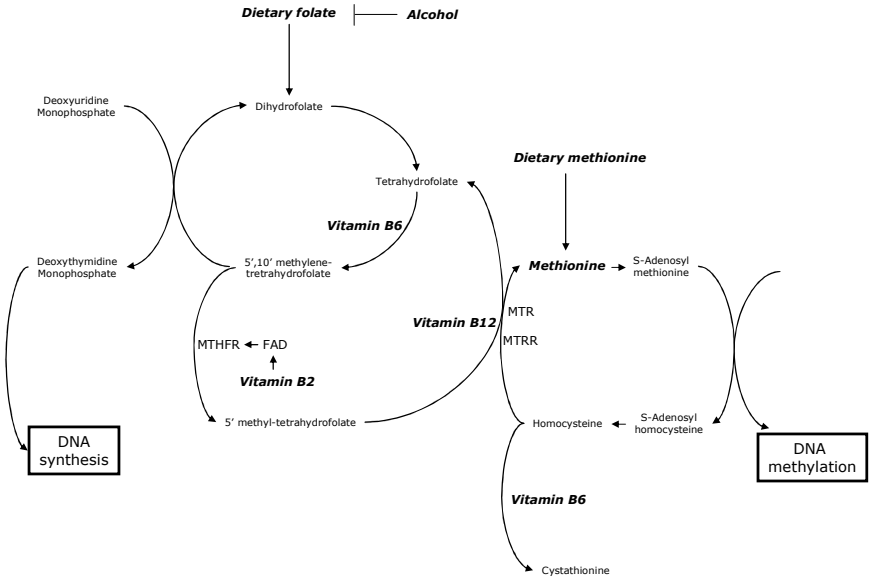

Figure 1 The role of folate, methionine, vitamins B2 and B6, and alcohol in the synthesis and methylation of DNA.

Dietary factors are displayed in *Italics.* Methyl donors are folate and methionine; factors that may modulate the bioavailability of methyl groups are vitamin B2, vitamin B6 and alcohol. FAD: flavine adenine dinucleotide, MTHFR: methylene tetrahydrofolate reductase, MTR: methionine synthase; MTRR: methionine synthase reductase.

Since folate plays an important role in both DNA synthesis and -methylation, one would expect that adequate folate status or sufficient folate intake can also reduce the risk of tumors harboring gene mutations. Opposite to this however, we have previously observed that men in the third tertile of folate intake may be at increased risk of CRCs with truncating *APC* mutations (19). The relation between diet and *BRAF* mutations has been studied, but no associations were observed with folate, vitamins B6 and B12, and methionine (20).

Here, we investigate associations between dietary folate, vitamin B2, vitamin B6, methionine and alcohol in relation to *MLH1* promoter methylation and the associated molecular characteristics of absence of MLH1 protein expression, MSI and *BRAF* mutations in CRCs. This allows us to establish which of these endpoints associated with the MSI phenotype is most sensitive to dietary exposure, and whether the effect of methyl donor intake through folate and methionine may be modulated by vitamins B2 and B6, or alcohol. We also describe the occurrence and overlap between *MLH1* promoter methylation, MLH1 protein expression, MSI and *BRAF* mutations. This study is carried out within the Netherlands Cohort Study on diet and cancer (NLCS), among a large group of unselected CRC patients.

Subjects and methods

Study population

The participants in this study are incident colon and rectal cancer patients and subcohort members from the Netherlands Cohort Study (NLCS), which has been described in detail elsewhere (21). Briefly, the study was initiated in 1986 and includes 58,279 men and 62,573 women aged 55-69 years at baseline, who originated from 204 Dutch municipalities with computerized population registers. At baseline, participants completed a self-administered food-frequency questionnaire that also provided information about age, sex and other risk factors for cancer. The entire cohort is being monitored for cancer occurrence by annual record linkage to the Netherlands Cancer Registry (NCR, nine cancer registries in The Netherlands) and to PALGA (Pathologisch Anatomisch Landelijk Geautomatiseerd Archief), a nationwide network and registry of histopathology and cytopathology reports (22). Accumulation of person-time in the cohort has been estimated through biennial vital status follow-up of a subcohort of 5,000 men and women who were randomly selected after baseline exposure measurement. Cases with prevalent cancer other than non-melanoma skin cancer were excluded from this subcohort, which left 4,774 men and women eligible for analysis.

In 1986, the PALGA registry was not yet implemented in some of the municipalities included in the study, but reached full coverage by the end of 1988. Incomplete coverage may introduce selection bias, and in addition, possible preclinical disease may have affected exposure status. For these reasons we excluded the first 2.3 years of follow-up from the analyses. A total of 101 subcohort members were either deceased or diagnosed with cancer other than non-melanoma skin cancer within this period, leaving 4,673 men and women for analysis. From 1989 to 1994, 925 incident cases were identified with histologically confirmed CRC of whom 815 could also be linked to a PALGA report of the lesion. The PALGA database was used to identify and locate tumor tissue in Dutch pathology laboratories. CRC was classified according to disease site as follows: colon, i.e. proximal colon (ICD-O-1 codes 153.0, 153.1, 153.4, 153.5, 153.6) and distal colon (153.2, 153.3, 153.7), rectosigmoid (154.0), rectum (154.1), or ICD-O-1 codes 153.8 and 153.9 if information of the disease site was not available.

Tissue samples

Tumor material of the colorectal cancer patients was collected after approval by the ethical review boards of Maastricht University, the NCR and PALGA (23). In addition, all pathology laboratories in the Netherlands agreed to make relevant tissue samples available from PALGA upon request. Of the 815 tissue samples that were scattered over 54 pathology laboratories in the Netherlands, 734 samples (90%) could be traced and were retrieved between August 1999 and December 2001 (23).

MLH1 promoter methylation

DNA methylation in the CpG islands of the *MLH1* gene was determined by chemical modification of genomic DNA with sodium bisulfite and subsequent Methylation Specific PCR (MSP, described in detail elsewhere (24)). In brief, 500 ng of DNA was denatured

by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega), again treated with NaOH, precipitated with ethanol and resuspended in H_2O.

To facilitate MSP analysis on DNA retrieved from formalin-fixed, paraffin-embedded tissue, DNA was first amplified with flanking PCR primers (described in ref (8)) that amplify bisulfite modified DNA but do not preferentially amplify methylated or unmethylated DNA. The resulting fragment was used as a template for the MSP-reaction (8,25).

All PCRs were performed with controls for unmethylated alleles (DNA from normal lymphocytes), methylated alleles (normal lymphocyte DNA treated in vitro with SssI methyltransferase (New England Biolabs)) and a control without DNA. Ten µl of each MSP reaction was directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide and visualized under UV illumination. MSP analyses of *MLH1* were successfully performed for 686 (93%) out of 734 patients. Reproducibility was high, with duplo anlyses performed on a random subset of 72 samples yielding the same result in 89% of the cases.

MLH1 protein expression status

Immunohistochemical analyses were performed and scored on 4 µm sections of formalin-fixed, paraffin-embedded cancer tissue and adjacent normal tissue using a monoclonal antibody against MLH1, as previously described (17). Staining was evaluated with normal cells as internal control. Two investigators reviewed the immunohistochemical staining profiles independently and discrepancies were re-examined and discussed with a pathologist until consensus was reached. MLH1 protein expression status was determined successfully in 721 (98%) of the 734 patients.

Microsatellite instability

Microsatellite instability (MSI) was determined by a pentaplex PCR, using the MSI markers BAT-26, BAT-25, NR-21, NR-22 and NR-24, as described in detail by Suraweera et al (26). MSI analyses were successful on 662 (90%) out of the 734 available samples. The reproducibility was 100%, since all of the 53 duplo analyses had identical results.

BRAF mutations

The common V600E *BRAF* mutation in exon 15 was analyzed by a semi-nested PCR and subsequent RFLP analyses as previously described (27). *BRAF* mutation status could be analyzed successfully in 697 (95%) out of 734 tissue samples. The analyses could be reproduced in a satisfactory 88% of the additional duplo analyses on 33 randomly drawn samples.

Food frequency questionnaire

The self-administered questionnaire was a 150-item semi-quantitative food frequency questionnaire (FFQ), which concentrated on habitual consumption of food and beverages during the year preceding the start of the study, and also contained questions about body weight and -length, smoking status, physical activity, and family history of colorectal cancer. Daily mean nutrient intakes were calculated as the

cumulated product of the frequencies and portion sizes of all food items and their tabulated nutrient contents from the Dutch Food Composition Table (NEVO table, 1986 (28)). The validity and reproducibility of the FFQ were determined (29,30). Questionnaire data were key-entered twice for all incident cases in the cohort and for all subcohort members in a blinded manner with respect to case/subcohort status. This was done in order to minimize observer bias in coding and interpretation of the data.

Folate data were derived from a validated liquid chromatography trienzyme method (31) used to analyze the 125 most important Dutch foods contributing to folate intake (32). Mean daily intakes of all other relevant nutrients were calculated using the computerized Dutch Food Composition Table (28). Dietary supplement data were also obtained via the food frequency questionnaire. However, the use of B-vitamin supplements was low (7%) and folic acid was generally not included in these supplements in the Netherlands in the late 1980s. Therefore, folic acid supplement use most likely plays a very minor role in our study population, and supplement use was not further accounted for in the analyses.

Statistical analyses

The occurrence and relative overlap of *MLH1* hypermethylation with any of the three other characteristics - absence of MLH1 protein expression, MSI, and *BRAF* mutations - were calculated and tested with Chi-square tests. Dietary factors and other baseline characteristics were summarized for men and women separately, for subcohort members and colorectal cancer cases by calculating means and standard deviations for continuous variables and distributions of the categorical variables. Differences in dietary folate, vitamins B2 and B6, methionine and alcohol intake were tested between CRC cases and the non-cases in the subcohort using Student t-tests or Chi-square tests where appropriate. This comparison was also made for the four other molecular characteristics.

Cox proportional hazards regression models were used to estimate multivariate-adjusted incidence rate ratios (RR) and corresponding 95% confidence intervals (CI) over tertiles of folate intake, vitamin B2, vitamin B6 and methionine, and over categories of alcohol intake, using the lowest intake categories as reference. Standard errors (SE) of the RRs were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling from the cohort (33). The proportional hazards assumption was tested using the scaled Schoenfeld residuals (34). Tests for dose response trends over the different categories of intake were estimated by fitting the ordinal exposure variables as continuous variables and evaluated using the Wald test. For each endpoint, a model was used that included all of these 5 dietary variables. Folate, vitamins B2 and B6, and methionine were adjusted for total energy intake by calculating nutrient residuals from the regression of nutrient intake on total energy intake, as described by Willet *et al* (35). These nutrient residuals are uncorrelated with total energy intake, and the effect of the variation in nutrient intake can subsequently be estimated independently of a potential effect of energy intake. The RRs were calculated for all colorectal tumors, for tumors with *MLH1* hypermethylation, absence of MLH1 protein expression, MSI, or *BRAF* mutations. Because tumors presenting MSI usually occur more frequently among women, and may have a different etiology than in men, we have chosen to present gender-specific results. In addition to these analyses, we also performed overall analyses for men and

women combined in order to create larger subgroups and thereby to reduce the probability of reporting chance findings.

Tests for heterogeneity were performed to evaluate differences between subtypes of tumors (e.g. *MLH1* methylated versus unmethylated), using the competing risks procedure in Stata. However, the SE for the difference of the logHRs from this procedure assumes independence of both estimated HRs, which would underestimate that SE and thus overestimate the p-values for their difference. Therefore, these p-values and the associated confidence intervals were estimated based on a bootstrapping method that was developed for the case-cohort design (36). For each bootstrap sample, X subcohort members were randomly drawn from the subcohort of X subjects and Y cases from the total of Y cases outside the subcohort, both with replacement, out of the dataset of X + Y observations. The logHRs were obtained from this sample using Stata's competing risks procedure and recalculated for each bootstrap-replication. The confidence interval and p-value of the differences in hazard ratio of the subtypes were then calculated from the replicated statistics. Each bootstrap analysis was based on 1,000 replications.

The covariates included in the multivariate analyses were those found to influence the RR by more than 10 percent, or those observed to be associated with CRC in previous studies. This applied for the variables age, family history of CRC, smoking, Body Mass Index (BMI), and dietary intakes of energy, meat, fat, fiber, vitamin C, iron and calcium. After excluding subjects with missing information on these covariates or subjects who only partly filled out the questionnaire, 4,059 subcohort members remained for statistical analyses as well as 648 CRC cases, irrespective of the available molecular analyses.

We determined possible interactions between dietary intakes of methyl donors (folate and methionine) and the potentially "modulating" factors vitamin B2, vitamin B6 and alcohol, for each of the individual endpoints. This was done by first testing, in separate models, the gender-specific interaction terms between folate and methionine, with vitamin B2, vitamin B6 and alcohol, i.e. folate*methionine, folate*B2, folate*B6, folate*alcohol, methionine*B2, methionine*B6 and methionine*alcohol. The Cox proportional hazard analyses without the interaction terms were subsequently stratified by low or high intake of folate, vitamins B2 and B6, and methionine using the median intakes as cut-off values to define both strata within each variable. The strata used for alcohol intake were 1: abstainers, 2: subjects with intake <30 g/day, and 3: subjects who consumed ≥30 g/day.

All statistical analyses were performed with the Stata statistical software package (version 9.1).

Results

Among the CRC patients in our study, we first assessed the occurrence of *MLH1* hypermethylation, MLH1 protein expression, MSI, and *BRAF* mutations. The analyses of these four characteristics were complete of 648 patients. The frequencies and percentages, as well as the overlap between these molecular phenotypes are shown in Table 1 and Figure 2.

Table 1 Frequencies of molecular phenotypes and overlap of *MLH1* promoter methylation with MLH1 expression, MSI and *BRAF* mutations

		Frequency of molecular phenotype		*MLH1* promoter methylation				
				Methylated		Unmethylated		
		N	(%)	N*	(%)	N*	(%)	*P*-value
***MLH1* methylation**	Methylated	152	(22.1%)	-	-	-	-	
	Unmethylated	534	(77.9%)	-	-	-	-	-
MLH1 expression	No	61	(8.5%)	41	(74.6%)	14	(25.4%)	
	Yes	660	(91.5%)	97	(17.3%)	463	(82.7%)	*<0.001*
MSI	Present	84	(12.7%)	50	(65.8%)	26	(34.2%)	
	Absent	578	(87.3%)	88	(16.3%)	452	(83.7%)	*<0.001*
BRAF	Mutation	112	(16.1%)	48	(47.5%)	53	(52.5%)	
	Wild Type	585	(83.9%)	90	(17.5%)	425	(82.5%)	*<0.001*

* Numbers and percentages are based on tumors with complete analyses of all four molecular characteristics (tumors of 648 cases in total)

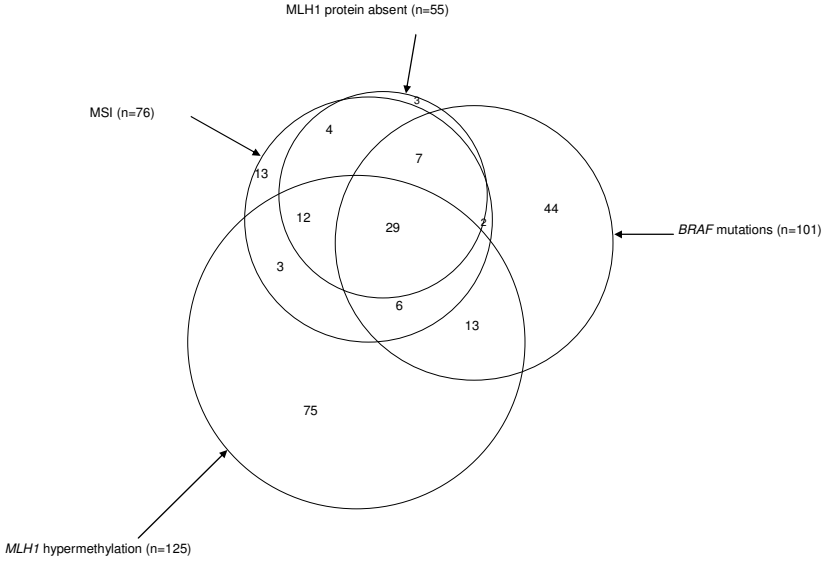

Figure 2 Overlap between *MLH1* hypermethylation, absence of MLH1 protein expression, MSI and *BRAF* mutations in tumors showing at least one of these four aberrations.

In total, there were n=238 tumors harboring at least one aberration. Numbers are based on tumors with complete analyses of all four molecular characteristics (tumors of n=648 cases in total). The sizes of the different areas in this figure do not exactly reflect the numbers of the applicable subsets.

In 152 (22.1%) of the patients with successful *MLH1* MSP analyses promoter methylation of this gene was found. The percentages of tumors that lacked the MLH1 protein, those with MSI as determined by the MSI pentaplex assay, or those harboring *BRAF* mutations were 8.5%, 12.7% and 16.1% respectively. Although the associations between *MLH1* hypermethylation and the three other aberrations individually were

highly significant (all Chi-square tests had *P*-values <0.001), the overlap between the four molecular phenotypes, which was based on patients with all molecular analyses available, was not complete. The highest relative overlap was observed with MLH1 protein expression since out of the 55 tumors lacking the MLH1 protein, 41 (74.6%) also showed *MLH1* hypermethylation. The overlap between MSI and *MLH1* hypermethylation was lower, with 50 (65.8%) out of 76 tumors harboring MSI also having *MLH1* hypermethylation. The lowest percentage of overlap was observed between *BRAF* mutations and *MLH1* hypermethylation, as 48 (47.5%) out of 101 *BRAF*-mutated tumors had a hypermethylated *MLH1* gene.

We then explored dietary intakes and other baseline characteristics of subcohort members and cancer cases with *MLH1* promoter methylation, absence of MLH1 protein expression, MSI, or *BRAF* mutations among men and women (Table 2). The percentage of male patients with a family history of colorectal cancer was substantially lower among men with a hypermethylated *MLH1* gene, as compared to all CRC tumors combined in men. It was also clear that tumors with *MLH1* hypermethylation, without MLH1 protein expression, with MSI or *BRAF* mutations all occurred more often in the proximal colon if compared to the total group of tumors in both men and women. The intakes of folate, vitamin B2, vitamin B6, methionine and alcohol were similar among cases in the different subgroups compared to subcohort members.

We subsequently estimated the associations between intakes of folate, vitamins B2 and B6, methionine and alcohol on the one hand, with colorectal carcinoma risk with or without *MLH1* hypermethylation, absence of MLH1 protein expression, MSI or *BRAF* mutations on the other. Among men, folate intake was not associated with overall colorectal cancer, with tumors showing *MLH1* hypermethylation, lacking MLH1 protein expression or with MSI. However, it was positively associated with colorectal tumors harboring *BRAF* mutations in men (RR=3.04, CI=1.13-8.20, P_{trend}=0.03 for the highest versus the lowest tertile of intake, Table 3). Conversely, there was an inverse association between methionine intake and risk of *BRAF* mutated tumors (highest vs. lowest tertile: RR=0.28, CI=0.09-0.86, P_{trend}=0.02). Both vitamin B2 and alcohol were not associated with any of the endpoints studied in men. However, dietary intake of vitamin B6 was positively associated with overall colorectal cancer in men (RR=1.54, CI=1.01-2.36, P_{trend}=0.06 for the highest versus the lowest tertile of intake). The association between vitamin B6 and tumors with *MLH1* hypermethylation in men was even stronger (RR=3.23, CI=1.15-9.06, P_{trend}=0.03).

Among women, dietary intakes of folate, vitamin B2, vitamin B6, methionine and alcohol were not associated with overall colorectal cancer or any of the endpoints studied (Table 4). Although the RRs of tumors with *BRAF* mutations and those with *MLH1* hypermethylation were also increased in the highest tertiles of folate and vitamin B6 intake respectively, these associations were not statistically significant among women.

Table 2 Baseline dietary intake (mean ± SD) and other characteristics of cancer cases and subcohort members from the Netherlands Cohort Study on diet and cancer

	MEN						WOMEN					
	Sub-cohort	All tumors	*MLH1* Methylation	No MLH1 protein	MSI	*BRAF* Mutation	Subcohort	All tumors	*MLH1* Methylation	No MLH1 protein	MSI	*BRAF* Mutation
Patient characteristics												
N *	2017	367	65	24	38	49	2042	281	73	32	38	52
Age(y)	61.3 ± 4.2	62.7 ± 4.1	62.5 ± 4.1	63.4 ± 4.4	62.9 ± 4.6	62.1 ± 4.2	61.3 ± 4.3	62.9 ± 3.9	62.5 ± 4.4	62.7 ± 4.6	63.6 ± 4.3	62.9 ± 4.0
Family History of CRC (% yes)	5.5	12.0	4.6	9.4	7.9	8.2	5.9	10.0	9.6	9.4	7.9	11.5
Tumor sublocalisation (%)												
Proximal	-	29.0	52.4	83.3	73.7	62.5	-	39.9	57.8	84.4	89.2	68.6
Distal	-	33.2	22.2	8.3	18.4	14.6	-	30.2	25.4	15.6	10.8	19.6
Rectosigmoid	-	10.5	9.5	8.3	7.9	2.1	-	11.5	7.0	0	0	2.0
Rectum	-	27.3	15.9	0	0	20.8	-	18.4	9.8	0	0	9.8
BMI (kg/m^2)	25.0 ± 2.6	25.4 ± 2.7	25.6 ± 2.5	25.7 ± 2.5	25.3 ± 2.3	25.2 ± 2.4	25.0 ± 3.6	25.6 ± 3.6	25.2 ± 3.4	25.5 ± 4.0	25.9 ± 4.2	25.6 ± 3.5
Smoking Status (%)												
Never	12.9	9.8	7.7	4.2	7.9	14.3	57.6	61.6	61.6	53.1	55.3	55.8
Ex Smoker	51.7	63.2	61.5	50.0	60.5	53.1	20.8	22.4	21.9	25.0	18.4	32.7
Current Smoker	35.4	27.0	30.8	45.8	31.6	32.6	21.6	16.0	16.4	21.9	26.3	11.5
Dietary factors												
Energy (kjoules/day)	9091 ± 21.37	8909 ± 1866	8911 ± 1715	8674 ± 1412	9211 ± 1863	8969 ± 1410	7062 ± 1655	6997 ± 1695	6774 ± 1741	6685 ± 1381	6851 ± 1516	6675 ± 1373
Folate (µg/day)	225.0 ± 75.3	225.5 ± 75.4	225.0 ± 73.9	209.5 ± 50.3	228.4 ± 89.1	233.5 ± 81.3	198.8 ± 67.6	197.6 ± 70.5	187.1 ± 64.5	198.4 ±101.9	191.8 ± 97.3	190.8 ±81.9
Vitamin B2 (mg/day)	1.58 ± 0.37	1.57 ± 0.35	1.62 ± 0.38	1.70 ± 0.38	1.65 ± 0.39	1.59 ± 0.36	1.45 ± 0.35	1.44 ± 0.34	1.37 ± 0.31	1.42 ± 0.35	1.38 ± 0.33	1.39 ± 0.31
Vitamin B6 (mg/day)	1.54 ± 0.27	1.56 ± 0.27	1.58 ± 0.28	1.56 ± 0.22	1.52 ± 0.26	1.56 ± 0.25	1.33 ± 0.24	1.34 ± 0.23	1.31 ± 0.22	1.33 ± 0.31	1.32 ± 0.31	1.33 ± 0.27
Methionine (mg/day)	1713 ± 293	1701 ± 284	1693 ± 274	1729 ± 252	1676 ± 266.2	1699 ± 2.68	1491 ± 276	1492 ± 271	1436 ± 254	1451 ± 263	1452 ± 261	1487 ± 243
Alcohol (%)												
0 g/day	14.3	13.4	12.3	25	15.8	10.2	32.3	36.7	31.5	34.4	34.2	36.5
< 30 g/day	71.2	69.2	72.3	66.7	73.7	75.5	64.3	58.7	61.6	56.2	57.9	55.8
≥ 30 g/day	14.6	17.4	15.4	8.3	10.5	14.3	3.4	4.6	6.9	9.4	7.9	7.7
Meat (g/day)	137.3 ± 52.2	132.2 ± 47.3	132.1 ± 45.6	128.5 ± 41.6	125.0 ± 42.5	135.1 ± 44.4	116.1 ± 46.0	118.2 ± 42.8	113.0 ± 43.3	116 ± 42.5	117.3 ± 40.5	116.6 ± 48.1
Fat (g/day)	94.0 ± 14.2	93.5 ± 12.8	96.8 ± 12.0	99.0 ± 12.0	95.0 ± 11.7	95.3 ± 10.3	74.0 ± 10.3	74.9 ± 10.5	74.8 ± 10.6	73.2 ± 11.9	74.8 ± 12.5	74.1 ± 11.6
Fiber (g/day)	28.7 ± 7.3	29.6 ± 6.7	29.4 ± 7.0	28.6 ± 5.9	28.8 ± 8.0	29.2 ± 7.3	25.3 ± 5.8	24.8 ± 5.6	25.4 ± 6.5	24.9 ± 6.8	24.7 ± 7.2	24.6 ± 5.8
Vitamin C (mg/day)	98.8 ± 41.6	106.8 ± 44.9	102.6 ± 45.9	95.2 ± 30.2	95.8 ± 42.5	105.4 ± 46.3	108.4 ± 42.7	107.0 ± 43.2	102.6 ± 45.0	103.7 ± 47.0	99.0 ± 42.4	102.0 ± 44.2
Iron (mg/day)	13.2 ± 2.4	13.7 ± 2.4	13.6 ± 2.5	13.4 ± 1.7	13.4 ± 2.3	13.7 ± 2.6	11.7 ± 2.0	11.6 ± 2.0	11.6 ± 1.9	11.6 ± 1.9	11.5 ± 2.0	11.7 ± 1.6
Calcium (mg/day)	947 ± 293	943 ± 281	964 ± 279	1048 ± 364	1006 ± 344	950 ± 306	903 ± 268	899 ± 256	839 ± 216	860 ± 243	863 ± 245	858 ± 233

* Numbers of subcohort members and patients are based on complete availability of dietary information and/or complete analyses of *MLH1* methylation, *MLH1* expression, MSI and *BRAF* mutations

Table 3 Associations between dietary factors and *MLH1* hypermethylation, absence of the MLH1 protein, MSI and *BRAF* mutations in colorectal tumors among men

		Overall			*MLH1* hypermethylation			No MLH1 protein			MSI			*BRAF* mutations		
Tertile (median within tertile)	**PY ***	**N †**	**RR ‡**	**95% CI §**	**N**	**RR**	**95% CI**	**N**	**RR**	**95% CI**	**N**	**RR**	**95% CI**	**N**	**RR**	**95% CI**
Folate (µg/day)																
1 (163.2)	3207	125	1.00		24	1.00		8	1.00		13	1.00		10	1.00	
2 (211.6)	3248	110	0.79	0.57-1.08	18	0.66	0.31-1.40	10	1.42	0.51-3.99	14	1.02	0.42-2.51	19	2.33	1.07-5.06
3 (279.9)	3258	132	0.97	0.65-1.44	23	0.88	0.36-2.14	6	1.00	0.20-5.10	11	0.78	0.23-2.67	20	3.04	1.13-8.20
P-value for linear trend			*0.88*			*0.83*			*0.95*			*0.66*			*0.03*	
Vitamin B2 (mg/day)																
1 (1.26)	3282	126	1.00		20	1.00		6	1.00		11	1.00		16	1.00	
2 (1.53)	3259	128	0.95	0.70-1.31	24	1.19	0.56-2.55	8	1.15	0.35-3.75	11	1.04	0.42-2.57	16	0.80	0.36-1.76
3 (1.90)	3172	113	0.80	0.53-1.21	21	0.93	0.35-2.46	10	1.13	0.24-5.31	16	1.59	0.56-4.53	17	0.79	0.28-2.24
P-value for linear trend			*0.32*			*0.92*			*0.88*			*0.39*			*0.67*	
Vitamin B6 (mg/day)																
1 (1.29)	3242	104	1.00		15	1.00		7	1.00		14	1.00		16	1.00	
2 (1.53)	3258	128	1.32	0.94-1.85	25	2.26	1.02-5.04	8	1.53	0.47-4.99	11	1.09	0.42-2.81	16	0.94	0.42-2.09
3 (1.79)	3212	135	1.54	1.01-2.36	25	3.23	1.15-9.06	9	2.49	0.57-10.86	13	1.82	0.57-5.80	17	1.04	0.35-3.08
P-value for linear trend			*0.06*			*0.03*			*0.24*			*0.31*			*0.89*	
Methionine (mg/day)																
1 (1445)	3243	127	1.00		26	1.00		8	1.00		15	1.00		19	1.00	
2 (1697)	3290	127	0.98	0.69-1.40	18	0.46	0.22-0.99	8	0.51	0.16-1.56	13	0.65	0.26-1.64	17	0.60	0.27-1.34
3 (1986)	3180	113	0.93	0.56-1.52	21	0.42	0.14-1.25	8	0.25	0.03-1.90	10	0.35	0.07-1.83	13	0.28	0.09-0.86
P-value for linear trend			*0.77*			*0.12*			*0.17*			*0.20*			*0.02*	
Alcohol																
0 g/day (0)	1370	49	1.00		8	1.00		6	1.00		6	1.00		5	1.00	
< 30 g/day (6.7)	6907	254	1.05	0.74-1.50	47	1.14	0.54-2.41	16	0.60	0.19-1.88	28	1.02	0.39-2.67	37	1.52	0.57-4.05
≥ 30 g/day (40.6)	1437	64	1.25	0.78-2.00	10	1.61	0.61-4.21	2	0.54	0.09-3.17	4	0.74	0.19-2.89	7	1.40	0.39-4.94
P-value for linear trend			*0.36*			*0.36*			*0.42*			*0.68*			*0.57*	

* Number of accumulated Person Years (PY) within categories of dietary intake

† Number of cases within categories of dietary intake

‡ Incidence Rate Ratio (RR) from a Cox regression model including the variables folate, vitamins B2 and B6, methionine and alcohol. Adjusted for age, family history of colorectal cancer, smoking behavior, BMI, energy, meat, total fat, fiber, vitamin c, total iron and calcium

§ 95% Confidence Interval (CI)

Table 4 Associations between dietary factors and *MLH1* hypermethylation, absence of the MLH1 protein, MSI and *BRAF* mutations in colorectal tumors among women

		Overall			*MLH1* hypermethylation			No MLH1 protein			MSI			*BRAF* mutations		
Tertile (median within tertile)	**PY ***	**N †**	**RR ‡**	**95% CI §**	**N**	**RR**	**95% CI**	**N**	**RR**	**95% CI**	**N**	**RR**	**95% CI**	**N**	**RR**	**95% CI**
Folate (µg/day)																
1 (142.4)	3295	98	1.00		31	1.00		12	1.00		16	1.00		20	1.00	
2 (186.6)	3384	99	1.02	0.72-1.47	22	0.79	0.39-1.58	11	1.27	0.50-3.19	13	0.93	0.37-2.34	17	1.23	0.58-2.61
3 (247.9)	3359	84	0.92	0.57-1.48	20	0.88	0.33-2.32	9	1.22	0.31-4.74	9	0.72	0.19-2.72	15	1.42	0.51-3.95
P-value for linear trend			*0.76*			*0.76*			*0.68*			*0.67*			*0.47*	
Vitamin B2 (mg/day)																
1 (1.12)	3324	97	1.00		32	1.00		14	1.00		16	1.00		22	1.00	
2 (1.42)	3358	90	0.99	0.69-1.41	22	0.84	0.45-1.56	7	0.66	0.22-1.99	10	0.85	0.34-2.13	14	0.75	0.33-1.71
3 (1.76)	3356	94	1.08	0.68-1.71	19	0.94	0.39-2.26	11	1.18	0.31-4.48	12	1.26	0.37-4.23	16	0.93	0.30-2.91
P-value for linear trend			*0.95*			*0.78*			*0.94*			*0.92*			*0.68*	
Vitamin B6 (mg/day)																
1 (1.12)	3330	90	1.00		23	1.00		13	1.00		15	1.00		21	1.00	
2 (1.32)	3416	92	1.16	0.80-1.69	26	1.39	0.70-2.75	7	0.57	0.17-1.86	10	0.78	0.28-2.18	12	0.56	0.23-1.34
3 (1.54)	3292	99	1.45	0.93-2.28	24	1.61	0.70-3.71	12	0.93	0.29-3.01	13	1.10	0.36-3.39	19	0.97	0.39-2.46
P-value for linear trend			*0.11*			*0.27*			*0.88*			*0.89*			*0.96*	
Methionine (mg/day)																
1 (1232)	3370	96	1.00		28	1.00		13	1.00		15	1.00		17	1.00	
2 (1476)	3331	85	0.83	0.56-1.22	25	1.08	0.51-2.30	7	0.69	0.21-2.30	9	0.69	0.24-2.00	16	1.44	0.59-3.49
3 (1738)	3338	100	0.89	0.53-1.51	20	1.13	0.39-2.29	12	1.19	0.34-4.13	14	1.15	0.33-4.01	19	2.06	0.67-6.32
P-value for linear trend			*0.64*			*0.83*			*0.89*			*0.88*			*0.23*	
Alcohol																
0 g/day (0)	3210	103	1.00		23	1.00		11	1.00		13	1.00		19	1.00	
< 30 g/day (3.9)	6478	165	0.82	0.62-1.10	45	1.10	0.62-1.94	18	0.93	0.41-2.11	22	1.02	0.48-2.17	29	0.77	0.41-1.47
≥ 30 g/day (36.8)	351	13	1.58	0.78-3.20	5	3.81	1.27-11.47	3	3.38	0.97-11.87	3	3.96	1.09-4.40	4	2.54	0.70-9.19
P-value for linear trend			*0.54*			*0.29*			*0.64*			*0.53*			*0.99*	

* Number of accumulated Person Years (PY) within categories of dietary intake

† Number of cases within categories of dietary intake

‡ Incidence Rate Ratio (RR) from a Cox regression model including the variables folate, vitamins B2 and B6, methionine and alcohol. Adjusted for age, family history of colorectal cancer, smoking behavior, BMI, energy, meat, total fat, fiber, vitamin c, total iron and calcium

§ 95% Confidence Interval (CI)

Increased relative risks could be observed in the highest category of alcohol intake for most of the endpoints, however, trends were not statistically significant and the numbers of cases in the highest category were low for both men and women.

When performing overall analyses for men and women together (data not shown), we observed that, although borderline significant, the highest tertile of folate intake was associated with colorectal tumors harboring *BRAF* mutations (RR=1.85, CI=0.92-3.73, P_{trend}=0.08). There clearly were positive associations between vitamin B6 intake and overall CRC (RR=1.51, CI=1.11-2.04, P_{trend}=0.01) and risk of tumors with *MLH1* promoter methylation (RR=2.32, CI=1.21-4.43, P_{trend}=0.01). The tests for heterogeneity did not show significant differences between tumors with or without *MLH1* hypermethylation, MLH1 protein expression, MSI or *BRAF* mutations (data not shown).

After testing potential interactions, it appeared that there was no interaction between folate and methionine, vitamin B2 or alcohol in either men or women. However, among men we observed an interaction between folate and vitamin B6 for overall CRC. In this respect, stratified analyses revealed that relatively high vitamin B6 intake was associated with an increased risk of overall CRC among men who had a folate intake in the category above the median (RR=1.96, CI=1.06-3.60, P_{trend}=0.02 for the highest vs. lowest tertile of B6 intake, data not shown), whereas the RR was not increased among subjects with folate intake below the median of the distribution ($P_{interaction}$=0.06). Similarly, men having tumors with *MLH1* hypermethylation also had higher RRs in the 3rd tertile of vitamin B6 intake in the subgroup of high folate intake (RR=3.36, CI=0.81-13.93, P_{trend}=0.08, data not shown), which was not observed among men with low folate intake. However, the interaction term for this endpoint was not statistically significant. Among women, an interaction between vitamin B6 and folate was not observed, though it was present for men and women combined for overall CRC ($P_{interaction=}$0.07). We observed no interactions between methionine and any of the other dietary variables under study.

Discussion

In this study we investigated the associations between dietary folate, vitamins B2 and B6, methionine, alcohol and colorectal cancer risk while accounting for *MLH1* promoter methylation, MLH1 protein expression, MSI and *BRAF* mutations. It was observed that the highest tertiles of folate, vitamins B2, B6 and methionine, and low alcohol intake were not associated with decreased colorectal cancer risk, irrespective of the presence of *MLH1* hypermethylation, the MLH1 protein or MSI. On the other hand, we observed a positive association between dietary folate and *BRAF* mutations in men. Moreover, vitamin B6 increased overall CRC risk, and especially *MLH1*-hypermethylated tumors among men.

We studied four different aberrations that have previously been suggested to be associated with the MSI pathway. Despite the strong and statistically significant correlations between these characteristics, they were not always concurrently present in tumors. Whereas the percentages of overlap between absence of MLH1 protein expression and *MLH1* methylation, and between MSI and *MLH1* methylation were relatively high (74.6% and 65.8% respectively), less than half of the tumors with *BRAF*

mutations (47.5%) appeared to have *MLH1* methylation. In this respect, it is appreciated that individual techniques were used for each endpoint, each having specific sensitivities, which may partly account for the observed incomplete overlap. However, an additional, even more obvious reason may lie in potential differences in tumor biology. For example, within the subset of sporadic CRCs showing MSI, there may be differences in the frequency of *MLH1* hypermethylation depending on the localization of the tumor. In this respect, *MLH1* hypermethylation was observed less frequently in distal sporadic CRCs with MSI compared to proximal MSI cancers, suggesting that an epigenetic pathway has played a smaller role in the development of distal MSI cancers (37). Next to *MLH1* hypermethylation, *MLH1* mutations may result in loss of the MLH1 protein as well. Although such mutations are mainly observed in tumors of HNPCC patients with MSI (38), it has been suggested that they may also occur in sporadic colorectal carcinomas, and that MSI may be present concurrently (39). The occurrence of *MLH1* hypermethylation, lack of MLH1 protein expression or *BRAF* mutations may apparently differ among tumors that develop through the MSI pathway. In addition to the observed incomplete overlap in our study, this is an important reason to study these molecular phenotypes separately in relation to methyl donor intake. From our results, it appeared that the endpoint of MSI is least related to the intake of the studied methyl donors, but that there rather may be an effect on methylation and mutations which are associated with MSI. Moreover, the relation between folate intake and MSI in colorectal cancer was previously investigated (18), but no association was observed in that particular study.

Promoter hypermethylation and global hypomethylation are alternative types of aberrant methylation, and it has been suggested that both of these patterns may contribute separately to the process of colorectal carcinogenesis (40,41). Moreover, they may also specifically affect DNA stability, given the observed strong associations between promoter hypermethylation and MSI and between global hypomethylation and CIN in colorectal carcinomas (42,43). However, the relative contribution of methyl donor intake to either hypermethylation or hypomethylation is unknown. Promoter hypermethylation may occur in normal colorectal mucosa of patients with hyperplastic polyposis (44) or colorectal cancer (45), suggesting that dietary methyl donors may play a role in the prevention or initiation of neoplastic formation. Moreover, folic acid supplementation may decrease global hypomethylation in the normal-appearing colonic mucosa of patients with adenomas (46). Furthermore, in a pilot study we previously observed an indication for an inverse association between relatively high folate intake in combination with low alcohol intake and promoter methylation in colorectal tumors (8). However, this association was only weak, and the conclusion in this study was based on an outcome measure defined as hypermethylation of at least one out of six genes. Others observed positive associations between high alcohol consumption and risk of MSI-H tumors (17,18,20), indicating that disruption of the folate metabolism may lead to colorectal cancer, possibly partly through increasing promoter hypermethylation.

Although the above suggests a protective effect of adequate folate status or intake on aberrant methylation, it has been hypothesized that its actual influence may also differ depending on the stage of tumor development in colorectal carcinogenesis. Whereas folate deficiency might increase the risk of neoplastic transformation of normal tissue by inducing genomic hypomethylation, it may have an inhibitory effect

on progression of neoplasms to cancer. Conversely, folate supplementation may prevent aberrant DNA methylation in normal tissue, but could promote established lesions by increasing promoter hypermethylation (47). Results of a recent study suggest that folic acid and vitamin B12 supplementation in subjects with a history of colorectal adenoma may indeed increase promoter hypermethylation, although this association was statistically borderline significant (9). Moreover, folic acid supplementation was associated with increased risk of advanced lesions or recurrence of multiple adenomas in patients with available follow-up information (48). Interestingly, the participants in that study also had a recent history of colorectal adenomas, and possibly, undetected neoplasms were present in these subjects which may have had a growth advantage in the presence of high concentrations of folic acid. However, one might wonder whether intake of dietary folate has a similar effect as supplementation with folic acid. Nevertheless, our data revealed the strongest increased risk among patients in the highest tertile of vitamin B6 intake of tumors with *MLH1* hypermethylation, suggesting that vitamin B6 may have had a tumor-promoting effect by increasing promoter methylation. We also observed that the increased RRs in the highest tertiles of vitamin B6 of overall CRC and tumors harboring *MLH1* hypermethylation were present only among men who also had a relatively high folate intake. The latter association suggests that the transfer of methyl donors provided by folate may at least partly depend on the availability of vitamin B6 as a "modulating" factor, and that the combination of relatively high vitamin B6 and folate intake may increase promoter methylation and thereby enhance the development of tumors with a methylation-associated phenotype. Our results therefore contribute to the hypothesis that relatively high methyl donor intake potentially increases aberrant promoter hypermethylation rather than preventing it.

Several other studies showed inverse associations between vitamin B6 intake and CRC risk. However, in these studies, the levels of vitamin B6 intake were generally higher, and the distributions sometimes even lie largely above the median intake of the highest tertile of the Dutch population included in our study (49-52). One could hypothesize that, if vitamin B6 is protective above a certain threshold of intake, this may have been a reason why we did not observe an inverse association. However, vitamin B6 was also associated with decreased CRC risk in a recent study in a Japanese population, in which intake of vitamin B6 was comparable to that in our study (53). There is one study that has previously demonstrated a positive association between vitamin B6 intake and CRC risk, among women who had a much higher vitamin B6 intake than in our study population (54). Apparently, a potential positive association between vitamin B6 and colorectal cancer should be taken into account and obviously needs further attention in future studies.

Another interesting finding in our study is the positive association between dietary folate and *BRAF* mutations among men, which was not expected considering the importance of folate for nucleotide synthesis. We have previously observed that folate may increase the risk of tumors harboring truncating *APC* mutations in men (19). *BRAF* and *APC* mutations are inversely correlated in this study population, which was also the case in a previous study (55), indicating that relatively high folate intake may give a growth advantage to mutated tumors independent of the type of mutation. Moreover, folate was not associated with *MLH1* hypermethylation in men, suggesting that it exerts a specific effect on tumors with gene mutations.

A limitation of our study is that some of the molecular subgroups present relatively small numbers of cases, and the possibility that some of the gender-specific results are partly based on chance can therefore not be excluded. However, overall analyses, with subgroups then containing substantial numbers of cases (at least 30) within tertiles of intake, showed similar positive associations of folate with *BRAF* mutations and of vitamin B6 with *MLH1* hypermethylation. Furthermore, residual confounding may have been present for the associations observed, although methyl donor intake was comparable between molecular subgroups and associations were further adjusted for a number of potential confounders or known risk factors of CRC. We consider it unlikely that changes in dietary intake over time have influenced disease outcome, since the reproducibility of the baseline FFQ over a period of 5 years was relatively high (30) and the follow-up of 7.3 years was only 2.3 years longer in the current study.

In this Dutch population, relatively high intakes of folate, vitamins B2 and B6 and methionine, or low alcohol intake were not associated with a decreased risk of CRC, or with tumors harboring *MLH1* hypermethylation, lack of MLH1 protein expression or with MSI. The occurrence of MSI does not seem to be sensitive to methyl donor intake; however, folate increased the risk of *BRAF* mutations whereas vitamin B6 increased the risk of tumors with *MLH1* hypermethylation among men. This may indicate that dietary folate and vitamin B6 have different effects, but may both enhance colorectal carcinogenesis by exerting an effect on genetic or epigenetic alterations.

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6

Genetic variants of methyl metabolizing enzymes and epigenetic regulators: associations with promoter CpG island hypermethylation in colorectal cancer

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Abstract

Aberrant DNA methylation affects carcinogenesis of colorectal cancer (CRC). Folate metabolizing enzymes may influence the bioavailability of methyl groups whereas DNA- and histonemethyltransferases are involved in epigenetic regulation of gene expression.

We studied associations between genetic variants of folate metabolizing enzymes (*MTHFR, MTR* and *MTRR*), the DNA methyltransferase *DNMT3b*, and histone methyltransferases (*EHMT1, EHMT2* and *PRDM2*) with CRC, accounting for the CpG island methylator phenotype (CIMP), *MLH1* hypermethylation and microsatellite instability (MSI). Incidence rate ratios (RR) were calculated in case-cohort analyses, with common alleles as reference, among 659 cases and 1,736 subcohort members of the Netherlands Cohort Study on diet and cancer (n=120,852).

Men with the *MTHFR* 677TT genotype were at decreased CRC risk (RR=0.49; P=0.01), but the T allele was associated with increased risk in women (RR=1.39; P=0.02). The *MTR* 2756GG genotype was associated with increased CRC risk (RR=1.58; P=0.04), and inverse associations were observed among women carrying rare variants of the DNMT3b C>T (rs406193, RR=0.72; P=0.04) or EHMT2 G>A (rs535586, RR=0.76; P=0.05) polymorphisms. Although significantly correlated (P<0.001), only 41.5% and 33.3% of CIMP tumors harbored *MLH1* hypermethylation or MSI, respectively. We observed inverse associations between *MTR* A2756G and CIMP among men (RR=0.58; P=0.04), and between *MTRR* A66G and *MLH1* hypermethylation among women (RR=0.55; P=0.02).

In conclusion, genetic variants of *MTHFR*, *MTR*, *DNMT3b* and *EHMT2* are associated with CRC risk and rare variants of *MTR* and *MTRR* may reduce promoter hypermethylation. Importantly, the incomplete overlap between CIMP, *MLH1* hypermethylation and MSI indicates that these related "methylation phenotypes" may not be similar and should be investigated separately.

Introduction

Aberrant DNA methylation is an important epigenetic modification which may affect cancer susceptibility (1). Folate metabolizing enzymes are involved in the provision of methyl groups, and the bioavailability of methyl groups is dependent on the activity of these enzymes. Single nucleotide polymorphisms (SNPs) in these genes have been reported to alter enzymatic activity. For example, the C677T and A1298C polymorphisms in the methylenetetrahydrofolate (*MTHFR*) gene result in reduced enzymatic activity (2,3) and were inversely associated with colorectal cancer (CRC) in several observational studies (4,5). However, it was suggested that subjects having the *MTHFR* 677TT or 1298CC genotypes were more likely to develop CRC showing a CpG island hypermethylation phenotype (6,7) or microsatellite instability (MSI) (8). Genetic variants of methionine synthase (*MTR*) have been studied less extensively, but the *MTR* A2756G SNP was suggested to decrease CRC risk in two studies (9,10). In contrast, it was suggested that some of the rare genetic variants of methionine synthase reductase (*MTRR*) may be associated with an increased risk of colorectal adenomas (CRA) and carcinomas (11-13).

Whereas the above mentioned one-carbon metabolizing enzymes are involved in the provision of methyl groups, DNA methyltransferases catalyze the transfer of these methyl groups from S-adenosylmethionine into CpG dinucleotides of DNA. The DNA methyltransferase 3b (*DNMT3b*) is involved in *de novo* methylation (14), and may influence epigenetic regulation of gene expression and cancer cell growth. In this respect, experimental research suggested that *DNMT3b* depletion can reduce aberrant promoter CpG island hypermethylation in cancer cells (15-17), while DNMT3b overexpression initiated promoter hypermethylation of tumor suppressor genes and the formation of colonic miroadenomas (18). In addition, it was observed that expression of DNMT3b was associated with *p16* and *RASSF1A* promoter methylation in non-small cell lung cancer (19), and with a promoter hypermethylator phenotype in breast cancer (20). Several SNPs of the *DNMT3b* gene have been identified, which may affect catalytic activity of the DNMT3b enzyme. For example, the *DNMT3b* C>T (rs2424913) polymorphism was found to significantly increase *DNTM3b* promoter activity, and was associated with an increased risk of lung cancer (21), prostate cancer (22), colorectal polyps including CRAs (23) or with prognosis of head and neck cancer (24). Moreover, subjects with hereditary non-polyposis colorectal cancer (HNPCC) carrying the TT genotype developed CRC at a younger age compared to those homozygous for the wild type *DNMT3b* CC allele (25). However, the association between the *DNMT3b* C>T (rs2424913) polymorphism and CRC risk has not previously been investigated.

It has been hypothesized that DNA methyltransferases may only have an effect on the chromatin if histone H3 lys-9 (H3K9) is first methylated by histone methyltransferases (HMTs; Figure 1), and that DNA methylation may thus depend on the activity of histone methyltransferases (26). Experimental research also indicated that methylation of H3K9 and other histones play a critical role in maintaining epigenetic silencing by promoter hypermethylation of genes involved in CRC (27). The retinoblastoma protein (Rb) interacting zinc finger gene (*RIZ* or *PRDM2)* is a HMT which may act as a tumor suppressor and *PRDM2* frameshift mutations have been observed in CRCs showing microsatellite instability (MSI) (28).

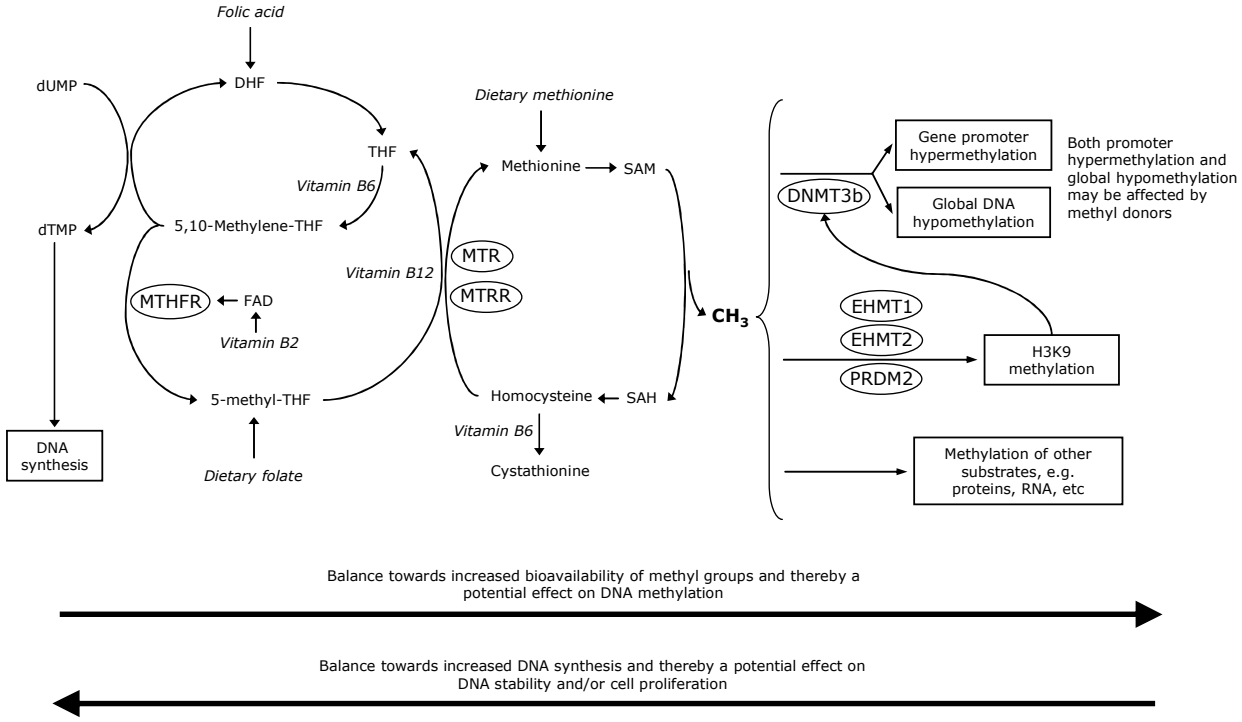

Figure 1 Folate metabolizing enzymes, DNA methyltransferases, histone methyl transferases and DNA methylation

Potential targets of methyl groups are DNA, lysines (amongst which lysine 9 of Histone 3 (H3K9)), or other substrates e.g. proteins, RNA, etc. DNMT3b activity and promoter CpG island hypermethylation may depend on prior methylation of H3K9. Ovals represent the enzymes of which SNPs are investigated in this study. dUMP: deoxyuridine monophosphate, dTMP: deoxythymidine monophosphate, DHF: dihydrofolate, THF: tetrahydrofolate, FAD: flavine adenine dinucleotide, SAM: S-adenosyl methionine, SAH: S-adenosyl homocysteine, MTHFR: methylene tetrahydrofolate reductase, MTR: methionine synthase, MTRR: methionine synthase reductase, DNMT3b: DNA methyltransferase 3b, PRDM2: PR domain 2, EHMT: euchromatine histone methyltransferase.

Genetic variation of *PRDM2* may be hypothesized to affect its gene activity, and SNPs in *PRDM2* were observed to be inversely associated with lung cancer (29). However, an increased risk of breast cancer was suggested (30). Other genes that have been identified as histone methyltransferases are Euchromatin Histone Methyltransferase-1 (*EHMT1*) and -2 (*EHMT2*), and genetic variants of these genes were modestly associated with breast cancer risk in a large case-control study (30). However, the potential impact of genetic variants of *PRDM2*, *EHMT1* and *EHMT2*, have not previously been studied in relation to CRC.

The aim of this study was to determine the occurrence of SNPs in genes encoding folate metabolizing enzymes (*MTHFR, MTR* and *MTRR*), the DNA methyltransferase *DNMT3b*, and histone methyltransferases (*EHMT1, EHMT2* and *PRDM2*) in a large population-based prospective cohort study in the Netherlands. We estimated associations of these SNPs with overall CRC risk and with CRCs with or without the CpG island methylator phenotype (CIMP), *MLH1* hypermethylation or MSI.

Subjects and methods

Study population and tumour tissue samples

Tumor material was obtained from incident CRC patients from the Netherlands cohort study (NLCS), which has been described in detail elsewhere (31). Briefly, this prospective cohort study was initiated in September 1986 and includes 58,279 men and 62,573 women aged 55-69 years and free of disease at baseline. The cohort is followed for cancer occurrence by annual record linkage to the Netherlands Cancer Registry (NCR) and to PALGA (Pathologisch Anatomisch Landelijk Geautomatiseerd Archief), a nationwide network and registry of histopathology and cytopathology reports (32,33). At baseline, participants filled out a self administered food frequency questionnaire, by which also information was obtained about age, sex, family history of CRC, smoking behaviour and body mass index (BMI). A subcohort of 5,000 subjects was randomly selected after baseline exposure measurement, to estimate accumulation of person-time in the cohort through biennial follow-up of vital status. Cases with prevalent cancer other than non-melanoma skin cancer were excluded from this subcohort, which left 4,774 men and women eligible for analysis. Tumor material of the CRC patients was collected after approval by the ethical review boards of Maastricht University, the NCR and PALGA. During a follow-up period of 7.3 years after baseline, 734 incident CRC patients were identified who had an available PALGA report of the lesion as well as a sufficient amount of isolated DNA.

Collection of mouth swabs of subcohort members

Subcohort members still alive in December 2000 (n=3,579) were contacted and asked to collect mouth swabs. Four cotton swabs in a small non-woven polyethylene envelope were mailed to each subject, including a simple protocol on how to use them. After receipt, the swabs were placed in a falcon tube containing 2.0 ml buffer solution (100mM NaCl, 10 mM EDTA, 10 mM Tris, Ph 8 with 0.2 mg/ml proteinase K and 0.5% w/v SDS). The lysed solution was kept stored at room temperature in the dark. In total, 1,929 subcohort members (54%) returned the mouth swabs with informed

consent. The average DNA yield per cotton swab was 0.1-10 µg, which corresponded with data from literature (34). In total, DNA could successfully be isolated from 1,829 subcohort members who also had complete follow-up information.

Genotyping analyses

Nine fragments containing the *MTHFR, MTR, MTRR, DNMT3b, EHMT1, EHMT2* and *PRDM2* SNPs were amplified, using multiplex polymerase chain reaction (PCR) amplification and single base extension (SBE) reactions as described previously by Knaapen et al (35). Genomic DNA (50 ng) was added to 1 x PCR buffer (Invitrogen, Breda, the Netherlands), 1.75 mM $MgCl_2$ (Invitrogen), 0.4 µmol/L dNTPs (Amersham Bioscience, Buckinghamshire, England), 100 nM of each primer (Eurogentec, Maastricht, The Netherlands) and 0.25 U Platinum Taq (Invitrogen) in a final volume of 10µl. PCR conditions were as follows: 3 min at 94 °C, 35 cycles of 30 sec at 94 °C, 30 sec at 62 °C and 30 sec at 72 °C, and a final extension for 5 minutes at 72 °C and 4 minutes at 20 °C. To degrade excess PCR primers and dNTPs, 5 µl multiplex PCR product was incubated with 2 µl EXO-SAP IT (Amersham Bioscience) at 37 °C for 15 minutes followed by 80 °C for 15 minutes to deactivate the enzyme. The multiplex SBE reaction was performed using a SNaPShot multiplex kit as described by the manufacturer (Applied Biosystems, Foster City, CA, USA). SBE primers were designed to bind immediately adjacent 5' to the SNP of interest with a template specific part of 20 to 33 bp and a Tm of 60 °C. Rs-numbers of the SNPs and primer sequences are shown in Supplementary Table 1.

During thermal cycling, the primers are extended at their 3' end with a single dideoxyribonucleoside triphosphate labelled with a distinct fluorophore, revealing the genotype of the SNP. SBE was performed using 25 cycles of 96 °C for 10 s and 60 °C for 30 s. Following cycling, the reaction was treated with 1 U Shrimp Alkaline Phosphatase (Amersham Bioscience) at 37 °C for 1 h to degrade the unincorporated dideoxynucleotide triphosphates, followed by enzyme deactivation at 75 °C for 15 min. One µl of SBE product was mixed with 13 µl of Hi-Di formamide (Applied Biosystems) and 0.4 µl of Genescan-120 size standard (Applied Biosystems), subsequently denatured at 95 °C for 5 min and then analyzed on an ABI Prism 3100 genetic analyzer using Genemapper Analysis software (version 4.0).

To validate the genotype data, we sequenced every fragment containing a specific SNP in a subset of 30 samples including 10 CRC cases, 10 female and 10 male subcohort members with mouth swabs. Sequencing was performed using the BigDye ®Terminater v1.1 cycle sequencing kit, following the manufacturer's recommendations using the ABI 3700 genetic analyzer. The sequencing results were similar to the SNaPShot results for all but one (99.6%) of the 9 SNPs within these 30 samples. Reproducibility of the SNaPShot analysis was established by subjecting 93 samples of which 31 CRC cases, 31 female and 31 male subcohort members with mouth swabs, twice to the complete SNaPShot analysis procedure, from multiplex PCR of genomic DNA to the genetic analysis of the samples. We observed that the analyses could be reproduced in 99.5% of these cases.

Supplementary Table 1 Overview of polymorphisms of the studied genes and primers used for genotyping analyses

Gene	SNP *	Rs number	Type of primer ‡	Primers (5' → 3')	Primer length §
Folate metabolizing enzymes					
MTHFR	C677T	rs1801133	*PCR primers:*	Forward: CTT TGA GGC TGA CCT GAA GC	20bp
				Reverse: TCA CAA AGC GGA AGA ATG TG	20bp
			SBE primer:	AAAGCTGCGTGATGATGAAATCG	23bp
MTHFR	A1298C	rs1801131	*PCR primers:*	Forward: AGG AGG AGC TGC TGA AGA TG	20bp
				Reverse: CCT TGT GAC CAT TCC GGT TT	20bp
			SBE primer:	AACTGACTAGAGGTAAAGAACAAA--GACTTCAAAGACACTT	40bp
MTR	A2756G	rs1805087	*PCR primers:*	Forward: CAG TGT TCC CAG CTG TTA GAT G	22bp
				Reverse: CAA GCA AAA ATC TGT TTC TAC CAC	24bp
			SBE primer:	TCATGGAAGAATATGAAGATATTAGA--CAGG	30bp
MTRR	A66G	rs1801394	*PCR primers:*	Forward: GCT ACA CAG CAG GGA CAG G	19bp
				Reverse: GCA CAA AAC GGT AAA ATC CAC T	22bp
			SBE primer:	AACAGACTAAATCCATGTACCACAGCT--TGCTCACA	35bp
DNA methyltransferase					
DNMT3b	C>T †	rs2424913	*PCR primers:*	Forward: TTG TCC TGA AGC TGG CTA CC	20bp
				Reverse: CGA GTT CGG ACC TAG AAG CA	20bp
			SBE primer:	CTGGCCCCGCCAGACCC	17bp
DNMT3b	C>T †	rs406193	*PCR primers:*	Forward: AAG TGC TTT GCC TGA CAC CT	20bp
				Reverse: CGT GCA TCC AGT CTT CAT TG	20bp
			SBE primer:	AACTGACTAAACTACCAGAGATGGCAT--ATTGTAAAAATGAGACC	45bp
Histone methyltransferases					
EHMT1	G>A †	rs4634736	*PCR primers:*	Forward: CTT AGC CTC CCA AAC TGC TG	20bp
				Reverse: GCG CTG CCA AGT CTC CTT	18bp
			SBE primer:	AACTCTCTAAAGATCGTCGGTCGTCGAT--TGCCAAGTCTCCTTATGGCTGA	50bp
EHMT2	G>A	rs535586	*PCR primers:*	Forward: TGC ATC CCA TCC TCA GTA GA	20bp
				Reverse: TTC GTC AGG GTC ACT TCT CC	20bp
			SBE primer:	AACTGACTAAACTATGTTCCACGTCGT--CCGGGCAGAACCTAACTCCTC	48bp
PRDM2	G>A †	rs2235515	*PCR primers:*	Forward: GAT GGA AGA ATG GAA AGT GAG G	22bp
				Reverse: AGA GCT GGC TCT GCA AAC A	19bp
			SBE primer:	CCTCCTGCTTTCCTACCTGCC	21bp

* SNP: Single Nucleotide Polymorphism
† SNP ocurring in intron
‡ Primers used for Polymerase Chain Reaction (PCR) and Single Base Extension (SBE) SNaPShot analysis
§ Primer length, number of base pairs (bp)

Promoter methylation analyses

The CpG island methylator phenotype (CIMP) was defined by promoter hypermethylation of at least 3 out of 5 methylation markers (*CACNA1G, IGF2, NEUROG1, RUNX3* and *SOCS1*), as suggested by Weisenberger *et al* (36). DNA methylation in the CpG islands of these five CIMP markers and of the *MLH1* gene was determined by Methylation Specific PCR (MSP) (37). Bisulfite modification was carried out on 500ng DNA using a commercially available kit (Zymo Research). To facilitate

MSP analysis on DNA retrieved from formalin-fixed, paraffin-embedded tissue, DNA was first amplified with flanking PCR primers that amplify bisulfite modified DNA but do not preferentially amplify methylated or unmethylated DNA.

The resulting fragment was used as a template for the MSP-reaction (38,39). All PCRs were performed with controls for unmethylated alleles (DNA from normal lymphocytes), methylated alleles (normal lymphocyte DNA treated in vitro with SssI methyltransferase (New England Biolabs)) and a control without DNA. Ten µl of each MSP reaction was directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide and visualized under UV illumination. The MSP analyses were successful of 81%, 79%, 79%, 90%, 83% and 93% out of the 734 patients for *CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1* and *MLH1*, respectively.

Microsatellite instability

MSI was determined by a pentaplex PCR, using the MSI markers BAT-26, BAT-25, NR-21, NR-22 and NR-24, as described in detail by Suraweera et al (40). MSI analyses were successful on 662 (90%) out of the 734 available samples.

Statistical analyses

Data analyses were conducted overall, and for men and women separately. The prevalence of the genotypes and minor allele frequencies (MAF) of the SNPs were calculated for subcohort members and CRC cases. Chi square tests were used to test differences in prevalence between CRC cases and subcohort members who did not develop CRC. The Hardy-Weinberg equilibrium was tested among subcohort members to evaluate whether for each SNP, the individual alleles combined into the observed genotypes in a random manner. Although this subcohort was a random sample of the total cohort, and thus selection would not be expected, the DNA samples were collected from individuals still alive several years after baseline. If survival of these subjects was due to presence of specific genotypes, this may bias the estimated associations.

However, since genetic status is unknown of the remaining subcohort members it is impossible to compare survival between genotypes, and we therefore alternatively compared mean age between genotypes of each gene.

The overlap between the three methylation endpoints, i.e. CIMP, *MLH1* hypermethylation and MSI, was compared and tested by Chi-square tests. Cox proportional hazards regression models were used to estimate age-adjusted incidence rate ratios (RR) and 95% confidence intervals (CI) taking homozygotes for common alleles as reference. Analyses were performed overall, and stratified by CIMP status, *MLH1* hypermethylation and MSI, allowing us to compare the effects between the recently defined CIMP phenotype (36), the "classic" methylation marker *MLH1* and MSI, and to account for a potential incomplete overlap that may exist between related molecular phenotypes (41). Since two SNPs were determined for *MTHFR* and *DNMT3b*, we estimated RRs for combinations of genotypes within these genes. In addition, RRs for combinations of genotypes per functional group were estimated (i.e. based on the number of rare alleles in any of the folate metabolizing enzymes *MTHFR*, *MTR* and *MTRR*, or in any of the histone methyltransferases *EHMT1*, *EHMT2* and *PRDM2*), or the combination of all the studied genes. Standard errors (SE) of the RRs were estimated using the robust Huber-White sandwich estimator to account for additional variance

introduced by sampling from the cohort (42). The proportional hazards assumption was tested using the scaled Schoenfeld residuals (43). Multivariate-adjusted analyses were additionally conducted including the co-variates dietary folate, vitamin B2, vitamin B6, methionine, alcohol, energy intake, family history of CRC, smoking behaviour and body mass index. Interactions with sex were tested for each of the SNPs. Statistical analyses were performed with the Stata statistical software package (version 10).

Results

The overall and gender-specific prevalences of genotypes were calculated and compared between subcohort members and CRC cases. We observed that the rare *MTHFR* 677TT variant occurred more often in subcohort members than in cases, and that this difference was present among men (prevalences in subcohort and cases were 9.7% and 5.0%, respectively). Prevalences of other genotypes did not significantly differ between subcohort members and cases (Table 1). The distribution of the *MTHFR* A1298C, *MTRR* A66G and *DNMT3b* C>T (rs406193) genotypes deviated from the Hardy-Weinberg equilibrium ($P<0.05$). However, mean age was similar between genotypes of any of the SNPs, suggesting that there was no survivorship effect due to genetic status.

Overall, subjects with the heterozygous *MTHFR* 677CT genotype were at modestly increased CRC risk compared to participants with the common CC genotype (RR=1.23, $P=0.04$, Table 2). Similarly, positive associations existed among women with this genotype (RR=1.43, $P=0.02$), and for the occurrence of the rare T allele (i.e. the combination of the CT and TT genotypes compared to CC, RR=1.39, $P=0.02$).

Table 1 Prevalence of genotypes of subcohort members and colorectal cancer patients in the Netherlands Cohort Study

			Men and women		Men		Women	
Gene and SNP *	dbSNP number	Genotype (MAF) ‡	Subcohort *(HWE)* §	CRC cases	Subcohort *(HWE)* §	CRC cases	Subcohort *(HWE)* §	CRC cases
***MTHFR* C677T**	rs1801133	CC	876 (48.9 %)	318 (46.2 %)	409 (45.4%)	179 (46.8%)	467 (52.3%)	139 (45.3%)
		CT	750 (41.8 %)	320 (46.4 %)	405 (44.9%)	184 (48.2%)	345 (38.7%)	136 (44.3%)
		TT	167 (9.3 %)	51 (7.4 %)	87 (9.7%)	19 (5.0%)	80 (9.0%)	32 (10.4%)
		(0.30)	*(0.72)*	¶	*(0.36)*	¶	*(0.16)*	
***MTHFR* A1298C**	rs1801131	AA	735 (41.6%)	299 (43.7%)	345 (39.3%)	167 (43.8%)	390 (43.9%)	132 (43.6%)
		AC	774 (43.8%)	275 (40.2%)	423 (48.2%)	166 (43.6%)	351 (39.5%)	109 (36.0%)
		CC	258 (14.6%)	110 (16.1%)	110 (12.5%)	48 (12.6%)	148 (16.6%)	62 (20.4%)
		(0.37)	*(0.02)*		*(0.26)*		*(<0.001)*	
***MTR* A2756G**	rs1805087	AA	1190 (65.9%)	449 (64.5%)	589 (65.1%)	257 (66.4%)	601 (66.8%)	192 (62.1%)
		AG	543 (30.1%)	204 (29.3%)	281 (31.0%)	108 (27.9%)	262 (29.1%)	96 (31.1%)
		GG	72 (4.0%)	43 (6.2%)	35 (3.9%)	22 (5.7%)	37 (4.1%)	21 (6.8%)
		(0.19)	*(0.31)*		*(0.84)*		*(0.22)*	
***MTRR* A66G**	rs1801394	AA	367 (20.4%)	136 (19.5%)	193 (21.4%)	65 (16.7%)	174 (19.4%)	71 (23.0%)
		AG	833 (46.4%)	338 (48.3%)	399 (44.2%)	196 (50.2%)	434 (48.5%)	142 (45.9%)
		GG	597 (33.2%)	225 (32.2%)	310 (34.4%)	129 (33.1%)	287 (32.1%)	96 (31.1%)
		(0.56)	*(0.01)*		*(0.003)*		*(0.67)*	
***DNMT3b* C>T †**	rs2424913	CC	597 (32.9%)	240 (34.1%)	303 (33.4%)	132 (33.9%)	294 (32.5%)	108 (34.4%)
		CT	895 (49.5%)	348 (49.5%)	449 (49.6%)	184 (47.3%)	446 (49.4%)	164 (52.2%)
		TT	318 (17.6%)	115 (16.4%)	154 (17.0%)	73 (18.8%)	164 (18.2%)	42 (13.4%)
		(0.42)	*(0.58)*		*(0.57)*		*(0.82)*	
***DNMT3b* C>T †**	rs406193	CC	1331 (74.3%)	528 (75.6%)	686 (76.7%)	291 (74.6%)	645 (71.9%)	237 (76.9%)
		CT	415 (23.1%)	152 (21.8%)	190 (21.2%)	88 (22.6%)	225 (25.1%)	64 (20.8%)
		TT	46 (2.6%)	18 (2.6%)	19 (2.1%)	11 (2.8%)	27 (3.0%)	7 (2.3%)
		(0.14)	*(0.05)*		*(0.18)*		*(0.18)*	
***EHMT1* G>A †**	rs4634736	GG	1444 (80.6%)	568 (81.7%)	730 (81.4%)	320 (83.1%)	714 (79.8%)	248 (80.0%)
		GA	334 (18.6%)	121 (17.4%)	163 (18.2%)	64 (16.6%)	171 (19.1%)	57 (18.4%)
		AA	14 (0.8%)	6 (0.9%)	4 (0.4%)	1 (0.3%)	10 (1.1%)	5 (1.6%)
		(0.10)	*(0.27)*		*(0.11)*		*(0.95)*	
***EHMT2* G>A**	rs535586	GG	755 (42.2%)	297 (42.9%)	390 (43.3%)	155 (40.3%)	365 (41.2%)	142 (46.3%)
		GA	810 (45.3%)	311 (44.9%)	414 (45.9%)	185 (48.0%)	396 (44.6%)	126 (41.0%)
		AA	223 (12.5%)	84 (12.2%)	97 (10.8%)	45 (11.7%)	126 (14.2%)	39 (12.7%)
		(0.35)	*(0.80)*		*(0.41)*		*(0.27)*	
***PRDM2* G>A †**	rs2235515	GG	1065 (59.0%)	377 (55.7%)	527 (58.5%)	210 (56.0%)	538 (59.6%)	167 (55.3%)
		GA	640 (35.5%)	262 (38.7%)	320 (35.5%)	141 (37.6%)	320 (35.4%)	121 (40.1%)
		AA	99 (5.5%)	38 (5.6%)	54 (6.0%)	24 (6.4%)	45 (5.0%)	14 (4.6%)
		(0.23)	*(0.82)*		*(0.56)*		*(0.77)*	

* SNP: Single Nucleotide Polymorphism
† SNP occurring in an intron of the gene
‡ MAF: Minor Allele Frequency among subcohort members (men and women combined)
§ *P*-value for test for Hardy-Weinberg equilibrium (HWE), based on the distribution of genotypes among subcohort members
¶ *P*-value < 0.05 for the difference in genotype frequencies between CRC cases and subcohort members who did not develop CRC

Table 2 Associations of SNPs in folate metabolizing enzymes and epigenetic regulators with CRC risk

Gene and SNP *	dbSNP number	Genotype	Men and women			Men			Women			Interaction with sex
			N †	RR (95% CI) ‡	*P*	N	RR (95% CI) §	*P*	N	RR (95% CI) §	*P*	*P*
***MTHFR* C677T**	rs1801133	CC	318	1.00		179	1.00		139	1.00		
		CT	320	1.23 (1.02-1.50)	0.04	184	1.10 (0.84-1.43)	0.49	136	1.43 (1.07-1.91)	0.02	
		TT	51	0.80 (0.56-1.15)	0.23	19	0.49 (0.28-0.85)	0.01	32	1.26 (0.78-2.05)	0.34	*0.03*
		CT+TT *vs* CC	371	1.15 (0.95-1.39)	0.15	203	0.98 (0.76-1.27)	0.89	168	1.39 (1.06-1.83)	0.02	
***MTHFR* A1298C**	rs1801131	AA	299	1.00		167	1.00		132	1.00		
		AC	275	0.89 (0.72-1.09)	0.26	166	0.81 (0.62-1.07)	0.14	109	1.00 (0.73-1.35)	0.98	
		CC	110	1.05 (0.79-1.38)	0.74	48	0.89 (0.59-1.35)	0.58	62	1.23 (0.85-1.78)	0.28	*0.42*
		AC+CC *vs* AA	385	0.93 (0.77-1.12)	0.45	214	0.83 (0.64-1.07)	0.15	171	1.07 (0.81-1.41)	0.64	
***MTR* A2756G**	rs1805087	AA	449	1.00		257	1.00		192	1.00		
		AG	204	1.03 (0.84-1.27)	0.77	108	0.95 (0.72-1.26)	0.73	96	1.14 (0.85-1.54)	0.38	
		GG	43	1.58 (1.03-2.43)	0.04	22	1.45 (0.79-2.66)	0.23	21	1.76 (0.97-3.21)	0.06	*0.64*
		AG+GG *vs* AA	247	1.10 (0.90-1.33)	0.35	130	1.01 (0.77-1.32)	0.94	117	1.22 (0.92-1.62)	0.17	
***MTRR* A66G**	rs1801394	AA	136	1.00		65	1.00		71	1.00		
		AG	338	1.08 (0.85-1.39)	0.53	196	1.36 (0.96-1.92)	0.09	142	0.83 (0.59-1.18)	0.31	
		GG	225	1.03 (0.79-1.34)	0.83	129	1.23 (0.85-1.78)	0.27	96	0.85 (0.58-1.24)	0.39	*0.15*
		AG+GG *vs* AA	563	1.06 (0.84-1.34)	0.62	325	1.30 (0.94-1.81)	0.11	238	0.84 (0.60-1.16)	0.29	
***DNMT3b* C>T**	rs2424913	CC	240	1.00		132	1.00		108	1.00		
		CT	348	0.95 (0.78-1.17)	0.66	184	0.91 (0.68-1.20)	0.50	164	1.01 (0.75-1.36)	0.94	
		TT	115	0.90 (0.68-1.19)	0.46	73	1.00 (0.69-1.45)	0.99	42	0.76 (0.50-1.16)	0.20	*0.37*
		CT+TT *vs* CC	463	0.94 (0.77-1.14)	0.54	257	0.93 (0.71-1.22)	0.60	206	0.95 (0.71-1.26)	0.72	
***DNMT3b* C>T**	rs406193	CC	528	1.00		291	1.00		237	1.00		
		CT	152	0.91 (0.73-1.14)	0.43	88	1.13 (0.83-1.54)	0.43	64	0.71 (0.51-0.99)	0.05	
		TT	18	1.05 (0.59-1.87)	0.87	11	1.32 (0.59-2.95)	0.50	7	-	-	*0.10*
		CT+TT *vs* CC	170	0.93 (0.75-1.15)	0.49	99	1.15 (0.86-1.54)	0.36	71	0.72 (0.52-0.99)	0.04	
***EHMT1* G>A**	rs4634736	GG	568	1.00		320	1.00		248	1.00		
		GA	121	0.93 (0.73-1.18)	0.55	64	0.92 (0.66-1.28)	0.62	57	0.95 (0.67-1.34)	0.76	
		AA	6	-	-	1	-	-	5	-	-	*0.41*
		GA+AA *vs* GG	127	0.93 (0.73-1.18)	0.55	65	0.89 (0.64-1.24)	0.49	62	0.98 (0.70-1.37)	0.90	
***EHMT2* G>A**	rs535586	GG	297	1.00		155	1.00		142	1.00		
		GA	311	0.92 (0.76-1.13)	0.44	185	1.10 (0.84-1.44)	0.48	126	0.74 (0.55-0.99)	0.05	
		AA	84	1.01 (0.74-1.37)	0.95	45	1.17 (0.76-1.80)	0.47	39	0.84 (0.55-1.27)	0.44	*0.13*
		GA+AA *vs* GG	395	0.94 (0.78-1.14)	0.53	230	1.12 (0.86-1.44)	0.41	165	0.76 (0.58-1.01)	0.05	
***PRDM2* G>A**	rs2235515	GG	377	1.00		210	1.00		167	1.00		
		GA	262	1.14 (0.93-1.38)	0.20	141	1.09 (0.83-1.43)	0.54	121	1.20 (0.90-1.60)	0.21	
		AA	38	1.09 (0.72-1.66)	0.67	24	1.02 (0.60-1.75)	0.93	14	1.20 (0.63-2.29)	0.58	*0.85*
		GA+AA *vs* GG	300	1.13 (0.94-1.37)	0.20	165	1.08 (0.83-1.40)	0.57	135	1.20 (0.91-1.58)	0.20	

* SNP: Single Nucleotide Polymorphism
† Number of cases. Subgroups of 10 or less cases were considered too small for precise estimates and were therefore omitted from the table
‡ Adjusted for age and sex
§ Adjusted for age

Conversely, we observed that men homozygous for the *MTHFR* 677TT genotype were at reduced CRC risk (RR=0.49, *P*=0.01). There was a significant interaction between sex and *MTHFR* C677T genotypes (*P*=0.03). Whereas *MTHFR* A1298C was not associated with CRC, we observed a positive association between the *MTR* 2756GG genotype and CRC (RR=1.58, *P*=0.04) particularly among women (RR=1.76, *P*=0.06). Among men, the *MTR* 2756GG genotype was associated with a non-significant increase of CRC risk (RR=1.45, *P*=0.23). *MTRR* A66G genotypes were not associated with overall CRC.

Among women, the rare alleles of *DNMT3b* C>T (rs406193) and *EHMT2* G>A (rs535586) were inversely associated with CRC (RR=0.72, *P*=0.04 and RR=0.76, *P*=0.05, respectively), but such associations were not observed among men. No associations were observed between individual genotypes of the remaining genes and overall CRC risk. Combining *MTHFR* and *DNMT3b* genotypes, genotypes per functional group or genotypes of all studied genes did not reveal any clear associations with CRC. Multivariate-adjusted analyses resulted in similar findings compared to age-adjusted analyses (data not shown).

In total, 167 (27.7%) out of the 603 CRCs with available CIMP analyses showed the CIMP phenotype (Table 3 and Figure 2), with no significant difference between men and women (data not shown). As reported previously for *MLH1* hypermethylation, MSI and *BRAF* mutations (41), the overlap between CIMP, *MLH1* hypermethylation and MSI was also incomplete. Although statistically significant ($P_{\text{chi square}}$<0.001), 41.5% of the CRCs with CIMP showed *MLH1* methylation, whereas only 33.3% harbored MSI.

Table 3 Frequency of CRCs harboring CIMP and overlap with *MLH1* promoter hypermethylation and MSI

	Frequency of molecular phenotype N (%)	CIMP		*P*-value *
		CIMP+	CIMP-	
CIMP				
CIMP+	167 (27.7)	-	-	-
CIMP–	436 (72.3)	-	-	
MLH1 promoter methylation				
Methylated	152 (22.1)	68 (41.5)	59 (14.0)	*<0.001*
Unmethylated	534 (77.9)	96 (58.5)	363 (86.0)	
Microsatellite instability				
MSI	84 (12.7)	52 (33.3)	17 (4.2)	*<0.001*
MSS	578 (87.3)	104 (67.7)	390 (95.8)	

* Chi-square test

MTHFR C677T polymorphisms were not associated with any of the methylation end-points among men (Table 4). However, we observed that women carrying the rare allele of *MTHFR* C677T were at significantly increased risk of developing a tumor without CIMP (CIMP$^-$, RR=1.40; P=0.04), without *MLH1* hypermethylation (RR=1.49; P=0.01) or without MSI (RR=1.36; P=0.04), but also those with CIMP (CIMP$^+$, RR=1.65; P=0.04, Table 5). Among men the *MTHFR* A1298C polymorphism was inversely associated with CRCs without CIMP (RR=0.72; P=0.03).

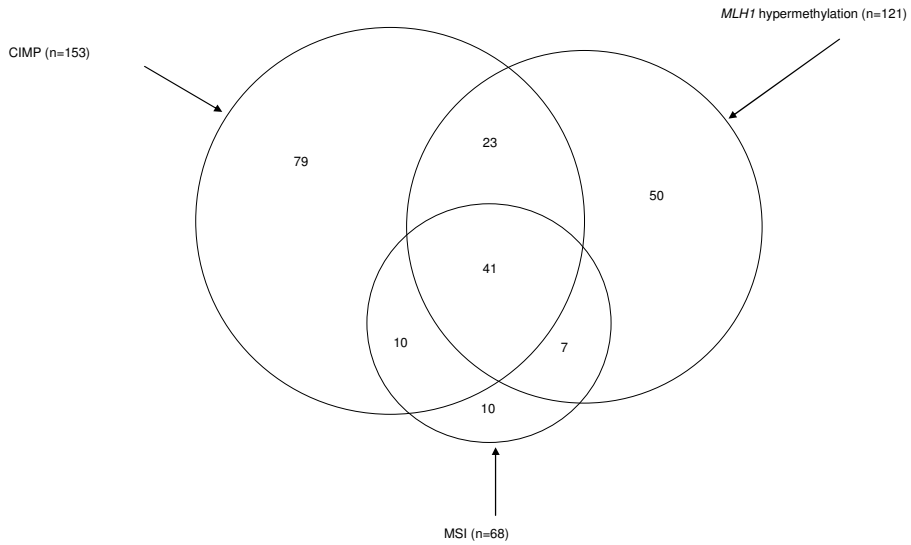

Figure 2 Overlap between CIMP, *MLH1* hypermethylation and MSI in CRCs showing at least one of these three aberrations

In total, there were n=271 CRCs harboring at least one aberration. Numbers are based on CRCs with complete analyses of all three molecular characteristics (n=547 CRCs). The sizes of the different areas in this figure may not exactly reflect the numbers of the applicable subsets.

Inverse associations were also observed between *MTR* A2756G and CIMP$^+$ among men (RR=0.58; P=0.04), and between *MTRR* A66G and *MLH1* hypermethylation among women (RR=0.55; P=0.02).

The *DNMT3b* C>T (rs406193) SNP was associated with decreased risks of CRCs without CIMP (RR=0.67; P=0.04), CRCs without MSI (RR=0.70; P=0.04), and a non-significant decreased risk of CRCs without *MLH1* hypermethylation (RR=0.73; P=0.09) among women. However, although not statistically significant, RRs were also decreased for CRCs harboring CIMP or *MLH1* hypermethylation. Similarly, *EHMT2* G>A (rs535586) was inversely associated CRCs without *MLH1* methylation (RR=0.73), CRCs without MSI (RR=0.73) or non-significantly with CRCs without CIMP (RR=0.75) in women. Conversely, we observed a positive association (RR=1.99) of *EHMT2* G>A with *MLH1* hypermethylation among men.

Table 4 Associations of SNPs in folate metabolizing enzymes and epigenetic regulators with CRC risk according to methylation status of the tumor among men

		CpG Island Methylator Phenotype (CIMP) ¶				*MLH1* promoter hypermethylation				Microsatellite instability (MSI)			
		CIMP+		CIMP–		Methylated		Unmethylated		MSI		No MSI	
Gene and SNP*	Genotype	N ‡	RR (95% CI) §	N	RR (95% CI) §	N	RR (95% CI) §	N	RR (95% CI) §	N	RR (95% CI) §	N	RR (95% CI) §
***MTHFR* C677T**	CC	43	ref.	103	ref.	32	ref.	143	ref.	21	ref.	145	ref.
(rs1801133) †	CT+TT	40	0.81 (0.51-1.28)	135	1.14 (0.84-1.54)	41	1.11 (0.68-1.81)	153	0.93 (0.70-1.22)	20	0.83 (0.44-1.58)	166	0.99 (0.75-1.30)
***MTHFR* A1298C**	AA	30	ref.	112	ref.	32	ref.	132	ref.	16	ref.	139	ref.
(rs1801131)	AC+CC	54	1.16 (0.72-1.87)	125	0.72 (0.53-0.97)	40	0.81 (0.49-1.32)	165	0.81 (0.61-1.07)	24	0.97 (0.50-1.88)	173	0.80 (0.61-1.06)
***MTR* A2756G**	AA	66	ref.	153	ref.	49	ref.	199	ref.	25	ref.	209	ref.
(rs1805087)	AG+GG	19	0.58 (0.34-0.99)	87	1.14 (0.83-1.55)	24	0.98 (0.58-1.65)	100	1.01 (0.75-1.35)	16	1.31 (0.68-2.51)	107	1.02 (0.77-1.36)
***MTRR* A66G**	AA	10	ref.	45	ref.	12	ref.	49	ref.	5	ref.	56	ref.
(rs1801394)	AG+GG	75	-	197	1.14 (0.79-1.66)	61	1.32 (0.69-2.52)	252	1.34 (0.93-1.92)	36	-	262	1.22 (0.87-1.72)
***DNMT3b* C>T**	CC	29	ref.	81	ref.	31	ref.	95	ref.	17	ref.	101	ref.
(rs2424913)	CT+TT	56	0.92 (0.60-1.49)	161	0.95 (0.69-1.30)	42	0.65 (0.39-1.06)	205	1.03 (0.77-1.38)	24	0.66 (0.35-1.28)	216	1.03 (0.77-1.36)
***DNMT3b* C>T**	CC	59	ref.	182	ref.	54	ref.	223	ref.	30	ref.	239	ref.
(rs406193)	CT+TT	26	1.49 (0.90-2.45)	60	1.11 (0.79-1.57)	19	1.19 (0.68-2.08)	78	1.18 (0.86-1.62)	11	1.25 (0.61-2.56)	79	1.12 (0.82-1.53)
***EHMT1* G>A**	GG	74	ref.	197	ref.	57	ref.	253	Ref.	36	ref.	259	ref.
(rs4634736)	GA+AA	11	0.65 (0.34-1.26)	43	0.96 (0.65-1.40)	16	1.23 (0.69-2.22)	46	0.84 (0.58-1.21)	4	-	57	0.96 (0.68-1.36)
***EHMT2* G>A**	GG	34	ref.	98	ref.	20	ref.	130	Ref.	15	ref.	124	ref.
(rs535586)	GA+AA	50	1.11 (0.69-1.76)	143	1.10 (0.81-1.48)	53	1.99 (1.16-3.41)	168	0.97 (0.73-1.28)	26	1.30 (0.67-2.51)	190	1.15 (0.88-1.52)
***PRDM2* G>A**	GG	46	ref.	126	ref.	41	ref.	160	Ref.	47	ref.	166	ref.
(rs2235515)	GA+AA	36	1.07 (0.67-1.71)	109	1.19 (0.88-1.60)	31	1.04 (0.63-1.71)	130	1.11 (0.84-1.47)	34	0.73 (0.37-1.44)	140	1.16 (0.88-1.52)

* SNP: Single Nucleotide Polymorphism
† dbSNP number
‡ Number of cases. Subgroups of ≤10 cases were considered too small for precise estimates and were therefore omitted from the table
§ Age-adjusted incidence rate ratios (95% Confidence Interval)
¶ CIMP+: ≥3 out of 5 CIMP markers methylated; CIMP–: 0-2 out of 5 CIMP markers methylated

Table 5 Associations of SNPs in folate metabolizing enzymes and epigenetic regulators with CRC risk according to methylation status of the tumor among women

		CpG Island Methylator Phenotype (CIMP) ¶				*MLH1* promoter hypermethylation				Microsatellite instability (MSI)			
		CIMP+		CIMP–		Methylated		Unmethylated		MSI		No MSI	
Gene and SNP*	Genotype	N ‡	RR (95% CI) §	N	RR (95% CI)	N	RR (95% CI)	N	RR (95% CI)	N	RR (95% CI)	N	RR (95% CI)
***MTHFR* C677T**	CC	33	ref.	82	ref.	41	ref.	94	ref.	18	ref.	115	ref.
(rs1801133) †	CT+TT	47	1.65 (1.02-2.65)	100	1.40 (1.01-1.94)	37	1.03 (0.64-1.65)	121	1.49 (1.09-2.03)	23	1.49 (0.78-2.84)	136	1.36 (1.02-1.82)
***MTHFR* A1298C**	AA	34	ref.	80	ref.	32	ref.	92	ref.	22	ref.	102	ref.
(rs1801131)	AC+CC	46	1.12 (0.70-1.80)	99	1.01 (0.73-1.41)	45	1.15 (0.71-1.85)	120	1.08 (0.79-1.48)	19	0.73 (0.38-1.39)	146	1.18 (0.87-1.59)
***MTR* A2756G**	AA	54	ref.	115	ref.	47	ref.	137	ref.	25	ref.	156	ref.
(rs1805087)	AG+GG	26	0.96 (0.59-1.58)	70	1.22 (0.87-1.71)	31	1.32 (0.82-2.13)	60	1.17 (0.85-2.61)	16	1.28 (0.67-2.45)	98	1.26 (0.93-1.70)
***MTRR* A66G**	AA	16	ref.	46	ref.	24	ref.	44	ref.	11	ref.	58	ref.
(rs1801394)	AG+GG	64	1.00 (0.56-1.79)	139	0.75 (0.51-1.11)	53	0.55 (0.33-0.92)	252	0.99 (0.68-1.46)	29	0.66 (0.32-1.37)	196	0.84 (0.59-1.20)
***DNMT3b* C>T**	CC	23	ref.	66	ref.	23	ref.	80	ref.	10	ref.	92	ref.
(rs2424913)	CT+TT	57	1.24 (0.74-2.06)	121	0.91 (0.65-1.27)	55	1.18 (0.71-1.97)	141	0.88 (0.64-1.21)	31	-	165	0.89 (0.66-1.21)
***DNMT3b* C>T**	CC	61	ref.	145	ref.	60	ref.	167	ref.	30	ref.	196	ref.
(rs406193)	CT+TT	19	0.74 (0.43-1.29)	40	0.67 (0.45-0.98)	17	0.69 (0.39-1.21)	51	0.73 (0.51-1.05)	10	-	57	0.70 (0.50-0.98)
***EHMT1* G>A**	GG	69	ref.	142	ref.	62	ref.	175	ref.	37	ref.	199	ref.
(rs4634736)	GA+AA	11	0.62 (0.32-1.21)	43	1.19 (0.81-1.75)	15	1.01 (0.56-1.82)	44	0.98 (0.67-1.44)	3	-	56	1.10 (0.78-1.57)
***EHMT2* G>A**	GG	35	ref.	86	ref.	35	ref.	102	ref.	15	ref.	119	ref.
(rs535586)	GA+AA	44	0.82 (0.51-1.32)	143	0.75 (0.54-1.04)	42	0.80 (0.50-1.28)	114	0.73 (0.54-1.00)	25	1.07 (0.56-2.07)	132	0.73 (0.54-0.98)
***PRDM2* G>A**	GG	49	ref.	99	ref.	39	ref.	122	ref.	21	ref.	137	ref.
(rs2235515)	GA+AA	31	0.94 (0.58-1.51)	109	1.22 (0.87-1.69)	37	1.41 (0.88-2.26)	91	1.11 (0.81-1.51)	20	1.41 (0.75-2.66)	111	1.20 (0.90-1.61)

* SNP: Single Nucleotide Polymorphism
† dbSNP number
‡ Number of cases. Subgroups of ≤10 cases were considered too small for precise estimates and were therefore omitted from the table
§ Age-adjusted incidence rate ratios (95% Confidence Interval)
¶ ≥3 out of 5 CIMP markers methylated
** 0-2 out of 5 CIMP markers methylated
¶ CIMP+: ≥3 out of 5 CIMP markers methylated; CIMP–: 0-2 out of 5 CIMP markers methylated

Discussion

Here we studied associations between SNPs in folate metabolizing enzymes, a DNA methyltransferase and histone methyltransferases with CRC risk, accounting for related methylation phenotypes in a large prospective cohort study in the Netherlands. We observed that *MTHFR* C677T may have opposite effects in men and women, and that *MTR* A2756G potentially increases CRC risk. Also, genetic variants of *DNMT3b* and *EHMT2* may reduce CRC risk among women. Regarding hypermethylation phenotypes, we observed that *MTR* and *MTRR* rare variants were inversely associated with CIMP in men and *MLH1* hypermethylation in women, respectively, possibly by lowering methyl group availability. Although methylation-associated characteristics were significantly related, the overlap between CIMP and *MLH1* hypermethylation or MSI was limited.

The *MTHFR* C677T and A1298C polymorphisms have previously been investigated and were generally inversely associated with CRC risk (4,5,12,44,45), or with CRA recurrence (46). Conversely, associations with increased CRC risk (47-49), CRA risk (50), or increased risk of CRA recurrence (51,52) have been observed as well. Analyses were stratified by sex in some studies, and positive associations with CRC were reported among men for *MTHFR* C677T (47) and A1298C (48,50). In addition, similar to our study, the *MTHFR* C677T polymorphism has been observed to be associated with increased CRC risk among women, but not among men (48). *MTHFR* polymorphisms may reduce enzymatic activity and thereby decrease the pool of 5-methyltetrahydrofolate. The resulting lower availability of methyl groups may lead to global DNA hypomethylation (53). It may also be associated with increased promoter hypermethylation in CRC (7), with CIMP (6), or with MSI (8,54,55) which is highly correlated with CIMP in CRC (36). Although these two opposite methylation patterns may develop independently of one another, global DNA hypomethylation and gene promoter hypermethylation are observed concurrently in CRC (56-58). However, it is currently unknown to what extent alterations in methyl group metabolism influence this imbalance in the distribution of cytosine methylation, and neither do we know to what extent this influences colorectal carcinogenesis. Moreover, methyl groups may have different targets, e.g. DNA, RNA or proteins (Figure 1), and the relative contribution of methyl groups to either of these targets is unknown.

Although in our study *MTHFR* C677T tended to increase overall CRC risk among women, this effect could not be explained by a distinct positive association with CIMP only. Among men on the other hand, *MTHFR* C677T reduced overall CRC risk and *MTHFR* A1298C was inversely associated with CRCs without CIMP. A decreased pool of 5-methyl tetrahydrofolate by *MTHFR* polymorphisms may be associated with an increased or normal pool of 5,10-methylenene tetrahydrofolate which optimizes DNA synthesis. However, this may have dual consequences for carcinogenesis since increased DNA synthesis possibly prevents DNA instability and thereby protects against carcinogenesis, but may also increase cell proliferation in tumors. Nonetheless, we hypothesize that MTHFR may act as a switch being able to shift the balance between DNA methylation and DNA synthesis (Figure 1), both of which may have distinct consequences for carcinogenesis. Our observations suggest that *MTHFR* polymorphisms, albeit in different directions, may influence CRC risk in both men and women but that a change in promoter hypermethylation, as measured by CIMP or

MLH1 hypermethylation, may not be the primary contributor to carcinogenesis in these individuals.

Among subjects in our study population, the *MTR* A2756G polymorphism was associated with increased CRC risk. Genetic variants of *MTR* were not associated with CRC in a number of previous studies (12,59,60). Modest risk reductions among *MTR* 2756GG homozygotes were found in one CRA study (9) and in one CRC study (10), whereas also non-significant increased risks were observed between this SNP and CRA (11) or CRC (13). SNPs in *MTR* were associated with lower plasma homocysteine (tHcy) concentrations (61), suggesting lower catalytic activity of the MTR enzyme and lower availability of methyl groups. Interestingly, we observed that the *MTR* A2756G SNP was associated with reduced risk of CRCs with CIMP among men.

It was previously observed that the *MTRR* A66G SNP may be inversely associated with CRA recurrence (46), but with increased CRC risk (13). In addition, other SNPs in *MTRR* were associated with an increased risk of CRA (11) and CRC (12). We did not observe an association with overall CRC risk in our study, but *MTRR* A66G was associated with reduced risk of CRCs with *MLH1* hypermethylation among women. Since the *MTRR* A66G SNP may also lead to reduced catalytic activity of the MTRR enzyme (62), our data suggest that it may result in decreased promoter hypermethylation in CRC similar to *MTR* A2756G, which is involved in the same metabolic conversion in the one-carbon metabolism.

The CIMP phenotype has not uniquely been defined, since various sets of promoters have previously been used with different cut-off values (63). In order to investigate an optimal set of markers for CIMP, Weisenberger *et al* (36) screened 195 CpG island methylation markers, and proposed a robust new panel of 5 markers to define CIMP in CRC. This new set has been successfully validated in a large group of incident CRC patients (64), and we have used these new markers in our study. However, we have shown that the overlap between CIMP and *MLH1* or MSI may be incomplete. It has also been suggested that CIMP may consist of three molecular subtypes based on presence or absence of MSI, *BRAF*, *KRAS* or *p53* mutations (65), but these characteristics are not mutually exclusive. In addition, we previously observed incomplete overlap between *MLH1* hypermethylation or –expression, MSI and *BRAF* mutations (41). The current study suggests that lower enzymatic activity of folate metabolizing enzymes *MTR* and *MTRR* is inversely associated with either CIMP or *MLH1* hypermethylation. In view of these observations, it remains important to investigate associations with CIMP and other methylation endpoints separately.

The *DNMT3b* C>T (rs2424913) SNP was not associated with CRC risk. However, an increased risk of CRC could be expected since this polymorphism increases enzymatic activity of DNMT3b (21), and DNMT3b overexpression was associated with increased promoter hypermethylation in different types of cancers (15-17,19,20). Moreover, positive associations were observed with colorectal polyps and early onset of CRC in HNPCC patients (23,25) and several other cancers (21,22,24). Other genetic variants of *DNMT3b* that have been identified are the -283T>C and -579G>T polymorphisms, of which the latter was associated with reduced CRC risk (66). In addition, lung cancer risk was reduced for occurrence of the rare alleles of each of these SNPs (67), which was contrary to the increased lung cancer risk associated with *DNMT3b* C>T (rs2424913) observed by Shen et al (21). Interestingly, enzymatic activity of DNMT3b was decreased when the rare allele of -283T>C was present (67). Apparently, different

polymorphisms in *DNMT3b* may have opposite effects and individuals may be more susceptible to carcinogenesis with higher DNMT3b enzymatic activity (21-23,25), whereas decreased activity may protect against carcinogenesis (66,67). While a similar but weak inverse association was previously observed with breast cancer for the *DNMT3b* C>T (rs406193) polymorphism (30), we observed an inverse association with CRC among women. However, this protective effect was not substantially different between CRCs with or without CIMP, *MLH1* hypermethylation or MSI.

Regarding the histone methyltransferases, we observed an inverse association between *EHMT2* G>A (rs535586) and CRC risk among women. A significant positive association was observed with breast cancer although the RR was small for that particular polymorphism (30). These observations seem contradictive, but may nonetheless suggest that this *EHMT2* polymorphism affects carcinogenesis of different cancer types. In our study, it was associated with increased risk of CRCs with *MLH1* hypermethylation in men and a decreased risk of those without *MLH1* hypermethylation among women. However, like for the other genes studied here, it lacks a biological explanation why an effect on methylation would be different for men and women. Moreover, it is currently unknown to which extent SNPs in histone methyltransferases such as *EHMT2* lead to altered enzymatic activity. Obviously, the exact role of *EHMT2* in colorectal carcinogenesis needs further investigation in the future.

In conclusion, genetic variants of methyl metabolism enzymes or epigenetic regulators may affect colorectal carcinogenesis and our observations suggest that reduced enzymatic activity of some folate metabolizing enzymes may result in decreased gene promoter hypermethylation in CRC. In view of this, it would be interesting to study whether methyl donor intake affects promoter hypermethylation and the potential modifying effect of polymorphisms in folate metabolizing enzymes, DNA methyltransferases and histone methyltransferases. We observed that the overlap of CpG island hypermethylation markers and associated characterstics may be incomplete, indicating that these characteristics should be considered separately when studying potential causes of a "methylation phenotype" in CRC.

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7

Dietary methyl donors, methyl metabolizing enzymes and epigenetic regulators: diet-gene interactions and promoter CpG island hypermethylation in colorectal cancer

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Abstract

Methyl group intake may influence DNA methylation during carcinogenesis and this effect is possibly modulated by genetic variation in methyl metabolizing enzymes or methyltransferases.

Here, we studied associations of dietary folate, methionine, vitamins B2 and B6 with colorectal cancer (CRC) risk among genotypes of folate metabolizing enzymes (*MTHFR, MTR* and *MTRR*), DNA methyltransferase *DNMT3b*, and histone methyltransferases *EHMT1, EHMT2* and *PRDM2*. Incidence rate ratios (RR) were calculated in case-cohort analyses over tertiles of intake among 609 cases and 1,663 subcohort members of the Netherlands Cohort Study on diet and cancer (n=120,852).

Methionine intake was associated with reduced CRC risk among subjects carrying common variants of both of the rs2424913 and rs406193 C>T polymorphisms in *DNMT3b* (highest versus lowest tertile: RR=0.44; P_{trend}=0.05). Vitamin B2 was inversely associated with CRC among individuals with the *MTHFR* 677CC genotype (RR=0.66; P_{trend}=0.08), while a strong inverse association was observed when ≤1 rare allele occurred in the combination of folate metabolizing enzymes *MTHFR*, *MTR* and *MTRR* (RR=0.30; P_{trend}=0.005). Folate or vitamin B6 were not inversely associated with CRC, neither did we observe associations between methyl donor intake and the CpG island methylator phenotype (CIMP).

In conclusion, *MTHFR* and *DNMT3b* polymorphisms may modulate the bioavailability of dietary methyl group donors and thereby affect colorectal carcinogenesis. The effect of methyl donors on CRC may be more pronounced if folate metabolizing enzymes or DNA methyltransferases are left unaffected by rare variants of their encoding polymorphic genes. Combining genotypes may reveal diet associations with CRC and should be considered in association studies.

Introduction

In the preceding manuscript by de Vogel *et al* (part 1), associations were investigated between genetic variants of folate metabolizing enzymes, a DNA methyltransferase and histone methyltransferases with sporadic colorectal cancer (CRC) risk, accounting for the CpG island methylator phenotype (CIMP), *MLH1* hypermethylation or microsatellite instability (MSI) within the Netherlands Cohort Study on diet and cancer (NLCS). In the present study, we investigate associations between dietary methyl donor intake and these endpoints within the same genotypes among subjects of the NLCS.

CRC may arise through several distinct molecular pathways, which are based on the presence or absence of for example CpG island promoter hypermethylation or specific types of DNA instability. CRCs showing the CpG island methylator phenotype (CIMP) are characterized by frequent hypermethylation of promoter CpG islands (1), which is associated with silencing of tumor suppressor and DNA repair genes thereby contributing to carcinogenesis (2). However, although chromomal instability (CIN) or microsatellite instability (MSI) may be two alternative mechanisms in CRC, CpG island promoter methylation may be associated with both MSI and with specific patterns of chromosomal aberrations in CIN CRCs (3), suggesting that these phenotypes are not mutually exclusive.

Folate and methionine are dietary methyl group donors and may influence DNA methylation, whereas vitamins B2 and B6 may modulate the bioavailability of methyl groups (4,5). A low folate status, which is likely to reflect low dietary folate intake, may decrease genomic DNA methylation (6,7), whereas folate supplementation resulted in increased global DNA methylation in the colonic mucosa (8). Although it may be hypothesized that adequate methyl donor intake also reduces CpG island promoter hypermethylation, the association with gene promoter hypermethylation in CRC was only weak (9), while no inverse associations with CIMP were observed among incident colon cancer patients of a large population-based case-control study (10). High vitamin B6 intake was even suggested to be associated with increased *MLH1* promoter methylation in CRC (11) while folate supplementation may increase promoter hypermethylation of multiple genes in colorectal mucosa (12). Apparently, the precise effect of methyl group bioavailability on gene promoter hypermethylation is still unclear and should be investigated further. High alcohol consumption, which reduces the bioavailability of folate, has been reported to be associated with DNA hypomethylation (13,14), and increased risk of CRCs harboring promoter hypermethylation-associated MSI (15). Notably, high alcohol intake was associated with increased risk of CRCs harboring MSI whereas no association was observed with CIMP in the same study population (10,16), suggesting that an effect on these related hypermethylation-associated phenotypes may not be similar. Moreover, we have previously observed that the overlap between CIMP and MSI may be incomplete (part1), which underscores the need to investigate these related endpoints separately.

The effect of methyl donor intake on DNA methylation may be modified by genetic variants of folate metabolizing enzymes, which may influence enzymatic activity and thereby the bioavailability of methyl groups. In the accompanying article in this issue (part1), we observed that the methionine synthase (*MTR*) A2756G polymorphism was associated with decreased risk of CRCs harboring CIMP among men, and that women with the methionine synthase reductase (*MTRR*) A66G polymorphism were at reduced

risk of developing CRC harboring *MLH1* hypermethylation. Furthermore, DNA methyltransferases (DNMTs) and histone methyltransferases (HMTs) are other enzymes involved in epigenetic regulation of gene expression which may modify the effect of methyl donor intake on DNA methylation in CRC. We previously observed inverse associations of *DNMT3b* C>T (rs406193) and the HMT *EHMT2* G>A (rs535586) with CRC among women (part 1). However, whether the effect of methyl donor intake is modified by these DNMT and HMT polymorphisms has not previously been studied in relation to CRC.

Here, we aimed to investigate associations between dietary folate, methionine, vitamins B2 and B6 with overall CRC, and risk of CRCs harboring CIMP, *MLH1* hypermethylation or MSI, accounting for the occurrence of any, or combinations of polymorphisms in folate metabolizing enzymes *MTHFR, MTR* and *MTRR*, the DNA methyltransferase *DNMT3b*, and histone methyltransferases *EHMT1, EHMT2* and *PRDM2* in the Netherlands Cohort Study on diet and cancer.

Subjects and methods

Study population

The participants of this study were incident CRC patients from the Netherlands Cohort Study on diet and cancer (NLCS), which has been described in detail elsewhere (17). Briefly, this prospective cohort study was initiated in September 1986 and includes 58,279 men and 62,573 women aged 55-69 years and free of disease at baseline. The cohort is followed for cancer occurrence by annual record linkage to the Netherlands Cancer Registry (NCR) and to PALGA (Pathologisch Anatomisch Landelijk Geautomatiseerd Archief), a nationwide network and registry of histopathology and cytopathology reports (18,19). A subcohort of 5,000 subjects was randomly selected after baseline exposure measurement, to estimate accumulation of person-time in the cohort through biennial follow-up of vital status. Cases with prevalent cancer other than non-melanoma skin cancer were excluded from this subcohort, which left 4,774 men and women eligible for analysis.

Food frequency questionnaire

At baseline, participants filled out a self-administered, 150-item semi-quantitative food frequency questionnaire (FFQ), which concentrated on habitual consumption of food and beverages during the year preceding the start of the study, and also contained questions about age, sex, body weight and -length, smoking status and family history of CRC. Daily mean nutrient intakes were calculated as the cumulated product of the frequencies and portion sizes of all food items and their tabulated nutrient contents from the Dutch Food Composition Table (NEVO table, 1986) (20). The questionnaire was validated through comparison with a 9-day diet record (21). Reproducibility and stability of dietary habits were determined by five annually repeated measurements (22). In order to minimize observer bias in coding and interpretation of the data, questionnaire data were key-entered twice for all incident cases in the cohort and for all subcohort members in a blinded manner with respect to case/subcohort status.

Folate data were derived from a validated liquid chromatography trienzyme method (23) used to analyze the 125 most important Dutch foods contributing to folate intake (24). Dietary supplement data were also obtained via the food frequency questionnaire. However, the use of B-vitamin supplements was low (7%) and folic acid was generally not included in these supplements in the Netherlands in the late 1980s. Therefore, folic acid supplement use most likely plays a very minor role in our study population, and supplement use was not further accounted for in the analyses.

Sample collection

Subcohort members still alive in December 2000 (n=3,579) were contacted and asked to collect mouth swabs, of whom 1,929 (54%) responded and returned the mouth swab with informed consent. In total, DNA could successfully be isolated of 1,829 subcohort members who also had complete follow-up information (part 1).

Tumor material of the CRC patients was collected after approval by the ethical review boards of Maastricht University, the NCR and PALGA. During a follow-up period of 7.3 years after baseline, 734 incident CRC patients were identified who had an available PALGA report of the lesion as well as a sufficient amount of isolated DNA needed for molecular analyses.

Genotyping analyses

MTHFR, MTR, MTRR, DNMT3b, EHMT1, EHMT2 and *PRDM2* genotypes were determined using multiplex polymerase chain reaction (PCR) amplification and single base extension (SBE) reactions as described previously (25) (part1). Genotype data were validated by sequencing of fragments containing specific SNPs, which were similar to the SNaPShot results for all but one (99.6%) of the 9 SNPs within a subset of 30 samples (part 1). Reproducibility of the SNaPShot analysis was established among 93 samples and we observed that the analyses could be reproduced in 99.5% of these cases (part 1). In total, genotyping analyses were successful from 1,736 subcohort members and 659 CRC patients.

Promoter methylation analyses

The CpG island methylator phenotype (CIMP) was defined by promoter hypermethylation of at least 3 out of 5 methylation markers (*CACNA1G, IGF2, NEUROG1, RUNX3* and *SOCS1*), as suggested by Weisenberger *et al* (1). Hypermethylation of the CpG islands of these five CIMP markers and of the *MLH1* gene was determined by Methylation Specific PCR (MSP) (26), and described in detail in (part 1). The MSP analyses were successful of 81%, 79%, 79%, 90%, 83% and 93% out of the 734 patients for *CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1* and *MLH1*, respectively.

Microsatellite instability

MSI was determined by a pentaplex PCR, using the MSI markers BAT-26, BAT-25, NR-21, NR-22 and NR-24, as described in detail by Suraweera et al (27). MSI analyses were successful on 662 (90%) out of the 734 available samples.

Statistical analyses

Cox proportional hazards regression models were used to estimate multivariate-adjusted incidence rate ratios (RR) and corresponding 95% confidence intervals (CI) over tertiles of dietary folate, methionine, vitamins B2 and B6, using the lowest tertiles as reference. Tests for dose response trends over the tertiles of intake were estimated by fitting the ordinal exposure variables as continuous variables and evaluated using the Wald test. Standard errors of the RR were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling from the cohort (28). The proportional hazards assumption was tested using the scaled Schoenfeld residuals (29), and by fitting the main determinants as time-dependent variables. The dietary variables were adjusted for total energy intake by calculating nutrient residuals from the regression of nutrient intake on total energy intake, as described by Willett et al (30).

The analyses were stratified according to genetic status of individuals, i.e. among those homozygous to common genetic variants and among subjects carrying rare alleles. Interactions were tested between dietary folate, methionine, vitamins B2 and B6, and each of the genetic variants. Associations between dietary factors and CRC were also estimated for combinations of genotypes per functional group (i.e. based on the number of rare alleles in any of the folate metabolizing enzymes *MTHFR*, *MTR* and *MTRR*, in the DNA methyltransferase *DNMT3b*, or in any of the histone methyltransferases *EHMT1*, *EHMT2* and *PRDM2*.

To investigate whether dietary methyl donors have an effect on promoter hypermethylation in CRC, associations of folate, methionine, vitamins B2 and B6 with the CIMP phenotype were estimated. The associations with *MLH1* hypermethylation and MSI were reported previously (31). Furthermore, it was investigated whether these effects would be modified by genetic status by estimating the associations with methylation endpoints within genotypes of folate metabolizing enzymes, *DNMT3b* and histone methyltransferases.

All models included the co-variates dietary folate, methionine, vitamin B2 and B6, and were additionally adjusted for age, sex, family history of CRC, smoking status, body mass index (BMI), alcohol consumption and energy intake. After excluding subjects with missing information on these covariates or subjects who only partly filled out the questionnaire, 1,663 subcohort members and 609 CRC cases remained for statistical analyses. All analyses were performed with the Stata statistical software package (version 10).

Results

CRC risk was estimated over tertiles of folate intake, methionine, vitamins B2 and B6 among subjects homozygous for common wild type alleles and among carriers of the rare allele. Folate or methionine intakes were not associated with CRC within either common homozygotes or within heterozygotes and rare homozygotes of any of the genotypes (Table 1).

However, we observed a non-significant inverse association between vitamin B2 intake and CRC risk among subjects with the *MTHFR* 677CC common genotype (RR for the highest versus the lowest tertile of intake=0.66, P_{trend}=0.08, Table 2), and a significant inverse association among subjects with the common GG genotype of *PRDM2* G>A (rs2235515, RR=0.67, P_{trend}=0.05). In addition, vitamin B2 was associated with reduced CRC risk in individuals carrying the variant allele of *DNMT3b* C>T (rs2424913, RR=0.69, P_{trend}=0.05). These associations for vitamin B2 were even more pronounced among men (RRs were 0.44, 0.56 and 0.55 for *MTHFR* 677CC, *PRDM2* GG (rs2235515) and *DNMT3b* TT (rs2424913), respectively), but were not observed among women (data not shown).

Conversely, subjects in the third tertile of vitamin B6 intake were at increased CRC risk when they carried the rare allele of *DNMT3b* C>T (rs406193, RR=1.90, P_{trend}=0.04), or the common allele of *PRDM2* G>A (rs2235515, RR=1.49, P_{trend}=0.03). However, interactions between these dietary factors and genotypes were not statistically significant.

Table 1 Intake of folate and methionine and CRC risk stratified by genetic status

		Folate †				Methionine ‡			
		Common homozygotes		Heterozygotes and rare homozygotes		Common homozygotes		Heterozygotes and rare homozygotes	
Gene and SNP (rs number, MAF) *	Tertile of intake	N §	RR (95% CI) ¶	N	RR (95% CI)	N	RR (95% CI)	N	RR (95% CI)
MTHFR C677T	1	90	ref.	133	ref.	86	ref.	123	ref.
(rs1801133, 0.30)	2	83	0.80 (0.52-1.21)	78	0.51 (0.35-0.75)	101	1.43 (0.96-2.13)	101	0.84 (0.57-1.24)
	3	100	1.22 (0.81-1.85)	123	0.82 (0.56-1.21)	86	0.92 (0.61-1.41)	110	1.08 (0.70-1.67)
	P_{trend} **		*0.28*		*0.30*		*0.63*		*0.90*
MTHFR A1298C	1	103	ref.	116	ref.	95	ref.	113	ref.
(rs1801131, 0.37)	2	72	0.61 (0.40-0.92)	89	0.73 (0.50-1.07)	85	1.00 (0.65-1.54)	115	1.14 (0.80-1.64)
	3	98	1.03 (0.68-1.56)	124	1.02 (0.69-1.51)	93	1.02 (0.65-1.60)	101	0.92 (0.62-1.38)
	P_{trend}		*0.97*		*0.86*		*0.99*		*0.58*
MTR A2756G	1	136	ref.	89	ref.	128	ref.	83	ref.
(rs1805087, 0.19)	2	107	0.55 (0.39-0.79)	57	0.84 (0.53-1.33)	141	1.22 (0.87-1.71)	62	0.87 (0.55-1.38)
	3	154	0.99 (0.70-1.39)	71	0.94 (0.57-1.54)	128	1.03 (0.71-1.48)	72	0.94 (0.57-1.54)
	P_{trend}		*0.90*		*0.83*		*0.98*		*0.69*
MTRR A66G	1	56	ref.	172	ref.	49	ref.	164	ref.
(rs1801394, 0.56)	2	24	0.29 (0.14-0.59)	140	0.78 (0.58-1.05)	38	1.00 (0.52-1.93)	165	1.08 (0.80-1.46)
	3	49	0.62 (0.32-1.22)	177	1.06 (0.78-1.45)	42	1.22 (0.53-2.78)	160	0.96 (0.70-1.33)
	P_{trend}		*0.23*		*0.69*		*0.94*		*0.72*
DNMT3b C>T	1	89	ref.	139	ref.	84	ref.	129	ref.
(rs2424913, 0.42)	2	60	0.60 (0.38-0.95)	106	0.70 (0.49-0.99)	61	0.61 (0.37-1.00)	144	1.37 (0.99-1.90)
	3	68	0.73 (0.44-1.20)	159	1.12 (0.80-1.57)	72	0.66 (0.39-1.11)	131	1.20 (0.83-1.74)
	P_{trend}		*0.20*		*0.40*		*0.15*		*0.43*
DNMT3b C>T	1	170	ref.	56	ref.	162	ref.	49	ref.
(rs406193, 0.14)	2	123	0.67 (0.48-0.93)	41	0.56 (0.32-0.98)	157	1.10 (0.80-1.51)	46	1.05 (0.60-1.82)
	3	175	1.04 (0.75-1.44)	51	0.72 (0.39-1.31)	149	0.91 (0.64-1.28)	53	1.43 (0.78-2.59)
	P_{trend}		*0.72*		*0.34*		*0.45*		*0.32*
EHMT1 G>A	1	187	ref.	38	ref.	172	ref.	39	ref.
(rs4634736, 0.10)	2	129	0.62 (0.45-0.84)	34	0.95 (0.49-1.82)	167	1.09 (0.81-1.48)	35	1.12 (0.57-2.22)
	3	186	0.99 (0.73-1.35)	40	0.97 (0.48-1.97)	163	0.95 (0.69-1.32)	38	1.24 (0.54-2.86)
	P_{trend}		*0.97*		*0.90*		*0.58*		*0.63*
EHMT2 G>A	1	103	ref.	122	ref.	89	ref.	122	ref.
(rs535586, 0.35)	2	73	0.55 (0.35-0.84)	89	0.73 (0.50-1.05)	83	1.17 (0.76-1.81)	119	0.99 (0.69-1.42)
	3	89	0.88 (0.57-1.35)	135	1.09 (0.76-1.59)	93	1.24 (0.80-1.91)	105	0.80 (0.53-1.22)
	P_{trend}		*0.50*		*0.59*		*0.48*		*0.25*
PRDM2 G>A	1	116	ref.	106	ref.	120	ref.	85	ref.
(rs2235515, 0.23)	2	93	0.67 (0.46-0.96)	65	0.61 (0.39-0.96)	107	0.95 (0.66-1.35)	90	1.29 (0.83-2.01)
	3	121	0.95 (0.65-1.39)	95	0.93 (0.61-1.43)	103	0.97 (0.65-1.43)	91	1.02 (0.64-1.64)
	P_{trend}		*0.81*		*0.86*		*0.69*		*0.91*

* SNP: Single Nucleotide Polymorphism, MAF: Minor Allele Frequency among subcohort members
† Among subcohort members within tertiles: median folate intake: 162, 200 and 255 µg/day; accumulated time at risk: 4131, 4091 and 4093 person years
‡ Among subcohort members within tertiles: median methionine intake: 1316, 1583 and 1881 mg/day; accumulated time at risk: 4110, 4105 and 4100 person years
§ Number of colorectal cancer cases
¶ RRs based on a model containing the variables folate, methionine, vitamin B2, vitamin B6, and further adjusted for age, sex, family history of colorectal cancer, body mass index, smoking status, alcohol consumption and total energy intake
** *P*-value for linear trend.

Table 2 Intake of vitamins B2 and B6 and CRC risk stratified by genetic status

		Vitamin B2 †				Vitamin B6 ‡			
		Common homozygotes		Heterozygotes and rare homozygotes		Common homozygotes		Heterozygotes and rare homozygotes	
Gene and SNP (rs number, MAF) *	Tertile of intake	N §	RR (95% CI) ¶	N	RR (95% CI)	N	RR (95% CI)	N	RR (95% CI)
MTHFR C677T	1	103	ref.	117	ref.	81	ref.	105	ref.
(rs1801133, 0.30)	2	92	0.80 (0.54-1.17)	112	1.02 (0.70-1.49)	96	1.24 (0.85-1.83)	111	1.11 (0.76-1.61)
	3	78	0.66 (0.42-1.04)	105	0.89 (0.58-1.35)	96	1.50 (0.98-2.28)	118	1.05 (0.69-1.61)
	P_{trend} **		*0.08*		*0.54*		*0.12*		*0.63*
MTHFR A1298C	1	100	ref.	118	ref.	90	ref.	98	ref.
(rs1801131, 0.37)	2	87	0.72 (0.47-1.09)	116	1.06 (0.75-1.49)	87	1.11 (0.74-1.66)	116	1.16 (0.81-1.68)
	3	86	0.76 (0.48-1.19)	95	0.85 (0.57-1.27)	96	1.19 (0.77-1.85)	115	1.23 (0.82-1.85)
	P_{trend}		*0.29*		*0.42*		*0.53*		*0.35*
MTR A2756G	1	133	ref.	88	ref.	115	ref.	74	ref.
(rs1805087, 0.19)	2	137	0.95 (0.68-1.31)	68	0.85 (0.54-1.34)	145	1.33 (0.95-1.86)	64	0.91 (0.58-1.44)
	3	127	0.81 (0.56-1.17)	61	0.87 (0.53-1.44)	137	1.17 (0.80-1.72)	79	1.31 (0.84-2.05)
	P_{trend}		*0.30*		*0.52*		*0.49*		*0.22*
MTRR A66G	1	49	ref.	174	ref.	39	ref.	153	ref.
(rs1801394, 0.56)	2	44	0.78 (0.39-1.56)	162	0.93 (0.70-1.25)	43	1.48 (0.80-2.74)	165	1.03 (0.76-1.38)
	3	36	0.59 (0.27-1.32)	153	0.89 (0.65-1.23)	47	1.49 (0.74-3.00)	171	1.11 (0.80-1.53)
	P_{trend}		*0.12*		*0.56*		*0.19*		*0.55*
DNMT3b C>T	1	79	ref.	146	ref.	68	ref.	125	ref.
(rs2424913, 0.42)	2	72	1.03 (0.64-1.69)	135	0.84 (0.61-1.15)	71	1.60 (0.96-2.67)	138	1.04 (0.76-1.42)
	3	66	1.07 (0.63-1.82)	123	0.69 (0.48-0.99)	78	1.42 (0.85-2.37)	141	1.18 (0.82-1.70)
	P_{trend}		*0.81*		*0.05*		*0.21*		*0.44*
DNMT3b C>T	1	171	ref.	52	ref.	145	ref.	45	ref.
(rs406193, 0.14)	2	151	0.86 (0.63-1.17)	53	0.86 (0.50-1.48)	163	1.12 (0.83-1.52)	45	1.28 (0.70-1.36)
	3	146	0.84 (0.59-1.19)	43	0.66 (0.37-1.19)	160	1.12 (0.80-1.58)	58	1.90 (1.00-3.60)
	P_{trend}		*0.33*		*0.20*		*0.60*		*0.04*
EHMT1 G>A	1	180	ref.	42	ref.	161	ref.	30	ref.
(rs4634736, 0.10)	2	165	0.93 (0.69-1.25)	40	0.94 (0.49-1.82)	169	1.07 (0.80-1.44)	38	1.33 (0.68-2.58)
	3	157	0.85 (0.62-1.18)	30	0.69 (0.32-1.50)	172	1.15 (0.84-1.58)	44	1.52 (0.71-3.26)
	P_{trend}		*0.38*		*0.38*		*0.40*		*0.29*
EHMT2 G>A	1	98	ref.	125	ref.	81	ref.	108	ref.
(rs535586, 0.35)	2	88	0.93 (0.62-2.38)	116	0.85 (0.59-1.21)	85	0.98 (0.65-1.48)	123	1.25 (0.88-1.78)
	3	79	0.85 (0.54-1.34)	105	0.75 (0.50-1.12)	99	1.17 (0.75-1.83)	115	1.25 (0.85-1.86)
	P_{trend}		*0.53*		*0.21*		*0.45*		*0.30*
PRDM2 G>A	1	123	ref.	95	ref.	101	ref.	83	ref.
(rs2235515, 0.23)	2	113	0.84 (0.59-1.19)	84	0.94 (0.62-1.44)	105	1.31 (0.92-1.88)	99	0.95 (0.63-1.42)
	3	94	0.67 (0.44-1.00)	87	1.02 (0.65-1.60)	124	1.49 (1.00-2.22)	84	0.89 (0.56-1.41)
	P_{trend}		*0.05*		*0.88*		*0.03*		*0.41*

* SNP: Single Nucleotide Polymorphism, MAF: Minor Allele Frequency among subcohort members

† Among subcohort members within tertiles: median vitamin B2 intake: 1.19, 1.50 and 1.84 mg/day; accumulated time at risk: 4116, 4109 and 4090 person years

‡ Among subcohort members within tertiles: median vitamin B6 intake: 1.20, 1.44 and 1.70 mg/day; accumulated time at risk: 4125, 4118 and 4073 person years

§ Number of colorectal cancer cases

¶ RRs based on a model containing the variables folate, methionine, vitamin B2, vitamin B6, and further adjusted for age, sex, family history of colorectal cancer, body mass index, smoking status, alcohol consumption and total energy intake

** *P*-value for linear trend.

We also investigated the associations between methyl donor intake and CRC risk according to the number of rare alleles within each functional group (i.e. folate metabolizing enzymes, *DNMT3b* and histon methyltransferases). It appeared that methionine was inversely associated with CRC if subjects were homozygous to both of the common variants of *DNMT3b* rs2424913 and rs406193 C>T SNPs (RR=0.44, P_{trend}=0.05, Table 3).

Table 3 Dietary folate, methionine, vitamins B2 and B6 and CRC risk for combinations of genotypes in folate metabolizing enzymes, DNA methyltransferase 3b or histone methyltransferases

		Folate metabolizing enzymes					
	Tertile	≤ 1 *		2		≥ 3	
	of intake	N	RR (95% CI)	N	RR (95% CI)	N	RR (95% CI)
Folate	1	41	ref.	95	ref.	83	ref.
	2	32	0.52 (0.24-1.14)	66	0.56 (0.37-0.86)	61	0.76 (0.44-1.31)
	3	42	1.23 (0.58-2.63)	102	0.91 (0.60-1.40)	75	0.89 (0.51-1.54)
	P_{trend}		*0.51*		*0.97*		*0.66*
Methionine	1	40	ref.	85	ref.	81	ref.
	2	41	1.50 (0.70-3.21)	89	1.24 (0.82-1.90)	70	0.73 (0.44-1.21)
	3	34	0.99 (0.38-2.53)	89	1.06 (0.68-1.65)	68	0.70 (0.40-1.24)
	P_{trend}		*0.90*		*0.90*		*0.18*
Vitamin B2	1	43	ref.	91	ref.	83	ref.
	2	40	0.76 (0.37-1.54)	90	0.95 (0.63-1.43)	71	0.94 (0.58-1.53)
	3	32	0.30 (0.11-0.81)	82	0.88 (0.55-1.39)	65	1.05 (0.61-1.80)
	P_{trend}		*0.005*		*0.96*		*0.87*
Vitamin B6	1	31	ref.	83	ref.	71	ref.
	2	46	0.94 (0.94-4.03)	88	1.05 (0.70-1.56)	69	1.07 (0.63-1.80)
	3	38	2.32 (1.00-5.36)	92	1.06 (0.68-1.65)	79	1.39 (0.80-2.42)
	P_{trend}		*0.07*		*1.00*		*0.21*
		DNA methyltransferase 3b (DNMT3b)					
	Tertile	0		1		2	
	of intake	N	RR (95% CI)	N	RR (95% CI)	N	RR (95% CI)
Folate	1	53	ref.	151	ref.	22	ref.
	2	37	0.72 (0.39-1.35)	107	0.63 (0.45-0.89)	20	0.67 (0.27-1.67)
	3	39	0.76 (0.39-1.48)	163	1.10 (0.78-1.55)	23	0.78 (0.31-1.93)
	P_{trend}		*0.37*		*0.54*		*0.79*
Methionine	1	53	Ref.	137	ref.	20	ref.
	2	37	0.74 (0.37-1.47)	142	1.18 (0.85-1.63)	24	1.71 (0.77-3.79)
	3	39	0.44 (0.21-0.94)	142	1.12 (0.79-1.60)	21	1.48 (0.56-3.90)
	P_{trend}		*0.05*		*0.66*		*0.47*
Vitamin B2	1	47	Ref.	154	ref.	22	ref.
	2	41	1.09 (0.56-2.12)	137	0.78 (0.57-1.08)	25	1.30 (0.57-2.98)
	3	41	1.52 (0.71-3.22)	130	0.72 (0.50-1.05)	18	0.79 (0.29-1.74)
	P_{trend}		*0.36*		*0.10*		*0.41*
Vitamin B6	1	42	Ref.	127	ref.	21	ref.
	2	42	1.80 (0.88-3.70)	147	1.13 (0.83-1.54)	18	0.98 (0.37-2.57)
	3	45	1.37 (0.68-2.75)	147	1.15 (0.81-1.63)	26	2.12 (0.79-5.72)
	P_{trend}		*0.44*		*0.46*		*0.18*
		Histone methyltransferases					
	Tertile	0		1		≥ 2	
	of intake	N	RR (95% CI)	N	RR (95% CI)	N	RR (95% CI)
Folate	1	43	Ref.	103	ref.	72	ref.
	2	30	0.47 (0.24-0.90)	76	0.72 (0.47-1.10)	51	0.71 (0.43-1.17)
	3	44	0.91 (0.46-1.80)	90	0.92 (0.61-1.39)	78	1.11 (0.67-1.85)
	P_{trend}		*0.64*		*0.69*		*0.65*
Methionine	1	41	ref.	93	ref.	68	ref.
	2	35	1.03 (0.51-2.06)	89	1.08 (0.71-1.64)	70	1.12 (0.68-1.85)
	3	41	1.14 (0.58-2.23)	87	1.12 (0.71-1.76)	63	0.77 (0.44-1.35)
	P_{trend}		*0.80*		*0.76*		*0.34*
Vitamin B2	1	45	ref.	96	ref.	75	ref.
	2	37	0.71 (0.37-1.37)	94	0.94 (0.63-1.40)	64	0.81 (0.50-1.30)
	3	35	0.55 (0.26-1.13)	79	0.86 (0.54-1.37)	62	0.78 (0.47-1.31)
	P_{trend}		*0.22*		*0.45*		*0.49*
Vitamin B6	1	36	ref.	85	ref.	60	ref.
	2	29	0.85 (0.44-1.64)	100	1.38 (0.94-2.04)	73	1.11 (0.68-1.79)
	3	52	1.69 (0.79-3.60)	84	1.01 (0.66-1.55)	68	1.39 (0.81-2.39)
	P_{trend}		*0.10*		*0.93*		*0.34*

* Number of variant alleles (i.e. heterozygotes or homozygotes for the rare allele)

Moreover, relatively high vitamin B2 intake was associated with reduced CRC risk in subjects with less than one SNP in folate metabolizing enzymes (RR=0.30, P_{trend}=0.005).

With respect to CpG island promoter hypermethylation, we observed no overall associations between folate, methionine, vitamins B2 or B6 with CIMP (Table 4). Moreover, there were no clear associations between methyl donor intake and CIMP, *MLH1* hypermethylation or MSI when accounting for genetic status of individuals (data not shown).

Table 4 Associations of folate, methionine, vitamins B2 and B6 with CIMP in CRC *

		CIMP+ ¶		CIMP- **	
Tertile (median within tertile)	**PY †**	**N ‡**	**RR (95% CI) §**	**N**	**RR (95% CI)**
Folate (µg/day)					
1 (151.4)	6502	54	ref.	128	ref.
2 (200.1)	6631	57	1.05 (0.71-1.57)	124	0.92 (0.69-1.23)
3 (264.6)	6618	42	0.83 (0.52-1.35)	134	1.05 (0.75-1.47)
P-value for linear trend			*0.54*		*0.73*
Methionine (mg/day)					
1 (1323)	6613	55	ref.	134	ref.
2 (1587)	6621	48	0.80 (0.51-1.26)	128	0.88 (0.67-1.17)
3 (1880)	6518	50	0.80 (0.49-1.31)	124	0.81 (0.59-1.10)
P-value for linear trend			*0.42*		*0.18*
Vitamin B2 (mg/day)					
1 (1.19)	6607	53	ref.	131	ref.
2 (1.48)	6617	48	0.96 (0.63-1.46)	134	1.06 (0.81-1.39)
3 (1.83)	6528	52	1.16 (0.72-1.87)	121	0.97 (0.72-1.31)
P-value for linear trend			*0.62*		*0.82*
Vitamin B6 (mg/day)					
1 (1.18)	6573	48	ref.	115	ref.
2 (1.43)	6675	56	1.21 (0.79-1.85)	129	1.15 (0.87-1.54)
3 (1.70)	6504	49	1.13 (0.71-1.80)	142	1.33 (0.97-1.83)
P-value for linear trend			*0.72*		*0.11*

* Associations are irrespective of genetic status, and are therefore based on a larger number of subcohort members and CRC cases compared to tables 1-3.
† Number of accumulated Person Years (PY) within categories of dietary intake
‡ Number of cases within tertiles of dietary intake
§ Incidence Rate Ratio (RR) from a Cox regression model including the variables folate, methionine, vitamins B2 and B6. Adjusted for age, sex, family history of colorectal cancer, Body Mass Index, smoking behavior, alcohol consumption and energy intake
¶ CpG Island Methylator Phenotype (CIMP); ≥3 out of 5 CIMP markers methylated
** 0-2 out of 5 CIMP markers methylated

Discussion

In the current prospective case-cohort study among incident CRC cases, we observed no associations between dietary folate and CRC risk when accounting for genetic variants of folate metabolizing enzymes, DNA methyltransferases or histone methyltransferases. However, relatively high methionine intake may protect against CRC if enzymatic activity of DNMT3b is not affected by two C>T SNPs in its encoding gene. In addition, subjects with high vitamin B2 intake may be at reduced CRC risk in combination with optimal MTHFR activity in those homozygous for the common 677CC variant, the common *PRDM2* GG (rs2235515) genotype and among those with the variant allele of *DNMT3b* C>T (rs2424913), and these associations were most pronounced among men. High vitamin B2 intake may reduce CRC risk among individuals carrying ≤1 rare allele in the combination of any of the folate metabolizing

enzymes *MTHFR*, *MTR* or *MTRR*. There were no associations with the CIMP phenotype or within strata of the studied genotypes.

It has been suggested that the *MTHFR* C677T polymorphism reduces binding of the MTHFR enzyme to its cofactor flavin adenine dinucleotide (FAD), a metabolite of vitamin B2, resulting in loss of enzymatic activity to approximately 30% when compared to wild types (32,33). The potentially resulting reduced bioavailability of methyl groups may induce DNA hypomethylation in for example blood cells (34,35) or CpG island promoter hypermethylation in CRC (36-38). We observed an inverse association between vitamin B2 and CRC risk, predominantly among men homozygous for the *MTHFR* 677CC variant, suggesting that vitamin B2 may maximize the catalytic activity of MTHFR when binding to FAD is optimal. Similarly, it was recently observed that high vitamin B2 plasma concentrations, in combination with *MTHFR* 677CC or CT genotypes, may reduce risk of CRA recurrence, whereas such an inverse association was not observed among individuals with the *MTHFR* 677TT variant (39). We also observed a strong inverse association between vitamin B2 and CRC risk in individuals carrying ≤ 1 variant allele out of the four studied SNPs of folate metabolizing enzymes, suggesting that the combination of common wild-type genotypes, which possibly results in higher bioavailability of methyl groups, protects against CRC in these people. However, we did not observe an association between methyl donor intake and CRC characterized by CIMP.

In addition to a potential protective effect of vitamin B2 among subjects with the *MTHFR* 677CC genotype and associated optimal enzymatic MTHFR activity, vitamin B2 may also reduce CRC risk among subjects carrying variant alleles of the *DNMT3b* C>T (rs2424913) polymorphism. Interestingly, enzymatic activity of DNMT3b is significantly increased among individuals with this polymorphism (40), and it may be hypothesized that normal or increased metabolic activity these enzymes may protect against CRC. Possibly, this may also be true for the histone methyltransferase PRDM2 since high vitamin B2 intake reduced CRC risk in the common GG variant. However, the consequence of the *PRDM2* G>A (rs2235515) polymorphism for the PRDM2 enzyme has not previously been studied. Nevertheless, although *PRDM2* G>A (rs2235515) was not directly associated with CRC (part 1), the association of vitamin B2 for wild-type carriers in the current study underscores its potential role in methyl metabolism and colorectal carcinogenesis.

Our study suggests that relatively high methionine intake may protect against CRC if enzymatic DNMT3b activity is not affected by two polymorphisms. Increased DNMT3b activity by the *DNMT3b* rs2424913 C>T polymorphism was associated with increased risks of various types of cancer (40-42). Moreover, it was associated with increased risk of CRA in individuals with low folate and methionine intakes (43), suggesting a nutrient-gene interaction in colorectal carcinogenesis. In view of the function of the DNMT3b enzyme of incorporating methyl groups into DNA, an interaction between methionine intake, *DNMT3b* polymorphisms and CpG island hypermethylation may be expected, but we did not clearly observe associations between methyl donor intake and CIMP, *MLH1* hypermethylation or MSI when accounting for *DNMT3b* genotypes.

Interestingly, the potential protective effects of vitamin B2 or methionine may only be present among individuals with ≤ 1 polymorphism in folate metabolizing enzymes or among those with common wild-type genotypes of *DNMT3b*, respectively. This suggests that the occurrence of only one polymorphism is compensated for, but that

the combination of several polymorphic genes may lead to disruption of a particular metabolic or regulatory function and to the abolishment of beneficial effects of nutrients. Moreover, these data indicate that a gene-nutrient interaction may be present and that combining genotypes is important to reveal associations of dietary factors with cancer risk. Such an approach has not been followed in previous studies investigating associations between genetic factors and cancer risk, and we recommend that combinations of genotypes are considered in future studies.

Although *MTHFR* C677T may be associated with increased promoter hypermethylation in CRC (36-38), and DNMT3b expression (which is increased by the *DNMT3b* C>T rs2424913 polymorphism) with increased risk of promoter hypermethylation in lung cancer (44) and breast cancer (45), we did not observe associations of folate, methionine, vitamins B2 or B6 with risk of CRC with CIMP. Moreover, associations between methyl donor intake and CIMP in CRC were not observed in a previous study (10), and an association of one-carbon SNPs with CIMP was only observed for the *MTHFR* A1298C polymorphism (36). However, these previous studies, as well as our study, may have lacked adequate power to demonstrate such associations, and particularly our study had relatively low numbers of cases within subgroups of genetic variants and methylation endpoints combined. Nonetheless, the effect of methyl donor intake on gene promoter hypermethylation may indeed be weak, and to demonstrate whether such an effect is modified by genetic variability of the methyl metabolism requires studies with large numbers of cases.

In conclusion, the present study suggests that a potential protective effect of vitamin B2 and methionine intakes depends on genetic variants of folate metabolizing enzymes and a DNA methyltransferase in CRC. Combining genotypes is a logical approach of investigating multiple genes and may reveal associations of dietary methyl donors and CRC. However, larger studies are needed to investigate a potential interaction between dietary methyl donor intake, genetic variation of folate metabolizing enzymes and epigenetic regulators, and methylation endpoints in CRC with more precision.

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8

Epilogue

The main aim of the current study was to investigate associations between dietary methyl donor intake and colorectal cancer risk, accounting for gene mutations and gene promoter hypermethylation in the tumor, and for genetic variants of enzymes involved in methyl group metabolism and epigenetic regulation. In this epilogue, I will briefly discuss the main findings of the study and relate these to the currently debated potential dual role of folate in colorectal carcinogenesis. Finally, I propose some recommendations for public health and future research.

Main findings

The findings of this study (Figure 1) suggest that in colorectal carcinogenesis, *MGMT* hypermethylation may succeed *APC* mutations but precedes *KRAS* mutations, and that tumors with *MGMT* hypermethylation may develop distinctly from those showing *MLH1* hypermethylation (chapter 2).

Although we did not observe an association between dietary folate and colorectal cancer, methionine and vitamin B2 were associated with reduced proximal colon cancer risk among men and women, respectively. Conversely, vitamin B6 intake was associated with increased colorectal cancer risk, being most pronounced for rectal cancer among women (chapter 3).

Predominantly among men, folate intake was associated with increased risk of colorectal tumors harboring *APC* mutations (chapter 4) or *BRAF* mutations (chapter 5), suggesting that folate may enhance the growth of such lesions.

Occurrence of polymorphisms in methyl metabolizing enzymes *MTHFR* and *MTR*, the DNA methyltransferase 3b (*DNMT3b*) and histone methyltransferase *EHMT2* were associated with overall colorectal cancer risk. Our study suggested that *MTR* and *MTRR* polymorphisms may reduce the risk of colorectal tumors with a promoter hypermethylation phenotype (chapter 6).

A protective effect of methionine and vitamin B2 against colorectal cancer appeared to be more pronounced if no polymorphisms occurred in *DNMT3b*, or when ≤1 rare allele occurred in the combination of methyl metabolizing enzymes *MTHFR*, *MTR* and *MTRR*, respectively. We therefore concluded that a protective effect of dietary methyl donors may be stronger if methyl metabolizing enzymes and DNA methyltransferases are left unaffected by polymorphisms in their encoding genes (chapter 7).

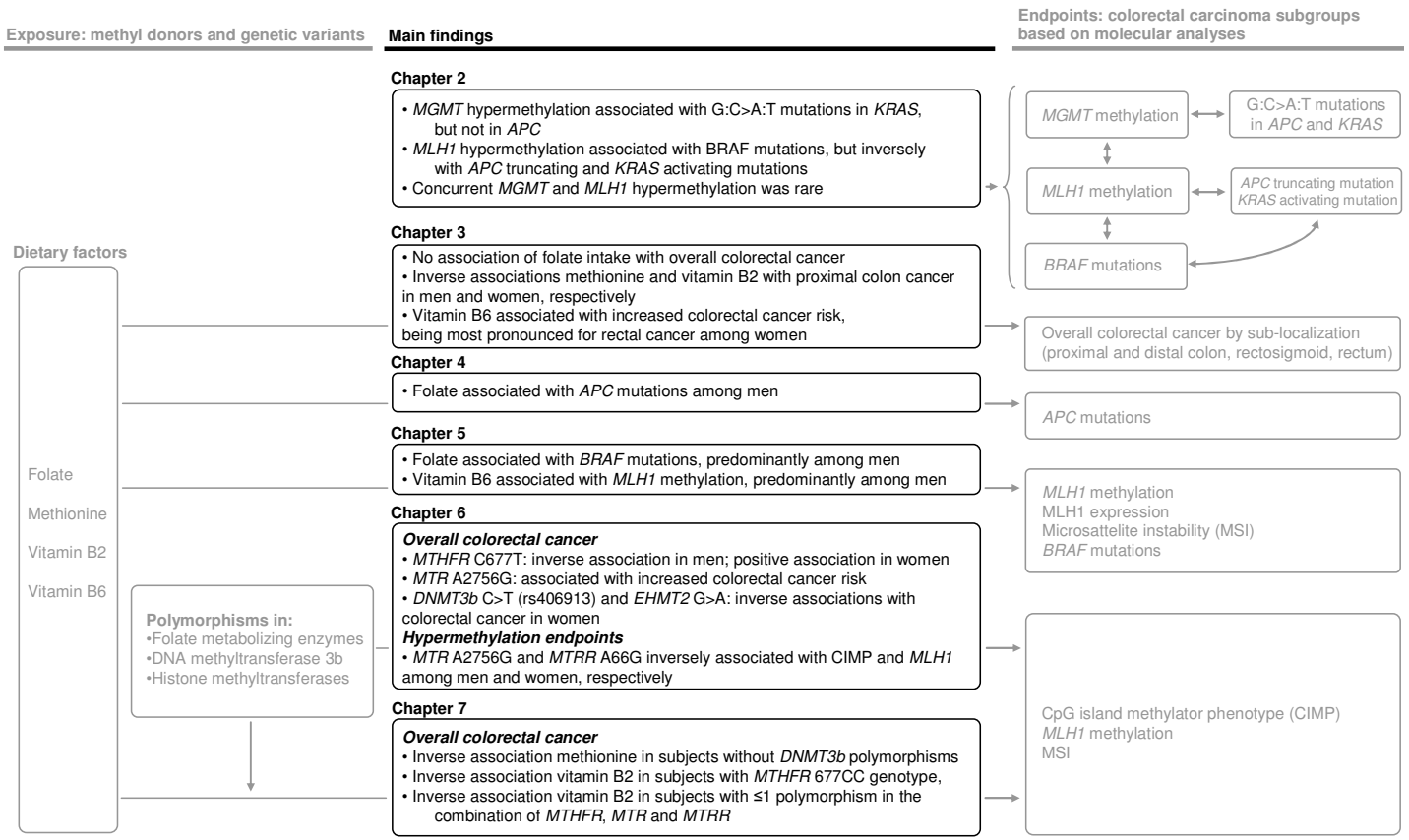

Figure 1 Overview of the main findings

Correlation between hypermethylation-associated tumor characteristics

Concurrent hypermethylation of *MLH1* and *MGMT* was rare in our study, and associations with related molecular aberrations suggested that these colorectal cancers may develop through distinct pathways (chapter 2). In addition, we observed that *MLH1* hypermethylation was significantly correlated with MSI, *BRAF* mutations and CIMP (chapters 2,5 and 6). However, the overlap between these characteristics was incomplete, which raises the question whether colorectal cancers with *MLH1* hypermethylation, those with MSI, *BRAF* mutations or CIMP actually develop through one distinct hypermethylation-associated pathway.

In sporadic colorectal cancer, *MLH1* hypermethylation is the predominant cause of MSI (1-3). However, germline mutations or loss of expression of other mismatch repair genes may also contribute to MSI, and *MLH1* hypermethylation was observed in only 60% of colorectal carcinomas with MSI in a large population-based study (n=1,061) (4). Moreover, although MSI and CIN are considered as two alternative mechanisms in colorectal cancer (5), CpG island promoter methylation may be associated with both MSI and with specific patterns of chromosomal aberrations in CIN colorectal cancers (6). This potential overlap suggests that a hypermethylation phenotype may, at least in part, develop independently from both types of DNA instability. Furthermore, tumors with different molecular aberrations may not be equally sensitive to environmental factors and genetic variation. This uncertainty prompted us to study the associations of methyl donor intake with CIMP and the related molecular characteristics *MLH1* hypermethylation, MSI and *BRAF* mutations separately.

Dietary methyl donors and promoter hypermethylation in colorectal cancer

Associations with overall colorectal cancer risk

The *a priori* hypothesis was that adequate intakes of methyl donors (folate and methionine) or nutrients that are involved in folate metabolism (vitamins B2 and B6) may prevent CpG island promoter hypermethylation in colorectal cancer, which was based on limited and preliminary evidence from literature (7). To test this hypothesis, we first studied associations of these dietary factors with overall colorectal cancer (chapter 3). We observed inverse associations between intakes of methionine and vitamin B2 with tumors in the proximal colon, but folate was not associated with colorectal cancer risk. Vitamin B6 was associated with an unexpected increased overall colorectal cancer risk, and the positive association was particularly strong with rectal tumors among women.

The reason why dietary methyl donors would have a site-specific effect in colorectal cancer remains unclear. Possibly, molecular characteristics differ between sub-sites, for example the observation that gene promoter hypermethylation may occur more often in proximal colon cancer and among women (4,8,9). Although in our study, the majority of the tumors with *MLH1* hypermethylation (54%) and CIMP (60%)

occurred in the proximal colon, we did not observe that methionine and vitamin B2 were associated with promoter hypermethylation (chapters 5 and 7). Furthermore, it lacks a biological explanation why hypermethylation would occur more often in the proximal colon. Possibly, the increasing thickness of bowel contents and a decreasing speed of passage through the colorectum moving from proximal colon to rectum may not equally influence molecular aberrations in colorectal carcinogenesis.

Although it has previously been observed that hypermethylation may occur more often in women than in men, the associations of our study did not clearly differ between the sexes. In addition, *MLH1* hypermethylation tended to occur only slightly more often in women than in men, while the occurrence of CIMP was similar. One interesting previous observation was that men may have higher folate requirement than women (10). In this respect, it is conceivable that high folate intake would be more protective against colorectal cancer in men than in women, because men in the lowest reference quintile might more often have insufficient folate intake. However, this is only speculative, and our data did not indicate that men with high folate intake, or of the other studied nutrients, were systematically at lower risk of developing colorectal cancer than women.

Associations with promoter hypermethylation and related characteristics

We did not observe that high intakes of dietary folate, methionine or vitamin B2 were inversely associated with *MLH1* promoter hypermethylation or CIMP (chapters 5 and 7). Conversely, in addition to a positive association with overall risk, vitamin B6 tended to increase the risk of colorectal tumors harboring *MLH1* hypermethylation (chapter 5). However, we should be cautious in drawing definite conclusions, since a protective effect of vitamin B6 on colorectal cancer has previously been suggested in several observational studies (11-15). Moreover, we observed a positive association with *MLH1* hypermethylation only and not with related molecular characteristics such as CIMP, MSI or *BRAF* mutations. In addition, the other dietary methyl donors did not seem to have an effect on promoter hypermethylation. However, because a smaller proportion of patients is generally characterized by *MLH1* methylation (22% of the cases in our study), this subgroup of patients may have been of inadequate size for precise analyses, and the findings therefore need replication in future studies.

Nevertheless, a true positive association between vitamin B6 and *MLH1* hypermethylation may indicate that high bioavailability of methyl groups increases promoter hypermethylation in colorectal cancer, rather than decreasing it. Interestingly, although previous studies suggested only weak inverse, or no associations between dietary methyl donors and hypermethylation in colorectal cancer (7,16), positive associations have been observed between plasma folate levels and promoter hypermethylation in colorectal tumors (17,18). Moreover, in a randomized intervention trial, folate supplementation increased promoter hypermethylation in rectal mucosa of patients with a history of colorectal adenoma (19). These observations indicate that the *a priori* hypothesis of high methyl donor intake to be inversely associated with promoter hypermethylation may have to be rejected.

Polymorphisms in folate metabolizing enzymes and epigenetic regulators

Associations with overall colorectal cancer and hypermethylation endpoints

In the current study, we investigated the combinations of dietary methyl donors, genetic variants of folate metabolizing enzymes and methyltransferases, and CpG island promoter hypermethylation in colorectal cancer. In addition to associations between polymorphisms in some folate metabolizing enzymes and colorectal cancer, we observed that genetic variants of the DNA methyltransferase *DNMT3b* and histone methyltransferase *EHMT2*, which were not previously investigated in relation to colorectal cancer in a prospective cohort study, were associated with reduced colorectal cancer risk among women. Therefore, it may be concluded that these enzymes potentially play an important role in colorectal cancer.

Moreover, polymorphisms in folate metabolizing enzymes *MTR* and *MTRR* were associated with reduced risk of developing colorectal tumors harboring promoter hypermethylation. The occurrence of both of these polymorphisms was associated with lower plasma homocysteine concentrations (20,21), suggesting lower catalytic activity of the MTR and MTRR enzymes. Therefore, this may be a second indication that the resulting lower bioavailability of methyl groups reduces promoter hypermethylation in colorectal cancer, rather than leading to an increase.

Interaction between dietary methyl donors and genotypes

Regarding the dietary methyl group donors, we observed that vitamin B2 and methionine intake were both strongly associated with reduced colorectal cancer risk if the enzymatic activity of folate metabolizing enzymes (for the effect of vitamin B2) and DNMT3b (effect of methionine) was not affected by polymorphisms in their encoding genes. Although interactions were not statistically significant, these findings suggest that the effect of vitamin B2 and methionine may depend on genetic status of folate metabolizing enzymes and a DNA methyltransferase. Importantly, our study demonstrates that not only polymorphisms in separate genes, but also combinations of genotypes should be considered within separate functional metabolic pathways, and that dietary associations with cancer may otherwise remain undetected.

Interpretation of associations

It is important to realize that polymorphisms in folate metabolizing enzymes and methyltransferases may have different consequences for the change in enzymatic activity. Of the genes that were included in this study, there is evidence that enzymatic activity of the folate metabolizing enzyme MTHFR is substantially reduced in individuals with the C677T or A1298C polymorphisms (22,23). As mentioned above, MTR and MTRR polymorphisms were associated with decreased homocysteine concentration, which is indirect evidence for decreased enzymatic activity. In contrast, DNMT3b activity increased due to the occurrence of a rare genetic variant (24), whereas changes of enzymatic activity of histone methyltransferases due to polymorphisms have not yet been investigated. However, the precise consequences of genetic variants need to be clear for the interpretation of biologically plausible mechanisms in genetic association studies.

Moreover, other epigenetic changes may contribute to colorectal carcinogenesis. For example, global DNA hypomethylation may play a role in colorectal carcinogenesis, but the relative contribution of global hypomethylation and promoter hypermethylation in carcinogenesis, and the effect of methyl donors, are unknown.

Potential dual role of folate in colorectal carcinogenesis

Observational studies

After a considerably long follow up period of 13.3 years after baseline, 2349 colorectal cancer patients were identified in the NLCS. With this large number of cases and thereby high power to demonstrate an effect, the hypothesis that adequate dietary folate intake may protect against colorectal cancer was not supported. Several previous observational studies have been conducted to investigate the effect of folate on colorectal carcinogenesis, but only a part of these studies actually suggested a protective effect (25). Furthermore, when estimating the pooled association of observational studies, it appeared that dietary folate, but not total folate (i.e. the combination of dietary folates and folic acid supplements), was inversely associated with colorectal cancer risk (26). However, in the NLCS, in which we only studied the association of dietary folate, this was not observed.

Intervention trials

The effect of folic acid has been studied in randomized controlled trials among individuals with a history of colorectal adenoma. Folic acid supplementation of 5 mg/day for 3 years significantly reduced the number of recurrent adenomas, although a rather small number of patients (n=94) was enrolled in that study (27). However, a larger study (n=853) did not suggest a lower risk of colorectal adenoma recurrence in combination with folic acid supplementation of 0.5 mg/day during a period of approximately 3 years (28). In another recent trial (the Aspirin/Folate Polyp Prevention Study; n=987), it was observed that baseline folate levels were inversely associated with colorectal adenoma recurrence among individuals in the placebo group, but not in the folic acid intervention group (1 mg/day), suggesting that folic acid supplementation does not have an additional protective effect on colorectal adenoma recurrence (29). Interestingly, the folic acid intervention itself was associated with increased risk of developing advanced lesions after a second follow up (30). This is the first trial suggesting a potential tumor promoting effect of folic acid supplementation in humans.

Animal studies

Previous animal studies indicated that, in rats with normal colonic mucosa, low folate intake or folate deficiency may increase the formation of colonic neoplasias (31), and that high folate intake suppresses intestinal and colorectal carcinogenesis (32-34). However, folate deficiency has an inhibitory effect, and folic acid supplementation may enhance tumor progression in animals with established neoplasms (33-35). It has therefore been proposed that folate may have a dual role in colorectal carcinogenesis, i.e. that the effect depends on the timing of intervention in the process of colorectal carcinogenesis (36). A possible explanation for the observed increased risk of adenoma

recurrence in the Aspirin/Folate Polyp Prevention Study (30) may be that undetected small lesions were still present in treated patients, and that folic acid enhanced growth of these lesions (37). However, a dual role of folate in colorectal carcinogenesis is still hypothetical and evidence from human studies is limited to date.

Mechanisms of a dual role

Biological mechanisms of a potential dual role of folate in carcinogenesis have not been well established. It is known that adequate folate levels provide nucleotide precursors for sufficient DNA synthesis, which may prevent DNA instability in normal mucosa but enhance proliferation of neoplastic cells (36). We observed that high dietary folate intake was associated with increased risk of tumors harboring *APC* mutations (chapter 4) or *BRAF* mutations (chapter 5). Possibly, these mutations arise early in the carcinogenic process and folate may have resulted in growth advantage of these lesions by ensuring adequate DNA synthesis needed for replicating and proliferating tissues. On the other hand, it is also reasonable to expect that adequate folate intake prevents the introduction of gene mutations and would thereby reduce the risk of tumors with gene mutations. However, previous observational studies addressing the association between folate intake and mutations in colorectal cancer are limited in number and were inconsistent (16,38-40). A recent intervention study suggested that supplementation of folic acid and vitamin B12 in subjects with a history of colorectal adenoma increased uracil mis-incorporation and promoter hypermethylation in rectal mucosa (19), suggesting that a high dosis of folic acid may enhance colorectal carcinogenesis.

Implications for folic acid fortification of foods

Folic acid versus naturally occurring folates

Results of animal studies (31-35) and randomized controlled trials in humans (27,28,30) were based on an effect of synthetic folic acid and not of folates that occur naturally in our diet. It is questionable however, whether high intake of dietary folates would have a comparable adverse effect on colorectal carcinogenesis as compared to folic acid, and a potential harmful effect may be attributable to supplemented folic acid. One possible explanation may be that naturally occurring folates, as polyglutamates, first have to be cleaved to monoglutamates by enzymes in the intestinal brush border prior to absorption. Since folic acid is a monoglutamate, it may be absorbed directly without enzymatic conversion and thus may have a higher bioavailability than dietary folates (36,41). Nonetheless, as we mentioned above, we observed that naturally occurring folates may also enhance colorectal carcinogenesis depending on molecular characteristics of the tumor.

Nation-wide fortification of foods with folic acid

Mean daily folate intake in the NLCS was 225 µg/d among men and 199 µg/d among women (chapter 3, table 1). In addition, mean folate intake in the Netherlands was estimated to be 282 µg/d among men and 230 µg/d among women in 1998 (42). The

Recommended Dietary Allowance (RDA) for folate intake in the Netherlands is 300 µg/d for adult men and women (42).

However, in the US, the RDA for adult men and women is 400 µg of folate per day (43), and folate intake is substantially higher than in the Netherlands (44). Due to mandatory fortification in the US, additional folic acid intake has been estimated to be 100-200µg/d (44 45). The increase appeared most pronounced among Caucasians, in whom the median total folate intakes increased from around 330 µg/d to at least 470 µg/d among men, and from 264-303 µg/d to 380-535 µg/d among women, depending on the age category (44). In addition, in a cross-sectional survey it has been estimated that more than half of the US adult population uses dietary supplements, which also contributes to total folate intake (46).

After the introduction of nation-wide folic acid fortification in the US and Canada in 1998, there has been an increase in colorectal cancer incidence compared to the pre-fortification period (1986 to 1998) (47). The observation may indicate a potential adverse effect of nation-wide fortification, though causality is not proven in such an ecological study and therefore requires further investigation. With respect to a potential tumor promoting effect of folic acid, mandatory nation-wide fortification of foods with folic acid in the US and Canada is of concern. Therefore, other countries that are currently discussing the potential introduction of a nation-wide folic acid fortification program should be very reserved in deciding to do so, because potential long term adverse effects on a population level can not be overseen at this moment.

Recommendations for public health and future research

Based on results described in the present thesis and the current debate on folate fortification, I would like to propose the following:

1. This is the first prospective cohort study investigating associations of genetic variability of DNA methyltransferases and histone methyltransferases with colorectal cancer risk, and these findings should be replicated in future studies.

2. The complicated interplay between genetic variability and consequences for methyl group metabolism in colorectal cancer warrants future research which should include measurements of global DNA hypomethylation, gene promoter hypermethylation and enzymatic activity of folate metabolizing enzymes and epigenetic regulators. Moreover, there is a need for large studies in order to investigate the relationship between dietary methyl donors, DNA methylation and genetic variants. In addition, existing data and future studies should preferably be pooled to increase power.

3. Despite the proven favorable effect of sufficient folate status in the prevention of neural tube defects (48,49) and stroke (50), we recommend that nation-wide fortification of foods with folic acid should not be introduced given the current state of knowledge about a possible harmful effect on colorectal carcinogenesis and because future long term public health consequences cannot be overseen at present.

4. It should be considered to decrease the concentration of folic acid in fortified foods in the US and Canada in order to reduce a potential adverse effect on colorectal carcinogenesis.

5. Patients with a history of colorectal adenoma or carcinoma should not be advised to take supplemental folic acid, and medical staff should be aware of a potential harmful effect.

6. Finally, to increase our understanding about the role of folate in colorectal cancer, future studies should focus on the role of folate in DNA synthesis and methylation in normal colorectal epithelium in addition to colorectal adenomas or carcinomas. This will allow conclusions on timing of events and a potential dual role of folate and other dietary methyl donors.

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Summary

Folate is a B-vitamin that occurs naturally in our diet, but may also exist as folic acid which is the synthetic form of folate used in dietary supplements and fortified foods. Folic acid supplementation has been observed to reduce neural tube defects during embryonic development. In addition, adequate folate intake may possibly contribute to preventing colorectal cancer. However, previous studies have been inconsistent in demonstrating a protective effect, and such inconsistencies may be partially explained by the hypothesis that folate has a dual role in colorectal carcinogenesis, i.e. that it may protect against neoplastic development in normal colorectal mucosa whereas folate supplementation might enhance growth of existing pre-malignant lesions.

During colorectal carcinogenesis, the colorectal epithelium transforms into aberrant crypt foci, through colorectal adenomas, colorectal carcinomas and finally metastasis. Several molecular aberrations are thought to contribute to this process, such as accumulation of gene mutations in the tumor suppressor- and oncogenes *APC*, *KRAS*, *P53* and *BRAF*. In addition, promoter hypermethylation of CpG islands is a predominant epigenetic alteration that results in inactivation of transcriptional activity. A distinct subset of colorectal cancers harbours widespread promoter hypermethylation and is referred to as the CpG island methylator phenotype (CIMP). Furthermore, colorectal tumors may harbour microsatellite instability (MSI) – genetic instability characterized by length alterations in repeat sequences – often caused by promoter CpG island hypermethylation of the mismatch repair (MMR) gene *MLH1*. In colorectal cancer, CIMP has been observed to be associated with *MLH1* hypermethylation, MSI and *BRAF* mutations.

Folate has two important biological functions that are relevant with respect to the process of carcinogenesis. First, since it is essential for the biosynthesis of nucleotide precursors, folate deficiency may result in DNA instability, and possibly the introduction of gene mutations thereby contributing to carcinogenesis. Furthermore, folate is a methyl group donor which may influence DNA methylation such as CpG island promoter hypermethylation or global DNA hypomethylation, both of which are often observed in colorectal cancer. Like folate, methionine is a methyl donor. In addition, vitamins B2, B6 and B12 are involved in folate metabolism as co-factors, and may therefore modulate the bioavailability of methyl groups. Adequate intakes of these nutrients ensure a sufficient supply of methyl groups, and may be hypothesized to prevent aberrant DNA methylation, and thus to protect against colorectal cancer.

In this thesis, we investigated the associations between dietary methyl group donors and of other B-vitamins involved in folate metabolism with colorectal cancer, with or without gene mutations or promoter CpG island hypermethylation. We also studied whether genetic variants of folate metabolizing enzymes and methyltransferases (i.e. enzymes that are involved in incorporation of methyl groups into DNA) modify these associations. In addition, we studied the occurrence and correlations of gene mutations and gene promoter hypermethylation in colorectal carcinomas.

The studies described in this thesis were conducted within the Netherlands Cohort Study on diet and cancer (NLCS), which includes 120,852 men and women who filled out a food frequency questionnaire at baseline in 1986. The cohort was followed-up for cancer incidence and a subcohort of 5,000 subjects was randomly selected after baseline exposure measurement to estimate accumulation of person-time in the cohort through biennial follow-up of vital status. Overall colorectal cancer could be

investigated among 2,349 cases from a follow-up period of 13.3 years after baseline. Tumor material was collected of colorectal cancer patients identified within the first 7.3 years of follow up after baseline. In total, 734 patients were identified of whom sufficient tumor DNA could be extracted needed for genotyping and other molecular analyses.

In chapter 2, we investigated associations between *MGMT* promoter methylation and G:C>A:T mutations in *KRAS* and *APC*, and between *MLH1* promoter methylation and *APC*, *KRAS* and *BRAF* mutations. We observed that concurrent hypermethylation of *MGMT* and *MLH1* was rare. *MGMT* hypermethylation occurred more frequently in tumors with G:C>A:T *KRAS* mutations compared to those without these mutations, whereas no such difference was observed for G:C>A:T mutations in *APC*. *MLH1* hypermethylation was less common in tumors with *APC* or *KRAS* mutations, but was positively associated with *BRAF* mutations. The findings of this study suggest that in colorectal carcinogenesis, *MGMT* hypermethylation may succeed *APC* mutations but precedes *KRAS* mutations, and that tumors with *MGMT* hypermethylation may develop distinctly from those showing *MLH1* hypermethylation.

The associations between dietary folate, methionine, vitamin B2 and vitamin B6 with overall colorectal cancer risk are presented in chapter 3. Although we did not observe an association between dietary folate and colorectal cancer, higher methionine and vitamin B2 intakes were associated with reduced proximal colon cancer risk among men and women, respectively. Conversely, vitamin B6 intake was associated with increased colorectal cancer risk, being most pronounced for rectal cancer among women.

Whether folate intake is associated with *APC* mutations in colorectal cancer was investigated in chapter 4. Men with relatively high folate intake were at reduced risk of developing tumors without *APC* mutations, but folate was positively associated with tumors harbouring *APC* mutations. In chapter 5, we investigated the associations between dietary folate, methionine, vitamins B2 and B6 with *MLH1* hypermethylation, MLH1 expression, MSI and *BRAF* mutations. Predominantly among men, folate was associated with increased risk of tumors harbouring *BRAF* mutations, whereas vitamin B6 was associated with *MLH1* hypermethylation. These observations suggest that folate may enhance the growth of lesions harbouring gene mutations, and that vitamin B6 may do so by increasing promoter hypermethylation. Intake of folate, methionine, vitamins B2 and B6 were not associated with CIMP (chapter 7).

Chapter 6 addresses the associations between genetic variants of folate metabolizing enzymes *MTHFR*, *MTR* and *MTRR*, the DNA methyltransferase 3b (*DNMT3b*) and histone methyltransferases *PRDM2*, *EHMT1* and *EHMT2* with overall colorectal cancer and with tumors with or without CIMP, *MLH1* hypermethylation or MSI. The *MTHFR* 677TT variant was inversely associated with colorectal cancer risk among men, whereas the rare T allele was associated with increased risk in women. The *MTR* 2756GG genotype was associated with increased colorectal cancer risk, and inverse associations were observed among women carrying rare variants of the *DNMT3b* C>T or *EHMT2* G>A polymorphisms. We also observed inverse associations between *MTR* A2756G and CIMP among men, and between *MTRR* A66G and *MLH1* hypermethylation among women, suggesting that the occurrence of rare variants of these *MTR* and *MTRR* polymorphisms may reduce the risk of colorectal tumors with a promoter hypermethylation phenotype.

Finally, in chapter 7 we investigated whether the association between methyl donor intake with overall colorectal cancer, or with CIMP, *MLH1* hypermethylation or MSI, may be modified by the genetic variants. An inverse association of methionine with colorectal cancer appeared to be more pronounced if no rare variants occurred in the polymorphic *DNMT3b* gene. Similarly, we observed that vitamin B2 was inversely associated with CRC among individuals with the *MTHFR* 677CC genotype, while a strong inverse association existed when ≤1 rare allele occurred in the combination of methyl metabolizing enzymes *MTHFR*, *MTR* and *MTRR*, respectively. We therefore concluded that dietary methyl donors may be more protective against colorectal carcinogenesis if methyl metabolizing enzymes and DNA methyltransferases are left unaffected by rare variants of their encoding polymorphic genes. In addition, combining genotypes may reveal diet associations with colorectal cancer and should be considered in association studies.

This is the first prospective cohort study investigating associations of genetic variability of DNA methyltransferases and histone methyltransferases with colorectal cancer risk, and these findings should be replicated in future studies. In addition, the complicated interplay between genetic variability and consequences for methyl group metabolism in colorectal cancer warrants future research which should include measurements of global DNA hypomethylation, gene promoter hypermethylation and enzymatic activity of folate metabolizing enzymes and epigenetic regulators. Moreover, there is a need for large studies in order to investigate the relationship between dietary methyl donors, DNA methylation and genetic variants. In addition, existing data and future studies should preferably be pooled to increase power. In view of the hypothesized potential dual role of folate in colorectal carcinogenesis, and given our observations that naturally occurring folates may enhance colorectal tumors with specific molecular characteristics, it should not be recommended to introduce nation-widefortification of foods with folic acid.

Samenvatting

Folaat, of vitamine B11 komt voor in onder andere groene groenten, brood, aardappelen, vlees- en zuivelproducten. Naast deze natuurlijke vorm van folaat bestaat er een synthetische en chemisch stabielere variant die gebruikt wordt in supplementen en voor de verrijking van voedingsmiddelen. Hoewel de officiële benaming van deze synthetische variant foliumzuur is, wordt deze term in het Nederlands over het algemeen ook gebruikt voor folaat dat van nature in de voeding voorkomt. Om echter verdere verwarring te voorkomen zal in deze samenvatting de term folaat worden gebruikt voor folaten in de voeding, en foliumzuur voor de synthetische vorm.

Er zijn sterke aanwijzingen dat adequate folaatinname beschermt tegen neuraalbuisdefecten tijdens de embryonale ontwikkeling, waartoe Noord-Amerika en Canada in 1996 tot landelijke foliumzuurfortificatie van granen en graanproducten overgegaan zijn. Bovendien zou het tevens beschermen tegen dikkedarmkanker. Echter, de resultaten uit verschillende studies naar de relatie tussen folaat en dikkedarmkanker zijn inconsistent gebleken, mogelijk doordat folaat een tweeledige rol speelt bij het ontstaan van dikkedarmkanker. Zo word verondersteld dat het weliswaar beschermt tegen afwijkende weefselgroei in gezond darmepitheel, maar dat het de groei van bestaande neoplasma's juist zou kunnen bevorderen.

De carcinogenese van dikkedarmkanker is een langdurig proces waarbij gezond darmepitheel geleidelijk transformeert in een of meerdere carcinomen. Bepaalde moleculaire afwijkingen dragen waarschijnlijk in belangrijke mate bij aan dit proces, zoals somatische mutaties in de tumorsuppressorgenen en oncogenen *APC*, *KRAS*, *P53* en *BRAF*. Naast zulke genetische afwijkingen spelen epigenetische veranderingen – afwijkingen in het DNA die niet het gevolg zijn van een verandering in de DNA sequentie – een belangrijke rol. Zo kan genexpressie worden geblokkeerd door hypermethylering van CpG dinucleotiden in promotorregio's van genen. In ongeveer 25% van de dikkedarmtumoren komt dit type epigenetische verandering aanzienlijk meer voor, en tumoren met dit "methyleringsfenotype" worden daarom in de Engelse wetenschappelijke literatuur aangeduid met "CpG island methylator phenotype" of CIMP. Andere moleculaire afwijkingen die sterk correleren met dit fenotype zijn microsatelliet instabiliteit (MSI), een bepaalde vorm van DNA-instabiliteit die onder andere veroorzaakt wordt door hypermethylering van het DNA-reparatiegen *MLH1*, en mutaties in het oncogen *BRAF*.

Folaat heeft twee belangrijke functies in het lichaam die ook relevant zijn voor het proces van carcinogenese. Het is enerzijds essentieel voor de synthese van DNA. Folaatdeficiëntie kan DNA-instabiliteit tot gevolg hebben, wat genmutaties zou kunnen induceren. Daarnaast is folaat een donor van methylgroepen en is dus mogelijk van invloed op DNA methylering. Naast folaat is methionine een methyldonor in onze voeding, terwijl de vitaminen B2, B6 en B12 betrokken zijn bij verschillende omzettingen in het folaatmetabolisme. We nemen aan dat voldoende inname van deze nutriënten zorgt voor een adequate toelevering van methylgroepen, wat afwijkingen in DNA methylering en daarmee de carcinogenese van dikkedarmkanker kan voorkomen.

In de studies beschreven in dit proefschrift zijn de associaties onderzocht tussen methylgroepdonoren en andere B-vitaminen uit de voeding en dikkedarmkanker, waarbij specifiek naar de relatie gekeken is met tumoren met of zonder genmutaties of CIMP. Er is tevens onderzocht wat de mogelijk modificerende werking is van een aantal folaat metaboliserende enzymen en methyltransferases (enzymen die zorgen voor het incorporeren van methylgroepen in het DNA). Daarnaast

onderzochten we het vóórkomen en correlaties tussen promotor hypermethylering en genmutaties in dikkedarmtumoren.

De studies zijn uitgevoerd binnen de Nederlandse Cohort Studie naar voeding en kanker (NLCS). Dit cohort bestaat uit 120.852 mannen en vrouwen die bij aanvang van de studie in 1986 een voedselfrequentievragenlijst ingevuld hebben. Voor het schatten van de opgebouwde persoonstijd is bij aanvang een subcohort geselecteerd van 5.000 willekeurige personen, terwijl het gehele cohort gevolgd wordt voor het optreden van kanker. In de eerste 7,3 jaar na aanvang van de studie waren er 734 patiënten met dikkedarmkanker geregistreerd, van wie tumormateriaal uit pathologielaboratoria was opgevraagd dat tevens voldoende DNA bleek te bevatten voor moleculaire analyses. Gedurende de eerste 13,3 jaar na aanvang van de studie waren er in totaal 2.349 patiënten met dikkedarmkanker geregistreerd die de voedselfrequentievragenlijst volledig ingevuld hadden.

In hoofdstuk 2 onderzochten we de correlatie tussen hypermethylering van DNA-reparatiegenen en mutaties in tumorsuppressorgenen en oncogenen in dikkedarmtumoren. Het *O^6-methylguanine DNA methyltransferase* (*MGMT*)-gen codeert voor een enzym dat alkyl adducten van de O6-positie van guanine verwijdert, waarmee G:C>A:T mutaties kunnen worden voorkomen. We onderzochten de correlatie tussen *MGMT* hypermethylering en zulke mutaties in *APC* en *KRAS*, en tussen *MLH1* hypermethylering en mutaties (ongeacht het type mutatie) in *APC*, *KRAS* en *BRAF*. Het bleek dat gelijktijdige hypermethylering van *MGMT* en *MLH1* in slechts 10% van de tumoren voorkwam. *MGMT* hypermethylering kwam vaker voor in tumoren met G:C>A:T *KRAS* mutaties dan in tumoren zonder deze mutaties, terwijl een dergelijk verschil niet bleek te bestaan voor G:C>A:T mutaties in het *APC* gen. *MLH1* hypermethylering was geassocieerd met *BRAF* mutaties, maar kwam juist minder vaak voor in tumoren met *APC* – of *KRAS* mutaties. Deze resultaten zouden erop kunnen wijzen dat *MGMT* hypermethylering ontstaat na de introductie van *APC* mutaties, maar voorafgaat aan het ontstaan van *KRAS* mutaties tijdens de carcinogenese van dikkedarmkanker. Tumoren met *MGMT* hypermethylering zouden zich via een ander moleculair mechanisme kunnen ontwikkelen dan tumoren met *MLH1* hypermethylering.

De associaties tussen innamen van folaat, methionine, vitamines B2 en B6, en dikkedarmkanker staan beschreven in hoofdstuk 3. Folaatinname was niet geassocieerd met dikkedarmkanker. Echter, methionine en vitamine B2 waren geassocieerd met een lager risico op het krijgen van een tumor in het proximale colon onder respectievelijk mannen en vrouwen. Vitamine B6-inname was echter geassocieerd met een hoger risico, van met name rectumtumoren bij vrouwen.

Of folaatinname geassocieerd was met *APC* mutaties in dikkedarmkanker is onderzocht in hoofdstuk 4. Mannen met een relatief hoge folaatinname hadden een lager risico op het krijgen van een tumor zonder *APC* mutatie, maar een hoger risico op tumoren met *APC* mutatie. In hoofdstuk 5 zijn de associaties onderzocht tussen innamen van folaat, methionine, vitaminen B2 en B6, en de gerelateerde moleculaire afwijkingen *MLH1* hypermethylering, MLH1 expressie, MSI en *BRAF* mutaties. Met name onder mannen was folaatinname geassocieerd met verhoogd risico op tumoren met *BRAF* mutaties, terwijl vitamine B6 met geassocieerd was met *MLH1* hypermethylering. Het zou zo kunnen zijn dat folaat de groei van beginnende tumoren met genmutaties als in *APC* en *BRAF* stimuleert, en dat vitamine B6 dat kan doen door het bevorderen van promotor hypermethylering. Folaat, methionine, vitaminen B2 en B6, waren niet geassocieerd met CIMP (hoofdstuk 7).

Hoofdstuk 6 beschrijft de associaties tussen genetische varianten van de folaat metaboliserende enzymen *MTHFR*, *MTR* en *MTRR*, het DNA methyltransferase 3b (*DNMT3b*), en de histonmethyltransferases *PRDM2*, *EHMT1* en *EHMT2* enerzijds; en dikkedarmkanker ongeacht moleculaire afwijkingen, of het risico op tumoren met of zonder CIMP, *MLH1* hypermethylering of MSI anderzijds. De *MTHFR* 677TT variant was geassocieerd met lager dikkedarmkankerrisico onder mannen, terwijl het zeldzamere T-allel juist geassocieerd was met verhoogd risico bij vrouwen. Het *MTR* 2756GG genotype was geassocieerd met een hoger risico, terwijl inverse associaties bestonden onder vrouwen met zeldzame varianten van de *DNMT3b* C>T en *EHMT2* G>A polymorfismen. Verder vonden we inverse associaties tussen *MTR* A2756G en CIMP onder mannen en tussen *MTRR* A66G en *MLH1* hypermethylering onder vrouwen, wat erop wijst dat het voorkomen van zeldzame varianten van deze *MTR* en *MTRR* polymorfismen het risico op dikkedarmtumoren met een "methyleringsfenotype" verlaagt.

Tenslotte is in hoofdstuk 7 onderzocht of de associatie tussen methyldonorinname en dikkedarmkanker met of zonder CIMP, *MLH1* hypermethylering of MSI, gemodificeerd wordt door het vóórkomen van de onderzochte genetische varianten. Het bleek dat de mogelijk beschermende werking van methionine tegen dikkedarmkanker sterker was indien er geen zeldzame varianten voorkwamen in het polymorfe *DNMT3b* gen. Analoog daaraan bestond de inverse associatie tussen vitamine B2 en dikkedarmkanker alleen onder individuen met het *MTHFR* 677CC genotype, terwijl een sterk invers verband bestond voor diegenen met minder dan 1 zeldzaam allel in de combinatie van de folaat metaboliserende enzymen *MTHFR*, *MTR* en *MTRR*. We concluderen daarom dat een inverse associatie tussen methyldonorinname en dikkedarmkanker sterker zou kunnen zijn in mensen bij wie folaat metaboliserende enzymen en methyltransferases niet zijn aangetast door zeldzame varianten van hun coderende polymorfe genen. Bovendien is blijkbaar het combineren van verschillende genotypen van belang om associaties tussen nutriënten en kankerrisico te kunnen ontdekken.

Dit is de eerste keer dat de associaties tussen genetische varianten van DNA methyltransferases en histonmethyltransferases en dikkedarmkanker zijn onderzocht in een prospectieve cohortstudie. Daarom moeten deze bevindingen in de toekomst worden herhaald, bij voorkeur in grote studies of door het samenvoegen van studies. Daarnaast zijn de exacte consequenties van polymorfismen in folaat metaboliserende enzymen en methyltransferases voor enzymactiviteit lang niet altijd bekend, en is het bovendien de vraag wat de gevolgen zijn voor veranderingen in DNA methylering. In toekomstig onderzoek zou daarom de verandering van enzymactiviteit gemeten moeten worden voor genetische varianten van enzymen in het folaatmetabolisme en methyltransferases, en eventuele veranderingen in DNA methylering. Gegeven de hypothese dat folaat een mogelijk tweeledige rol speelt in de carcinogenese van dikkedarmkanker, en gezien onze observaties dat natuurlijk voorkomend folaat het risico van tumoren met specifieke moleculaire afwijkingen kan verhogen, zou een fortificatieprogramma met foliumzuur op landelijk niveau moeten worden afgeraden.

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Stefan de Vogel
Bergen, april 2009

Curriculum vitae

About the author

Stefan de Vogel was born in Leidschendam in the Netherlands on May 2nd, 1975. After completing Athenaeum at the IJsselcollege, Zwolle in 1995, he started studying Human Nutrition at the Wageningen University. For his first master thesis, Stefan was involved in a randomized controlled trial on iron supplementation and malaria chemoprophylaxis to prevent anaemia in infants in a rural area in Kenya. His second thesis was an epidemiological study to determine the representativeness of subjects in a calibration study among Dutch participants of the European Investigation into Cancer and nutrition (EPIC). He performed work on this thesis at the National Institute of Public Health and the Environment in Bilthoven, the Netherlands. In June 2001, Stefan graduated and started working as a SAS programmer at the pharmaceutical company Chiron BV in Amsterdam. From 2004, he was employed as a PhD student at the departments of Pathology and Epidemiology of the Maastricht University, where he worked on a project entitled: "Epigenetic silencing of caretaker genes as a cause of gatekeeper gene defects in sporadic colorectal cancer: modulation by dietary factors and polymorphisms in methyl donor metabolic enzymes" which resulted in the present thesis. Stefan is currently working as a post-doctoral fellow at the department of Public Health and Primary Health Care of the University of Bergen, Norway, where he continues to work in the field of folate and cancer.

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