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

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 tumour 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 tumours 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. Tumour 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 tumour 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 tumours 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 tumours 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 tumours 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 tumours without *APC* mutations, but folate was positively associated with tumours 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 tumours 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 tumours 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 tumours 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 tumours with specific molecular characteristics, it should not be recommended to introduce nation-wide fortification of foods with folic acid.