# DIET, LIFESTYLE, AND MOLECULAR ALTERATIONS THAT DRIVE COLORECTAL CARCINOGENESIS

**Brenda Diergaarde**

**Promotor**

Prof. dr. ir. Frans J. Kok
Hoogleraar Voeding en Gezondheid
Wageningen Universiteit, Wageningen

**Co-promotoren**

Dr. ir. Ellen Kampman
Universitair Hoofddocent, sectie Humane Voeding
Wageningen Universiteit, Wageningen

Dr. Goos N.P. van Muijen
Universitair Hoofddocent, afdeling Pathologie
Universitair Medisch Centrum St. Radboud, Nijmegen

**Promotiecommissie**

Dr. ir. Sandra A. Bausch-Goldbohm
TNO Voeding, Zeist

Prof. dr. Riccardo Fodde
Universitair Medisch Centrum Rotterdam, Rotterdam

Dr. Fokko M. Nagengast
Universitair Medisch Centrum St. Radboud, Nijmegen

Prof. dr. Evert G. Schouten
Wageningen Universiteit, Wageningen

Dit onderzoek is uitgevoerd binnen de onderzoekschool VLAG (Voeding, Levensmiddelentechnologie, Agrobiotechnologie en Gezondheid).

# DIET, LIFESTYLE, AND MOLECULAR ALTERATIONS THAT DRIVE COLORECTAL CARCINOGENESIS

**Brenda B. M. E. Diergaarde**

**Proefschrift**
ter verkrijging van de graad van doctor
op gezag van de Rector Magnificus
van Wageningen Universiteit,
Prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op maandag 20 december 2004
des namiddags te half twee in de Aula.

Diergaarde, Brenda B.M.E.
Diet, lifestyle, and molecular alterations that drive colorectal carcinogenesis.
Thesis Wageningen University – with references – with summary in Dutch

ISBN 90-8504-103-1

*Aan mijn ouders*

# Abstract

Environmental factors have been repeatedly implicated in the etiology of colorectal cancer, and much is known about the molecular events involved in colorectal carcinogenesis. The relationships between environmental risk factors and the molecular alterations that drive colorectal carcinogenesis are less clear. Further insight into these relationships may prove useful for the development of effective colorectal cancer prevention and/or treatment strategies. In this thesis, we examine associations between dietary and lifestyle factors previously reported to be associated with colorectal cancer risk and the molecular alterations known to play important roles in colorectal carcinogenesis.

Data from a case-control study of sporadic colorectal polyps (278 cases; 414 polyp-free controls) were used to evaluate associations between dietary factors and truncating *APC* mutations in adenomas. High intake of red meat and fat seemed to increase the risk of polyps without truncating *APC* mutation ($APC^-$) in particular, whereas high intake of carbohydrates seemed to especially decrease the risk of $APC^-$ polyps.

Associations between dietary factors and truncating *APC* mutations in colorectal carcinomas were investigated in a population-based case-control study (184 cases; 259 controls) of sporadic colon cancer. Consumption of vegetables lowered the risk of tumors with truncating *APC* mutation ($APC^+$) as well as $APC^-$ tumors, most explicitly of the last. Alcohol intake was associated with an increased risk of $APC^-$ tumors only, whereas meat, fish and fat seemed to especially increase the risk of $APC^+$ tumors. The same study population was used to evaluate associations between dietary factors and MSI, hMLH1 expression and *hMLH1* hypermethylation. Intake of red meat seemed to increase the risk of MSI-L/MSS carcinomas in particular, whereas alcohol intake appeared to increase the risk of MSI-H tumors. Fruit consumption seemed to especially decrease the risk of MSI-H tumors with hypermethylated *hMLH1*. Associations between cigarette smoking and mutations in the *APC*, K-*ras* and *p53* genes, p53 overexpression, and MSI were also assessed in this study population. Our data suggest that smoking-related colon carcinomas develop through a p53 overexpression-negative pathway and that smoking results in colon tumor cells with transversion mutations in particular.

Finally, we used data from a case-control study of HNPCC-associated colorectal tumors (145 cases; 103 tumor-free controls) to gain insight into the effects of environmental factors on colorectal tumor risk in individuals with HNPCC. Fruit consumption and dietary fiber intake lowered the risk of ever developing HNPCC-associated colorectal tumors, whereas cigarette smoking and alcohol consumption seemed to increase this risk. This suggests that also HNPCC-associated outcomes may be modified by environmental factors.

# Contents

**Chapter 1** | Introduction **9**

**Chapter 2** | Dietary factors and truncating *APC* mutations in sporadic colorectal adenomas **27**

*Int. J. Cancer* 2004 Aug 25; Epub ahead of print

**Chapter 3** | Dietary factors and the occurrence of truncating *APC* mutations in sporadic colon carcinomas: a Dutch population-based study **49**

*Carcinogenesis* 2003; 24 (2): 283-290

**Chapter 4** | Dietary factors and microsatellite instability in sporadic colon carcinomas **73**

*Cancer Epidemiol. Biomarkers Prev.* 2003; 12 (11): 1130-1136

**Chapter 5** | Cigarette smoking and genetic alterations in sporadic colon carcinomas **91**

*Carcinogenesis* 2003; 24 (3): 565-571

**Chapter 6** | Environmental factors, truncating *APC* mutations, and HNPCC-associated colorectal tumors **113**

*Submitted for publication*

**Chapter 7** | General discussion **137**

**Summary** | **159**

**Samenvatting** | **165**

**Dankwoord/Acknowledgements** | **172**

**About the author** | **175**

# Abbreviations

**APC**: adenomatous polyposis coli

**CI**: confidence interval

**CIN**: chromosomal instability

**FAP**: familial adenomatous polyposis

**hMLH**: human MutL homologue

**hMSH**: human MutS homologue

**HNPCC**: hereditary nonpolyposis colorectal cancer

**MCR**: mutation cluster region

**MMR**: mismatch repair

**MSI**: microsatellite instability

**MSI-H**: microsatellite instability high

**MSI-L**: microsatellite instability low

**MSS**: microsatellite stable

**OR**: odds ratio

**PhIP**: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

**SSCP**: single-strand conformation polypmorphism

# Chapter 1

# Introduction

Colorectal cancer is one of the most common malignancies in the Western world and one of the major causes of cancer morbidity and mortality (1). In the Netherlands, the country in which the studies described in this thesis were conducted, approximately 8500 new cases are diagnosed and about 4000 people die of colorectal cancer each year (2). In 1998, colorectal cancer was after breast cancer the second most common cancer among women and after prostate and lung cancer the third most common cancer among men (2). The lifetime risk of colorectal cancer in the general population is approximately 5%, whereas in proven carriers of an inherited susceptibility for colorectal cancer the lifetime risk can be close to 100%. Colorectal cancer incidence rates in the Netherlands resemble the rates in other Western European countries (1).

## Risk factors for colorectal cancer

Colorectal cancer is a multifactorial disease, the etiology is complex and involves environmental (including diet and lifestyle) as well as genetic factors. Colorectal cancer incidence rates vary widely between countries and regions, with the highest rates observed in the Western world (including North America, Western Europe, Australia, New Zealand and Japan) and relatively low rates in Africa, Asia, and parts of Latin America and the Caribbean (1, 3). Studies in migrant populations have also shown that among immigrants and their descendants, colorectal cancer incidence rates rapidly reach those of the new country (4). This suggests that environmental factors influence colorectal cancer risk.

Known dietary and lifestyle risk factors for colorectal cancer include high intake of red meat, alcohol and fat, and, possibly, cigarette smoking. Inverse associations have been observed with vegetable consumption, dietary fiber, calcium, use of non-steroidal anti-inflammatory drugs, physical activity, and, although less consistently, fruit intake (5, 6).

Other important risk factors for colorectal cancer are age and a positive family history. The risk of colorectal cancer increases with advancing age. Most cases are diagnosed in individuals ≥ 55 years of age and colorectal adenomas, thought to be the precursor lesions of most colorectal carcinomas, are present in about 50% of the population older than 70 years of age (7). Regarding a positive family history, colorectal cancer seems to occur more frequently in certain families -- "to run in certain families". Epidemiological studies have suggested that the risk of colorectal cancer in first-degree relatives (that is, parent, sibling or offspring) of cases is increased by approximately two-fold (8). This risk increases further with multiple affected relatives and a younger age at diagnosis (8). It should be noted that family history not only captures shared genetic factors but also shared dietary, lifestyle and other non-genetic factors.

Although the majority of colorectal cancer cases are considered to be 'sporadic' (that is, are without a clear-cut inheritance pattern), approximately 5-10% occur as part of hereditary cancer syndromes among which familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) are the most common (reviewed in Ref. 9 and, in Dutch, Refs. 10 and 11). Interestingly, the genes responsible for the increased colorectal cancer risk in FAP and HNPCC, the adenomatous polyposis coli (*APC*) gene and the DNA mismatch repair (MMR) genes, respectively, also play key roles in the development of sporadic colorectal cancer.

## Molecular alterations involved in colorectal carcinogenesis

Carcinogenesis is a multistep process and although the etiology of colorectal cancer is complex and multifactorial, from the perspective of understanding the molecular events involved in carcinogenesis, it is one of the best-characterized epithelial tumors. Most colorectal carcinomas appear to arise through a series of well-defined histopathological stages, the so-called adenoma-

carcinoma sequence, as a result of the accumulation of genetic and/or epigenetic alterations in genes involved in the regulation of key cellular processes, *e.g.*, cell proliferation, cell-cycle control, apoptosis and DNA repair (12). In the general, 'average-risk' population, the progression from benign adenoma to malignant carcinoma may take 10 to 15 years. Not all adenomas will progress to malignancy, however, and whether an adenoma ends up as a carcinoma depends at least in part on the molecular alterations present.

Commonly observed molecular alterations in colorectal tumors that are thought to play important roles in colorectal carcinogenesis are: 1) mutations in the *APC* gene; 2) mutations in the K-*ras* gene; 3) mutations in the *p53* gene; and, 4) microsatellite instability (MSI). However, the pattern of alterations observed in colorectal tumors is heterogeneous, and several different molecular pathways to colorectal cancer have been proposed (see for instance Ref. 6). Not all colorectal carcinomas exhibit, for instance, mutations in *APC*, K-*ras* **and** *p53* -- alterations in these genes are necessary to progress from normal epithelium to carcinoma according to the genetic model for colorectal tumorigenesis proposed by Fearon and Vogelstein in 1990 (12). In fact, it seems that only in a very small fraction of the carcinomas all three genes are mutated (13). Moreover, significant inverse relationships have been reported between MSI and mutations in *APC*, K-*ras* and *p53*, which suggests that colorectal tumors with MSI develop via a different molecular pathway (14, 15). The studies described in this thesis mostly focus on *APC*, and MSI and the MMR genes, which are therefore discussed further in the sections below. More information on K-*ras* and *p53* can be found in Box 1 and Box 2, respectively.

### *APC*

Inactivating mutations in the *APC* tumor suppressor gene can be detected in many colorectal adenomas and carcinomas and are thought to be early, initiating events in sporadic as well as familial colorectal carcinogenesis (12, 16). The *APC* gene is located on chromosome 5q21 (17);

it is a relatively large gene encoding a multifunctional, 312-kDa protein (see Ref. 18 for a recent overview of the multiple functions of APC). The main tumor suppressing function of APC seems to reside in its ability to properly regulate levels of intracellular ß-catenin, a key mediator in the Wnt signaling pathway (19-22). Loss of APC function results in the accumulation of ß-catenin, which in turn results in constitutive transcriptional activation of Wnt target genes (*e.g.*, *c-Myc* and *cyclin D1*; see also Roel Nusse's Wnt website: www.stanford.edu/~rnusse/wntwindow.html). Typically, loss of APC function is the result of truncating mutations (that is, nonsense and frameshift mutations) in the *APC* gene (23). However, inactivation of APC through hypermethylation of the *APC* promoter region has also been reported (24, 25).

Germline mutations in *APC* are responsible for FAP, a rare autosomal dominant cancer syndrome characterized by the development of hundreds to thousands of adenomatous polyps in the large intestine early in life. If not removed, some of these benign tumors will (inevitably) progress to carcinomas (for more information on FAP see Ref. 9 or, in Dutch, Refs. 10 and 11). In contrast to the germline mutations, which are scattered throughout the 5' half of the *APC* gene, so-called somatic or acquired *APC* mutations seem to cluster within a small region in exon 15 (codons 1286-1513) known as the mutation cluster region (MCR) (26, 27). Most colorectal tumors in which *APC* is mutated exhibit at least one truncating mutation in this region (28). Results from several studies indicate that truncating mutations in the MCR probably provide cells with a selective growth advantage due to (partial) inactivation of the ß-catenin down-regulating function of APC (29-31). See Fodde *et al*. (32) for a recent review of APC.

**Box 1: K-*ras***

Activating point mutations in the oncogene K-*ras* are thought to occur early in the adenoma-carcinoma sequence and to accompany the conversion of small to larger adenomas. They have been reported to occur in 30-40% of the sporadic colorectal tumors (33, 34). The K-*ras* gene is located on the short arm of chromosome 12 and encodes a 21-kDa GTPase that is involved in several signaling pathways. Bound to GTP, the ras protein is active; it becomes inactive when GTP is hydrolyzed to GDP. Most (~90%) of the

K-*ras* mutations identified in colorectal tumors are located in codons 12 and 13 (33). Mutations in these codons, which are both in the GTP-binding domain, result in a constitutively active ras protein, which in turn leads to inappropriate signaling. For more information on K-*ras* see Bos (35) or Ellis and Clark (36).

**Box 2: *p53***

The *p53* tumor suppressor gene is the most frequently altered gene in human cancers. Regarding colorectal cancer, mutations in *p53* seem to be especially important in later stages of colorectal carcinogenesis (37). They have been reported to occur in ~45% of the colorectal tumors; interestingly, most are missense mutations (IARC p53 database: www.iarc.fr/p53). The *p53* gene is located on the short arm of chromosome 17 and encodes a protein that is involved in, among other things, cell cycle control and apoptosis. Under normal circumstances, p53 is activated and accumulates in response to DNA-damage and various other types of stress, resulting in either growth arrest or apoptosis. Most *p53* mutations are located in exons 4-8 (38), the DNA-binding domain of the protein. For more information on *p53* see May and May (39) or Guimaraes and Hainaut (40).

***Microsatellite instability – MMR genes***

Approximately 10-20% of the sporadic colorectal carcinomas and most colorectal tumors associated with the HNPCC syndrome (see Box 3 for more information on HNPCC) are characterized by the presence of alterations in the length of simple, repetitive microsatellite sequences, called microsatellite instability (MSI) [(41-45); and, see Boland *et al.* (46) for international criteria for the determination of MSI in colorectal cancer]. This molecular phenotype was previously, and sometimes still is, referred to as the replication error (RER) phenotype. It is a hallmark of DNA mismatch repair (MMR) deficiency, which in turn appears to be primarily due to inherited and/or acquired alterations in the MMR genes *hMLH1* and *hMSH2* (*hMLH* stands for human *MutL* homologue; *hMSH* for human *MutS* homologue) [(47-49); see also Mitchell *et al*. (50) for review of the evidence that support the role of hMLH1 and hMSH2 in colorectal carcinogenesis]. Mutations in other currently known MMR genes (*e.g.*, *hMSH3*, *hMSH6*, and *hPMS2*) have rarely, if at all, been found (51).

The primary role of the DNA MMR system is to remove base-base mismatches and insertion/deletion loops that arise during DNA replication (reviewed in for instance Refs. 51 and 52). Furthermore, MMR proteins also appear to be involved in DNA-damage signaling and apoptosis (53). Thus, alterations in the MMR genes may provide cells with both an increased mutation rate and a selective growth advantage (53).

**Box 3: Hereditary nonpolyposis colorectal cancer (HNPCC)**

Germline mutations in the DNA MMR genes are responsible for HNPCC, one of the most common cancer syndromes in humans. So far, the majority (~90%) of germline mutations have been detected in *hMLH1* (54) and *hMSH2* (55, 56); mutations in other MMR genes [*e.g.*, *hMSH6* (57)] have also been found but seem more rare (International Collaborative Group on HNPCC website: www. nfdht.nl). HNPCC, also called Lynch syndrome, is characterized by an autosomal dominant mode of inheritance and an early onset of carcinogenesis (mean age colorectal cancer, ~ 45 years). Colorectal tumors are the predominant form of cancer in HNPCC but extracolonic cancers (including endometrial, ovarian, and stomach cancer) are often observed. It is currently not clear why especially these tissues are affected. Most HNPCC-associated colorectal tumors exhibit MSI, the hallmark of DNA MMR deficiency.

The diagnosis of HNPCC has traditionally been based on family history, as unlike FAP, which can be diagnosed based on the presence of multiple colorectal adenomas, HNPCC cannot be diagnosed on the basis of personal clinical characteristics only. The clinical diagnostic criteria most often used to define HNPCC are summarized in Table 1.1. The Amsterdam criteria have been the most widely used for diagnostic and research purposes; the goal of the Bethesda Guidelines was to guide who should undergo MSI analysis [see Syngal *et al*. (58) for a discussion of the sensitivity and specificity of the different clinical criteria]. The molecular definition of HNPCC requires the identification of a pathogenic germline mutation in one of the MMR genes. Identifying a germline mutation within an HNPCC family can be useful for testing and counseling family members. In the Netherlands, most mutation carriers are currently identified by referral of individuals with a family history fulfilling the clinical criteria for HNPCC to so-called clinical genetic centers for counseling, mutation analyses and presymptomatic testing.

HNPCC is thought to account for 1-5% of all colorectal cancer cases (45, 59-61), and mutation carriers may have a lifetime risk of developing colorectal cancer of 60-80% [reviewed in Mitchell *et al*. (50)]. The incomplete penetrance of disease among mutation carriers and the observation that the clinical expression of HNPCC seems to vary between regions and to have changed over time suggest that also HNPCC-associated outcomes may be modified by environmental factors (62, 63). Epidemiological data

supporting this hypothesis are, however, currently limited. Thus far only one study, Voskuil *et al*. (64), has examined associations between environmental factors and HNPCC-associated colorectal tumors.

Colorectal polyps occur in HNPCC patients at the same frequency as in the general population; however, these polyps appear to develop into tumors more rapidly (2-3 years instead of the 10-15 years in the general population). Therefore, in the Netherlands, surveillance recommendations for known and suspected 'HNPCC-ers' currently include screening for colorectal tumors by complete colonoscopy at an interval of two years or less, starting at 20-25 years of age [http://www.nfdht.nl; (65)]. For reviews of HNPCC see Marra and Boland (63), in Dutch, Menko *et al*. (10, 11), Lynch and De La Chapelle (9) or Umar *et al*. (66).

**Table 1.1** Clinical criteria for HNPCC

| *Name* | *Criteria* |
|---|---|
| **Amsterdam I:** (Ref. 67) | At least three relatives with colorectal cancer, one of whom is a first-degree relative of the other two; at least two successive generations are affected; at least one of the relatives is diagnosed with colorectal cancer before age 50; and FAP is excluded. |
| **Amsterdam II:** (Ref. 68) | At least three relatives with an HNPCC-associated tumor (*i.e*., colorectal, endometrial, stomach, ovary, ureter or renal-pelvis, small bowel, hepatobiliary tract, and sebaceous skin tumors), one of whom is a first-degree relative of the other two; at least two successive generations are affected; at least one of the relatives is diagnosed with cancer before age 50; and FAP is excluded. |
| **Bethesda:** (Ref. 59) | (1) Individuals from families that fulfill the Amsterdam criteria. Or, (2) individuals with two HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated extracolonic cancers. Or, (3) individuals with colorectal cancer, plus colorectal and/or HNPCC-related extracolonic cancer and/or colorectal adenoma in a first-degree relative; at least one of the cancers diagnosed before age 45 years and the adenoma diagnosed before age 40 years. Or, (4) individuals with colorectal or endometrial cancer diagnosed before age 45 years. Or, (5) individuals with right-sided colorectal cancer with an undifferentiated histopathological pattern (solid/cribiform) diagnosed before age 45. Or, (6) individuals with signet-ring cell type colorectal cancer diagnosed before age 45 years. Or, (7) individuals with colorectal adenomas diagnosed before age 40 years. |

## Environmental factors and molecular alterations in colorectal tumors

Environmental factors have been repeatedly implicated in the etiology of colorectal cancer, and much is known about the molecular events involved in colorectal carcinogenesis (see above). However, the relationships between the two (*i.e.*, between environmental factors and the molecular alterations that drive colorectal carcinogenesis) are less clear (6). Further insight into these relationships may prove useful for the development of effective colorectal cancer prevention and/or treatment strategies.

The dietary and lifestyle factors previously reported to be associated with colorectal cancer risk may well exert their effect on this risk by affecting the occurrence of specific molecular alterations that drive colorectal carcinogenesis (*e.g.*, by being involved in the actual production or prevention of these alterations, or by creating an environment that favors the proliferation of certain cells and not others). And (thus), as proposed by Breivik and Gaudernack (69), the different molecular pathways to colorectal cancer may reflect different environmental exposures. Supporting this hypothesis, Bardelli *et al*. (70) demonstrated that exposure to the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanide produced tumor cells characterized by MSI, whereas exposure to the bulky-adduct-forming agent 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) produced tumor cells characterized by chromosomal instability (CIN). CIN is another type of genetic instability and most colorectal tumors exhibit CIN or MSI, but not both (71).

Associations between environmental factors and the occurrence of *APC* mutations in human colorectal tumors have, to our knowledge, not been previously examined. However, the carcinogen PhIP, which is abundantly present in meat prepared at high temperatures, has been linked to specific *Apc* mutations in rat colon tumors (72, 73). Moreover, dietary factors have been found to be associated with the occurrence of (specific) K-*ras* mutations in human

colorectal adenomas (74) and colon carcinomas (75-77). Associations between environmental factors and *p53* alterations in colorectal tumors have also been reported (78-82). Regarding MSI, Slattery *et al.* (83) recently (*i.e.*, after the start of this PhD project) reported a positive association between long-term alcohol consumption and the occurrence of MSI in colon carcinomas. In the same study population, Slattery *et al.* (84) also observed a positive association between cigarette smoking and the occurrence of MSI. Wu *et al*. (85) have reported a positive association between well-done red meat intake and MSI tumors in a case-only study.

## Outline of thesis

The overall aim of the studies described in this thesis was to gain further insight into the relationship(s) between dietary and lifestyle factors previously reported to be associated with colorectal cancer risk and molecular alterations known to play important roles in colorectal carcinogenesis. We examined associations between: (a) dietary factors and truncating *APC* mutations in sporadic colorectal tumors; (b) dietary factors and MSI in sporadic colon carcinomas; (c) cigarette smoking and *APC*, K-*ras* and *p53* alterations and MSI in sporadic colon carcinomas; and, (d) dietary factors, cigarette smoking, and HNPCC-associated colorectal tumors.

In *Chapter 2* and *Chapter 3* we evaluate associations between dietary factors previously reported to be associated with colorectal cancer risk and the occurrence of truncating mutations in the MCR of *APC* in sporadic colorectal adenomas and colon carcinomas, respectively. Associations between dietary factors and *APC* mutations in human colorectal tumors have, to our knowledge, not been examined before. In *Chapter 2*, data from a Dutch case-control study of sporadic colorectal adenomas with a polyp-free control group (278 cases; 414 controls) are used to

examine the associations. In *Chapter 3*, we use data from a Dutch population-based case-control study of sporadic colon carcinomas (184 cases; 259 controls). In *Chapter 4* the focus is on MSI. In this chapter we examine associations between dietary factors and the occurrence of MSI in sporadic colon carcinomas. To further explore the relationship between diet and the presence of MSI, we additionally assess associations with MMR protein expression and *hMLH1* promoter hypermethylation. In the study described in *Chapter 5*, we assess associations between cigarette smoking and the occurrence of mutations in the *APC*, K-*ras* and *p53* genes, p53 overexpression, and MSI in sporadic colon carcinomas in order to evaluate the hypothesis that smoking is primarily linked to a specific colon tumor subgroup(s). In *Chapter 6* the focus is on HNPCC. In this chapter, we use data from a Dutch case-control study on environmental factors and HNPCC-associated colorectal tumors (145 cases; 103 tumor-free controls) to gain insight into the effects of environmental risk factors known to be relevant in sporadic colorectal carcinogenesis (*i.e.*, diet and cigarette smoking) on colorectal tumor risk in individuals with HNPCC. Finally, in *Chapter 7* the main findings of the five different studies are summarized, and methodological considerations, public health implications and future research directions are discussed.

## References

1. Ferlay, J., Bray, F., Posani, P., and Parkin, D.M. (2001) *GLOBOCAN 2000: Cancer incidence, mortality and prevalence worldwide, version 1*. IARC CancerBase No. 5. Lyon (France): IARC Press.
2. Visser, O., Coebergh, J.W.W., Van Dijck, J.A.A.M., and Siesling, S. (Editors) (2002) *Incidence of cancer in the Netherlands 1998*. Utrecht (The Netherlands): Vereniging van Integrale Kankercentra.
3. Pisani, P., Bray, F., and Parkin, D.M. (2002) Estimates of the world-wide prevalence of cancer for 25 sites in the adult population. *Int. J. Cancer*, **97**, 72-81.
4. McMichael, A.J., and Giles, G.G. (1988) Cancer in migrants to Australia: extending the descriptive epidemiological data. *Cancer Res.*, **48**, 751-756.

5. World Cancer Research Fund (WCRF) Panel (Potter J.D., Chair). (1997) *Food, nutrition and the prevention of cancer: a global perspective*. WCRF/American Institute for Cancer Research, Washington, DC.
6. Potter, J.D. (1999) Colorectal cancer: molecules and populations. *J. Natl. Cancer Inst*., **91**, 916-932.
7. Peipins, L.A., and Sandler, R.S. (1994) Epidemiology of colorectal adenomas. *Epidemiol. Rev*., **16**, 273-297.
8. Johns, L.E., and Houlston, R.S. (2001) A systematic review and meta-analysis of familial colorectal cancer risk. *Am. J. Gastroenterology*, **96**, 2992-3003.
9. Lynch, H.T., and De la Chapelle, A. (2003) Hereditary colorectal cancer. *N. Engl. J. Med*., **348**, 919-932.
10. Menko, F.H., Griffioen, G., Wijnen, J. Th., Tops, C.M.J., Fodde, R., and Vasen, H.F.A. (1999) Genetica van darmkanker. I. Non-polyposis- en polyposisvormen van erfelijke darmkanker. *Ned. Tijdschr. Geneeskd*., **143**, 1201-1206.
11. Menko, F.H., Griffioen, G., Wijnen, J. Th., Tops, C.M.J., Fodde, R., and Vasen, H.F.A. (1999) Genetica van darmkanker. II. Erfelijke achtergrond van sporadische en familiaire darmkanker. *Ned. Tijdschr. Geneeskd*., **143**, 1207-1211.
12. Fearon, E.R., and Vogelstein, B. (1990) A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759-767.
13. Smith, G., Carey, F.A., Beattie, J., Wilkie, M.J.V., Lightfoot, T.J., Coxhead, J., Garner, R.C., Steele, R.J.C., and Wolf, C.R. (2002) Mutations in *APC*, Kirsten-*ras*, and *p53* – alternative genetic pathways to colorectal cancer. *Proc. Natl. Acad. Sci. USA*, **99**, 9433-9438.
14. Salahshor, S., Kressner, U., Pahlman, L., Glimelius, B., Lindmark, G., and Lindblom, A. (1999) Colorectal cancer with and without microsatellite instability involves different genes. *Genes Chromosomes Cancer*, **26**, 247-252.
15. Samowitz, W.S., Holden, J.A., Curtin, K., Edwards, S.L., Walker, A.R., Lin, H.A., Robertson, M.A., Nichols, M.F., Gruenthal, K.M., Lynch, B.J., Leppert, M.F., and Slattery, M.L. (2001) Inverse relationship between microsatellite instability and K-*ras* and *p53* gene alterations in colon cancer. *Am. J. Pathology*, **158**, 1517-1524
16. Powell, S.M., Zilz, N., Beazer-Barclay, Y., Bryan, T.M., Hamilton, S.R., Thibodeau, S.N., Vogelstein, B., and Kinzler, K.W. (1992) *APC* mutations occur early during colorectal tumorigenesis. *Nature*, **359**, 235-237.
17. Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., *et al*. (1991) Identification and characterization of the familial adenomatous polyposis coli gene. *Cell*, **66**, 589-600.
18. Fodde, R. (2003) The multiple functions of tumour suppressors: it's all in APC. *Nature Cell Biol*., **5**, 190-192.

19. Korinek, V., Barker, N., Morin, P.J., Van Wichen, D., De Weger, R., Kinzler, K.W., Vogelstein, B., and Clevers, H. (1997) Constitutive transcriptional activation by a ß-catenin-Tcf complex in APC$^{-/-}$ colon carcinoma. *Science*, **275**, 1784-1787.
20. Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K.W. (1997) Activation of ß-catenin-Tcf signaling in colon cancer by mutations in *ß-catenin* or *APC*. *Science*, **275**, 1787-1790.
21. Smits, R., Kielman, M.F., Breukel, C., Zurcher, C., Neufeld, K., Jagmohan-Changur, S., Hofland, N., Van Dijk, J., White, R., Edelmann, W., Kucherlapati, R., Khan, P.M., and Fodde, R. (1999) *Apc*$^{1638T}$: a mouse model delineating critical domains of the adenomatous polyposis coli protein involved in tumorigenesis and development. *Genes Dev*., **13**, 1309-1321.
22. Willert, K., and Nusse, R. (1998) Beta-catenin: a key mediator of Wnt signaling. *Curr. Opin. Genet. Dev.*, **8**, 95-102.
23. Laurent-Puig, P., Beroud, C., and Soussi, T. (1998) *APC* gene: database of germline and somatic mutations in human tumors and cell lines. *Nucl. Acids Res*., **26**, 269-270.
24. Hiltunen, M.O., Alhonen, L., Koistinaho, J., Myohanen, S., Paakkonen, M., Marin, S., Kosma, V.M., and Janne, J. (1997) Hypermethylation of the *APC* (adenomatous polyposis coli) gene promoter region in human colorectal carcinoma. *Int. J. Cancer*, **70**, 664-648.
25. Esteller, M., Sparks, A., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M.A., Gonzalez, S., Tarfa, G., Sidransky, D., Meltzer, S.J., Baylin, S.B., and Herman, J.G. (2000) Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. *Cancer Res.,* **60**, 4366-4371.
26. Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T., and Nakamura, Y. (1992) Somatic mutations of the *APC* gene in colorectal tumors: mutation cluster region in the *APC* gene. *Hum. Mol. Genet*., **1**, 229-233.
27. Miyaki, M., Konishi, M., Kikuchi-Yanoshita, R., Enomoto, M., Igari, T., Tanaka, K., Muraoka, M., Takahashi, H., Amada, Y., Fukayama, M., Maeda, Y., Iwama, T., *et al*. (1994) Characteristics of somatic mutation of the adenomatous polyposis coli gene in colorectal tumors. *Cancer Res*., **54**, 3011-3020.
28. Rowan, A.J., Lamlum, H., Ilyas, M., Straub, J., Papadopoulou, A., Bicknell, D., Bodmer, W.F., and Tomlinson, I.P.M. (2000) *APC* mutations in sporadic colorectal tumors: A mutational "hotspot" and interdependence of the "two hits". *Proc. Natl. Acad. Sci. USA*, **97**, 3352-3357.
29. Lamlum, H., Ilyas, M., Rowan, A., Clark, S., Johnson, V., Bell, J., Frayling, I., Efstathiou, J., Pack, K., Payne, S., Roylance, R., Gorman, P., *et al*. (1999) The type of somatic mutation at *APC* in familial adenomatous polyposis is determined by the site of the germline mutation: a new facet to Knudson's 'two-hit' hypothesis. *Nat. Med*., **5**, 1071-1075.

30. Smits, R., Hofland, N., Edelmann, W., Geugien, M., Jagmohan-Changur, S., Albuquerque, C., Breukel, C., Kucherlapati, R., Kielman, M.F., and Fodde, R. (2000) Somatic *Apc* mutations are selected upon their capacity to inactivate the ß-catenin downregulating activity. *Genes, Chromosomes Cancer*, **29**, 229-239.
31. Crabtree, M., Sieber, O.M., Lipton, L., Hodgson, S.V., Lamlum, H., Thomas, H.J.W., Neale, K., Phillips, R.K.S., Heinimann, K., and Tomlinson, I.P.M. (2003) Refining the relation between 'first hits' and 'second hits' at the *APC* locus: the 'loose fit' model and evidence for differences in somatic mutation spectra among patients. *Oncogene*, **22**, 4257-4265.
32. Fodde, R., Smits, R., and Clevers H. (2001) *APC*, signal transduction and genetic instability in colorectal cancer. *Nature Rev. Cancer*, **1**, 55-67.
33. Bos, J.L., Fearon, E.R., Hamilton, S.R., Verlaan-de Vries, M., Van Boom, J.H., Van der Eb, J.A., and Vogelstein, B. (1987) Prevalence of ras *gene* mutations in human colorectal cancers. *Nature*, **327**, 293-297.
34. Andreyev, H.J.N., Norman, A.R., Cunningham, D., Oates, J.R., and Clarke, P.A. for the RASCAL Group. (1998) Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" study. *J. Natl. Cancer Inst*., **90**, 675-684.
35. Bos, J.L. (1998) All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral. *EMBO J*., **17**, 6776-6782.
36. Ellis, C.A., and Clark, G. (2000) The importance of being K-ras. *Cellular Signalling*, **12**, 425-434.
37. Kikuchi-Yanoshita, R., Konishi, M., Ito, S., Seki, M., Tanaka, K., Maeda, Y., Lino, H., Fukayama, M., Koike, M, and Mori, T. (1992) Genetic changes of both *p53* alleles associated with the conversion from colorectal adenoma to early carcinoma in familial adenomatous polyposis and non-familial adenomatous polyposis patients. *Cancer Res*., **52**, 3965-3971.
38. Greenblatt, M.S., Bennett, W.P., Hollstein, M., and Harris, C.C. (1994) Mutations in the *p53* tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res*., **54**, 4855-4878.
39. May, P., and May, E. (1999) Twenty years of p53 research: structural and functional aspects of the p53 protein. *Oncogene*, **18**, 7621-7636.
40. Guimaraes, D.P., and Hainaut, P. (2002) TP53: a key gene in human cancer. *Biochimie*, **84**, 8393.
41. Aaltonen, L.A., Peltomäki, P., Leach, F., Sistonen, P., Pylkkänen, L., Mecklin, J-P., Järvinen, H., Powell, S.M., Jen, J., Hamilton, S.R., Petersen, G.M., Kinzler, K.W., *et al*. (1993) Clues to the pathogenesis of familial colorectal cancer. *Science*, **260**, 812-816.
42. Thibodeau, S.N., Bren, G., and Schaid, D. (1993) Microsatellite instability in cancer of the proximal colon. *Science*, **260**, 816-819.
43. Ionov, Y., Peinado, M.A., Malkhosyan, S., Shibata, D., and Perucho, M. (1993) Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature*, **363**, 558-561.

44. Aaltonen, L.A., Peltomäki, P., Mecklin, J-P., Järvinen, H., Jass, J.R., Green, J.S., Lynch, H.T., Watson, P., Tallqvist, G., Juhola, M., Sistonen, P., Hamilton, S.R., *et al*. (1994) Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. *Cancer Res*., **54**, 1645-1648.
45. Cunningham, J.M., Kim, C-Y, Christensen, E.R. Tester, D.J., Parc, Y., Burgart, L.J., Halling, K.C., McDonnell, S.K., Schaid, D.J., Walsh-Vockley, C. Kubly, V., Nelson, H., *et al*. (2001) The frequency of hereditary defective mismatch repair in a prospective series of unselected colorectal carcinomas. *Am. J. Hum. Genet*., **69**, 780-790.
46. Boland, C.R., Thibodeau, S.N., Hamilton, S.R., Sidransky, D., Eshleman, J.R., Burt, R.W., Meltzer, S.J., Rodrigues-Bigas, M.A., Fodde, F., Ranzani, G.N., and Srivastava, S. (1998) A National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res*., **58**, 5248-5257.
47. Dietmaier, W., Wallinger, S., Bocker, T., Kullman, F., Fishel, R., and Rüschoff, J. (1997) Diagnostic microsatellite instability: definition and correlation with mismatch repair expression. *Cancer Res*., **57**, 4749-4756.
48. Thibodeau, S.N., French, A.J., Cunningham, J.M., Tester, D., Burgart, L.J., Roche, P.C., McDonnel, S.K., Schaid, D.J., Walsh-Vockley, C., Michels, V.V., Farr, G.H., and O'Connell, M.J. (1998) Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of *hMLH1*. *Cancer Res*., **58**,1713-1718.
49. Lindor, N.M., Burgart, L.J., Leontovich, O., Goldberg, R.M., Cunningham, J.M., Sargent, D.J., Walsh-Vockley, C., Petersen, G.M., Walsh, M., Leggett, B.A., Young, J.P., Barker, M.A., *et al*. (2002) Immunohistochemistry versus microsatellite instability testing in phenotyping colorectal tumors. *Journal Clin. Oncology*, **20**, 1043-1048.
50. Mitchell, R.J., Farrington, S.M., Dunlop, M.G., and Campbell, H. (2002) Mismatch repair genes *hMLH1* and *hMSH2* and colorectal cancer: a HuGE review. *Am. J. Epidemiol*., **156**, 885-902.
51. Jiricny, J., and Nyström-Lahti, M. (2000) Mismatch repair defects in cancer. *Curr. Opin. Genet. Dev*., **10**, 157-161.
52. Aquilina, G., and Bignami, M. (2001) Mismatch repair in correction of replication errors and processing of DNA damage. *J. Cell. Physiol*., **187**, 145-154.
53. Fishel, R. (2001) The selection for mismatch repair defects in hereditary nonpolyposis colorectal cancer: revising the mutator hypothesis. *Cancer Res*., **61**, 7369-7374.
54. Papadopoulos, N., Nicolaides, N.C., Wei, Y.F., Ruben, S.M., Carter, K.C., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., Adams, M.D., Venter, J.C., Hamilton, S.R., *et al*. (1994) Mutation of a mutL homolog in hereditary colon cancer. *Science*, **263**, 1625-1629.

55. Fishel, R., Lescoe, M.K., Rao, M.R., Copeland, N.G., Jenkins, N.A., Garber, J., Kane, M., and Kolodner, R. (1993) The human mutator gene homolog *MSH2* and its association with hereditary nonpolyposis colon cancer. *Cell*, **75**, 1027-1038.
56. Leach, F.S., Nicolaides, N.C., Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomaki, P., Sistonen, P., Aaltonen, L.A., Nystrom-Lahti, M., Guan, X.Y., Zhang, J., *et al*. (1993) Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. *Cell*, **75**, 1215-1225.
57. Miyaki, M., Konishi, M., Tanaka, K., Kikuchi-Yanoshita, R., Muraoka, M., Yasuno, M., Igari, T., Koike, M., Chiba, M., and Mori, T. (1997) Germline mutation of *MSH6* as the cause of hereditary nonpolyposis colorectal cancer. *Nature Genet*., **17**, 271-272.
58. Syngal, S., Fox, E.A., Eng, C., Kolodner, R.D., and Garber, J.E. (2000) Sensitivity and specificity of clinical criteria for hereditary non-polyposis colorectal cancer associated mutations in *MSH2* and *MLH1*. *J. Med. Genet*., **37**, 641-645.
59. Rodriguez-Bigas, M.A., Boland, C.R., Hamilton, S.R, Henson, D.E., Jass, J.R., Meera Khan, P., Lynch, H., Perucho, M., Smyrk, T., Sobin, L., and Srivastava, S.A. (1997) A National Cancer Institute work shop on hereditary nonpolyposis colorectal cancer syndrome: meeting highlights and Bethesda guidelines. *J. Natl. Cancer Inst*., **89**, 1758-1762.
60. Aaltonen, L.A., Salovaara, R., Kristo, P., Canzian, F., Hemminki, A., Peltomaki, P., Chadwick, R.B., Kaariainen, H., Eskelinen, M., Jarvinen, H., Mecklin, J-P., and De la Chapelle, A. (1998) Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. *N. Engl. J. Med*., **338**, 1481-1487.
61. Samowitz, W., Curtin, K., Lin, H.H., Robertson, M.A., Schaffer, D., Nichols, M., Gruenthal, K., Leppert, M.F., and Slattery, M.L. (2001) The colon cancer burden of genetically defined hereditary nonpolyposis colon cancer. *Gastroenterology*, **121**, 830-838.
62. Park, J-G., Park, Y.J., Wijnen, J.T., and Vasen, H.F.A. (1999) Gene-environment interaction in hereditary nonpolyposis colorectal cancer with implications for diagnosis and genetic testing. *Int. J. Cancer*, **82**, 516-519.
63. Marra, G., and Boland, C.R. (1995) Hereditary nonpolyposis colorectal cancer: the syndrome, the genes, and historical perspectives. *J. Natl. Cancer Inst*., **87**, 1114-1125.
64. Voskuil, D.W., Kampman, E., Grubben, M.J.A.L., Kok, F.J., Nagengast, F.M., Vasen, H.F.A., and Van ‘t Veer, P. (2002) Meat consumption and meat preparation in relation to colorectal adenomas among sporadic and HNPCC family patients in the Netherlands. *Eur. J. Cancer*, **38**, 2300-2308.
65. De Vos tot Nederveen Cappel, W.H., Nagengast, F.M., Griffioen, G., Menko, F.H., Taal, B., Kleibeuker, J.H., and Vasen, H.F. (2002) Surveillance for hereditary nonpolyposis colorectal cancer. A long-term study on 114 families. *Dis. Col. Rectum*, **45**, 1588-1594.
66. Umar, A., Risinger, J.I., Hawk, E.T., and Barrett, J.C. (2004) Testing guidelines for hereditary nonpolyposis colorectal cancer. *Nature Rev. Cancer*, **4**, 153-158.

67. Vasen, H.F., Mecklin, J-P., Meera Khan, P., and Lynch, H.T. (1991) The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC). *Dis. Col. Rectum*, **34**, 424-425.
68. Vasen, H.F.A., Watson, P., Mecklin, J-P, and Lynch, H.T. (1999) New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative Group on HNPCC. *Gastroenterology*, **116**, 1453-1456.
69. Breivik, J, and Gaudernack, G. Carcinogenesis and natural selection: a new perspective to the genetics and epigenetics of colorectal cancer. *Adv. Cancer Res*., **76**, 187-212, 1999.
70. Bardelli, A., Cahill, D.P., Lederer, G., Speicher, M.R., Kinzler, K.W., Vogelstein, B., and Lengauer, C. Carcinogen-specific induction of genetic instability. *Proc. Natl. Acad. Sci. USA*, **98**, 5770-5775, 2001
71. Lengauer, C., Kinzler, K.W., and Vogelstein, B. Genetic instabilities in human cancers. *Nature*, **396**, 643-648, 1998.
72. Kakiuchi, H., Watanabe, M., Ushijima, T., Toyota, M., Imai, K., Weisburger, J.H., Sugimura, T., and Nagao, M. (1995) Specific 5'-GGGA-3' → 5'-GGA-3' mutation of the *Apc* gene in rat colon tumors induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Proc. Natl. Acad. Sci. USA*, **92**, 910-914.
73. Nagao, M., Ushijima, T., Toyota, M., Inoue, R., and Sugimura, T. (1997) Genetic changes induced by heterocyclic amines. *Mutat. Res*., **376** (1-2), 161-167.
74. Martinez, M.E., Maltzman, T., Marshall, J.R., Einspahr, J., Reid, M.E., Sampliner, R., Ahnen, D.J., Hamilton, S.R., and Alberts D.S. (1999) Risk factors for Ki-*ras* protooncogene mutation in sporadic colorectal adenomas. *Cancer Res*., **59**, 5181-5185.
75. Kampman, E., Voskuil, D.W., Van Kraats, A.A., Balder, H.F., Van Muijen, G.N.P., Goldbohm, R.A., and Van 't Veer, P. (2000) Animal products and K-*ras* codon 12 and 13 mutations in colon carcinomas. *Carcinogenesis*, **21**, 307-309.
76. Slattery, M.L., Curtin, K., Anderson, K., Ma, K-N, Edwards, S., Leppert, M., Potter, J., Schaffer, D., and Samowitz, W.S. (2000) Associations between dietary intake and Ki-*ras* mutations in colon tumors: a population-based study. *Cancer Res*., **60**, 6935-6941.
77. Moreno, V., Guino, E., Bosch, F., Peinado, M., Capella, G., Navarro, M., Marti, J., Cambray, M., and Lloberas, B., for the Bellvitge Colorectal Cancer Study Group. (2002) Diet and K-*ras* mutations in colorectal cancer. *IARC Sci. Publ*., **156**, 501-502.
78. Freedman, A.N., Michalek, A.M., Marshall, J.R., Mettlin, C.J., Petrelli, N.J., Zhang, Z-F, Black, J.D., Satschidanand, S., and Asirwatham, J.E. (1996) The relationship between smoking exposure and p53 overexpression in colorectal cancer. *Br. J. Cancer*, **73**, 902-908.

79. Freedman, A.N., Michalek, A.M., Marshall, J.R., Mettlin, C.J., Petrelli, N.J., Black, J.D., Zhang, Z-F, Satschidanand, S., and Asirwatham, J.E. (1996) Familial and nutritional risk factors for p53 overexpression in colorectal cancer. *Cancer Epidemiol. Biomark. Prev*., **5**, 285-291.
80. Voskuil, D.W., Kampman, E., Van Kraats, A.A., Balder, H.F., Van Muijen, G.N.P., Goldbohm, R.A., and Van 't Veer, P. (1999) P53 overexpression and *p53* mutations in colon carcinomas: relation to dietary risk factors. *Int. J. Cancer*, **81**, 675-681.
81. Slattery, M.L., Curtin, K., Ma, K-N, Edwards, S., Schaffer, D., Anderson, K., and Samowitz, W.S. (2002) Diet, activity, and lifestyle associations with *p53* mutations in colon tumors. *Cancer Epidemiol. Biomark. Prev*., **11**, 541-548.
82. Terry, M.B., Neugut, A.I., Mansukhani, M., Waye, J., Harpaz, N., and Hibshoosh, H. (2003) Tobacco, alcohol, and p53 overexpression in early colorectal neoplasia. *BMC Cancer*, **3**:29 (http://www.biomedcentral.com/1471-2407/3/29).
83. Slattery, M.L., Anderson, K., Curtin, K., Ma, K-N, Schaffer, D., and Samowitz, W. (2001) Dietary intake and microsatellite instability in colon tumors. *Int. J. Cancer*, **93**, 601-607.
84. Slattery, M.L., Curtin, K., Anderson, K., Ma, K-N, Ballard, L., Edwards, S., Schaffer, D., Potter, J., Leppert, M., and Samowitz, W.S. (2000) Associations between cigarette smoking, lifestyle factors, and microsatellite instability in colon tumors. *J. Natl. Cancer Inst*., **92**, 1831-1836.
85. Wu, A.H., Shibata, D., Yu, M. C., Lai, M-Y, and Ross, R.K. (2001) Dietary heterocyclic amines and microsatellite instability in colon adenocarcinomas. *Carcinogenesis*, **22**, 1681-1684.

# Chapter 2

# Dietary factors and truncating *APC* mutations in sporadic colorectal adenomas

Brenda Diergaarde

Edine W. Tiemersma

Hanneke Braam

Goos N. P. van Muijen

Fokko M. Nagengast

Frans J. Kok

Ellen Kampman

***International Journal of Cancer,* August 2004; Epub ahead of print**

## Abstract

Inactivating mutations in *APC* are thought to be early, initiating events in colorectal carcinogenesis. To gain insight into the relationship between diet and inactivating *APC* mutations, we evaluated associations between dietary factors and the occurrence of these mutations in a Dutch case-control study of sporadic colorectal adenomas (278 cases; 414 polyp-free controls). Direct-sequencing was used to screen adenomas for mutations in the mutation cluster region of *APC*; truncating mutations were detected in 161 (58%) of the adenomas. Red meat consumption was significantly differently related to polyps with truncating *APC* mutation ($APC^+$ polyps) compared with polyps without truncating *APC* mutation ($APC^-$ polyps) [OR (95% CI) for highest versus lowest tertile, 0.5 (0.3-1.0)]. High intake of red meat and fat seemed to increase the risk of $APC^-$ polyps only [$APC^+$ versus controls: red meat, 1.0 (0.6-1.6); fat, 1.1 (0.6-1.9). $APC^-$ versus controls: red meat, 1.8 (1.0-3.1); fat, 1.9 (1.0-3.7)]. Intake of carbohydrates was inversely associated with both polyp groups, most noticeably with $APC^-$ polyps. Most other evaluated dietary factors were not distinctively associated with a specific *APC* status. None of the dietary factors was specifically associated with a particular type of truncating *APC* mutation. Our data suggest that red meat and fat may increase the risk of $APC^-$ polyps in particular, whereas carbohydrates may especially decrease the risk of $APC^-$ polyps. However, most examined dietary factors do not appear to be specifically associated with the occurrence of truncating *APC* mutations in colorectal adenomas but seem to affect both pathways equally.

## Introduction

Colorectal cancer is one of the most common malignancies in the Western world and one of the major causes of cancer morbidity and mortality. Most colorectal carcinomas appear to arise through a series of well-characterized histopathological stages, the so-called adenoma-carcinoma sequence, as a result of the accumulation of genetic and epigenetic alterations (1).

Inactivating mutations in the adenomatous polyposis coli (*APC*) tumor suppressor gene can be detected in many colorectal adenomas and carcinomas and are thought to be early, initiating events in sporadic as well as familial colorectal carcinogenesis (1, 2). Typically, loss of APC function is the result of truncating mutations (that is, nonsense and frameshift mutations) in the *APC* gene although inactivation of APC through hypermethylation of the *APC* promoter

region has also been observed (3, 4). Germline *APC* mutations result in familial adenomatous polyposis (FAP), one of the major hereditary predispositions to colorectal cancer, and are scattered throughout the 5' half of the gene. However, somatic *APC* mutations seem to cluster within a small region of the gene (codons 1286-1513) known as the mutation cluster region (MCR) (5, 6). Most colorectal tumors in which *APC* is mutated exhibit at least one truncating mutation in the MCR (7). Truncating mutations in this area result in absence of the C-terminal portion of the APC protein. Results from several studies indicate that they probably provide cells with a selective growth advantage due to (partial) inactivation of the β-catenin down-regulating function of APC (8-10).

Diet has been repeatedly implicated in the etiology of colorectal cancer (11). Known dietary risk factors for colorectal cancer include high intake of red meat, alcohol, and fat. Inverse associations have been observed with vegetable consumption, dietary fiber, calcium, and, less consistently, fruit intake. These dietary factors may well exert their effect on colorectal cancer risk by affecting the occurrence of specific molecular alterations that drive colorectal carcinogenesis, and the different molecular pathways to colorectal cancer may well reflect different dietary exposures. Supporting this idea, the carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), which is present in meat prepared at high temperatures, has been linked to specific *Apc* mutations in rat colon tumors (12). Moreover, we previously observed a positive association between red meat, fish, and fat intake and colon carcinomas with truncating *APC* mutation (13). In the same study population, alcohol consumption seemed to particularly increase the risk of carcinomas without truncating *APC* mutation; vegetable consumption was found to be inversely associated with both carcinoma groups but the observed protective effect was significantly higher for carcinomas without truncating *APC* mutation (13). Dietary factors have also been found to be associated with the occurrence of (specific) K-*ras*

mutations in colorectal adenomas and colon carcinomas (14-16) and with the occurrence of microsatellite instability (17, 18).

Associations between dietary factors and *APC* mutations in colorectal adenomas have, to our knowledge, not been previously examined. Because adenomas are an early stage in colorectal tumorigenesis, examining the relationship between diet and the occurrence of truncating *APC* mutations in adenomas may provide further insight into how, and which, dietary factors are involved in the production of these specific, early occurring mutations. Therefore, in this study, we evaluate associations between dietary factors previously reported to be associated with colorectal cancer risk and the occurrence of truncating mutations in the MCR region of *APC* in a Dutch case-control study of sporadic colorectal adenomas with a polyp-free control group.

## Material and methods

### *Study population*

For this study, cases and controls participating in an ongoing case-control study on diet and colorectal adenomatous polyps were used. Details of the ongoing case-control study have been described elsewhere (19). In short, both cases and controls underwent colonoscopy in the outpatient clinics of seven hospitals, all located in the Netherlands, between June 1997 and December 2001. Potential participants were recruited at the time of colonoscopy by the endoscopy staff (53% of the participants), or were identified by regular review of the medical records of individuals who had undergone colonoscopy and invited by mail to participate within three months of their colonoscopy (47% of the participants). Eligible subjects were Dutch speaking, Caucasian, between 18 and 75 years old at time of colonoscopy, mentally competent to participate, and had no known personal history of colorectal cancer or (partial) bowel resection, FAP, hereditary nonpolyposis colorectal cancer, ulcerative colitis, Crohn’s disease, or serious disabling morbidity. Cases (*n*=295) were women and men diagnosed with at least one histologically confirmed colorectal adenomatous polyp, with polyp(s) diagnosed either during the index colonoscopy (*i.e.*, the colonoscopy that resulted in recruitment for this study) or during a colonoscopy performed maximally three years before the index colonoscopy. Controls (*n*=414) were women and men never diagnosed with any type of colorectal polyp and who had all undergone a complete colonoscopy (*i.e.*,

reaching the cecum) or sigmoidoscopy followed by colon X-ray. For cases, the most common indications for the index colonoscopy were bleeding (33%), large bowel complaints (including defecation problems) (27%) and routine screening for adenoma recurrence (26%). Controls mostly underwent colonoscopy because of large bowel complaints (including defecation problems) (59%) and bleeding (14%). Written informed consent was obtained from all participants. The study protocol was reviewed and approved by the Medical Ethical committees of the participating hospitals and Wageningen University.

### *Data collection*

Information on lifestyle factors including smoking habits, medical history, reproductive history, physical activity, and family history of colorectal cancer was collected using a self-administered, structured questionnaire. Medical records were checked for information on history of colorectal polyps and colorectal cancer and other relevant medical history. Usual dietary intake was assessed with a validated, semi-quantitative food frequency questionnaire that was originally developed for the Dutch cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC) [for details see Ocké *et al*. (20, 21)]. Briefly, this self-administered questionnaire contained questions on the average consumption frequency during one year for 79 main food items. Referent period for the current study was the year preceding the index colonoscopy or start of complaints. For 21 foods, the questionnaire contained photographs of 2-4 differently sized portions. For most other items the consumption frequency was asked in number of specified units (*e.g.*, slices or glasses); for a few foods a standard portion size was assumed. Frequencies per day and portion sizes were multiplied to obtain grams per day for each food item. In total, the average daily consumption of 178 foods was estimated with the information obtained by the questionnaire. Energy and nutrient intakes were calculated using an adapted version of the computerized Dutch food composition table. When questionnaires were returned incomplete, study staff followed up with the participants by phone. Information on completeness of colonoscopy (*i.e.*, whether cecum was reached or not) and location (proximal: cecum through transverse colon; distal: splenic flexure through sigmoid; rectum: first 15cm from anal opening), size, number, and type of polyps detected was obtained from endoscopy and pathology reports. Formalin-fixed, paraffin-embedded polyp tissue of the cases was obtained from the pathology departments of the seven collaborating hospitals. Regarding cases for which tissue was available from more than one histologically confirmed adenomatous polyp, the largest adenomatous polyp obtained was selected for mutation analysis. The study pathologist re-evaluated the histology of all obtained polyps.

### *DNA extraction*

DNA was extracted from formalin-fixed, paraffin-embedded polyp tissue (10-12 10µm thick sections) using the Puregene$^{TM}$ DNA isolation kit (Gentra Systems, Minneapolis, MN). Microdissection was

performed, guided by a hematoxylin and eosin-stained 4μm section, and only those areas containing >60% tumor cells were used. Isolated tissue was incubated overnight at 55ºC in 500μl cell lysis solution containing 0.5mg/ml proteinase K (Roche Diagnostics, Mannheim, Germany), followed by 72 hours at 37ºC. Proteins were removed with the protein precipitation solution according to the manufacturer's protocol. DNA was precipitated with 500μl 100% isopropanol at 4ºC for 30 minutes. The pellet was washed with 500μl 70% ethanol, air- dried, and subsequently the DNA was rehydrated in 30μl DNA hydration solution.

### *APC mutation analysis*

Codons 1286 to 1585 (this area includes the MCR) of the *APC* gene were analyzed for truncating mutations by direct-sequencing. First, the region was divided into five overlapping fragments (codons 1286-1358, 1337-1404, 1387-1455, 1437-1526, and 1509-1585, respectively), which were separately amplified in two consecutive PCRs as described previously (13). PCR products were checked on an ethidium bromide stained 2% agarose gel and purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Subsequently, the PCR products were sequenced using ABI PRISM® BigDye™ Terminators v3.0 (Applied Biosystems, Foster City, CA) and cycle sequencing with AmpliTaq® DNA polymerase, FS (Applied Biosystems, Foster City, CA). Sequencing was performed in both directions using the same primers as in the second PCR. Samples were analyzed on an ABI PRISM® 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Mutation analysis started in all samples with fragment 1, and only if no truncating mutations were detected was fragment 2 screened for mutations, and so on. We focused on truncating mutations because these mutations indisputably result in function loss of the APC protein whereas the biological significance of missense mutations in *APC* is uncertain. We were unable to perform mutation analysis on 17 polyps due to bad quality DNA and/or insufficient amounts of DNA. The cases exhibiting these polyps did not differ significantly from the other cases (data not shown). They were excluded from the analyses leaving 278 cases. Polyps were classified as $APC^+$ (with truncating mutation in *APC*) or $APC^-$ (without truncating mutation in *APC* and all five fragments completely analyzed for mutations).

### *Statistical analyses*

The distribution of truncating *APC* mutations in the polyps was determined. Energy-adjusted nutrient intake was computed, for women and men separately, with the residual method (22). To convey the sense of an actual nutrient intake, we added the mean nutrient intake to each residual (22). Differences in characteristics between groups were assessed using *t*-tests for continuous variables and Chi-square tests or Fisher's exact tests for categorical variables. *P* values <0.05 were considered significant. All significance tests were two-sided. The categorization of the dietary factors, in tertiles, was based on the

distribution of intake in the control group. Case-control comparisons, separately comparing the $APC^+$ cases and $APC^-$ cases with the polyp-free controls, were conducted to estimate the relative risk of developing adenomatous polyps with and without truncating *APC* mutations, respectively. Case-case comparisons were conducted to evaluate heterogeneity in dietary risk factors for the different polyp groups. Odds ratios (OR) and the corresponding 95% confidence intervals (95% CI) were calculated using multiple logistic regression models. Linear trend was assessed using the tertile medians as continuous variables in multiple logistic regression models. All analyses were adjusted for age (continuously), sex, and total energy intake (continuously). Alcohol intake was also adjusted for cigarette smoking (never, ever). Additional adjustment for family history of colorectal cancer, polyp location (proximal, distal, rectum), polyp size (≤1cm, >1cm), polyp number (single, multiple), polyp type (tubular, tubulovilleus, villous), cigarette smoking (never, ever), body mass index (continuously), and other dietary factors (continuously) did not change the estimates by more than 10%. All analyses were performed using the SAS® statistical software package (SAS version 8.2, SAS Institute Inc. Cary, NC). With a power of 0.80 and $\alpha$=0.05 (two-sided) the present study is able to detect the following ORs: all cases versus controls, ≤0.61 and ≥1.56; $APC^+$ versus controls, ≤0.55 and ≥1.69; $APC^-$ versus controls, ≤0.50 and ≥1.80; and $APC^+$ versus $APC^-$, ≤0.46 and ≥2.00 (23).

## Results

One hundred and sixty-one (57.9%) of the 278 colorectal adenomatous polyps included in this study exhibited a truncating *APC* mutation. Frameshift mutations were observed most often (*n*=107, 66.5% of all truncating *APC* mutations); nonsense mutations were identified in 54 polyps (26 transitions, 28 transversions). Codon 1309 was the most frequently mutated *APC* codon in this study (21 frameshifts and 5 nonsense mutations).

Characteristics of the study population are given in Table 2.1. The mean age of the controls was significantly lower than the mean age of the cases. The control group contained significantly more women and less ever smokers than the case group. Alcohol, fat, dietary fiber, and folate intake were higher among the cases than among the controls. $APC^-$ cases consumed more red meat and proteins than the controls. However, $APC^+$ cases and $APC^-$ cases

**Table 2.1** Characteristics of the study population[a]

| | Controls | Cases (*n*=278) | |
|---|---|---|---|
| | | APC$^+$ | APC$^-$ |
| | (*n*=414) | (*n*=161; 57.9%) | (*n*=117; 42.1%) |
| Age (in years, mean ± SD) | 50.5 ± 14.0 [b] | 57.7 ± 10.5 [c] | 57.6 ± 9.9 [d] |
| Sex [women, *n* (%)] | 252 (60.9) [b] | 74 (46.0) [c] | 47 (40.2) [d] |
| Family history of colorectal cancer [*n* (%)] | 94 (22.7) | 33 (20.5) | 32 (27.4) |
| Body mass index (kg/m$^2$, mean ± SD) | 25.6 ± 4.0 | 26.1 ± 4.1 | 25.9 ± 3.7 |
| Total energy intake (kJ/day, mean ± SD) | 8554.1 ± 2468.1 | 8849.6 ± 2366.7 | 8662.8 ± 2149.0 |
| Ever smoked [*n* (%)] | 218 (52.7) [b] | 107 (66.5) [c] | 88 (75.2) [d] |
| *Food groups* (g/day; mean ± SD) | | | |
| Vegetables | 116.5 ± 43.5 | 118.6 ± 43.9 | 124.3 ± 48.6 |
| Fruit | 182.7 ± 128.8 | 204.3 ± 136.7 | 192.7 ± 141.2 |
| Cereals | 42.9 ± 39.2 | 41.0 ± 35.1 | 38.1 ± 34.2 |
| Red meat | 55.9 ± 32.4 | 56.3 ± 31.8 | 65.4 ± 34.3 [d,e] |
| Poultry | 11.8 ± 11.8 | 12.7 ± 12.7 | 11.8 ± 11.7 |
| Fish | 10.9 ± 10.3 | 11.5 ± 13.7 | 12.6 ± 11.2 |
| Dairy products | 399.1 ± 280.7 | 365.1 ± 243.8 | 380.4 ± 258.1 |
| *Nutrients*[f] (g/day; mean ± SD) | | | |
| Alcohol | 10.0 ± 14.3 [b] | 14.2 ± 17.3 [c] | 15.9 ± 18.8 [d] |
| Fat | 79.9 ± 14.3 [b] | 82.1 ± 15.0 | 86.1 ± 15.6 [d,e] |
| Protein | 77.9 ± 12.9 | 78.6 ± 11.9 | 80.5 ± 12.4 [d] |
| Carbohydrates | 231.8 ± 35.8 | 235.2 ± 41.4 | 228.1 ± 37.1 |
| Dietary fiber | 23.4 ± 5.1 [b] | 24.5 ± 4.8 [c] | 24.0 ± 4.8 |
| Calcium (mg/day) | 1093.7 ± 337.3 | 1073.1 ± 308.4 | 1084.0 ± 341.7 |
| Vitamin C (mg/day) | 105.5 ± 44.7 | 109.8 ± 43.7 | 111.2 ± 49.4 |
| ß-Carotene (µg/day) | 1432.4 ± 517.8 | 1391.0 ± 469.8 | 1474.3 ± 533.6 |
| Folate (µg/day) | 193.4 ± 39.6 [b] | 202.1 ± 40.1 [c] | 206.5 ± 40.2 [d] |

[a] APC$^+$: adenomas with truncating *APC* mutation; APC$^-$: adenomas without truncating *APC* mutation. Differences between the groups were assessed with *t*-tests for continuous variables and Chi-square tests for categorical variables. [b] All cases versus controls, $P < 0.05$. [c] APC$^+$ cases versus controls, $P < 0.05$. [d] APC$^-$ cases versus controls, $P < 0.05$. [e] APC$^+$ cases versus APC$^-$, $P < 0.05$. [f] Adjusted for total energy intake by the residual method.

only differed markedly from each other in red meat and fat intake. $APC^{+}$ cases consumed less red meat and fat than $APC^{-}$ cases. The frequency of individuals with personal history of colorectal polyps did not differ between the two case groups (data not shown). In addition, cases with a polyp exhibiting a frameshift mutation in *APC* ($APC^{frameshift}$ polyps, *n*=107) differed significantly from cases with a polyp exhibiting a nonsense mutation in *APC* ($APC^{nonsense}$ polyps, *n*=54) only in cereal and β-carotene intake. $APC^{frameshift}$ cases consumed more cereals and β-carotene than $APC^{nonsense}$ cases (data not shown).

Polyp size was positively associated with the occurrence of a truncating *APC* mutation (Table 2.2). In addition, compared with $APC^{-}$ polyps, $APC^{+}$ polyps seemed to be more frequently located in the distal part of the colon than in the proximal part or in the rectum, though the association was not statistically significant. Most polyps were tubular adenomas, but $APC^{+}$ polyps seemed to be more often tubulovillous or villous than $APC^{-}$ polyps (not statistically significant) (Table 2.2). Additional adjustment for the other adenoma characteristics (*e.g.*, size and histological type when evaluating location) did not change the observed estimates significantly. $APC^{frameshift}$ polyps were comparable with $APC^{nonsense}$ polyps with regard to size, location, and histological type (data not shown).

Results of case-control and case-case comparisons conducted to evaluate associations between the various dietary factors and the occurrence of truncating *APC* mutations in sporadic adenomas are presented in Tables 2.3 (total energy intake and food groups) and 2.4 (nutrients). Intake of dairy products was inversely associated with $APC^{+}$ polyps as well as $APC^{-}$ polyps when the two polyp groups were separately compared with the polyp-free controls. Fruit intake was positively associated with $APC^{+}$ polyps. Red meat consumption was positively associated with $APC^{-}$ polyps; no clear association was observed with $APC^{+}$ polyps. Case-case comparison showed that red meat intake was significantly differently related to $APC^{+}$ polyps compared with

APC$^-$ polyps. Additional adjustment for vegetables and cigarette smoking did not change the observed estimates for red meat consumption significantly.

**Table 2.2** Adenoma characteristics and truncating *APC* mutations[a]

| | *n* (%) | | | |
|---|---|---|---|---|
| | APC$^+$ (*n*=161) | APC$^-$ (*n*=117) | *P*[b] | OR (95% CI)[c] |
| *Location*[d] | | | 0.11 | |
| Proximal | 21 (13.0) | 16 (13.7) | | 1.0 |
| Distal | 115 (71.4) | 68 (58.1) | | 1.2 (0.6-2.6) |
| Rectum | 21 (13.0) | 25 (21.4) | | 0.6 (0.3-1.5) |
| *Size*[e] | | | 0.07 | |
| ≤1 cm | 78 (48.5) | 69 (59.0) | | 1.0 |
| >1 cm | 68 (42.2) | 37 (31.6) | | 1.6 (1.0-2.7) |
| *Histological type* | | | 0.53 | |
| Tubular | 114 (70.8) | 90 (76.9) | | 1.0 |
| Tubulovillous | 25 (15.5) | 15 (12.8) | | 1.3 (0.6-2.6) |
| Villous | 22 (13.7) | 12 (10.3) | | 1.5 (0.7-3.2) |

[a] APC$^+$: adenomas with truncating *APC* mutation; APC$^-$: adenomas without truncating *APC* mutation. [b] APC$^+$ versus APC$^-$, Fisher's exact test. [c] Adjusted for age, sex, and total energy intake. [d] The location of 12 colorectal adenomas was unknown (4 APC$^+$, 8 APC$^-$). [e] The size of 26 colorectal adenomas was unknown (15 APC$^+$, 11 APC$^-$).

Assessment of nutrients (Table 2.4) showed that consumption of carbohydrates was inversely associated with both polyp groups, especially with APC$^-$ polyps. Additional analyses of carbohydrate subgroups (*i.e.*, mono/disaccharides and polysaccharides) suggested that in particular polysaccharides lower the risk of APC$^-$ polyps [APC$^-$ versus controls, OR (95%CI) increment of 45g/day: mono/disaccharides, 0.8 (0.6-1.1); polysaccharides, 0.6 (0.4-1.0). Not in table]. High intake of fat seemed to increase the risk of APC$^-$ polyps in particular.

**Table 2.3** Associations between total energy intake, food groups, and adenomas with (APC$^+$) and without (APC$^-$) truncating *APC* mutations: case-control and case-case comparisons

| | Odds ratios (95% confidence intervals)[a] | | | |
|---|---|---|---|---|
| | T1 | T2 | T3 | $P_{trend}$[b] |
| *Total energy intake* (kJ/day) | ≤7418.2 | 7418.2-9377.3 | ≥9377.3 | |
| No. APC$^+$/APC$^-$/controls | 45/34/138 | 57/39/138 | 59/44/138 | |
| All cases versus controls | 1.0 | 1.3 (0.9-1.9) | 1.2 (0.8-1.9) | 0.44 |
| APC$^+$ versus controls | 1.0 | 1.4 (0.9-2.3) | 1.3 (0.8-2.3) | 0.33 |
| APC$^-$ versus controls | 1.0 | 1.1 (0.6-1.9) | 1.0 (0.6-1.8) | 0.99 |
| APC$^+$ versus APC$^-$ | 1.0 | 1.2 (0.6-2.2) | 1.1 (0.6-2.2) | 0.74 |
| *Vegetables* (g/day) | ≤93.6 | 93.6-130.8 | ≥130.8 | |
| No. APC$^+$/APC$^-$/controls | 48/33/138 | 56/39/138 | 57/45/138 | |
| All cases versus controls | 1.0 | 1.2 (0.8-1.8) | 1.2 (0.8-1.8) | 0.35 |
| APC$^+$ versus controls | 1.0 | 1.2 (0.7-1.8) | 1.1 (0.7-1.8) | 0.62 |
| APC$^-$ versus controls | 1.0 | 1.3 (0.8-2.3) | 1.4 (0.8-2.4) | 0.26 |
| APC$^+$ versus APC$^-$ | 1.0 | 0.9 (0.5-1.7) | 0.8 (0.4-1.4) | 0.37 |
| *Fruit* (g/day) | ≤118.1 | 118.1-237.4 | ≥237.4 | |
| No. APC$^+$/APC$^-$/controls | 47/36/138 | 41/39/138 | 73/42/138 | |
| All cases versus controls | 1.0 | 1.0 (0.6-1.5) | 1.3 (0.9-2.0) | 0.16 |
| APC$^+$ versus controls | 1.0 | 1.0 (0.6-1.6) | 1.5 (1.0-2.3) | 0.10 |
| APC$^-$ versus controls | 1.0 | 1.1 (0.6-1.9) | 1.1 (0.7-1.9) | 0.70 |
| APC$^+$ versus APC$^-$ | 1.0 | 0.8 (0.4-1.6) | 1.3 (0.7-2.2) | 0.38 |
| *Cereals* (g/day) | ≤19.8 | 19.8-50.7 | ≥50.7 | |
| No. APC$^+$/APC$^-$/controls | 53/41/138 | 61/44/138 | 47/32/138 | |
| All cases versus controls | 1.0 | 1.5 (1.0-2.2) | 1.3 (0.8-2.0) | 0.48 |
| APC$^+$ versus controls | 1.0 | 1.6 (1.0-2.6) | 1.3 (0.8-2.3) | 0.44 |
| APC$^-$ versus controls | 1.0 | 1.5 (0.9-2.5) | 1.2 (0.7-2.2) | 0.74 |
| APC$^+$ versus APC$^-$ | 1.0 | 1.1 (0.6-2.0) | 1.2 (0.6-2.4) | 0.59 |
| *Red meat* (g/day) | ≤39.2 | 39.2-70.7 | ≥70.7 | |
| No. APC$^+$/APC$^-$/controls | 53/30/138 | 53/33/138 | 55/54/138 | |
| All cases versus controls | 1.0 | 1.1 (0.7-1.7) | 1.3 (0.8-1.9) | 0.25 |
| APC$^+$ versus controls | 1.0 | 1.0 (0.6-1.7) | 1.0 (0.6-1.6) | 0.85 |
| APC$^-$ versus controls | 1.0 | 1.2 (0.7-2.2) | 1.8 (1.0-3.1) | 0.03 |
| APC$^+$ versus APC | 1.0 | 0.8 (0.4-1.6) | 0.5 (0.3-1.0) | 0.03 |

| *Table 2.3 cont.* | T1 | T2 | T3 | $P_{trend}$[b] |
|---|---|---|---|---|
| *Poultry* (g/day) | ≤5.3 | 5.3-13.8 | ≥13.8 | |
| No. APC$^+$/APC$^-$/controls | 49/41/138 | 64/41/138 | 48/35/138 | |
| All cases versus controls | 1.0 | 1.3 (0.9-1.9) | 0.9 (0.6-1.4) | 0.56 |
| APC$^+$ versus controls | 1.0 | 1.4 (0.9-2.2) | 1.0 (0.6-1.6) | 0.81 |
| APC$^-$ versus controls | 1.0 | 1.2 (0.7-1.9) | 0.8 (0.5-1.4) | 0.40 |
| APC$^+$ versus APC$^-$ | 1.0 | 1.3 (0.7-2.3) | 1.2 (0.6-2.1) | 0.74 |
| *Fish* (g/day) | <4.4 | 4.4-14.0 | ≥14.0 | |
| No. APC$^+$/APC$^-$/controls | 52/34/132 | 58/34/144 | 51/49/138 | |
| All cases versus controls | 1.0 | 0.9 (0.6-1.3) | 0.9 (0.6-1.3) | 0.51 |
| APC$^+$ versus controls | 1.0 | 1.0 (0.6-1.5) | 0.7 (0.4-1.1) | 0.14 |
| APC$^-$ versus controls | 1.0 | 0.8 (0.5-1.4) | 1.1 (0.6-1.9) | 0.62 |
| APC$^+$ versus APC$^-$ | 1.0 | 1.2 (0.7-2.2) | 0.7 (0.4-1.2) | 0.14 |
| *Dairy products* (g/day) | ≤238.9 | 238.9-495.0 | ≥495.0 | |
| No. APC$^+$/APC$^-$/controls | 62/40/138 | 49/45/138 | 50/32/138 | |
| All cases versus controls | 1.0 | 0.8 (0.5-1.2) | 0.7 (0.4-1.0) | 0.04 |
| APC$^+$ versus controls | 1.0 | 0.6 (0.4-1.0) | 0.6 (0.4-1.0) | 0.05 |
| APC$^-$ versus controls | 1.0 | 1.0 (0.6-1.6) | 0.7 (0.4-1.2) | 0.15 |
| APC$^+$ versus APC$^-$ | 1.0 | 0.7 (0.4-1.2) | 0.9 (0.5-1.7) | 0.69 |

[a] Adjusted for age, sex, and total energy intake; total energy intake adjusted for age and sex only.

[b] Trend was assessed using the median values of the tertiles as continuous variables.

Although statistically non-significant, alcohol intake seemed to increase the risk of APC$^+$ polyps as well as APC$^-$ polyps. Comparable results were observed for unadjusted (*i.e.*, intake not adjusted with the residual method) alcohol intake (data not shown). We also assessed associations with absolute amounts of calcium, vitamin C, ß-carotene, and folate because for these nonenergy-bearing nutrients the absolute amount may be more relevant than an energy-adjusted amount. Results from the 'absolute amount' approach were comparable with those from the 'energy-adjusted' approach (data not shown).

We additionally conducted case-control and case-case comparisons to assess associations between the various dietary factors and the presence of a specific type of truncating *APC*

mutation (*i.e.*, frameshift or nonsense mutation). None of the evaluated dietary factors was distinctively associated with the occurrence of a specific type of truncating *APC* mutation (data not shown).

**Table 2.4** Associations between nutrients and adenomas with ($APC^+$) and without ($APC^-$) truncating *APC* mutations: case-control and case-case comparisons

| | Odds ratios (95% confidence intervals)[a] | | | $P_{trend}$[b] |
|---|---|---|---|---|
| | T1 | T2 | T3 | |
| *Alcohol* (g/day) | ≤2.0 | 2.0-9.3 | ≥9.3 | |
| No. $APC^+$/$APC^-$/controls | 46/23/138 | 36/35/138 | 79/59/138 | |
| All cases versus controls | 1.0 | 0.9 (0.6-1.5) | 1.4 (0.9-2.1) | 0.04 |
| $APC^+$ versus controls | 1.0 | 0.7 (0.4-1.2) | 1.2 (0.7-2.0) | 0.12 |
| $APC^-$ versus controls | 1.0 | 1.3 (0.7-2.5) | 1.6 (0.9-2.9) | 0.15 |
| $APC^+$ versus $APC^-$ | 1.0 | 0.6 (0.3-1.2) | 0.8 (0.4-1.5) | 0.96 |
| *Fat* (g/day) | ≤72.4 | 72.4-85.6 | ≥85.6 | |
| No. $APC^+$/$APC^-$/controls | 41/24/138 | 57/31/138 | 63/62/138 | |
| All cases versus controls | 1.0 | 1.3 (0.8-2.0) | 1.4 (0.8-2.2) | 0.26 |
| $APC^+$ versus controls | 1.0 | 1.3 (0.8-2.2) | 1.1 (0.6-1.9) | 0.94 |
| $APC^-$ versus controls | 1.0 | 1.2 (0.6-2.2) | 1.9 (1.0-3.7) | 0.05 |
| $APC^+$ versus $APC^-$ | 1.0 | 1.0 (0.5-2.1) | 0.5 (0.2-1.2) | 0.07 |
| *Protein* (g/day) | ≤71.8 | 71.8-82.6 | ≥82.6 | |
| No. $APC^+$/$APC^-$/controls | 45/27/138 | 62/39/138 | 54/51/138 | |
| All cases versus controls | 1.0 | 1.0 (0.6-1.5) | 0.8 (0.5-1.3) | 0.40 |
| $APC^+$ versus controls | 1.0 | 1.0 (0.6-1.6) | 0.7 (0.4-1.2) | 0.18 |
| $APC^-$ versus controls | 1.0 | 0.9 (0.5-1.6) | 0.9 (0.5-1.7) | 0.86 |
| $APC^+$ versus $APC^-$ | 1.0 | 1.0 (0.5-1.9) | 0.7 (0.3-1.3) | 0.20 |
| *Carbohydrates* (g/day) | ≤216.8 | 216.8-244.4 | ≥244.4 | |
| No. $APC^+$/$APC^-$/controls | 57/44/138 | 38/34/138 | 66/39/138 | |
| All cases versus controls | 1.0 | 0.6 (0.4-0.9) | 0.7 (0.4-1.0) | 0.05 |
| $APC^+$ versus controls | 1.0 | 0.6 (0.4-1.0) | 0.8 (0.5-1.4) | 0.48 |
| $APC^-$ versus controls | 1.0 | 0.7 (0.4-1.1) | 0.5 (0.3-0.9) | 0.02 |
| $APC^+$ versus $APC^-$ | 1.0 | 1.0 (0.5-1.9) | 1.7 (0.9-3.4) | 0.10 |

| *Table 2.4 cont.* | T1 | T2 | T3 | $P_{trend}$[b] |
|---|---|---|---|---|
| *Dietary fiber* (g/day) | ≤21.0 | 21.0-25.6 | ≥25.6 | |
| No. APC+/APC-/controls | 34/34/138 | 64/40/138 | 63/43/138 | |
| All cases versus controls | 1.0 | 1.4 (0.9-2.1) | 1.1 (0.7-1.6) | 0.83 |
| APC+ versus controls | 1.0 | 1.8 (1.1-2.9) | 1.3 (0.8-2.2) | 0.39 |
| APC- versus controls | 1.0 | 1.0 (0.6-1.7) | 0.8 (0.5-1.4) | 0.41 |
| APC+ versus APC- | 1.0 | 1.6 (0.9-3.1) | 1.6 (0.8-2.9) | 0.23 |
| *Calcium* (mg/day) | ≤924.8 | 924.8-1222.3 | ≥1222.3 | |
| No. APC+/APC-/controls | 44/40/138 | 61/40/138 | 56/37/138 | |
| All cases versus controls | 1.0 | 0.9 (0.6-1.4) | 0.8 (0.5-1.2) | 0.31 |
| APC+ versus controls | 1.0 | 1.1 (0.7-1.8) | 0.9 (0.6-1.5) | 0.68 |
| APC- versus controls | 1.0 | 0.7 (0.4-1.3) | 0.6 (0.4-1.1) | 0.11 |
| APC+ versus APC- | 1.0 | 1.4 (0.8-2.5) | 1.3 (0.7-2.4) | 0.37 |
| *Vitamin C* (mg/day) | ≤80.4 | 80.4-119.0 | ≥119.0 | |
| No. APC+/APC-/controls | 50/34/138 | 53/41/138 | 58/42/138 | |
| All cases versus controls | 1.0 | 1.1 (0.8-1.7) | 1.3 (0.8-1.9) | 0.28 |
| APC+ versus controls | 1.0 | 1.0 (0.6-1.6) | 1.2 (0.7-1.9) | 0.50 |
| APC- versus controls | 1.0 | 1.2 (0.7-2.0) | 1.3 (0.8-2.3) | 0.32 |
| APC+ versus APC- | 1.0 | 0.9 (0.5-1.6) | 0.9 (0.5-1.6) | 0.66 |
| *ß-Carotene* (µg/day) | ≤1203.4 | 1203.4-1553.9 | ≥1553.9 | |
| No. APC+/APC-/controls | 56/36/138 | 59/39/138 | 46/42/138 | |
| All cases versus controls | 1.0 | 1.1 (0.8-1.7) | 1.0 (0.6-1.4) | 0.78 |
| APC+ versus controls | 1.0 | 1.1 (0.7-1.7) | 0.8 (0.5-1.3) | 0.34 |
| APC- versus controls | 1.0 | 1.2 (0.7-2.0) | 1.2 (0.7-2.1) | 0.44 |
| APC+ versus APC- | 1.0 | 1.0 (0.5-1.8) | 0.7 (0.4-1.3) | 0.21 |
| *Folate* (µg/day) | ≤174.3 | 174.3-205.1 | ≥205.1 | |
| No. APC+/APC-/controls | 41/23/138 | 56/42/138 | 64/52/138 | |
| All cases versus controls | 1.0 | 1.3 (0.9-2.0) | 1.3 (0.8-2.0) | 0.29 |
| APC+ versus controls | 1.0 | 1.3 (0.8-2.1) | 1.2 (0.7-1.9) | 0.57 |
| APC- versus controls | 1.0 | 1.5 (0.9-2.8) | 1.5 (0.9-2.8) | 0.20 |
| APC+ versus APC- | 1.0 | 0.8 (0.4-1.5) | 0.7 (0.4-1.4) | 0.34 |

[a] Adjusted for age, sex, and total energy intake; alcohol additionally adjusted for cigarette smoking.

[b] Trend was assessed using the median values of the tertiles as continuous variables.

## Discussion

In this study, we evaluated associations between dietary factors previously reported to be associated with colorectal cancer risk and truncating *APC* mutations in sporadic colorectal adenomas. Red meat consumption was significantly differently related to $APC^{+}$ polyps compared with $APC^{-}$ polyps. High intake of red meat and high fat intake seemed to increase the risk of $APC^{-}$ polyps in particular. High intake of carbohydrates seemed to especially decrease the risk of $APC^{-}$ polyps. Consumption of dairy products decreased the risk of $APC^{+}$ polyps as well as $APC^{-}$ polyps, while alcohol intake seemed to increase the risk of both polyp groups. However, like most other evaluated dietary factors, dairy products and alcohol were not distinctively associated with a specific *APC* status. In addition, none of the dietary factors was specifically associated with a particular type of truncating *APC* mutation.

The histology of all obtained polyps was re-evaluated by the study pathologist and only adenomatous polyps were included in the current study. Direct-sequencing was used to screen codons 1286 through 1585 of the *APC* gene for mutations. As discussed in more detail elsewhere (13), we do not expect that our decision to focus on truncating mutations in this region has resulted in extensive misclassification because most colorectal tumors in which *APC* is mutated exhibit at least one truncating mutation in the MCR (7). Truncating *APC* mutations were identified in 57.9% of the colorectal adenomas. This is in line with previous studies, all smaller than the current study, which reported mutation frequencies for this region between 30 and 60% (2, 5, 6, 7, 24). In addition, the characteristics of the mutations identified were consistent with those reported by others (6, 7; APC database: http://perso.curie.fr/Thierry.Soussi /APC.html). Microdissection was performed and only areas containing >60% tumor cells were used for DNA extraction. However, it remains possible that, due to contaminating normal tissue,

alterations were missed eventually resulting in misclassification (*i.e.*, polyps with a truncating *APC* mutation in the APC$^-$ group), which in turn may have attenuated some of our results.

Both cases and controls were recruited among those undergoing colonoscopy. To minimize the possibility of misclassification, controls included in the current study had all undergone a complete colonoscopy or a sigmoidoscopy followed by colon X-ray. In the Netherlands, unlike for instance in the United States, routine screening (*i.e.*, screening without having complaints) for colorectal tumors is not (yet) recommended for the general population. Consequently, in our study, colonoscopies were mostly conducted because of complaints. Therefore, our study population may not be representative of the general Dutch population.

Cases and controls were asked to recall their diets from the past and differential recall is possible. However, because cases are unaware of the mutational status of their polyps, systematic errors in dietary recall are less likely to bias results from case-case comparisons. In addition, results of our 'all cases versus controls' comparisons were generally consistent with those reported by others (11, 25, 26). The control group contained significantly more women, possibly because women are more likely than men to undergo colonoscopy for bowel complaints. Cases were also older than controls and age is a known risk factor for colorectal tumors. Therefore, all analyses were adjusted for sex and age.

The main tumor suppressing function of APC seems to reside in its ability to properly regulate levels of intracellular ß-catenin, a key mediator of Wnt signaling (27-30). Loss of APC function results in the accumulation of ß-catenin, which in turn results in constitutive transcriptional activation of Wnt target genes. Oncogenic activation of the *ß-catenin* gene itself also results in constitutive activation of the Wnt signaling pathway (28). However, while loss of APC and oncogenic activation of ß-catenin appear equally potent with regard to transcriptional activation and are both early events in colorectal carcinogenesis (28, 31), truncating *APC*

mutations seem to have additional functional consequences and may, unlike mutations in *ß-catenin*, contribute to tumor progression as well (31, 32).

Thus far, most epidemiological studies used sporadic colorectal carcinomas to investigate the relationship between diet and the occurrence of specific molecular alterations (13, 15-18). This is, to our knowledge, the first study that has evaluated associations between dietary factors and the occurrence of *APC* mutations in sporadic colorectal adenomas. Most dietary factors evaluated in this study were not specifically associated with $APC^+$ polyps or $APC^-$ polyps. They seemed, instead, to influence the development of these two distinct early stages in colorectal carcinogenesis equally, which suggests that they are probably not specifically involved in the initiation of truncating mutations in the *APC* gene. In general, a possible explanation for the observed associations between dietary factors and specific molecular alterations in colon carcinomas (13, 15-18) might be that dietary factors affect progression into later stages differently depending on the advantage for tumor formation exerted by the mutations already present; although, some genes may indeed also be more mutation sensitive than others.

In this study, red meat and fat seemed to especially increase the risk of $APC^-$ polyps. No clear associations were observed with $APC^+$ polyps. Red meat has been reported to be associated with increased risk of colorectal cancer and adenomas (11, 33-35). It is an important source of known carcinogens such as heterocyclic amines and nitrosamines, and may affect risk of colorectal cancer by being involved in the production of somatic alterations. Red meat is also a major contributor to total fat intake, which may explain the observed association with fat in this study. Also, the type of fat that has been most strongly associated with colorectal cancer is fat from red meat sources (33).

Contrary to our current results, a population-based case-control study of sporadic colon carcinomas suggested that red meat and fat were more notably, positively associated with carcinomas with truncating *APC* mutation than with carcinomas without truncating *APC*

mutation (13). The dissimilarity in results might be caused by chance. Alternatively, and admittedly speculatively, the gene(s), *e.g.*, *ß-catenin*, involved in the development of APC$^-$ polyps may be more susceptible to mutations caused by red meat consumption than *APC*, but alterations in *APC* may provide cells with a higher advantage for carcinoma formation. The oncogene *ß-catenin*, for instance, requires only one 'hit' to be activated whereas for loss of APC function two 'hits' are necessary. Polyps with truncating *APC* mutations seem, however, much more likely to progress into carcinomas than polyps with activating mutations in *ß-catenin* (31, 32).

To conclude, our data suggest that red meat and fat intake may increase the risk of APC$^-$ polyps in particular, while carbohydrates may especially decrease the risk of APC$^-$ polyps. Most evaluated dietary factors were, however, not specifically associated with APC$^+$ polyps or APC$^-$ polyps but seemed to affect both pathways equally, if at all. Our study population was relatively small, and it is possible that some associations were not detected due to insufficient power. In addition, although all examined dietary factors were reported previously to be associated with colorectal cancer risk, it should be noted that multiple comparisons might lead to chance findings. Therefore, confirmation of our results by other studies is necessary. Adenomas are relatively prevalent in the general population. Many seem to be initiated by truncating *APC* mutations and may progress into carcinomas. Because successful targeting of early molecular events can have a high impact on outcomes, enhanced insight into the relationship between diet, the occurrence of truncating *APC* mutations, and tumor progression may prove useful for the development of effective colorectal cancer prevention strategies. Especially since *APC* mutations seem to greatly increase cells' chances of tumor formation (32).

## Acknowledgements

We thank Maria van Vugt and Elly Monster for assistance with data collection and participant recruitment; Dr. Marga Ocké (National Institute of Public Health and the Environment, Bilthoven) for calculation of dietary intake; and, Dr. Han van Krieken (UMC St Radboud, Nijmegen) for re-evaluation of polyp histology. We gratefully acknowledge the endoscopy staff of the following hospitals for assistance with participant recruitment: Gelderse Vallei hospital, Ede-Wageningen; Bosch MediCentrum, Den Bosch; Eemland hospital, Amersfoort; Slingeland hospital, Doetinchem; Rivierenland hospital, Tiel; UMC St. Radboud, Nijmegen; Rijnstate hospital, Arnhem. The Pathology Departments of the above mentioned hospitals are thanked for providing polyp tissue. This study was supported by the Dutch Cancer Society (grants LUW 96-1374 and LUW 98-1687).

## References

1. Fearon, E.R., and Vogelstein, B. (1990) A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759-767.
2. Powell, S.M., Zilz, N., Beazer-Barclay, Y., Bryan, T.M., Hamilton, S.R., Thibodeau, S.N., Vogelstein, B., and Kinzler, K.W. (1992) *APC* mutations occur early during colorectal tumorigenesis. *Nature*, **359**, 235-237.
3. Hiltunen, M.O., Alhonen, L., Koistinaho, J., Myohanen, S., Paakkonen, M., Marin, S., Kosma, V.M., and Janne, J. (1997) Hypermethylation of the *APC* (adenomatous polyposis coli) gene promoter region in human colorectal carcinoma. *Int. J. Cancer*, **70**, 664-648.
4. Esteller, M., Sparks, A., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M.A., Gonzalez, S., Tarfa, G., Sidransky, D., Meltzer, S.J., Baylin, S.B., and Herman, J.G. (2000) Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. *Cancer Res.,* **60**, 4366-4371.
5. Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T., and Nakamura, Y. (1992) Somatic mutations of the *APC* gene in colorectal tumors: mutation cluster region in the APC gene. *Hum. Mol. Genet*., **1**, 229-233.
6. Miyaki, M., Konishi, M., Kikuchi-Yanoshita, R., Enomoto, M., Igari, T., Tanaka, K., Muraoka, M., Takahashi, H., Amada, Y., Fukayama, M., Maeda, Y., Iwama, T., *et al*. (1994) Characteristics of somatic mutation of the adenomatous polyposis coli gene in colorectal tumors. *Cancer Res*., **54**, 3011-3020.
7. Rowan, A.J., Lamlum, H., Ilyas, M., Wheeler, J., Papadopoulou, A., Bicknell, D., Bodmer, W.F., and Tomlinson, I.P.M. (2000) *APC* mutations in sporadic colorectal tumors: A mutational “hotspot” and interdependence of the “two hits”. *Proc. Natl. Acad. Sci. USA*, **97**, 3352-3357.

8. Lamlum, H., Ilyas, M., Rowan, A., Clark, S., Johnson, V., Bell, J., Frayling, I., Efstathiou, J., Pack, K., Payne, S., Roylance, R., Gorman, P., *et al*. (1999) The type of somatic mutation at *APC* in familial adenomatous polyposis is determined by the site of the germline mutation: a new facet to Knudson's 'two-hit' hypothesis. *Nature. Medicine*, **5**, 1071-1075.
9. Smits, R., Hofland, N., Edelmann, W., Geugien, M., Jagmohan-Changur, S., Albuquerque, C., Breukel, C., Kucherlapati, R., Kielman, M.F., and Fodde, R. (2000) Somatic *Apc* mutations are selected upon their capacity to inactivate the ß-catenin downregulating activity. *Genes Chromosomes Cancer*, **29**, 229-239.
10. Crabtree, M., Sieber, O.M., Lipton, L., Hodgson, S.V., Lamlum, H., Thomas, H.J.W., Neale, K., Phillips, R.K.S., Heinimann, K., and Tomlinson, I.P.M. (2003) Refining the relation between 'first hits' and 'second hits' at the *APC* locus: the 'loose fit' model and evidence for differences in somatic mutation spectra among patients. *Oncogene*, **22**, 4257-4265.
11. World Cancer Research Fund (WCRF) Panel (Potter, J.D., Chair). (1997) *Food, nutrition and the prevention of cancer: a global perspective.* WCRF/American Institute for Cancer Research, Washington, DC.
12. Kakiuchi, H., Watanabe, M., Ushijima, T., Toyota, M., Imai, K., Weisburger, J.H., Sugimura, T., and Nagao, M. (1995) Specific 5'-GGGA-3' $\rightarrow$ 5'-GGA-3' mutation of the *Apc* gene in rat colon tumors induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Proc. Natl. Acad. Sci. USA*, **92**, 910-914.
13. Diergaarde, B., Van Geloof, W.L., Van Muijen, G.N.P., Kok, J.F., and Kampman, E. (2003) Dietary factors and the occurrence of truncating *APC* mutations in sporadic colon carcinomas: a Dutch population-based study. *Carcinogenesis*, **24**, 283-290.
14. Martinez, M.E., Maltzman, T., Marshall, J.R., Einspahr, J., Reid, M.E., Sampliner, R., Ahnen, D.J., Hamilton, S.R., and Alberts D.S. (1999) Risk factors for Ki-*ras* protooncogene mutation in sporadic colorectal adenomas. *Cancer Res*., **59**, 5181-5185.
15. Kampman, E., Voskuil, D.W., Van Kraats, A.A., Balder, H.F., Van Muijen, G.N.P., Goldbohm, R.A., and Van 't Veer, P. (2000) Animal products and K-*ras* codon 12 and 13 mutations in colon carcinomas. *Carcinogenesis*, **21**, 307-309.
16. Slattery, M.L., Curtin, K., Anderson, K., Ma, K-N, Edwards, S., Leppert, M., Potter, J., Schaffer, D., and Samowitz, W.S. (2000) Associations between dietary intake and Ki-*ras* mutations in colon tumors: a population-based study. *Cancer Res*., **60**, 6935-6941.
17. Slattery, M.L., Anderson, K., Curtin, K., Ma, K-N, Schaffer, D., and Samowitz, W. (2001) Dietary intake and microsatellite instability in colon tumors. *Int. J. Cancer*, **93**, 601-607.

18. Diergaarde, B., Braam, H., Van Muijen, G.N.P., Ligtenberg, M.J.L., Kok, F.J., and Kampman, E. (2003) Dietary factors and microsatellite instability in sporadic colon carcinomas. *Cancer Epidemiol., Biomarkers & Prev.,* **12***,* 1130-1136.
19. Tiemersma, E.W., Wark, P.A., Ocké, M.C., Bunschoten, A., Otten, M.H., Kok, F.J., and Kampman, E. (2003) Alcohol consumption, alcohol dehydrogenase 3 polymorphism, and colorectal adenomas. *Cancer Epidemiol., Biomarkers & Prev*., **12**, 419-425.
20. Ocké, M.C, Bueno de Mesquita, H.B., Goddijn, H.E., Jansen, A., Pols, M.A., Van Staveren, W.A., and Kromhout, D. (1997) The Dutch EPIC food frequency questionnaire. I. Description of the questionnaire, and relative validity and reproducibility for food groups. *Int. J. Epidemiol*., **26** (Suppl. 1), S37-S48.
21. Ocké, M.C, Bueno de Mesquita, H.B., Pols, M.A., Smit, H.A., Van Staveren, W.A., and Kromhout, D. (1997) The Dutch EPIC food frequency questionnaire. II. Relative validity and reproducibility for nutrients. *Int. J. Epidemiol*., **26** (Suppl. 1), S49-S58.
22. Willett, W.C., Howe, G.R., and Kushi, L.H. (1997) Adjustment for total energy intake in epidemiologic studies. *Am J. Clin. Nutr*., **65** (suppl), 1220S-1228S.
23. Schlesselman, J.J. (1982) *Case-control studies. Design, conduct, analysis*. Oxford University Press.
24. Smith, G., Carey, F.A., Beattie, J., Wilkie, M.J.V., Lightfoot, T.J., Coxhead, J., Garner, R.C., Steele, R.J.C., and Wolf, C.R. (2002) Mutations in *APC*, Kirsten-*ras*, and *p53* – alternative genetic pathways to colorectal cancer. *Proc. Natl. Acad. Sci. USA,* **99**, 9433-9438.
25. Platz, E.A., Giovannucci, E., Rimm, E.B., Rockett, H.R., Stampfer, M.J., Colditz. G.A., and Willett, W.C. (1997) Dietary fiber and distal colorectal adenoma in men. *Cancer Epidemiol., Biomarkers & Prev*., **6**, 661-670.
26. Smith-Warner, S.A., Elmer, P.J., Fosdick, L., Randall, B., Bostick, R.M., Grandits, G., Grambsch, P., Louis, T.A., Wood, J.R., and Potter J.D. (2002) Fruits, vegetables, and adenomatous polyps. The Minnesota cancer prevention research unit case-control study. *Am. J. Epidemiol*., **155**, 1104-1113.
27. Korinek, V., Barker, N., Morin, P.J., Van Wichen, D., De Weger, R., Kinzler, K.W., Vogelstein, B., and Clevers, H. (1997) Constitutive transcriptional activation by a ß-catenin-Tcf complex in APC$^{-/-}$ colon carcinoma. *Science*, **275**, 1784-1787.
28. Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K.W. (1997) Activation of ß-catenin-Tcf signaling in colon cancer by mutations in *ß-catenin* or *APC*. *Science*, **275**, 1787-1790.
29. Smits, R., Kielman, M.F., Breukel, C., Zurcher, C., Neufeld, K., Jagmohan-Changur, S., Hofland, N., Van Dijk, J., White, R., Edelmann, W., Kucherlapati, R., Khan, P.M., and Fodde, R. (1999) *Apc*$^{1638T}$: a mouse model delineating critical domains of the adenomatous polyposis coli protein involved in tumorigenesis and development. *Genes Dev*., **13**, 1309-1321.

30. Willert, K., and Nusse, R. (1998) Beta-catenin: a key mediator of Wnt signaling. *Curr. Opin. Genet. Dev.*, **8**, 95-102.
31. Samowitz, W.S., Powers, M.D., Spirio, L.N., Nollet, F., Van Roy, F., and Slattery, M.L. (1999) *ß-Catenin* mutations are more frequent in small colorectal adenomas than in large adenomas and invasive carcinomas. *Cancer Res*., **59**, 1442-1444.
32. Fodde, R., Smits, R., and Clevers, H. (2001) *APC*, signal transduction and genetic instability in colorectal cancer. *Nat. Rev. Cancer*, **1**, 55-67.
33. Potter, J.D. (1999) Colorectal cancer: molecules and populations. *J. Natl. Cancer Inst*., **91**, 916-932.
34. Norat, T., and Riboli, E. (2001) Meat consumption and colorectal cancer: a review of epidemiologic evidence. *Nutr. Rev*., **59**, 37-47.
35. Sandhu, M.S., White, I.R., and McPherson, K. (2001) Systematic review of the prospective cohort studies on meat consumption and colorectal cancer risk: a meta-analytical approach. *Cancer Epidemiol., Biomarkers & Prev.*, **10**, 439-446.

# Chapter 3

# Dietary factors and the occurrence of truncating *APC* mutations in sporadic colon carcinomas: a Dutch population-based study

Brenda Diergaarde

Winny L. van Geloof

Goos N. P. van Muijen

Frans J. Kok

Ellen Kampman

***Carcinogenesis,* February 2003; 24 (2): 283-290**

## Abstract

The interactions between environmental factors and the genetic and epigenetic changes that drive colon carcinogenesis are not clear. Dietary factors reported previously to be associated with colon cancer risk may well influence the occurrence of specific somatic alterations in colon tumors. To explore this idea, data from a Dutch population-based case-control study (184 cases; 259 controls) of sporadic colon cancer were used to assess associations between dietary factors and the occurrence of truncating mutations in the adenomatous polyposis coli (*APC*) gene in carcinomas. Single-strand conformation polymorphism analysis and DNA sequencing were used to screen tumors for mutations in the mutation cluster region of *APC*. Usual dietary habits were assessed by an interview-based questionnaire. Truncating *APC* mutations were detected in 63 (34%) of the tumors. Vegetable consumption was inversely associated with $APC^+$ (*with* mutation) tumors [odds ratio (OR) and 95% confidence interval (CI) for highest versus lowest tertile, OR: 0.6, 95% CI: 0.3-1.3] as well as $APC^-$ (*without* mutation) tumors (OR: 0.3, 95% CI: 0.2-0.5). Alcohol intake was positively associated with $APC^-$ tumors (OR: 1.7, 95% CI: 1.0-3.0) and inversely associated with $APC^+$ tumors (OR: 0.5, 95% CI: 0.3-1.1). Positive associations were observed for meat, fish and fat with $APC^+$ tumors (OR: 1.7, 95% CI: 0.8-3.6; OR: 1.4, 95% CI: 0.7-2.8; OR: 4.5, 95% CI: 1.6-12.8, respectively). Of the dietary factors examined, vegetable consumption and alcohol intake were significantly different related to $APC^+$ tumors than to $APC^-$ tumors ($APC^+$ versus $APC^-$, OR: 2.3, 95% CI: 1.0-5.3; OR: 0.3, 95% CI: 0.2-0.7, respectively). Our data suggest that vegetables play a protective role in the etiology of both tumor subsets, although this role appears to be less influential in the $APC^+$ group. Alcohol seems to especially promote the development of $APC^-$ tumors whereas meat, fish and fat appear to enhance the development of $APC^+$ tumors.

## Introduction

Colon cancer is one of the most common types of cancer in the Western world. The etiology of colon cancer is complex and involves both genetic and environmental factors. Known risk factors include a positive family history, age, meat and alcohol consumption, fat intake, and, possibly, smoking. Inverse associations are reported with vegetable consumption, physical activity, use of non-steroidal anti-inflammatory drugs and, although less consistent, with fruit consumption and calcium intake (1).

Although the etiology of colon cancer is complex and multifactorial, from the perspective of understanding the genetic events involved in carcinogenesis, it is one of the best-characterized epithelial tumors. Mutations in the adenomatous polyposis coli (*APC*) tumor suppressor gene, *i.e.*, those resulting in loss of APC function, are thought to be a key initiating event in familial as well as in sporadic colorectal cancer. They can be detected in many sporadic adenomas and carcinomas, including adenomas as small as 5 mm in diameter (2). Recently, both Fodde *et al*. (3) and Kaplan *et al*. (4) showed that APC is also involved in chromosomal segregation and that truncation of *APC* causes chromosomal instability in embryonic stem cells. This suggests that loss of APC function is not only important for tumor initiation but may play a role in later stages of malignant progression as well.

In contrast to the germline mutations, which are scattered over a large part of the gene, the majority of the somatic *APC* mutations seem to cluster within a small region in exon 15 (codon 1286-1513), the so-called mutation cluster region (MCR) (5-7). Truncating mutations (that is, nonsense and frameshift mutations) in the MCR have been reported to occur in 30-45% of the sporadic colon tumors (2, 5, 6, 8). The observed clustering of somatic mutations in *APC* could be caused by hypermutability of this specific region, a selective advantage for tumor formation exerted by mutations in this region, or a combination of these two. Results from several studies indicate that truncating mutations in the MCR indeed provide cells with a selective growth advantage, probably due to inactivation of the ß-catenin down-regulating function of APC (9, 10).

The relationships between environmental factors and the genetic and epigenetic [*e.g.*, DNA methylation, see Refs. 11 and 12] alterations that drive colon carcinogenesis are not (yet) clear. However, dietary factors reported previously to be associated with colon cancer risk may well, directly and/or indirectly, influence the occurrence of somatic truncating *APC* mutations in colon tumors. Bardelli *et al*. (13) demonstrated recently that exposure to specific carcinogens

can indeed select for tumor cells with distinct forms of genetic instability. Dietary factors have been found associated with the occurrence of specific K-*ras* mutations in human colon carcinomas (14, 15). Moreover, the carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), an in cooked meat abundantly present heterocyclic amine, has been linked to specific *Apc* mutations in rat colon tumors (16, 17).

Therefore, in this study, we assess the associations between specific dietary factors (all reported previously to be associated with colon cancer risk) and the occurrence of truncating mutations in the MCR region of *APC* in a population-based case-control study of incident cases of sporadic colon carcinomas. This is, to our knowledge, the first study that examines these associations in a human population.

## Materials and methods

### *Study population*

A population-based case-control study on diet and colon cancer was conducted in the Netherlands between 1989 and 1993. Details were described previously (18). In short, cases (n=204) were women and men newly diagnosed with a first primary incident colon carcinoma. Sixty percent of the eligible cases invited, agreed to participate. Controls (n=259), frequency matched to the cases by age (5 year intervals), sex, region and degree of urbanization, were randomly recruited by the general practitioners of the cases. Of the controls invited, 57% agreed to participate. All subjects were Caucasian, up to 75 years old at time of diagnosis, mentally competent to complete the interview, and had no known personal history of cancer, familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer, ulcerative colitis, or Crohn's disease. Except for a more favorable Dukes' stage in cases, participants did not differ importantly from non-participants. Formalin-fixed, paraffin-embedded colon tumor tissue, collected before chemo- or radiotherapy started, was available from 185 cases. From 19 patients, tissue could not be obtained due to administrative reasons. In 2000 it became known that one of the cases actually had hereditary nonpolyposis colorectal cancer, *i.e.*, exhibited a germline mutation in one of the mismatch repair genes. This case was therefore excluded from the analyses, leaving a final number of 184 cases.

### *Data collection*

Usual dietary habits were assessed by an interview-based questionnaire. The questionnaire covered the complete dietary pattern. The interval between diagnosis and interview was, for cases, three to six months. The consumption frequency per month in the preceding year (for cases, the year preceding diagnosis or symptoms), number of months during which the item was used, number of portions per consumption and portion sizes of 289 food items were collected. To check for internal consistency, the consumption frequencies of various meal components were compared with the total meal pattern. Frequently used household utensils and cups were weighed to be able to estimate portion sizes. Average daily intake of nutrients was calculated using the Dutch National Food Table (19). The interviewing of cases and controls was balanced over seasons to account for seasonal fluctuations in food patterns. During the interview, information was also obtained on current and previous smoking habits, aspirin and non-steroidal anti-inflammatory drug use, family history of colorectal cancer, and medical history, see also Kampman *et al*. (20).

### *APC mutation detection*

DNA was isolated from tumor-rich areas (>60% tumor cells) as described elsewhere (21). Single-strand conformation polymorphism (SSCP) analysis was used to screen the *APC* gene for nonsense and frameshift mutations. Most studies on somatic mutations in *APC* focus on the MCR of the gene (codons 1286-1513). Our analysis covers codons 1286 to 1585 (extended-MCR) of *APC* and includes codon 1554 in which somatic mutations are also often observed (7) (APC database: http://perso.curie.fr/Thierry.Soussi/APC/html). The region was divided into five, ~220 base pairs long, overlapping fragments (codons 1286-1358, 1337-1404, 1387-1455, 1437-1526, and 1509-1585, respectively) which were amplified separately in two consecutive PCRs using the following primer sets (primer sequence: 5'→3'):

| | | codon: |
|---|---|---|
| Fragment 1: | | |
| 1.1 F-CAGACTTATTGTGTAGAAG | R-CGCTCCTGAAGAAAATTCAAG | 1260-1358 |
| 1.2 F-GAAATAGGATGTAATCAGACG | R-CGCTCCTGAAGAAAATTCAAC | 1286-1358 |
| Fragment 2: | | |
| 2.1 F-ACTGCAGGGTTCTAGTTTATC | R-TCTGCTTGGTGGCATGGTTT | 1337-1436 |
| 2.2 F-ACTGCAGGGTTCTAGTTTATC | R-GAGCTGGCAATCGAACGACT | 1337-1404 |
| Fragment 3: | | |
| 3.1 F-CTCAGACACCCAAAAGTCC | R-ATTTTTAGGTACTTCTCGCTTG | 1366-1455 |
| 3.2 F-TACTTCTGTCAGTTCACTTGATA | R-ATTTTTAGGTACTTCTCGCTTG | 1387-1455 |
| Fragment 4: | | |
| 4.1 F-AAACACCTCCACCACCTCC | R-TCATTCCCATTGTCATTTTCC | 1437-1536 |
| 4.2 F-AAACACCTCCACCACCTCC | R-GCATTATTCTTAATTCCACATC | 1437-1526 |

Fragment 5:

| | | |
|---|---|---|
| 5.1 F-ACTCCAGATGGATTTTTCTTG | R-GGCTGGCTTTTTGCTTTAC | 1497-1596 |
| 5.2 F-GAGCCTCGATGAGCCATTTA | R-TGTTGGCATGGCAGAAATAA | 1509-1585 |

PCR reaction mixtures (total volume 50µl) contained 50ng DNA or 2µl of the 1:100 diluted product of the first PCR, 0.2µM of both primers, 0.2mM dNTPs, 10mM Tris-HCL pH 9.0, 1.5 to 2.5mM $MgCl_2$, 50mM KCl, 0.01% Tween, 10% glycerol, and 0.3U *Taq* DNA polymerase. Reaction conditions first PCR: 25 cycles of 30 s at 94°C, 45 s at 55°C (53°C for primer set 1.1; 57°C for primer set 4.1), 1 min at 72°C, followed by 5 min at 72°C. Conditions second PCR: 30 cycles of 30 s at 94°C, 45 s at 52°C (56°C for primer sets 3.2 and 4.2; 57°C for primer set 5.2), 1 min at 72°C, followed by 5 min at 72°C. Products were checked using an ethidium bromide stained 2% agarose gel. SSCP was performed as described earlier (21) with electrophoresis at 10 and 18°C. The original PCR products from the samples that displayed an abnormal pattern in the SSCP were subjected to sequencing in both directions using the same primers as in the second PCR. Sequencing was performed as described previously (21). Mutation analysis started in all samples with fragment 1, and only if no nonsense or frameshift mutations (resulting in truncated, non-functional APC protein) were detected fragment 2 was screened for mutations, and so on.

### *Statistical analyses*

Cases were classified as $APC^+$ (carcinomas containing a nonsense or frameshift mutation in the extended-MCR of *APC*), or $APC^-$ (carcinomas without a nonsense or frameshift mutation in the extended-MCR of *APC*). Energy-adjusted nutrient intakes were computed, for women and men separately, as the residuals from the regression model with total energy as the independent variable and absolute nutrient intake as the dependent variable. As a constant, the mean of the nutrient intake was added to each residual (22, 23). Differences in characteristics between the groups were assessed using *t*-tests for continuous and Chi-square tests for categorical variables. The categorization of food groups and nutrients, in tertiles, was based on the distribution of intake in the control population. To look at the combined effect of vegetable consumption and red meat intake, mutually exclusive categories of combinations of the two exposures were defined based on median split. Case-control comparisons, separately comparing $APC^+$ cases and $APC^-$ cases with the population-based controls, were conducted to estimate the relative risk of developing carcinomas, respectively, with and without a truncating *APC* mutation. Case-case comparisons were conducted to evaluate heterogeneity in dietary risk factors for the two subsets of carcinomas. Odds ratios (ORs) and the corresponding 95% confidence intervals (95% CIs) were calculated using multiple logistic regression models. Linear trend was assessed using the tertile medians as continuous variables in multiple logistic regression models. To quantify the associations on a continuous scale and allow direct comparisons between the different vegetable subgroups, ORs and 95%

CIs were also calculated for a fixed amount (10g/day) of intake (24). All analyses were adjusted for age, sex and total energy intake. Alcohol intake was additionally adjusted for smoking. Additional adjustment for Dukes' stage, tumor location, smoking, body mass index and other dietary factors, did not change the estimates significantly (that is, not more than 10%). Analyses were performed with the use of the SAS® statistical software package (SAS version 6.12. SAS Institute Inc., Cary, NC, USA).

## Results

In 63 (34.2%) of the 184 colon tumors included in this study, a truncating mutation in the extended-MCR of the *APC* gene was identified. Frameshift mutations were the most common, 13 insertions and 32 deletions were detected together representing 71.4% of all truncating *APC* mutations. Among the nonsense mutations (18 in total), C→T transitions were the most frequent (50.0%). Mutational hotspots, *i.e.*, codons in which 4 or more of the tumors in this study showed a truncating mutation, were observed at codons 1309 (*n*=5), 1465 (*n*=4) and 1554 (*n*=7). In addition, 14 colon tumors exhibited a missense mutation (but no truncating mutation).

Characteristics of the study population are given in Table 3.1. Cases were divided into two groups: $APC^+$, individuals with a tumor exhibiting a truncating *APC* mutation; and $APC^-$, individuals with a tumor without a truncating *APC* mutation. Age, sex, and body mass index did not differ significantly between cases and controls, and neither between $APC^+$ and $APC^-$ cases. Total energy intake was higher among the cases, and highest among the $APC^-$ cases. The frequency of Dukes' stage C and D tumors did not differ significantly between the two case-groups; proximal tumors (cecum, ascending colon, hepatic flexure and transverse colon) were slightly more common among the $APC^-$ cases. There were more 'ever smokers' in the $APC^-$ group than in the $APC^+$ group.

Regarding dietary factors, there were marked differences between $APC^+$ and $APC^-$ cases in total vegetable, alcohol, dietary fiber and ß-carotene intake. $APC^-$ cases consumed less

vegetables, carbohydrates, dietary fiber, vitamin C and ß-carotene but more dairy products and alcohol than the controls. Alcohol intake was lower among the APC$^+$ cases than among the controls (see Table 3.1).

**Table 3.1** Characteristics of the study population

| | Controls | Cases[a] (*n*=184) | |
|---|---|---|---|
| | (*n*=259) | APC$^+$ (*n*=63) | APC$^-$ (*n*=121) |
| Age (in years, mean ± SD) | 61.8 ± 10.0 | 63.4 ± 8.8 | 60.9 ± 10.9 |
| Sex (% women) | 47.5 | 49.2 | 43.8 |
| Body mass index (kg/m$^2$, mean ± SD) | 26.0 ± 3.8 | 25.5 ± 3.8 | 26.1 ± 4.7 |
| Energy intake (kJ/day, mean ± SD) | 9361.7 ± 2844.1 | 9907.1 ± 2658.6 | 10541.7 ± 3464.6[b] |
| Ever smoked (%) | 69.1 | 60.3 | 74.8[c] |
| Dukes' stage (% CD) | n.a.[d] | 36.5 | 36.4 |
| Tumor location (% proximal) | n.a. | 39.0 | 48.2 |
| *Dietary factors* (g/day; mean ± SD) | | | |
| Vegetables & fruit | | | |
| Total vegetables | 207.9 ± 124.2 | 183.5 ± 76.5 | 163.2 ± 82.0[e] |
| Leafy greens | 31.3 ± 21.0 | 27.4 ± 18.9 | 25.5 ± 20.4[b] |
| Cruciferous | 34.5 ± 20.8 | 34.3 ± 26.4 | 32.5 ± 23.9 |
| Allium | 19.6 ± 18.2 | 18.3 ± 18.8 | 15.0 ± 15.3[b] |
| Root vegetables | 22.2 ± 29.3 | 19.5 ± 21.7 | 17.1 ± 15.6 |
| Fruit | 230.8 ± 180.6 | 227.5 ± 177.1 | 207.9 ± 153.3 |
| Animal products | | | |
| Red meat | 73.9 ± 34.1 | 79.7 ± 31.0 | 77.9 ± 38.5 |
| Poultry | 13.8 ± 13.7 | 13.4 ± 11.6 | 14.6 ± 16.7 |
| Fish | 18.2 ± 21.2 | 19.9 ± 18.5 | 22.8 ± 37.7 |
| Dairy products | 268.2 ± 244.3 | 319.7 ± 286.7 | 333.4 ± 352.9[b] |
| *Nutrients*[f] | | | |
| Alcohol | 13.7 ± 17.6 | 8.5 ± 12.3 | 20.7 ± 28.6[g] |
| Total fat | 99.6 ± 24.8 | 104.0 ± 24.4 | 102.7 ± 26.0 |
| Total protein | 83.1 ± 14.9 | 84.1 ± 16.0 | 84.4 ± 17.2 |
| Total Carbohydrate | 252.0 ± 52.0 | 247.3 ± 61.6 | 238.1 ± 58.9[b] |
| Dietary fiber | 28.8 ± 8.3 | 29.2 ± 9.2 | 26.1 ± 8.2[e] |

| *Table 3.1 cont.* | Controls (*n*=259) | APC$^{+}$ (*n*=63) | APC$^{-}$ (*n*=121) |
|---|---|---|---|
| Vitamin C (mg/day) | 117.5 ± 53.8 | 112.5 ± 61.9 | 98.0 ± 45.1[b] |
| ß-Carotene (mg/day) | 2.9 ± 2.3 | 2.7 ± 1.4 | 2.2 ± 1.4[e] |
| Calcium (mg/day) | 1254.3 ± 406.3 | 1238.2 ± 406.5 | 1290.3 ± 436.4 |

[a] APC$^{+}$: individuals with a carcinoma *with* a truncating *APC* mutation; APC$^{-}$: individuals with a carcinoma *without* a truncating *APC* mutation. [b] All cases versus controls $P<0.05$; APC$^{-}$ versus controls $P<0.05$. [c] APC$^{+}$ versus APC$^{-}$ $P<0.05$. [d] n.a.: not applicable. [e] All cases versus controls $P<0.05$; APC$^{-}$ versus controls $P<0.05$; APC$^{+}$ versus APC$^{-}$ $P<0.05$. [f] Adjusted for total energy intake by regression analysis, for women and men separately. [g] APC$^{+}$ versus APC$^{-}$ $P<0.05$; APC$^{+}$ versus controls $P<0.05$; APC$^{-}$ versus controls $P<0.05$.

Table 3.2 presents results of case-control and case-case comparisons conducted to assess associations between the various food groups and APC$^{+}$ and APC$^{-}$ tumors. Total vegetable intake was inversely associated with APC$^{+}$ tumors (although here the association was not statistically significant) as well as APC$^{-}$ tumors when the two tumor subsets were separately compared with the population-based controls. Interestingly, case-case comparison showed that total vegetable intake was significantly different related to APC$^{+}$ tumors than to APC$^{-}$ tumors. Similar patterns of association were observed for the evaluated vegetable subgroups: leafy greens, cruciferous, allium and root vegetables. To allow direct comparisons between the different vegetable subgroups and to quantify associations on a continuous scale, ORs and 95% CIs were also calculated for a fixed amount (10g/day) of intake. The strongest inverse associations were observed for allium vegetables and leafy greens with APC$^{-}$ tumors (APC$^{-}$ versus controls, OR: 0.81, 95% CI: 0.70-0.94; OR: 0.86, 95% CI: 0.76-0.97, respectively; not in table). Additional adjustment for meat consumption did not alter the observed associations significantly.

**Table 3.2** Associations between food groups and carcinomas *with* ($APC^+$) and *without* ($APC^-$) a truncating *APC* mutation: case-control and case-case comparisons

| | Odds ratios (95% confidence intervals)[a] | | | |
|---|---|---|---|---|
| | T1 | T2 | T3 | $P_{trend}$[b] |
| *Vegetables & fruit* | | | | |
| Total vegetables (g/day) | ≤166 | 166-223 | >223 | |
| No. $APC^+$/$APC^-$/controls | 25/65/87 | 21/36/87 | 17/20/85 | |
| All cases versus controls | 1.0 | 0.6 (0.4-1.0) | 0.4 (0.2-0.6) | <0.01 |
| $APC^+$ versus controls | 1.0 | 0.8 (0.4-1.6) | 0.6 (0.3-1.3) | 0.22 |
| $APC^-$ versus controls | 1.0 | 0.5 (0.3-0.9) | 0.3 (0.2-0.5) | <0.01 |
| $APC^+$ versus $APC^-$ | 1.0 | 1.5 (0.7-3.0) | 2.3 (1.0-5.3) | 0.04 |
| Leafy greens (g/day) | ≤21 | 21-36 | ≥36 | |
| No. $APC^+$/$APC^-$/controls | 29/59/88 | 14/36/84 | 20/26/87 | |
| All cases versus controls | 1.0 | 0.6 (0.4-1.0) | 0.5 (0.3-0.8) | <0.01 |
| $APC^+$ versus controls | 1.0 | 0.5 (0.3-1.1) | 0.7 (0.3-1.3) | 0.26 |
| $APC^-$ versus controls | 1.0 | 0.7 (0.4-1.1) | 0.4 (0.2-0.8) | <0.01 |
| $APC^+$ versus $APC^-$ | 1.0 | 0.8 (0.4-1.8) | 1.6 (0.7-3.5) | 0.24 |
| Cruciferous vegetables (g/day) | <24 | 24-41 | >41 | |
| No. $APC^+$/$APC^-$/controls | 23/48/85 | 20/41/88 | 20/31/86 | |
| All cases versus controls | 1.0 | 0.9 (0.5-1.4) | 0.6 (0.4-1.0) | 0.07 |
| $APC^+$ versus controls | 1.0 | 0.9 (0.5-1.7) | 0.8 (0.4-1.7) | 0.64 |
| $APC^-$ versus controls | 1.0 | 0.8 (0.5-1.4) | 0.6 (0.3-1.0) | 0.04 |
| $APC^+$ versus $APC^-$ | 1.0 | 1.0 (0.5-2.2) | 1.5 (0.7-3.2) | 0.34 |
| Allium vegetables (g/day) | ≤8 | 8-22 | >22 | |
| No. $APC^+$/$APC^-$/controls | 20/50/90 | 25/42/86 | 18/29/83 | |
| All cases versus controls | 1.0 | 1.0 (0.6-1.6) | 0.7 (0.4-1.1) | 0.09 |
| $APC^+$ versus controls | 1.0 | 1.4 (0.7-2.7) | 1.0 (0.5-2.0) | 0.88 |
| $APC^-$ versus controls | 1.0 | 0.9 (0.5-1.5) | 0.5 (0.3-1.0) | 0.03 |
| $APC^+$ versus $APC^-$ | 1.0 | 1.4 (0.7-2.9) | 1.8 (0.8-4.1) | 0.15 |
| Root vegetables (g/day) | ≤10 | 10-24 | >24 | |
| No. $APC^+$/$APC^-$/controls | 24/50/90 | 22/45/86 | 17/26/83 | |
| All cases versus controls | 1.0 | 1.0 (0.6-1.5) | 0.6 (0.4-1.0) | 0.04 |
| $APC^+$ versus controls | 1.0 | 0.9 (0.5-1.8) | 0.7 (0.3-1.4) | 0.26 |
| $APC^-$ versus controls | 1.0 | 1.0 (0.6-1.7) | 0.6 (0.3-1.0) | 0.04 |

| *Table 3.2 cont.* | T1 | T2 | T3 | $P_{trend}$[b] |
|---|---|---|---|---|
| APC$^+$ versus APC$^-$ | 1.0 | 1.0 (0.5-2.1) | 1.3 (0.6-2.9) | 0.52 |
| Fruit (g/day) | ≤142 | 142-269 | ≥269 | |
| No. APC$^+$/APC$^-$/controls | 21/45/87 | 23/41/85 | 19/35/87 | |
| All cases versus controls | 1.0 | 0.9 (0.6-1.5) | 0.7 (0.5-1.2) | 0.22 |
| APC$^+$ versus controls | 1.0 | 1.1 (0.5-2.1) | 0.8 (0.4-1.6) | 0.42 |
| APC$^-$ versus controls | 1.0 | 0.9 (0.5-1.5) | 0.7 (0.4-1.3) | 0.29 |
| APC$^+$ versus APC$^-$ | 1.0 | 1.2 (0.6-2.5) | 1.1 (0.5-2.4) | 0.83 |
| *Animal products* | | | | |
| Red meat (g/day) | <58 | 58-87 | ≥87 | |
| No. APC$^+$/APC$^-$/controls | 15/34/85 | 22/45/87 | 26/42/87 | |
| All cases versus controls | 1.0 | 1.3 (0.8-2.1) | 1.2 (0.7-1.9) | 0.60 |
| APC$^+$ versus controls | 1.0 | 1.5 (0.7-3.0) | 1.7 (0.8-3.6) | 0.18 |
| APC$^-$ versus controls | 1.0 | 1.2 (0.7-2.1) | 0.9 (0.5-1.7) | 0.80 |
| APC$^+$ versus APC$^-$ | 1.0 | 1.1 (0.5-2.5) | 1.7 (0.7-3.8) | 0.20 |
| Poultry (g/day) | ≤5 | 5-16 | >16 | |
| No. APC$^+$/APC$^-$/controls | 18/40/90 | 26/37/84 | 19/44/85 | |
| All cases versus controls | 1.0 | 1.1 (0.7-1.8) | 1.1 (0.7-1.7) | 0.86 |
| APC$^+$ versus controls | 1.0 | 1.5 (0.7-2.9) | 1.0 (0.5-2.2) | 0.97 |
| APC$^-$ versus controls | 1.0 | 1.0 (0.6-1.7) | 1.1 (0.6-1.8) | 0.83 |
| APC$^+$ versus APC$^-$ | 1.0 | 1.6 (0.7-3.4) | 1.0 (0.5-2.2) | 0.83 |
| Fish (g/day) | <7 | 7-19 | >19 | |
| No. APC$^+$/APC$^-$/controls | 17/41/82 | 17/33/91 | 29/47/86 | |
| All cases versus controls | 1.0 | 0.8 (0.5-1.3) | 1.1 (0.7-1.7) | 0.63 |
| APC$^+$ versus controls | 1.0 | 0.9 (0.4-1.9) | 1.4 (0.7-2.8) | 0.26 |
| APC$^-$ versus controls | 1.0 | 0.7 (0.4-1.3) | 0.9 (0.5-1.6) | 0.90 |
| APC$^+$ versus APC$^-$ | 1.0 | 1.2 (0.5-2.8) | 1.6 (0.7-3.4) | 0.23 |
| Dairy products (g/day) | ≤117 | 117-305 | >305 | |
| No. APC$^+$/APC$^-$/controls | 16/41/87 | 25/35/86 | 22/45/86 | |
| All cases versus controls | 1.0 | 1.0 (0.6-1.6) | 1.0 (0.6-1.6) | 0.87 |
| APC$^+$ versus controls | 1.0 | 1.5 (0.8-3.1) | 1.2 (0.6-2.6) | 0.77 |
| APC$^-$ versus controls | 1.0 | 0.7 (0.4-1.3) | 0.9 (0.5-1.5) | 0.73 |
| APC$^+$ versus APC$^-$ | 1.0 | 2.0 (0.9-4.4) | 1.4 (0.6-3.2) | 0.61 |

[a] Adjusted for age, sex, and total energy intake. [b] Trend was assessed using the median values of the tertiles as continuous variables.

No statistically significant associations were observed for the evaluated products of animal origin. However, red meat and fish did appear more notably, positively, associated with $APC^+$ tumors than with $APC^-$ tumors (Table 3.2). Additional adjustment for vegetable consumption did not alter the observed associations significantly.

To look at the combined effect of vegetable and red meat consumption, mutually exclusive categories of combinations of the two exposures were defined based on median split. Individuals with high meat (≥72g/day)/low vegetables (<191g/day) intake showed a substantially increased risk for $APC^+$ tumors ($APC^+$ versus controls, OR: 2.9, 95% CI: 1.2-7.2) as well as $APC^-$ tumors ($APC^-$ versus. controls, OR: 2.1, 95% CI: 1.1-4.2) compared to individuals with low meat (<72g/day)/high vegetables (≥191g/day) intake (not in table).

Assessment of nutrients (presented in Table 3.3) showed that alcohol intake was positively associated with $APC^-$ tumors whereas an inverse, statistically non-significant, association was observed with $APC^+$ tumors. Moreover, case-case comparison demonstrated that alcohol was significantly different related to $APC^+$ tumors than to $APC^-$ tumors. Total fat was positively associated with $APC^+$ tumors, and cases with high total fat intake do appear to more likely develop an $APC^+$ tumor than an $APC^-$ tumor (Table 3.3). Interestingly, no clear associations were observed for saturated fat but unsaturated fat was strongly, positively, associated with $APC^+$ tumors. Cholesterol was positively associated with both tumor groups, most pronounced and significantly with $APC^-$ tumors. Carbohydrate was inversely associated with both tumor groups, most pronounced and significantly with $APC^-$ tumors. Dietary fiber, vitamin C and ß-carotene showed association patterns similar to those observed for vegetable consumption. However, all three are related to vegetable intake and after inclusion of total vegetables in the multivariate model, the observed ORs were attenuated and dietary fiber was no longer significantly different related to $APC^+$ tumors than to $APC^-$ tumors (data not shown).

**Table 3.3** Associations between nutrients[a] and carcinomas *with* (APC$^+$) and *without* (APC$^-$) a truncating *APC* mutation: case-control and case-case comparisons

| | Odds ratios (95% confidence intervals)[b] | | | |
|---|---|---|---|---|
| | T1 | T2 | T3 | $P_{trend}$[c] |
| Alcohol (g/day) | <3.8 | 3.8-12.9 | >12.9 | |
| No. APC$^+$/APC$^-$/controls | 31/38/86 | 16/25/87 | 16/58/86 | |
| All cases versus controls | 1.0 | 0.7 (0.4-1.2) | 1.2 (0.7-1.9) | 0.29 |
| APC$^+$ versus controls | 1.0 | 0.6 (0.3-1.2) | 0.5 (0.3-1.1) | 0.18 |
| APC$^-$ versus controls | 1.0 | 0.9 (0.5-1.6) | 1.7 (1.0-3.0) | 0.02 |
| APC$^+$ versus APC$^-$ | 1.0 | 0.7 (0.3-1.6) | 0.3 (0.2-0.7) | <0.01 |
| Total fat (g/day) | <85 | 85-109 | ≥109 | |
| No. APC$^+$/APC$^-$/controls | 14/30/86 | 21/47/86 | 28/44/87 | |
| All cases versus controls | 1.0 | 1.8 (1.1-3.0) | 2.3 (1.2-4.4) | 0.02 |
| APC$^+$ versus controls | 1.0 | 1.8 (0.9-4.0) | 4.5 (1.6-12.8) | <0.01 |
| APC$^-$ versus controls | 1.0 | 1.7 (0.9-3.0) | 1.6 (0.7-3.3) | 0.28 |
| APC$^+$ versus APC$^-$ | 1.0 | 1.1 (0.4-2.7) | 3.0 (0.9-10.0) | 0.06 |
| Saturated fat (g/day) | <36 | 36-47 | ≥47 | |
| No. APC$^+$/APC$^-$/controls | 19/35/86 | 27/47/86 | 17/39/87 | |
| All cases versus controls | 1.0 | 1.4 (0.9-2.4) | 1.0 (0.5-1.8) | 0.73 |
| APC$^+$ versus controls | 1.0 | 1.5 (0.7-3.1) | 0.9 (0.4-2.2) | 0.67 |
| APC$^-$ versus controls | 1.0 | 1.3 (0.7-2.4) | 1.0 (0.5-1.9) | 0.78 |
| APC$^+$ versus APC$^-$ | 1.0 | 1.2 (0.5-2.6) | 0.9 (0.3-2.4) | 0.80 |
| Unsaturated fat (g/day) | <49 | 49-64 | >64 | |
| No. APC$^+$/APC$^-$/controls | 14/33/86 | 22/43/87 | 27/45/86 | |
| All cases versus controls | 1.0 | 1.7 (1.0-2.9) | 2.1 (1.1-3.9) | 0.03 |
| APC$^+$ versus controls | 1.0 | 2.0 (0.9-4.2) | 3.4 (1.3-8.6) | 0.01 |
| APC$^-$ versus controls | 1.0 | 1.5 (0.9-2.8) | 1.5 (0.7-3.1) | 0.29 |
| APC$^+$ versus APC$^-$ | 1.0 | 1.3 (0.6-3.2) | 2.2 (0.8-6.0) | 0.13 |
| Cholesterol (g/day) | <245 | 245-318 | >318 | |
| No. APC$^+$/APC$^-$/controls | 17/25/86 | 18/43/87 | 28/53/86 | |
| All cases versus controls | 1.0 | 1.7 (1.0-2.8) | 2.4 (1.4-4.1) | <0.01 |
| APC$^+$ versus controls | 1.0 | 1.2 (0.6-2.5) | 2.0 (0.9-4.3) | 0.07 |
| APC$^-$ versus controls | 1.0 | 2.0 (1.1-3.6) | 2.5 (1.3-4.7) | <0.01 |
| APC$^+$ versus APC$^-$ | 1.0 | 0.7 (0.3-1.6) | 0.8 (0.4-2.0) | 0.88 |

| *Table 3.3 cont.* | T1 | T2 | T3 | $P_{trend}$[c] |
|---|---|---|---|---|
| Total protein (g/day) | <77 | 77-89 | ≥89 | |
| No. $APC^+/APC^-$/controls | 19/41/86 | 23/36/86 | 21/44/87 | |
| All cases versus controls | 1.0 | 1.0 (0.6-1.6) | 1.1 (0.6-1.8) | 0.87 |
| $APC^+$ versus controls | 1.0 | 1.3 (0.6-2.8) | 1.2 (0.5-2.8) | 0.60 |
| $APC^-$ versus controls | 1.0 | 0.8 (0.5-1.5) | 1.0 (0.5-1.8) | 0.92 |
| $APC^+$ versus $APC^-$ | 1.0 | 1.5 (0.7-3.4) | 1.4 (0.6-3.4) | 0.47 |
| Animal protein (g/day) | <51 | 51-62 | ≥62 | |
| No. $APC^+/APC^-$/controls | 25/43/86 | 14/35/86 | 24/43/87 | |
| All cases versus controls | 1.0 | 0.8 (0.5-1.3) | 0.9 (0.6-1.6) | 0.88 |
| $APC^+$ versus controls | 1.0 | 0.6 (0.3-1.3) | 0.9 (0.5-1.9) | 0.90 |
| $APC^-$ versus controls | 1.0 | 0.9 (0.5-1.5) | 1.0 (0.5-1.7) | 0.88 |
| $APC^+$ versus $APC^-$ | 1.0 | 0.7 (0.3-1.6) | 1.1 (0.5-2.3) | 0.87 |
| Total carbohydrate (g/day) | <226 | 226-272 | >272 | |
| No. $APC^+/APC^-$/controls | 27/54/86 | 15/35/87 | 21/32/86 | |
| All cases versus controls | 1.0 | 0.6 (0.4-0.9) | 0.5 (0.3-0.9) | 0.02 |
| $APC^+$ versus controls | 1.0 | 0.6 (0.3-1.1) | 0.7 (0.3-1.6) | 0.40 |
| $APC^-$ versus controls | 1.0 | 0.6 (0.3-1.1) | 0.4 (0.2-0.8) | <0.01 |
| $APC^+$ versus $APC^-$ | 1.0 | 0.9 (0.4-2.1) | 1.8 (0.7-4.5) | 0.21 |
| Mono/disaccharide (g/day) | <113 | 113-141 | >141 | |
| No. $APC^+/APC^-$/controls | 26/54/86 | 18/32/87 | 19/35/86 | |
| All cases versus controls | 1.0 | 0.7 (0.4-1.1) | 0.6 (0.4-1.1) | 0.08 |
| $APC^+$ versus controls | 1.0 | 0.7 (0.4-1.4) | 0.7 (0.4-1.5) | 0.37 |
| $APC^-$ versus controls | 1.0 | 0.7 (0.4-1.2) | 0.6 (0.4-1.1) | 0.09 |
| $APC^+$ versus $APC^-$ | 1.0 | 1.1 (0.5-2.4) | 1.1 (0.5-2.4) | 0.75 |
| Dietary fiber (g/day) | <25 | 25-31 | >31 | |
| No. $APC^+/APC^-$/controls | 25/61/86 | 13/30/87 | 25/30/86 | |
| All cases versus controls | 1.0 | 0.5 (0.3-0.8) | 0.6 (0.4-1.0) | 0.02 |
| $APC^+$ versus controls | 1.0 | 0.5 (0.3-1.1) | 1.0 (0.5-1.9) | 0.99 |
| $APC^-$ versus controls | 1.0 | 0.5 (0.3-0.9) | 0.5 (0.3-0.8) | <0.01 |
| $APC^+$ versus $APC^-$ | 1.0 | 1.1 (0.5-2.4) | 2.5 (1.2-5.6) | 0.03 |
| Vitamin C (mg/day) | ≤89 | 89-133 | >133 | |
| No. $APC^+/APC^-$/controls | 21/58/87 | 26/41/86 | 16/22/86 | |
| All cases versus controls | 1.0 | 0.9 (0.5-1.4) | 0.5 (0.3-0.7) | <0.01 |
| $APC^+$ versus controls | 1.0 | 1.2 (0.6-2.3) | 0.7 (0.3-1.4) | 0.30 |

| *Table 3.3 cont.* | T1 | T2 | T3 | $P_{trend}$[c] |
|---|---|---|---|---|
| APC$^{-}$ versus controls | 1.0 | 0.7 (0.4-1.2) | 0.4 (0.2-0.7) | <0.01 |
| APC$^{+}$ versus APC$^{-}$ | 1.0 | 1.6 (0.8-3.3) | 1.9 (0.8-4.2) | 0.12 |
| ß-Carotene (mg/day) | <2.0 | 2.0-2.9 | >2.9 | |
| No. APC$^{+}$/APC$^{-}$/controls | 23/61/86 | 20/30/87 | 20/30/86 | |
| All cases versus controls | 1.0 | 0.6 (0.4-1.0) | 0.6 (0.4-1.0) | 0.04 |
| APC$^{+}$ versus controls | 1.0 | 0.9 (0.4-1.7) | 0.8 (0.4-1.6) | 0.58 |
| APC$^{-}$ versus controls | 1.0 | 0.5 (0.3-0.9) | 0.5 (0.3-0.9) | 0.02 |
| APC$^{+}$ versus APC$^{-}$ | 1.0 | 1.8 (0.9-3.9) | 1.7 (0.8-3.6) | 0.17 |
| Calcium (mg/day) | ≤1062 | 1062-1358 | >1358 | |
| No. APC$^{+}$/APC$^{-}$/controls | 20/40/87 | 18/30/86 | 25/51/86 | |
| All cases versus controls | 1.0 | 0.9 (0.6-1.5) | 1.3 (0.8-2.1) | 0.22 |
| APC$^{+}$ versus controls | 1.0 | 1.0 (0.5-2.0) | 1.3 (0.7-2.6) | 0.44 |
| APC$^{-}$ versus controls | 1.0 | 0.9 (0.5-1.6) | 1.4 (0.8-2.3) | 0.23 |
| APC$^{+}$ versus APC$^{-}$ | 1.0 | 1.2 (0.5-2.6) | 1.0 (0.5-2.1) | 0.98 |

[a] Adjusted for total energy intake by regression analysis, for women and men separately. [b] Adjusted for age, sex, and total energy intake. Alcohol adjusted for age, sex, total energy intake and smoking. [c] Trend was assessed using the median values of the tertiles as continuous variables.

## Discussion

In this study, we evaluated associations between various dietary factors reported previously as being associated with colon cancer risk and the occurrence of truncating *APC* mutations in sporadic colon carcinomas. Consumption of vegetables lowered the risk of APC$^{+}$ tumors as well as APC$^{-}$ tumors, but most explicitly of the last. Alcohol intake was associated with an increased risk of APC$^{-}$ tumors only, whereas meat, fish and (unsaturated) fat seemed to especially increase the risk of APC$^{+}$ tumors. Of the dietary factors examined, vegetable consumption and alcohol intake were significantly different related to APC$^{+}$ tumors than to APC$^{-}$ tumors, suggesting differences in the role these dietary factors play in the etiology of the two distinct colon carcinoma subsets.

We focused on nonsense and frameshift mutations in the MCR because these mutations indisputably result in truncated and non-functional APC protein whereas the biological significance of missense mutations in *APC* is uncertain. Allelic loss at *APC* also results in loss of APC function. At the moment, however, allelic loss is thought to be a primarily spontaneous event (25). Besides that, colon tumors in which *APC* is mutated usually show allelic loss plus a truncating mutation in the MCR or two truncating mutations (at least one in the MCR) but rarely only allelic loss or only mutations outside the MCR (7). We therefore do not expect that our decision to not consider allelic loss and to concentrate on truncating mutations in the MCR has resulted in extensive misclassification.

Using SSCP analysis and sequencing, we identified truncating mutations in the extended-MCR (codons 1286 to 1585) of *APC* in 34.2% of the carcinomas. Despite the fact that our frequency seems low when compared with the conventional wisdom that most colon tumors follow a genetic pathway involving *APC*, it is consistent with the mutation frequencies for this region, 30-45%, reported by most others (2, 5, 6, 8). Only Rowan *et al*. observed, in tumor cell lines, a higher frequency, 60% (7). The characteristics of the mutations and the hotspots we identified were also similar to those observed by others (6, 7,) (APC database: http://perso.curie.fr/Thierry.Soussi/APC/html). Although microdissection was performed, it remains possible that, due to contaminating normal tissue, mutations were missed eventually resulting in misclassification, *i.e.*, tumors with a truncating mutation in the $APC^-$ group. This may have attenuated some of our results.

The case-control study was initially designed to examine the role of dietary factors in the etiology of sporadic colon cancer in general (that is, colon cancer not categorized according to mutational status of the tumors). The results of the cases versus controls comparisons, previously reported in Kampman *et al*. (18, 20), were in line with those reported by others (1). As in any retrospective study, information bias and selection bias may have affected our results.

Cases and controls were asked to recall their diets from the past and differential recall is possible. One of the advantages of the conducted case-case comparisons is, however, that the cases are unaware of the mutational status of their tumors. Consequently, systematic errors in dietary recall are less likely to bias results from case-case comparisons. Recall of dietary habits can also be influenced by tumor stage or treatments that affect appetite. Our cases were relatively healthy. That is, the frequency of Dukes' A and B tumors among the cases was relatively high, 63%, compared to the frequency reported by the Dutch Cancer Registry, 51% (26). Adjusting the case-case comparisons for Dukes' stage did not change the estimates significantly.

We calculated the largest and smallest odds ratios detectable with a power of 0.90 for the four different study populations used here in order to determine what effects we were able to exclude (27). For all cases versus controls, the present study is able to detect odds ratios ≤0.5 and ≥1.9; for $APC^+$ versus controls, ≤0.3 and ≥2.5; for $APC^-$ versus controls, ≤0.4 and ≥2.0; and for $APC^+$ versus $APC^-$, ≤0.3 and ≥2.7. Although the dietary factors evaluated in this study were all previously identified risk factors for colon cancer, it should be noted that multiple comparisons might lead to chance findings.

Dietary factors may influence the occurrence of truncating *APC* mutations in colon carcinomas directly, *i.e.*, by being involved in the actual production or prevention of these mutations, and/or indirectly, *i.e.*, by being involved in the promotion or evasion of progression into later stages and eventually into carcinomas. In this study, we observed a protective effect of vegetables for $APC^+$ tumors as well as $APC^-$ tumors. Interestingly, the protective effect was markedly higher for $APC^-$ tumors. So far, to our knowledge, no other studies have evaluated dietary factors and *APC* mutations in human colon carcinomas. However, in line with our results, low vegetable intake was reported to also increase the risk of colon carcinomas with and

without a K-*ras* mutation in a large study on diet and K-*ras* mutations in sporadic colon carcinomas. And, there too, the increase was most pronounced for tumors without a mutation (15).

A possible explanation for the observed difference in protective effect of vegetables between the two tumor subsets is that, as also suggested by other observations reported previously (3, 4, 28), loss of APC function is not only important for tumor initiation but plays a role in later stages of malignant progression as well. Hence, the protective influence of vegetables on progression might be more limited after an *APC* mutation has occurred than in otherwise initiated tumors. The latter appear to follow a different pathway (29) and possibly need to undergo more and/or different genetic and epigenetic changes -- which may be prevented by vegetables -- before they eventually are able to develop into carcinomas.

Alcohol was also significantly different related to $APC^+$ tumors than to $APC^-$ tumors in our population. It increased the risk of $APC^-$ tumors but not of $APC^+$ tumors which points to a (negative) role in the pathway of tumors without a truncating *APC* mutation in particular. Alcohol possibly exerts its effect on colon cancer through interference with folic acid availability (30-32). Folate is involved in DNA methylation and appears to be essential for normal DNA synthesis and repair (33). Vegetables, especially green leafy and cruciferous vegetables, contain large amounts of folate and are the main dietary sources. Disturbances in DNA methylation pathways can result in chromosome breaks due to deficient methylation of uracil to thymine (34), but also in epigenetic silencing of genes (11, 12). Regarding the latter, hypermethylation of the promotor region of the *hMLH1* gene -- observed in the majority of sporadic colon carcinomas with microsatellite instability -- seems to be particularly important for tumor development as it results in the inactivation of the DNA mismatch repair system (35-37) which, in turn, can lead to the additional loss of other genes involved in carcinogenesis due to repairs not executed. Inverse relationships have been reported between microsatellite

instability and mutations in *APC* and *p53* which suggests that $APC^+$ tumors and tumors with microsatellite instability develop through different pathways (38). Thus, microsatellite instability positive tumors are probably most common in our $APC^-$ group. Interestingly, and consistent with our results, Slattery *et al*. (39) recently reported that long-term alcohol consumption increased the probability of developing sporadic colon carcinomas with microsatellite instability.

For animal products, no statistically significant associations were observed. However, red meat and fish did seem more notably associated with $APC^+$ tumors than with $APC^-$ tumors, which suggests that products of animal origin may influence the occurrence of truncating *APC* mutations in colon carcinomas positively. Red meat and fish, prepared at high temperatures, are major sources of heterocyclic amines (40). Heterocyclic amines are bulky-adduct-forming agents that can produce DNA strand breaks (41) and probably so contribute to carcinogenesis. They have been shown to be carcinogenic in rodents (42, 43) and to induce specific deletion mutations in the *Apc* gene in rat colons (16, 17). High intake of certain heterocyclic amines was also found associated with increased risk of colorectal adenomas in humans (44).

Interestingly, we observed that fat, especially unsaturated fat, increased the risk of $APC^+$ tumors in our population. Recent studies on specific fatty acids and colon cancer (in general) have reported no clear association with colon cancer risk (45, 46). However, intake of unsaturated fat (especially linoleic and 20-carbon poly-unsaturated fatty acids) does appear to be more strongly associated with colon cancer risk among those with a family history of colorectal cancer than among those without (45). Slattery *et al*. (15) also observed that fat (mono-unsaturated fat most pronounced) was related differently to carcinomas with a G→T transversion at the second base of K-*ras* codon 12 than to tumors without a K-*ras* mutation. In our own population, as reported earlier (14), we did not observe an association between dietary fat and K-*ras* mutations.

In summary, our data suggest that vegetables play a protective role in the etiology of $APC^+$ tumors as well as $APC^-$ tumors, although the protective effect of vegetables appears to be less influential in the $APC^+$ subset. Alcohol seems to promote the development of $APC^-$ tumors in particular whereas meat, fish and fat appear to enhance the development of $APC^+$ tumors. This supports the idea that $APC^+$ tumors and $APC^-$ tumors develop through different pathways affected by specific dietary factors. Our results, if confirmed in other studies, provide further clues to the relationships between dietary factors and the molecular alterations that drive colon carcinogenesis.

## Acknowledgements

We are indebted to the Departments of Pathology of the following hospitals for providing tumor tissue: Canisius-Wilhelmina Hospital, Nijmegen; UMC Utrecht, Utrecht; St Antonius Hospital, Nieuwegein; and Eemland Hospital, Amersfoort. The initial case-control study was conducted in collaboration with regional hospitals and cancer registries, and with financial support from the Dutch Dairy Foundation on Nutrition and Health. This study was supported by a grant from the Dutch Cancer Society (LUW 98-1687).

## References

1. World Cancer Research Fund (WCRF) Panel (Potter J.D., Chair). (1997) *Food, nutrition and the prevention of cancer: a global perspective*. WCRF/American Institute for Cancer Research, Washington, DC.
2. Powell, S.M., Zilz, N., Beazer-Barclay, Y., Bryan, T.M., Hamilton, S.R., Thibodeau, S.N., Vogelstein, B., and Kinzler, K.W. (1992) *APC* mutations occur early during colorectal tumorigenesis. *Nature*, **359**, 235-237.
3. Fodde, R., Kuipers, J., Rosenberg, C., Smits, R., Kielman, M., Gaspar, C., Van Est, J.H., Breukel, C., Wiegant, J., Giles, R.H., and Clevers, H. (2001) Mutations in the *APC* tumour suppressor gene cause chromosomal instability. *Nature Cell Biology*, **3**, 433-438.

4. Kaplan, K.B., Burds, A.A., Swedlow, J.R., Bekir, S.S., Sorger, P.K., and Näthke, I.S. (2001) A role for the adenomatous polyposis coli protein in chromosome segregation. *Nature Cell Biology*, **3**, 429-432.
5. Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T., and Nakamura, Y. (1992) Somatic mutations of the *APC* gene in colorectal tumors: mutation cluster region in the *APC* gene. *Hum. Mol. Genet*., **1**, 229-233.
6. Miyaki, M., Konishi, M., Kikuchi-Yanoshita, R., Enomoto, M., Igari, T., Tanaka, K., Muraoka, M., Takahashi, H., Amada, Y., Fukayama, M., Maeda, Y., Iwama, T., *et al*. (1994) Characteristics of somatic mutation of the adenomatous polyposis coli gene in colorectal tumors. *Cancer Res*., **54**, 3011-3020.
7. Rowan, A.J., Lamlum, H., Ilyas, M., Straub, J., Papadopoulou, A., Bicknell, D., Bodmer, W.F., and Tomlinson, I.P.M. (2000) *APC* mutations in sporadic colorectal tumors: A mutational “hotspot” and interdependence of the “two hits”. *Proc. Natl. Acad. Sci. USA*, **97**, 3352-3357.
8. Yashima, K., Nakamori, S., Murakami, Y., Yamaguchi, A., Ishikakawa, O., Konishi, Y., and Sekiya, T. (1994) Mutations of the adenomatous polyposis coli gene in the mutation cluster region: comparison of human pancreatic and colorectal cancers. *Int. J. Cancer*, **59**, 43-47.
9. Lamlum, H., Ilyas, M., Rowan, A., Clark, S., Johnson, V., Bell, J., Frayling, I., Efstathiou, J., Pack, K., Payne, S., Roylance, R., Gorman, P., *et al*. (1999) The type of somatic mutation at *APC* in familial adenomatous polyposis is determined by the site of the germline mutation: a new facet to Knudson’s ‘two-hit’ hypothesis. *Nature Medicine*, **5**, 1071-1075.
10. Smits, R., Hofland, N., Edelmann, W., Geugien, M., Jagmohan-Changur, S., Albuquerque, C., Breukel, C., Kucherlapati, R., Kielman, M.F., and Fodde, R. (2000) Somatic *Apc* mutations are selected upon their capacity to inactivate the ß-catenin downregulating activity. *Genes Chromosomes Cancer*, **29**, 229-239.
11. Jones, P.A., and Laird, P.W. (1999) Cancer epigenetics comes of age. *Nature Genetics*, **21**, 163-167.
12. Ballestar, E., and Esteller, M. (2002) The impact of chromatin in human cancer: linking DNA methylation to gene silencing. *Carcinogenesis*, **23**, 1103-1109.
13. Bardelli, A., Cahill, D.P., Lederer, G., Speicher, M.R., Kinzler, K.W., Vogelstein, B., and Lengauer,C. (2001) Carcinogen-specific induction of genetic instability. *Proc. Natl. Acad. Sci. USA*, **98**, 5770-5775.
14. Kampman, E., Voskuil, D.W., Van Kraats, A.A., Balder, H.F., Van Muijen, G.N.P., Goldbohm, R.A., and Van ‘t Veer, P. (2000) Animal products and K-*ras* codon 12 and 13 mutations in colon carcinomas. *Carcinogenesis*, **21**, 307-309.
15. Slattery, M.L., Curtin, K., Anderson, K., Ma, K-N, Edwards, S., Leppert, M., Potter, J., Schaffer, D., and Samowitz, W.S. (2000) Associations between dietary intake and Ki-*ras* mutations in colon tumors: a population-based study. *Cancer Res*., **60**, 6935-6941.

16. Kakiuchi, H., Watanabe, M., Ushijima, T., Toyota, M., Imai, K., Weisburger, J.H., Sugimura, T., and Nagao, M. (1995) Specific 5’-GGGA-3’ → 5’-GGA-3’ mutation of the *Apc* gene in rat colon tumors induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Proc. Natl. Acad. Sci. USA*, **92**, 910-914.
17. Nagao, M., Ushijima, T., Toyota, M., Inoue, R., and Sugimura, T. (1997) Genetic changes induced by heterocyclic amines. *Mutat. Res*., **376** (1-2), 161-167.
18. Kampman, E. Van ‘t Veer, P., Hiddink, G.J., Van Aken-Schneijder, P., Kok, F.J., and Hermus, R.J.J. (1994) Fermented dairy products, dietary calcium and colon cancer: a case-control study in the Netherlands. *Int. J. Cancer*, **59**, 170-176.
19. Voorlichtingsbureau voor de Voeding. (1990) Nevo Tabel. *Dutch Food Composition Table 1989-1990*. Stichting Nevo, The Hague, the Netherlands.
20. Kampman, E., Verhoeven, D., Sloots, L., and Van 't Veer, P. (1995) Vegetable and animal products as determinants of colon cancer risk in Dutch men and women. *Cancer Causes and Control*, **6**, 225-234.
21. Voskuil, D.W., Kampman, E., Van Kraats, A.A., Balder, H.F., Van Muijen, G.N.P., Goldbohm, R.A., and Van ‘t Veer, P. (1999) P53 overexpression and *p53* mutations in colon carcinomas: relation to dietary risk factors. *Int. J. Cancer*, **81**, 675-681.
22. Willett, W., and Stampfer, M.J. (1986) Total energy intake: implications for epidemiological analyses. *Am. J. Epidemiol*., **124**, 17-27.
23. Willett, W.C., Howe, G.R., and Kushi, L.H. (1997) Adjustment for total energy intake in epidemiologic studies. *Am. J. Clin. Nutr*., **65** (suppl), 1220S-8S.
24. Kulldorff, M., Sinha, R., Chow, W-H, and Rothman, N. (2000) Comparing odds ratios for nested subsets of dietary components. *Int. J. Epidemiology*, **29**, 1060-1064.
25. De Nooij-van Dalen, A.G., Van Buuren-van Seggelen, V.H.A., Lohman, P.H.M., and Giphart-Gassler, M. (1998) Chromosome loss with concomitant duplication and recombination both contribute most to loss of heterozygosity in vitro. *Genes Chromosomes Cancer*, **21**, 30-38.
26. Coebergh, J.W.W., Van der Heijden, L.H., and Janssen-Heijnen, M.L.G. (1995) *Cancer incidence and survival in the southeast of the Netherlands.* Comprehensive Cancer Centre South, Eindhoven.
27. Schlesselman, J.J. (1982) *Case-control studies. Design, conduct, analysis*. Oxford University Press.
28. Samowitz, W.S., Powers, M.D., Spirio, L.N., Nollet, F., Van Roy, F., and Slattery, M.L. (1999) ß-catenin mutations are more frequent in small colorectal adenomas than in large adenomas and invasive carcinomas. *Cancer Res*., **59**, 1442-1444.
29. Potter, J.D. (1999) Colorectal cancer: molecules and populations. *J. Natl. Cancer Inst*, **91**, 916-932.
30. Shaw, S., Jayatilleke, E., Herbert, V., and Colman, N. (1989) Cleavage of folates during ethanol metabolism. *Biochem. J*., **257**, 277-280.

31. Giovanucci, E., Rimm, E.B., Ascherio, A., Stampfer, M.J. Colditz, G.A., and Willett, W.C. (1995) Alcohol, low-methionine-low-folate diets and risk of colon cancer in men. *J. Natl. Cancer Inst*., **87**, 265-273.
32. Baron, J.A., Sandler, R.S., Haile, R., Mandel, J.S., Mott, L.A., and Greenberg, R. (1998) Folate intake, alcohol consumption, cigarette smoking, and risk of colorectal adenomas. *J. Natl. Cancer Inst*., **90**, 57-62.
33. Choi, S.W., and Mason, J.B. (2000) Folate and carcinogenesis: an integrated scheme. *J. Nutr*., **130**, 129-132.
34. Blount, B.C., Mack, M.M., Wehr, C., MacGregor, J., Hiatt, R., Wang, G., Wickramasinghe, S.N., Everson, R.B., and Ames, B.N. (1997) Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. *Proc. Natl. Acad. Sci. USA*, **94**, 3290-3295.
35. Xiong, Z., Wu, A.H., Bender, C.M., Tsao, J-L., Blake, C., Shibata, D., Jones, P.A., Yu, M.C., Ross, R.K., and Laird, P.W. (2001) Mismatch repair deficiency and CpG island hypermethylation in sporadic colon adenocarcinomas. *Cancer Epidemiol. Biomark. Prev*., **10**, 799-803.
36. Cunningham, J.M., Christensen, E.R., Tester, D.J., Kim, C-Y., Roche, P.C., Burgart, L.J., and Thibodeau, S.N. (1998) Hypermethylation of the *hMLH1* promotor in colon cancer with microsatellite instability. *Cancer Res*., **58**, 3455-3460.
37. Herman, J.G., Umar, A., Polyak, K., Graff, J.R.,Ahuja, N., Issa, J-P., Markowitz, S., Willson, J.K., Hamilton, S.R., Kinzler, K.W., Kane, M.F., Kolodner, R.D., *et al*. (1998) Incidence and functional consequences of *hMLH1* promotor hypermethylation in colorectal carcinoma. *Proc. Natl. Acad. Sci. USA*, **95**, 6870-6875.
38. Salahshor, S., Kressner, U., Pahlman, L., Glimelius, B., Lindmark, G., and Lindblom, A. (1999) Colorectal cancer with and without microsatellite instability involves different genes. *Genes Chromosomes Cancer*, **26**, 247-252.
39. Slattery, M.L., Anderson, K., Curtin, K., Ma, K-N., Schaffer, D., and Samowitz, W. (2001) Dietary intake and microsatellite instability in colon tumors. *Int. J. Cancer*, **93**, 601-607.
40. Wakabayashi, K., Nagao, M., Esumi, H., and Sugimura, T. (1992) Food derived mutagens and carcinogens. *Cancer Res*., **52**, 2092s-2098s.
41. Pfau, W., Martin, F.L., Cole, K.J., Venitt, S., Phillips, D.H., Grover, P.L., and Marquardt, H. (1999) Heterocyclic aromatic amines induce DNA atrand breaks and cell transformation. *Carcinogenesis*, **20**, 545-551.
42. Ohgaki, H., Hasegawa, H., Kato, T., Suenaga, M., Sata, S., Takayama, S., and Sugimura, T. (1985) Carcinogenicities in mice and rats of IQ, MeIQ, and MeIQxX. *Symp. Princess Takamatsu Symposium Fund*, **16**, 97-105.

43. Ito, N., Hasegawa, H., Sano, M., Tamano, S., Esumi, H., Takayama, S., and Sugimura, T. (1991) A new colon and mammary carcinogen in cooked food, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Carcinogenesis*, **12**, 1503-1506.
44. Sinha, R., Kulldorf, M., Chow, W-H., Denobile, J., and Rothman, N. (2001) Dietary intake of heterocyclic amines, meat-derived mutagenic activity, and risk of colorectal adenomas. *Cancer Epidemiol. Biomark. Prev.*, **10**, 559-362.
45. Slattery, M.L., Potter, J.D., Duncan, D.M., and Berry, T.D. (1997) Dietary fats and colon cancer: assessment of risk associated with specific fatty acids. *Int. J. Cancer*, **73**, 670-677.
46. Terry, P., Bergkvist, L., Holmberg, L., and Wolk, A. (2001) No association between fat and fatty acids intake and risk of colorectal cancer. *Cancer Epidemiol. Biomark. Prev.*, **10**, 913-914.

# Chapter 4

# Dietary factors and microsatellite instability in sporadic colon carcinomas

Brenda Diergaarde

Hanneke Braam

Goos N. P. van Muijen

Marjolijn J. L. Ligtenberg

Frans J. Kok

Ellen Kampman

**_Cancer Epidemiol. Biomarkers Prev._, November 2003; 12 (11): 1130-1136**

## Abstract

Microsatellite instability (MSI) occurs in 10-20% of the sporadic colon carcinomas and appears to be primarily due to alterations in *hMLH1* and *hMSH2*. Little is known about the role of diet in MSI-related colon carcinogenesis. We used data from a Dutch population-based case-control study of sporadic colon carcinomas (184 cases; 259 controls) to evaluate associations between dietary factors previously reported as being associated with colon cancer risk and MSI, hMLH1 expression and *hMLH1* hypermethylation. Red meat intake was significantly differently related to MSI-H tumors compared with MSI-L/MSS tumors [odds ratio (OR): 0.3, 95% confidence interval (CI): 0.1-0.9]. It was inversely associated with MSI-H tumors when compared with the population-based controls (OR: 0.5, 95% CI: 0.2-1.2), positively with MSI-L/MSS tumors (OR: 1.5, 95% CI: 0.9-2.6). A positive association was observed for alcohol intake with MSI-H tumors (OR: 1.9, 95% CI: 0.8-4.7). Fruit consumption seemed to especially decrease the risk of MSI-H tumors with hypermethylated *hMLH1* (Methyl$^+$ tumors) [Methyl$^+$ versus controls, OR: 0.4, 95% CI: 0.2-0.9; MSI-H tumors without hypermethylated *hMLH1* (Methyl$^-$ tumors) versus. controls, OR: 1.2, 95% CI: 0.8-1.7; Methyl$^+$ versus Methyl$^-$, OR: 0.2, 95% CI: 0.1-0.9]. Most other evaluated dietary factors were not distinctively associated with a specific MSI or *hMLH1* methylation status. Our data suggest that red meat consumption may enhance the development of MSI-L/MSS carcinomas in particular, whereas alcohol intake appears to increase the risk of MSI-H tumors. Fruit consumption may especially decrease the risk of MSI-H carcinomas exhibiting epigenetically silenced *hMLH1*.

## Introduction

Approximately 10-20% of the sporadic colon carcinomas and most colon tumors associated with the hereditary nonpolyposis colorectal cancer syndrome are characterized by microsatellite instability (MSI) (1-5). MSI is a hallmark of DNA mismatch repair (MMR) deficiency that in turn appears to be primarily due to inherited and/or acquired alterations in the MMR genes *hMLH1* and *hMSH2.* Presence of MSI correlates well with the absence of either hMLH1 or hMSH2 (6-8). In sporadic colon carcinomas, loss of hMLH1 expression is frequently the result of hypermethylation of the promoter region of *hMLH1*, whereas loss of hMSH2 expression seems to occur through genetic mutations only (9-11).

Diet has been repeatedly implicated in the etiology of colon cancer and certain dietary factors, especially those reported previously to be associated with colon cancer risk, may well specifically influence the development of microsatellite unstable colon carcinomas. Most colon cancers exhibit MSI or chromosomal instability (CIN), another type of genetic instability, but not both (12). This suggests different molecular pathways to colon cancer which may reflect different environmental exposures (13). Supporting this idea, Bardelli *et al*. (14) demonstrated that exposure to the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanide produced tumor cells characterized by MSI, whereas exposure to the bulky-adduct-forming agent 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine produced tumor cells characterized by CIN.

Thus far, few epidemiological studies have examined associations between diet and MSI and knowledge about the role of dietary factors in MSI-related sporadic colon carcinogenesis is limited. Slattery *et al*. (15) reported a positive association between long-term alcohol consumption and occurrence of MSI. The only other epidemiological study on dietary factors and occurrence of MSI in colon carcinomas published to date, reported a positive association between well-done red meat consumption and MSI (16). Associations between dietary factors and MMR protein expression or *hMLH1* promoter hypermethylation have, to our knowledge, not been previously examined.

In this study, we evaluate associations between dietary factors and the occurrence of MSI, as determined with the Bethesda reference panel markers (17), in a Dutch population-based case-control study of sporadic colon carcinomas. To further explore the relationship between diet and the presence of MSI, we additionally assess associations with MMR protein expression and *hMLH1* promoter hypermethylation.

## Materials and methods

### *Study population*

A population-based case-control study on diet and colon cancer was conducted in the Netherlands between 1989 and 1993. Details were described previously (18). In short, cases (*n*=204) were women and men newly diagnosed with first primary incident colon carcinoma. They were recruited in regional hospitals in the Netherlands and invited to participate by their medical specialists within three months of diagnosis. Sixty percent of those invited agreed to participate. Controls (*n*=259), frequency matched to the cases by age (5 year intervals), sex, region and degree of urbanization, were randomly recruited by the general practitioners of the cases. Of the controls invited, 57% agreed to participate. All subjects were Dutch speaking, Caucasian, up to 75 years old at time of diagnosis, mentally competent to complete the interview, and had no known personal history of cancer, familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer, ulcerative colitis or Crohn's disease. Except for a more favorable Dukes' stage in cases, participants did not differ importantly from non-participants. Formalin-fixed, paraffin-embedded colon tumor tissue, collected before chemo- or radiotherapy started, was available from 185 cases; normal tissue (that is, tumor-free colon tissue) from 159 cases. In 2000, it became known that one of the cases exhibited a germline mutation in one of the mismatch repair genes. This case was excluded from the analyses, leaving a final number of 184 cases.

### *Data collection*

Usual dietary habits were assessed by an interview-based questionnaire. The questionnaire covered the complete dietary pattern. The interval between diagnosis and interview was, for cases, three to six months. The consumption frequency per month in the preceding year (for cases, the year preceding diagnosis or symptoms), number of months during which the item was used, number of portions per consumption and portion sizes of 289 food items were collected. Average daily intake of nutrients was calculated using the Dutch National Food Table (19). The interviewing of cases and controls was balanced over seasons to account for seasonal fluctuations in food patterns. During the interview, information was also obtained on current and previous smoking habits, aspirin and non-steroidal anti-inflammatory drug use, family history of colorectal cancer and personal medical history. Information on the location of the tumors was obtained from pathology reports. TNM tumor stage was determined by re-evaluation of the information in the pathology reports.

### *DNA extraction*

Both tumor and normal DNA were extracted from formalin-fixed, paraffin-embedded tissue as described elsewhere (20). Microdissection was performed and for tumor DNA only those areas containing >60% tumor cells were used; normal DNA was isolated from tumor-free colon tissue.

### *Microsatellite instability*

Paired tumor and normal DNA were analyzed for MSI with the five Bethesda reference panel markers (17): *BAT25*, *BAT26*, *D5S346*, *D2S123*, and *D17S250*. When matching normal DNA was not available (*n*=25; *BAT25/26*-only group), only *BAT25* and *BAT26* were checked for instability. Methods have been discussed in detail elsewhere (21). Tumors were classified as MSI-H if two or more markers showed instability and as MSI-L/MSS if one or none of the markers examined showed instability (17).

### *Immunohistochemical analysis*

All tumors were subjected to immunohistochemical analysis to determine hMLH1 and hMSH2 expression. Immunohistochemical staining was performed on 4μm sections of formalin-fixed, paraffin-embedded tissue using standard procedures. After deparaffinization and rehydration, endogenous peroxidase activity was blocked by immersing the sections in 3% hydrogen peroxide for 30 min. Antigen retrieval was accomplished by boiling for 10 min in 1mM EDTA (pH 8.0) for hMLH1 and in 10mM citrate buffer (pH 6.0) for hMSH2. Nonspecific antibody binding was prevented by pre-incubating the sections with 10% normal horse serum in 1% BSA/PBS for 10 min. Subsequently, sections were incubated overnight at 4°C with monoclonal antibodies against human hMLH1 (clone: G168-15; BD Pharmingen International/Becton Dickinson), dilution 1:100; or, hMSH2 (clone: GB12; Oncogene Research Products), dilution 1:40. Antibody binding was detected using the DAKO Envision™ + System (DAKO corporation) for hMLH1 and the Vectastain® ABC KIT (Brunschwig – Amsterdam) for hMSH2. Diaminobenzidine was used for visualization and sections were counterstained with hematoxylin. Staining was evaluated using normal cells as internal control. Loss of expression was recorded when nuclear staining was present in surrounding normal cells and absent in tumor cells.

### *Methylation analysis*

A PCR-based *Hpa*II- *Msp*I restriction enzyme assay was used to determine hypermethylation of the promoter region of *hMLH1* in MSI-H tumors. The region analyzed, -593 to -312, includes 4 *Hpa*II/*Msp*I sites (at positions -339, -345, -525 and -565, relative to the transcription start site; GenBank accession no. U83845.1). Tumor DNA was digested, in separate reaction tubes, with *Hpa*II (Roche) and *Msp*I (Roche). Additionally a ‘digest’ was performed with $H_2O$ instead of restriction enzyme (‘undigested’). The digests (total volume 10μl) contained 40ng DNA, 1μl of *Hpa*II or *Msp*I or $H_2O$, and 1μl SuRE/Cut Buffer L (Roche). All samples were incubated overnight at 37°C. Subsequently, the samples were subjected to PCR which was performed in a total volume of 50μl containing: 5μl PCR buffer II (Applied Biosystems), 10pmol forward (sequence: 5’GACCAGGCACAGGGCCCCATCGC) and reverse (sequence: 5’ATATCCAGCCAATAGGAGCAGAGATG) primer, 0.3U AmpliTaq Gold® (Applied Biosystems) and the 10μl digested DNA. PCR reaction conditions: 10 min at 94°C, followed by 38

cycles of 40 s at 92°C, 45 s at 58°C, 1 min at 72°C, followed by 5 min at 72°C. The PCR products were loaded (undigested, *Hpa*II, and *Msp*I) on an ethidium bromide stained 2% agarose gel, PCR products originating from the same tumor in adjacent lanes. Tumors were scored positive for *hMLH1* promoter hypermethylation if PCR product was present in the undigested and *Hpa*II-treated but not in the *Msp*I-treated lane, negative if PCR product was present in the undigested lane only. Note, this method does not reveal whether only one or both *hMLH1* alleles are hypermethylated. The upstream region of *hMSH2* was not examined for hypermethylation because *hMSH2* does not seem to be prone to hypermethylation-associated inactivation (10).

### *Statistical analyses*

The distribution of MSI, hMLH1 and hMSH2 expression and *hMLH1* promoter hypermethylation in the colon carcinomas was determined. Differences in (tumor) characteristics between MSI-H and MSI-L/MSS cases were assessed using *t*-tests for continuous and Chi-square tests for categorical variables; *P* values <0.05 were considered significant. The categorization of the dietary factors in tertiles and the interquartile ranges ($Q_3$-$Q_1$; used to quantify associations on a continuous scale) were based on the distribution of intake in the control population. Energy-adjusted nutrient intake was computed, for women and men separately, as the residual from the regression model with total energy as the independent variable and absolute nutrient intake as the dependent variable. Subsequently, the mean nutrient intake was added to each residual (22). Case-control comparisons, comparing cases with a specific tumor status (*e.g*., MSI-H) with the population-based controls, were conducted to estimate the relative risk of developing carcinomas with this particular status. In addition, case-case comparisons were conducted to evaluate heterogeneity in dietary risk factors for the different tumor subsets. Odds ratios (OR) and corresponding 95% confidence intervals (95% CI) were calculated using multiple logistic regression models. Linear trend was assessed using the tertile medians as continuous variables in multiple logistic regression models. All analyses were adjusted for age (years, continuously), sex, body mass index ($kg/m^2$, continuously) and total energy intake (kJ/day, continuously). Alcohol intake was additionally adjusted for cigarette smoking (never, ever). Additional adjustment for TNM stage, tumor location, cigarette smoking and other dietary factors, did not change the estimates significantly (that is, not more than 10%). All analyses were performed with the use of the SAS® statistical software package (SAS version 8.0, SAS Institute Inc., Cary, NC, USA).

## Results

Forty (22%) of the 184 colon carcinomas included in this study were MSI-H. All other tumors showed either instability in one (*n*=20; 11%) or in none (*n*=124; 67%) of the markers and were

classified as MSI-L/MSS. In 10 of the tumors for which matching normal tissue was not available, *BAT25* and *BAT26* were both unstable; both markers were stable in the other 15 tumors. Characteristics of the study population are presented in Table 4.1. No statistically significant differences between cases with MSI-H tumors and cases with MSI-L/MSS tumors were observed. However, body mass index seemed higher and meat intake lower among

**Table 4.1** Characteristics of the study population

| | Controls | Cases | | |
|---|---|---|---|---|
| | | MSI-H | MSI-L/MSS | |
| | (*n*=259) | (*n*=40; 22%) | (*n*=144; 78%) | *P*[a] |
| Age (in years, mean ± SD) | 61.8 ± 10.0 | 61.9 ± 10.1 | 61.8 ± 10.4 | 0.97 |
| Sex (% women) | 47.5 | 45.0 | 45.8 | 0.93 |
| Family history of colorectal cancer (%) | 11.5 | 16.7 | 21.1 | 0.56 |
| Body mass index (kg/m$^2$, mean ± SD) | 26.0 ± 3.8 | 27.3 ± 5.4 | 25.5 ± 4.0 | 0.06 |
| Ever smoked (%) | 69.1 | 67.5 | 70.4 | 0.72 |
| *Dietary factors* (g/day, mean ± SD) | | | | |
| Vegetables | 207.9 ± 124.2 | 168.4 ± 75.4 | 170.6 ± 82.1 | 0.88 |
| Fruit | 230.8 ± 180.6 | 214.7 ± 167.1 | 214.6 ± 160.6 | 0.996 |
| Red meat | 73.9 ± 34.1 | 69.2 ± 38.0 | 81.1 ± 35.2 | 0.07 |
| Fish | 18.2 ± 21.2 | 20.2 ± 26.7 | 22.3 ± 33.8 | 0.72 |
| Dairy products | 268.2 ± 244.3 | 351.0 ± 337.4 | 322.5 ± 330.1 | 0.63 |
| Alcohol[b] | 13.7 ± 17.6 | 18.7 ± 23.6 | 15.9 ± 25.3 | 0.53 |
| Dietary fiber[b] | 28.8 ± 8.3 | 27.6 ± 10.0 | 27.0 ± 8.3 | 0.70 |
| Calcium[b] (mg/day) | 1254.3 ± 406.3 | 1263.4 ± 436.9 | 1275.0 ± 424.4 | 0.88 |
| Vitamin C[b] (mg/day) | 117.5 ± 53.8 | 93.5 ± 42.9 | 105.5 ± 53.8 | 0.19 |
| Total energy intake (kJ/day) | 9362 ± 2844 | 10783 ± 3762 | 10197 ± 3053 | 0.31 |
| *Tumor characteristics* | | | | |
| Tumor location (% proximal) | n.a.[c] | 67.5 | 34.7 | <0.01 |
| TNM stage (% I/II) | n.a. | 75.0 | 58.3 | 0.07 |

[a] MSI-H versus MSI-L/MSS, *t*-test for continuous variables and Chi-square test for categorical variables.
[b] Adjusted for total energy intake by regression analysis, for women and men separately.
[c] n.a.: not applicable.

participants with MSI-H tumors. Proximal tumors (that is, located in cecum, ascending colon, hepatic flexure, or transverse colon) and TNM stage I/II tumors were more common, but the difference of the latter was statistically non-significant, in the MSI-H subset.

Table 4.2 shows results of case-control and case-case comparisons conducted to assess associations between the various dietary factors and MSI-H tumors and MSI-L/MSS tumors. Vegetable intake was inversely associated with MSI-H tumors as well as MSI-L/MSS tumors when the two tumor subsets were separately compared with the population-based controls. Red meat consumption was inversely, but statistically non- significant, associated with MSI-H tumors, and positively, again statistically non-significant, with MSI-L/MSS tumors. Interestingly, case-case comparison showed that red meat consumption was significantly differently related to MSI-H tumors than to MSI-L/MSS tumors. Additional adjustment for vegetable and fruit intake and cigarette smoking did not change the observed associations for red meat consumption significantly. Vitamin C intake showed association patterns similar to those observed for vegetable consumption. Although statistically non-significant, alcohol intake seemed to increase the risk of MSI-H tumors in particular (Table 4.2).

To gain insight in the underlying cause(s) of MSI, expression of hMLH1 and hMSH2 was determined in all tumors by immunohistochemistry and all MSI-H tumors were examined for hypermethylation of the promoter region of *hMLH1*. Twenty-six of the MSI-H tumors showed absence of hMLH1 expression ($MLH1^{neg}$ tumors); six showed absence of hMSH2 expression ($MSH2^{neg}$ tumors); none of the tumors showed absence of both proteins; and, hMLH1 and hMSH2 were both present in all MSI-L/MSS tumors (Table 4.3). Twenty MSI-H tumors exhibited *hMLH1* promoter hypermethylation; hMSH2 was present in all hypermethylated tumors; hMLH1 was present in three hypermethylated tumors (Table 4.3). The *hMLH1* promoter methylation status of three MSI-H tumors could not be determined due to PCR failure. Two of these tumors (including one MSI-H tumor of the *BAT25/26*-only group)

**Table 4.2** Dietary factors and microsatellite instability[a] in sporadic colon carcinomas: case-control and case-case comparisons

| | Odds ratios (95% confidence intervals)[b] | | | | |
|---|---|---|---|---|---|
| | T1 | T2 | T3 | $P_{trend}$ | continuous[c] |
| *Vegetables* (g/day) | ≤166 | 166-223 | >223 | | /106 |
| No. MSI-H/MSS/controls | 20/70/87 | 12/45/87 | 8/29/85 | | 40/144/259 |
| All cases vs. controls | 1.0 | 0.6 (0.4-1.0) | 0.4 (0.2-0.6) | <0.01 | 0.6 (0.5-0.8) |
| MSI-H vs. controls | 1.0 | 0.5 (0.2-1.2) | 0.4 (0.1-0.9) | 0.02 | 0.6 (0.4-0.9) |
| MSS vs. controls | 1.0 | 0.7 (0.4-1.1) | 0.4 (0.2-0.7) | <0.01 | 0.6 (0.5-0.8) |
| MSI-H vs. MSS | 1.0 | 0.9 (0.4-2.0) | 0.8 (0.3-2.2) | 0.67 | 0.9 (0.6-1.5) |
| *Fruit* (g/day) | ≤142 | 142-269 | ≥269 | | /164 |
| No. MSI-H/MSS/controls | 15/51/87 | 15/49/85 | 10/44/87 | | 40/144/259 |
| All cases vs. controls | 1.0 | 0.9 (0.6-1.5) | 0.7 (0.5-1.2) | 0.22 | 0.9 (0.7-1.0) |
| MSI-H vs. controls | 1.0 | 1.0 (0.4-2.3) | 0.6 (0.2-1.4) | 0.21 | 0.8 (0.6-1.2) |
| MSS vs. controls | 1.0 | 0.9 (0.6-1.5) | 0.8 (0.5-1.3) | 0.40 | 0.9 (0.7-1.1) |
| MSI-H vs. MSS | 1.0 | 1.1 (0.5-2.6) | 0.7 (0.3-1.8) | 0.47 | 0.9 (0.7-1.4) |
| *Red meat* (g/day) | <58 | 58-87 | ≥87 | | /43 |
| No. MSI-H/MSS/controls | 16/33/85 | 11/56/87 | 13/55/87 | | 40/144/259 |
| All cases vs. controls | 1.0 | 1.3 (0.8-2.1) | 1.2 (0.7-1.9) | 0.60 | 1.1 (0.8-1.4) |
| MSI-H vs. controls | 1.0 | 0.6 (0.3-1.5) | 0.5 (0.2-1.2) | 0.11 | 0.6 (0.4-1.0) |
| MSS vs. controls | 1.0 | 1.6 (1.0-2.8) | 1.5 (0.9-2.6) | 0.19 | 1.2 (0.9-1.6) |
| MSI-H vs. MSS | 1.0 | 0.3 (0.1-0.8) | 0.3 (0.1-0.9) | 0.03 | 0.5 (0.3-0.9) |
| *Fish* (g/day) | <7 | 7-19 | >19 | | /20 |
| No. MSI-H/MSS/controls | 15/43/82 | 9/41/91 | 16/60/86 | | 40/144/259 |
| All cases vs. controls | 1.0 | 0.8 (0.5-1.3) | 1.1 (0.7-1.7) | 0.62 | 1.1 (0.9-1.2) |
| MSI-H vs. controls | 1.0 | 0.6 (0.2-1.5) | 0.9 (0.4-1.9) | 0.84 | 1.0 (0.7-1.3) |
| MSS vs. controls | 1.0 | 0.8 (0.5-1.4) | 1.2 (0.7-2.0) | 0.43 | 1.1 (0.9-1.3) |
| MSI-H vs. MSS | 1.0 | 0.6 (0.2-1.6) | 0.6 (0.3-1.4) | 0.27 | 0.9 (0.7-1.1) |
| *Dairy products* (g/day) | ≤117 | 117-305 | >305 | | /287 |
| No. MSI-H/MSS/controls | 12/45/87 | 12/48/86 | 16/51/86 | | 40/144/259 |
| All cases vs. controls | 1.0 | 1.0 (0.6-1.6) | 1.0 (0.6-1.6) | 0.87 | 1.1 (0.9-1.4) |
| MSI-H vs. controls | 1.0 | 0.9 (0.4-2.3) | 1.1 (0.5-2.7) | 0.70 | 1.2 (0.8-1.7) |
| MSS vs. controls | 1.0 | 1.0 (0.6-1.6) | 0.9 (0.5-1.6) | 0.77 | 1.1 (0.9-1.4) |
| MSI-H vs. MSS | 1.0 | 1.0 (0.4-2.5) | 1.3 (0.5-3.2) | 0.54 | 1.1 (0.8-1.5) |

| *Table 4.2 cont.* | T1 | T2 | T3 | $P_{trend}$ | continuous[c] |
|---|---|---|---|---|---|
| *Alcohol*[d] (g/day) | <3.8 | 3.8-12.9 | >12.9 | | /18.4 |
| No. MSI-H/MSS/controls | 12/57/86 | 10/31/87 | 18/56/86 | | 40/144/259 |
| All cases vs. controls | 1.0 | 0.8 (0.5-1.3) | 1.2 (0.7-1.9) | 0.30 | 1.1 (1.0-1.4) |
| MSI-H vs. controls | 1.0 | 1.2 (0.5-3.2) | 1.9 (0.8-4.7) | 0.13 | 1.2 (0.9-1.7) |
| MSS vs. controls | 1.0 | 0.7 (0.4-1.2) | 1.0 (0.6-1.8) | 0.49 | 1.1 (0.9-1.4) |
| MSI-H vs. MSS | 1.0 | 1.7 (0.6-4.6) | 1.7 (0.7-4.1) | 0.36 | 1.1 (0.9-1.4) |
| *Dietary fiber*[d] (g/day) | <25 | 25-31 | >31 | | /10.3 |
| No. MSI-H/MSS/controls | 17/69/86 | 11/32/87 | 12/43/86 | | 40/144/259 |
| All cases vs. controls | 1.0 | 0.5 (0.3-0.8) | 0.6 (0.4-1.0) | 0.03 | 0.7 (0.6-1.0) |
| MSI-H vs. controls | 1.0 | 0.8 (0.3-1.8) | 0.7 (0.3-1.7) | 0.48 | 0.8 (0.5-1.3) |
| MSS vs. controls | 1.0 | 0.5 (0.3-0.8) | 0.6 (0.3-1.0) | 0.03 | 0.7 (0.5-0.9) |
| MSI-H vs. MSS | 1.0 | 1.6 (0.6-3.9) | 1.2 (0.5-2.9) | 0.71 | 1.1 (0.7-1.8) |
| *Calcium*[d] (mg/day) | ≤1062 | 1062-1358 | >1358 | | /458.3 |
| No. MSI-H/MSS/controls | 12/48/87 | 13/35/86 | 15/61/86 | | 40/144/259 |
| All cases vs. controls | 1.0 | 0.9 (0.6-1.5) | 1.3 (0.8-2.1) | 0.22 | 1.0 (0.8-1.3) |
| MSI-H vs. controls | 1.0 | 1.4 (0.6-3.4) | 1.5 (0.6-3.5) | 0.42 | 1.1 (0.7-1.5) |
| MSS vs. controls | 1.0 | 0.8 (0.5-1.4) | 1.3 (0.8-2.2) | 0.24 | 1.0 (0.8-1.3) |
| MSI-H vs. MSS | 1.0 | 1.8 (0.7-4.5) | 1.1 (0.4-2.6) | 0.99 | 1.0 (0.7-1.5) |
| *Vitamin C*[d] (mg/day) | ≤89 | 89-133 | >133 | | /68.5 |
| No. MSI-H/MSS/controls | 17/62/87 | 18/49/86 | 5/33/86 | | 40/144/259 |
| All cases vs. controls | 1.0 | 0.9 (0.5-1.4) | 0.5 (0.3-0.8) | <0.01 | 0.7 (0.5-0.9) |
| MSI-H vs. controls | 1.0 | 1.2 (0.5-2.5) | 0.3 (0.1-0.8) | 0.03 | 0.5 (0.3-0.8) |
| MSS vs. controls | 1.0 | 0.8 (0.5-1.3) | 0.5 (0.3-0.9) | 0.02 | 0.7 (0.6-1.0) |
| MSI-H vs. MSS | 1.0 | 1.3 (0.6-2.9) | 0.5 (0.2-1.6) | 0.38 | 0.7 (0.4-1.2) |

[a] The subset MSI-L/MSS is, for shortness, called MSS in this table. [b] Adjusted for age, sex, body mass index and total energy intake. Alcohol is additionally adjusted for cigarette smoking. Trend was assessed using the median values of the tertiles as continuous variables. [c] Per interquartile range ($Q_3$-$Q_1$). [d] Adjusted for total energy intake by regression analysis, for women and men separately.

showed absence of hMLH1 expression; hMLH1 and hMSH2 were both present in the other tumor. Furthermore, six of the 10 MSI-H tumors of the *BAT25/26*-only group showed absence of hMLH1 expression; hMLH1 and hMSH2 were both present in the other four tumors. Four of

the MSI-H tumors of the *BAT25/26*-only group exhibited *hMLH1* promoter hypermethylation, hMLH1 was present in one of these tumors. MSI-H tumors with hypermethylated *hMLH1* and hMLH1 not present were classified as Methyl$^{+}$ tumors (*i.e.*, Methyl$^{+}$ tumors are a subset of the MLH1$^{neg}$ tumors) (*n*=17); MSI-H tumors without hypermethylated *hMLH1* were classified as Methyl$^{-}$ tumors (*n*=17).

**Table 4.3** Expression of hMLH1 and hMSH2, and *hMLH1* promoter methylation status[a]

| | | | | Hypermethylation | |
|---|---|---|---|---|---|
| *n* (%) | All tumors (*n*=184) | MSI-H (*n*=40) | MSI-L/MSS (*n*=144) | Yes (*n*=20) | No (*n*=17) |
| hMLH1 absent | 26 (14) | 26 (65) | 0 (0) | 17 (85) | 7 (41) |
| hMSH2 absent | 6 (3) | 6 (15) | 0 (0) | 0 (0) | 6 (35) |
| hMLH1 and hMSH2 present | 152 (83) | 8 (20) | 144 (100) | 3 (15) | 4 (24) |

[a] The *hMLH1* promoter methylation status of three MSI-H tumors could not be determined.

Subsequently, we assessed associations (quantified on a continuous scale) between the various dietary factors and absence of hMLH1 expression and *hMLH1* promoter methylation status. Associations observed with MLH1$^{neg}$ tumors generally did not differ significantly from those observed with MSI-H tumors (data not shown). We were unable to separately evaluate associations with absence of hMSH2 expression as only six tumors in our study population exhibited this phenotype. Fruit consumption was inversely associated with Methyl$^{+}$ tumors and positively with Methyl$^{-}$ tumors when the two subsets were separately compared with the population-based controls (Methyl$^{+}$, OR: 0.4, 95% CI: 0.2-0.9; Methyl$^{-}$, OR: 1.2, 95% CI: 0.8-1.7; per 164g/day). Case-case comparison showed that fruit consumption was significantly differently related to Methyl$^{+}$ than to Methyl$^{-}$ tumors (Methyl$^{+}$ versus Methyl$^{-}$, OR: 0.2, 95% CI: 0.1-0.9). Vegetable consumption and red meat intake were both inversely associated with Methyl$^{+}$ tumors as well as Methyl$^{-}$ tumors [Vegetables (per 106g/day): Methyl$^{+}$ versus controls,

OR: 0.5, 95% CI: 0.3-1.0; Methyl$^-$ versus controls, OR: 0.6, 95% CI: 0.3-1.1. Red meat (per 43g/day): Methyl$^+$ versus controls, OR: 0.4, 95% CI: 0.2-.09; Methyl$^-$ versus controls, OR: 0.5, 95% CI: 0.3-1.0]. They were, like most other evaluated dietary factors, not specifically associated with Methyl$^+$ tumors or Methyl$^-$ tumors but seemed to affect both pathways to MSI-H tumors equally (data not shown).

## Discussion

In this study, we evaluated associations between dietary factors reported previously to be associated with colon cancer risk and occurrence of MSI, hMLH1 expression and *hMLH1* promoter hypermethylation in sporadic colon carcinomas. Red meat intake was significantly differently related to MSI-H tumors than to MSI-L/MSS tumors. A positive association was observed with MSI-L/MSS tumors while an inverse association was observed with MSI-H tumors. Alcohol intake seemed to increase the risk of MSI-H tumors in particular. Interestingly, fruit consumption was significantly differently related to Methyl$^+$ tumors than to Methyl$^-$ tumors, suggesting differences in the role fruit intake plays in the etiology of these two distinct MSI-H colon carcinoma subsets. Vegetable consumption lowered the risk of MSI-H tumors as well as MSI-L/MSS tumors but like most other evaluated dietary factors was not distinctively associated with a specific MSI or *hMLH1* promoter methylation status.

Overall, 22% of the 184 sporadic colon carcinomas was MSI-H. This is consistent with frequencies reported previously (5, 7, 15). MSI-H tumors were more common in the *BAT25/26*-only group than in the group for which matching normal tissue was available. However, this is probably due to chance and we do not expect that our decision to use only *BAT25* and *BAT26* tumor results when matching normal tissue was not available has resulted in extensive misclassification or lead to serious misinterpretation of our results. Six of the 10 MSI-H tumors

of the *BAT25/26*-only group also showed loss of hMLH1 expression. Inclusion of the 4 tumors that expressed hMLH1 as well hMSH2 in the MSI-L/MSS group instead of the MSI-H group did not change the observed associations significantly (data not shown).

Immunohistochemistry showed that most (65%) MSI-H tumors in our study population had an *hMLH1*-associated etiology. In 17 (71%) of the 24 $MLH1^{neg}$ tumors in which promoter methylation status could be determined, *hMLH1* was inactivated by promoter hypermethylation. This is in line with what has been reported for sporadic colon cancers by others (5, 9-11, 23). MSI-H as determined with the Bethesda reference panel had a 100% sensitivity for identifying colon tumors having hMLH1 or hMSH2 loss of expression. HMLH1 and hMSH2 were both present in 8 of the MSI-H tumors. Possibly these tumors expressed altered, nonfunctional hMLH1 or hMSH2 protein that could be detected by immunohistochemistry (24), or the MSI may have been the result of alterations in one of the other MMR genes. Regarding the three MSI-H tumors with hMLH1 and hMSH2 expression that showed hypermethylation of the *hMLH1* promoter, hypermethylation possibly affected only one of the two *hMLH1* alleles in these tumors, or it affected some CpG sites but left other sites intact whose methylation might be necessary for inactivation of *hMLH1* (25).

As in any retrospective case-control study, the possibility of information and selection bias is an important concern. Cases and controls were asked to recall their diets from the past and differential recall is possible. However, since cases are unaware of the, for instance, MSI-status of their tumors, systematic errors in recall are less likely to bias results from case-case comparisons. Our cases were relatively healthy. That is, the frequency of TNM stage I/II tumors among the cases was relatively high, 62%, compared with the frequency reported by the Dutch Cancer Registry, 51% (26).

In this study, red meat intake was significantly differently related to MSI-H tumors than to MSI-L/MSS tumors. It increased the risk of MSI-L/MSS tumors, whereas an inverse

association was observed with MSI-H tumors. Red meat prepared at high temperatures is a major source of heterocyclic amines. Heterocyclic amines are bulky-adduct-forming agents. They are mutagenic and carcinogenic in animals (27) and those present in red meat have been found associated with increased risk of colorectal cancer in humans (28). A possible explanation, admittedly speculative, for the observed associations with red meat intake is that genes involved in the pathway that results in MSI-L/MSS tumors, *e.g.*, *APC* (29, 30), are more susceptible to mutations caused by red meat consumption than *hMLH1* and *hMSH2*, and/or that 'red meat' mutations in MSI-L/MSS pathway-related genes exert a higher selective growth advantage. In addition, most MSI-L/MSS tumors probably exhibit CIN (12) thus, viewing red meat consumption as a surrogate marker for exposure to heterocyclic amines, our results are in line with the observations of Bardelli *et al*. (14).

Previously, Slattery *et al*. (15) observed no associations between red meat intake and MSI status of colon carcinomas in a large population-based case-control study. Wu *et al*. (16) reported a positive association between well-done red meat consumption and MSI-H tumors in a case-only study but did not observe a significant association with red meat intake in general. The dissimilarities with our results might be caused by differences in meat cooking methods and/or differences in the composition of the study populations, *e.g.*, in the frequency of participants with a positive family history of colorectal cancer, or chance.

None of the other dietary factors evaluated in this study was significantly differently related to MSI-H tumors than to MSI-L/MSS tumors. Consistent with Slattery *et al*. (15), alcohol intake did seem more positively associated with MSI-H tumors than with MSI-L/MSS tumors. In our study, however, the associations were statistically non-significant. This may be due to the smaller size of our study population.

This is, to our knowledge, the first study that has evaluated associations between dietary factors and *hMLH1* promoter hypermethylation in colon carcinomas. Fruit consumption was

significantly differently related to Methyl$^{+}$ tumors than to Methyl$^{-}$ tumors. It decreased the risk of Methyl$^{+}$ tumors, whereas a positive association was observed with Methyl$^{-}$ tumors. This suggests, assuming that epigenetically silenced *hMLH1* and genetically inactivated hMLH1 or hMSH2 exert the same selective advantage for MSI-H tumor formation, that fruits, or their constituents, are specifically involved in the prevention of *hMLH1* promoter hypermethylation. The exact mechanisms responsible for silencing of specific genes by promoter hypermethylation are not (yet) clear. However, fruits may be interrelated with DNA methylation through involvement in the supply of methyl groups and/or through involvement in processes that modify utilization of methyl groups, *e.g.*, regulation of DNA methyltransferase activity.

To conclude, our data suggest that, in subjects not suspected of carrying an inherited mutation in one of the MMR genes, red meat consumption may promote the development of MSI-L/MSS carcinomas in particular, whereas alcohol intake appears to increase the risk of MSI-H tumors. Fruit consumption may especially decrease the risk of MSI-H carcinomas exhibiting epigenetically silenced *hMLH1*. Our study population was relatively small and, hence, the statistical power of the study was relatively low. This may explain why only a few statistically significant associations were observed. Although all evaluated dietary factors were reported previously to be associated with colon cancer risk, it should additionally be noted that multiple comparisons might lead to chance findings. Thus, confirmation of our results by other studies is necessary. Nonetheless, the observed relation between fruit consumption and *hMLH1* promoter methylation status is intriguing and calls for further investigation. Epigenetic events such as *hMLH1* promoter hypermethylation are, by definition, susceptible to change. Elucidation of the mechanisms through which dietary factors influence (gene-specific) epigenetic events may prove useful for the development of effective dietary intervention strategies for colon cancer prevention.

## Acknowledgements

We thank Winny van Geloof and Riki Willems for technical assistance; Dr. Han van Krieken for secondary review of the immunohistochemical analyses; Dr. Iris Nagtegaal for assistance with tumor stage re-evaluation; and the Pathology Departments of the following hospitals for providing tissue: Canisius-Wilhelmina Hospital, Nijmegen; UMC Utrecht, Utrecht; St Antonius Hospital, Nieuwegein; and Eemland Hospital, Amersfoort. The initial case-control study was conducted in collaboration with regional hospitals and cancer registries, and with financial support from the Dutch Dairy Foundation on Nutrition and Health. This study was supported by a grant from the Dutch Cancer Society (LUW 98-1687).

## References

1. Aaltonen, L.A., Peltomäki, P., Leach, F., Sistonen, P., Pylkkänen, L., Mecklin, J-P., Järvinen, H., Powell, S.M., Jen, J., Hamilton, S.R., Petersen, G.M., Kinzler, K.W., *et al*. (1993) Clues to the pathogenesis of familial colorectal cancer. *Science*, **260**, 812-816.
2. Thibodeau, S.N., Bren, G., and Schaid, D. (1993) Microsatellite instability in cancer of the proximal colon. *Science*, **260**, 816-819.
3. Ionov, Y., Peinado, M.A., Malkhosyan, S., Shibata, D., and Perucho, M. (1993) Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature*, **363**, 558-561.
4. Aaltonen, L.A., Peltomäki, P., Mecklin, J-P., Järvinen, H., Jass, J.R., Green, J.S., Lynch, H.T., Watson, P., Tallqvist, G., Juhola, M., Sistonen, P., Hamilton, S.R., *et al*. (1994) Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. *Cancer Res*., **54**, 1645-1648.
5. Cunningham, J.M., Kim, C-Y, Christensen, E.R. Tester, D.J., Parc, Y., Burgart, L.J., Halling, K.C., McDonnell, S.K., Schaid, D.J., Walsh-Vockley, C. Kubly, V., Nelson, H., *et al*. (2001) The frequency of hereditary defective mismatch repair in a prospective series of unselected colorectal carcinomas. *Am. J. Hum. Genet*., **69**, 780-790.
6. Dietmaier, W., Wallinger, S., Bocker, T., Kullman, F., Fishel, R., and Rüschoff, J. (1997) Diagnostic microsatellite instability: definition and correlation with mismatch repair expression. *Cancer Res*., **57**, 4749-4756.
7. Thibodeau, S.N., French, A.J., Cunningham, J.M., Tester, D., Burgart, L.J., Roche, P.C., McDonnel, S.K., Schaid, D.J., Walsh-Vockley, C., Michels, V.V., Farr, G.H., and O'Connell, M.J. (1998) Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of *hMLH1*. *Cancer Res*., **58**,1713-1718.

8. Lindor, N.M., Burgart, L.J., Leontovich, O., Goldberg, R.M., Cunningham, J.M., Sargent, D.J., Walsh-Vockley, C., Petersen, G.M., Walsh, M., Leggett, B.A., Young, J.P., Barker, M.A., *et al*. (2002) Immunohistochemistry versus microsatellite instability testing in phenotyping colorectal tumors. *Journal Clin. Oncology*, **20**, 1043-1048.
9. Kane, M.F., Loda, M., Gaida, G.M., Lipman , J., Mishra, R., Goldman, H., Jessup, J.M., and Kolodner, R. (1997) Methylation of the *hMLH1* promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res*., ***57***, 808-811.
10. Cunningham, J.M., Christensen, E.R., Tester, D.J., Kim, C-Y, Roche, P.C., Burgart, L.J., and Thibodeau, S.N. (1998) Hypermethylation of the *hMLH1* promoter in colon cancer with microsatellite instability. *Cancer Res*., **58**, 3455-3460.
11. Herman, J.G., Umar, A. Polyak, K., Graff, J.R., Ahuja, N., Issa, J.P., Markowitz, S., Willson, J.K., Hamilton, S.R., Kinzler, K.W., Kane, M.F., Kolodner, R.D., *et al*. (1998) Incidence and functional consequences of *hMLH1* promoter hypermethylation in colorectal carcinoma. *Proc. Natl. Acad. Sci. USA*, **95**, 6870-6875.
12. Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1998) Genetic instabilities in human cancers. *Nature*, **396**, 643-648.
13. Breivik, J, and Gaudernack, G. (1999) Carcinogenesis and natural selection: a new perspective to the genetics and epigenetics of colorectal cancer. *Adv. Cancer Res*., **76**, 187-212.
14. Bardelli, A., Cahill, D.P., Lederer, G., Speicher, M.R., Kinzler, K.W., Vogelstein, B., and Lengauer, C. (2001) Carcinogen-specific induction of genetic instability. *Proc. Natl. Acad. Sci. USA*, **98**, 5770-5775.
15. Slattery, M.L., Anderson, K., Curtin, K., Ma, K-N, Schaffer, D., and Samowitz, W. (2001) Dietary intake and microsatellite instability in colon tumors. *Int. J. Cancer*, **93**, 601-607.
16. Wu, A.H., Shibata, D., Yu, M. C., Lai, M-Y, and Ross, R.K. (2001) Dietary heterocyclic amines and microsatellite instability in colon adenocarcinomas. *Carcinogenesis*, **22**, 1681-1684.
17. Boland, C.R., Thibodeau ,S.N., Hamilton, S.R., Sidransky, D., Eshleman, J.R., Burt, R.W., Meltzer, S.J., Rodrigues-Bigas, M.A., Fodde, F., Ranzani, G.N., and Srivastava, S. (1998) A National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res*., **58**, 5248-5257.
18. Kampman, E., Van ‘t Veer, P., Hiddink, G.J., Van Aken-Schneijder, P., Kok, F.J., and Hermus, R.J.J. (1994) Fermented dairy products, dietary calcium and colon cancer: a case-control study in the Netherlands. *Int. J. Cancer*, **59**, 170-176.
19. Voorlichtingsbureau voor de Voeding. Nevo Tabel. (1990) *Dutch Food Composition Table 1989-1990*. Stichting Nevo, The Hague, The Netherlands.

20. Voskuil, D.W., Kampman, E., Van Kraats, A.A., Balder, H.F., Van Muijen, G.N.P., Goldbohm, R.A., and Van ‘t Veer, P. (1999) P53 overexpression and *p53* mutations in colon carcinomas: relation to dietary risk factors. *Int. J. Cancer*, **81**, 675-681.
21. Diergaarde, B., Vrieling, A., Van Kraats, A.A., Van Muijen, G.N.P., Kok, F.J., and Kampman, E. (2003) Cigarette smoking and genetic alterations in sporadic colon carcinomas. *Carcinogenesis*, **24**, 565-571.
22. Willett, W.C., Howe, G.R., and Kushi, L.H. (1997) Adjustment for total energy intake in epidemiologic studies. *Am. J. Clin. Nutr*., **65** *(suppl)*, 1220S-8S.
23. Kuismanen, S.A., Holmberg, M.T., Slovaara, R., De la Chapelle, A, and Peltomäki, P. (2000) Genetic and epigenetic modification of *MLH1* accounts for a major share of microsatellite-unstable colorectal cancers. *Am. J. Pathology*, **156**, 1773-1778.
24. Wahlberg, S.S., Schmeits, J., Thomas, G., Loda, M., Garber, J., Syngal, S., Kolodner, R.D., and Fox, E. (2002) Evaluation of microsatellite instability and immunohistochemistry for the prediction of germ-line *MSH2* and *MLH1* mutations in hereditary nonpolyposis colon cancer. *Cancer Res*., **62**, 3485-3492.
25. Deng, G., Chen, A., Hong, J., Chae, H.S., and Kim, Y.S. (1999) Methylation of CpG in a small region of the *hMLH1* promoter invariably correlates with the absence of gene expression. *Cancer Res*., **59**, 2029-2033.
26. Coebergh, J.W.W., Van der Heijden, L.H., and Janssen-Heijnen, M.L.G. (1995) *Cancer incidence and survival in the southeast of the Netherlands*. Comprehensive Cancer Centre South, Eindhoven.
27. Wakabayashi, K, Nagao, M, Esumi, H, and Sugimura, T. (1992) Food derived mutagens and carcinogens. *Cancer Res*., **52**, 2092s-2098s.
28. Nowel, S., Coles, B., Sinha, R., Macleod, S., Ratnasinghe, D.L., Stotts, C., Kadlubar, F.F., Ambrosone, C.B., and Lang, N.P. (2002) Analysis of total meat intake and exposure to individual heterocyclic amines in a case-control study of colorectal cancer: contribution of metabolic variation to risk. *Mut. Res*., **506-507**, 175-185.
29. Heinen, C.D., Richardson, D., White, R., and Groden, J. (1995) Microsatellite instability in colorectal adenocarcinoma cell lines that have full-length adenomatous polyposis coli protein. *Cancer Res*., **55**, 4797-4799.
30. Salahshor, S., Kressner, U., Pahlman, L., Glimelius, B., Lindmark, G., and Lindblom, A. (1999) Colorectal cancer with and without microsatellite instability involves different genes. *Genes Chromosomes Cancer*, **26**, 247-252.

# Chapter 5

# Cigarette smoking and genetic alterations in sporadic colon carcinomas

Brenda Diergaarde

Alina Vrieling

Annemieke A. van Kraats

Goos N. P. van Muijen

Frans J. Kok

Ellen Kampman

***Carcinogenesis*, March 2003; 24 (3): 565-571**

## Abstract

Cigarette smoking has been inconsistently associated with colon cancer risk. To evaluate the hypothesis that smoking is primarily linked to a specific colon tumor subgroup(s), we assessed associations between smoking and the occurrence of mutations in the *APC*, K-*ras* and *p53* genes, p53 overexpression, and microsatellite instability (MSI) in a Dutch population-based case-control study on sporadic colon carcinomas. The study population consisted of 176 cases and 249 controls. Smoking status (never, ever), number of cigarettes smoked per day (never, <15, ≥15), total years of smoking (never, ≤30, >30), and years since first started smoking (never, ≤35, >35) were all evaluated in this study. Cigarette smoking status was significantly different related to p53 overexpression-positive ($p53^{pos}$) tumors compared to p53 overexpression-negative ($p53^{neg}$) tumors (ever versus never, OR: 0.4, 95% CI: 0.2-0.9), as well as to tumors with transversion mutations in *APC*, K-*ras* or *p53* ($transv^{+}$) compared to tumors without transversion mutations in one of these genes ($transv^{-}$) (ever versus never, OR: 2.5, 95% CI: 1.0-5.9). Positive associations were observed with $p53^{neg}$ tumors and $transv^{+}$ tumors when compared to the population-based controls (ever versus never, OR: 1.5, 95% CI: 0.9-2.8; OR: 2.2, 95% CI: 0.9-5.6, respectively), inverse associations with $p53^{pos}$ tumors and $transv^{-}$ tumors (ever versus never, OR: 0.5, 95% CI: 0.3-1.0; OR: 0.8, 95% CI: 0.5-1.3, respectively). Similar patterns of association were observed for the other smoking variables evaluated. In addition, although non-significant, smoking was more notably, positively, associated with tumors that exhibit K-*ras* mutations, especially K-*ras* transversion mutations, than with tumors without K-*ras* mutations. An inverse relationship between smoking and the occurrence of *APC* mutations was suggested while no clear associations were observed with MSI. Our data suggest that smoking-related colon cancers develop through a p53 overexpression-negative pathway and that smoking particularly results in colon carcinomas with transversion mutations.

## Introduction

Cigarette smoking has been consistently associated with colorectal adenomas, the precursor lesions of most colon carcinomas. Most studies observed a two- to threefold increased risk of adenomas for long-term, heavy cigarette smoking (1). The association of smoking with colon cancer risk has, however, not been consistent (1). A possible explanation for this discrepancy is that cigarette smoking is only involved in the production of certain types of mutations in specific

genes and, thus, primarily affects, and results in, the development of a particular subset(s) of colon carcinomas.

Mutations in the *APC*, K-*ras* and *p53* genes as well as microsatellite instability (MSI) are commonly observed genetic alterations in colon cancer. Mutations in *APC* (that is, those mutations resulting in loss of APC function) are believed to be a key initiating event in colon cancer (2-4). K-*ras* mutations are thought to accompany the conversion of small to larger adenomas and have been reported to occur in 30-40% of sporadic colon tumors (5, 6), whereas mutation of *p53* seems to be especially important in later stages of colon tumorigenesis (7). MSI occurs in most colon tumors associated with the hereditary nonpolyposis colorectal cancer syndrome and in approximately 10-20% of the sporadic colon tumors (8-11).

Interestingly, significant inverse relationships have been observed between the presence of MSI and mutations in *APC*, K-*ras* and *p53* in sporadic colon tumors (12, 13). This suggests different molecular pathways to colon cancer which in turn may reflect different environmental exposures. Supporting this idea, Bardelli *et al.* (14) demonstrated recently that exposure to specific carcinogens can indeed select for colon tumor cells with distinct forms of genetic alterations. Regarding smoking, K-*ras* (15, 16) and *p53* mutations (17, 18), G→T transversions in particular, are significantly more prevalent in lung cancer from smokers than in lung cancer from non-smokers suggesting an etiological link between exposure to tobacco smoke carcinogens and these genetic alterations.

Few studies have examined associations between smoking and genetic alterations in colon cancer. Those that have (6, 19, 20, 21) generally limited their analyses to alterations in one specific gene. Intriguingly, Freedman *et al.* (19), who used p53 overexpression as an indicator of *p53* mutations, observed an increased risk of p53 overexpression-negative colon tumors for smokers. Slattery *et al.* (20) and Yang *et al*. (21) both reported a positive association between

cigarette smoking and sporadic colon tumors with MSI. To (further) explore the hypothesis that cigarette smoking is primarily associated with a specific colon tumor subgroup(s), in this study, we assess the associations between smoking and the occurrence of (specific) mutations in the *APC*, K-*ras* and *p53* genes, p53 overexpression, and MSI in sporadic colon carcinomas.

## Material and methods

### *Study population*

A population-based case-control study on diet and colon cancer was conducted in the Netherlands between 1989 and 1993. Details were described previously (22, 23). Briefly, cases (*n*=204) were women and men newly diagnosed with histologically confirmed, first primary incident colon carcinoma. They were recruited in regional hospitals located in the eastern and central regions of the Netherlands and invited to participate by their medical specialists within three months of diagnosis. Cancer registries were used to check for completeness. Of all eligible cases diagnosed in the cooperating hospitals, 47% were invited to participate. Sixty percent of those invited, agreed to participate. Controls (*n*=259), frequency matched to the cases by age (5 year intervals), sex, region and degree of urbanization, were randomly recruited by the general practitioners of the cases. Of the controls invited, 57% agreed to participate. All subjects were Dutch speaking, Caucasian, up to 75 years old at time of diagnosis, mentally competent to complete the interview, and had no known personal history of cancer, familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer, ulcerative colitis, or Crohn's disease. Except for a more favorable Dukes' stage among cases, participants did not differ importantly from non-participants. Cigar and/or pipe only smokers (*n*=18) and two subjects with missing data on smoking status were excluded from the analyses. From 18 cases, colon tumor tissue could not be obtained due to administrative reasons. In total, 176 cases and 249 controls were included in the analyses here presented.

### *Data collection*

Participants were interviewed in their own homes by trained dieticians using a structured questionnaire. The interval between diagnosis and the interview was, for cases, three to six months. Cigarette smoking status (never, ever, ex, current) was determined. Participants who had stopped smoking one year or more prior to the date of the interview were classified as ex-smokers; participants who had stopped less than one year prior to the interview date or were still smoking were classified as current smokers. Information about the number of years smoked and the number of cigarettes usually smoked per day (categorized in four categories: 1-<5, 5-<15, 15-<25, and 25 or more cigarettes) was obtained. Years since first started

smoking was calculated from information on duration of smoking and, if applicable, time since stopped smoking. The interview also consisted of a dietary history part in which information on the frequency and amount of foods consumed in the year prior to the interview (for cases, the year preceding diagnosis or complaints) was collected. Information on aspirin and non-steroidal anti-inflammatory drug use, family history of colorectal cancer, and medical history was also obtained during the interview.

### *DNA extraction*

Both tumor and normal DNA were extracted from formalin-fixed, paraffin-embedded colon tissue, collected before chemo- or radiotherapy started, as described elsewhere (24). Microdissection was performed and for tumor DNA only those areas containing >60% tumor cells were used. Corresponding normal DNA was isolated from tumor-free colon tissue.

### *APC mutation detection*

Single-strand conformation polymorphism (SSCP) analysis was used to screen the *APC* gene for mutations. The majority of the somatic mutations in *APC* seem to cluster within a small region in exon 15 (codons 1286-1513), the so-called mutation cluster region (MCR) (25-27). Our analysis covered codons 1286 to1585 (extended-MCR) of *APC*. The region was divided into five, ~220 base pairs long, overlapping fragments (codons 1286-1358, 1337-1404, 1387-1455, 1437-1526, and 1509-1585, respectively) which were separately amplified in two consecutive PCRs using the following primer sets (primer sequence: 5'→3'):

| Fragment 1: | | codon: |
|---|---|---|
| 1.1 F-CAGACTTATTGTGTAGAAG | R-CGCTCCTGAAGAAAATTCAAG | 1260-1358 |
| 1.2 F-GAAATAGGATGTAATCAGACG | R-CGCTCCTGAAGAAAATTCAAC | 1286-1358 |
| Fragment 2: | | |
| 2.1 F-ACTGCAGGGTTCTAGTTTATC | R-TCTGCTTGGTGGCATGGTTT | 1337-1436 |
| 2.2 F-ACTGCAGGGTTCTAGTTTATC | R-GAGCTGGCAATCGAACGACT | 1337-1404 |
| Fragment 3: | | |
| 3.1 F-CTCAGACACCCAAAAGTCC | R-ATTTTTAGGTACTTCTCGCTTG | 1366-1455 |
| 3.2 F-TACTTCTGTCAGTTCACTTGATA | R-ATTTTTAGGTACTTCTCGCTTG | 1387-1455 |
| Fragment 4: | | |
| 4.1 F-AAACACCTCCACCACCTCC | R-TCATTCCCATTGTCATTTTCC | 1437-1536 |
| 4.2 F-AAACACCTCCACCACCTCC | R-GCATTATTCTTAATTCCACATC | 1437-1526 |
| Fragment 5: | | |
| 5.1 F-ACTCCAGATGGATTTTTCTTG | R-GGCTGGCTTTTTGCTTTAC | 1497-1596 |
| 5.2 F-GAGCCTCGATGAGCCATTTA | R-TGTTGGCATGGCAGAAATAA | 1509-1585 |

PCR reaction mixtures (total volume of 50µl) contained: 50ng DNA (or 2µl of the 1:100 diluted product of the first PCR), 0.2µM of both primers, 0.2mM dNTPs, 10mM Tris-HCL pH 9.0, 1.5 to 2.5mM $MgCl_2$, 50mM KCl, 0.01% Tween, 10% glycerol, and 0.3U Taq DNA polymerase. Reaction conditions first PCR: 25 cycles of 30 s at 94°C, 45 s at 55°C (53 °C for primer set 1.1; 57°C for 4.1), 1 min at 72°C, followed by 5 min at 72°C. Conditions second PCR: 30 cycles of 30 s at 94°C, 45 s at 52°C (56°C for primer sets 3.2 and 4.2; 57°C for 5.2), 1 min at 72°C, followed by 5 min at 72°C. Products were checked using an ethidium bromide stained 2% agarose gel. SSCP was performed as described earlier (24) with electrophoresis at 10 and 18°C. The original PCR products from the samples that displayed an abnormal pattern in the SSCP were subjected to sequencing in both directions using the same primers as in the second PCR. Sequencing was performed as described previously (24). Mutation analysis started in all samples with fragment 1, and only if no truncating mutations (*i.e.*, nonsense or frameshift mutations) were detected, fragment 2 was screened for mutations, and so on. We focused on truncating mutations because these mutations indisputably result in function loss of the APC protein whereas the biological significance of missense mutations in *APC* is uncertain. Carcinomas were classified as $APC^+$ (with a truncating mutation in the extended-MCR of *APC*), or $APC^-$ (without a truncating mutation in the extended-MCR of *APC* and all five fragments completely analyzed for mutations).

### *K-ras mutation detection*

Codons 12 and 13 of the K-*ras* gene were examined for mutations (*i.e.*, those resulting in amino acid change) by mutant allele-specific amplification as described earlier (28).

### *p53 mutation detection*

To enable the evaluation of (specific) *p53* mutations, SSCP analysis was used to screen exons 5-8 of the *p53* gene for mutations as described previously (24). We focused on exons 5-8 as it has been observed that most *p53* mutations occur in this region of the gene (29). The original PCR products from the samples that displayed an abnormal pattern in the SSCP were subjected to sequencing in both directions. Samples in which a mutation (*i.e.*, one that resulted in amino acid change or truncation of the protein) was detected were excluded from analysis of the subsequent exons. The exons were screened in the following order: 7, 8, 5 and 6. Carcinomas were classified as $p53^+$ (with a mutation in codons 5-8 of *p53*), or $p53^-$ (without a mutation in codons 5-8 of *p53* and all 4 codons completely analyzed for mutations).

### *p53 immunohistochemistry*

Overexpression of p53 was determined using a mixture of two antibodies (DO-7 which recognizes both mutant and wildtype forms of p53, and PAb 240 which recognizes only mutant forms of p53) as

published earlier (24). Stained sections were scored independently by two investigators (A.A.v.K and G.N.P.v.M.). Tumors were scored as p53 overexpression-negative ($p53^{neg}$) if less than 20% of the cells displayed nuclear positivity and as p53 overexpression-positive ($p53^{pos}$) if otherwise, like in Freedman *et al*. (19).

### *Microsatellite instability*

For MSI analysis, paired tumor and normal DNA were investigated with the five Bethesda reference panel markers (30): *BAT25*, *BAT26*, *D5S346*, *D2S123*, and *D17S250*. PCR reaction mixtures (total volume of 25µl) contained: 100ng DNA, 10pmol forward (fluorescent labeled) and reverse primer, 2.0mM $MgCl_2$ (2.5mM $MgCl_2$ for *BAT26*), 0.2mM dNTPs, 75mM Tris-HCL (pH 9.0), 20mM $(NH_4)_2SO_4$, 0.01% Tween, 0.3U Thermoperfect DNA polymerase (Integro). PCR reaction conditions: 35 cycles of 30 s at 92°C, 45 s at 50°C, 1 min at 72°C, followed by 30 min at 72°C. Products were checked using an ethidium bromide stained 2% agarose gel. One µl of (diluted) PCR product was added to 10µl formamide and 0.5µl of ROX-500 (size standard), denatured at 95°C for 5 min, chilled on ice, and loaded on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Genotyper® ABI PRISM version 3.5 NT (Perkin Elmer) was used to analyze the data. MSI at a specific marker was defined as the presence of a novel length allele in tumor tissue when compared to corresponding normal tissue. When matching normal DNA was not available (*n*=22), only the mononucleotide repeat markers *BAT25* and *BAT26* were checked for instability. Tumors were classified as MSI-H if two or more markers showed instability, MSI-L if one marker showed instability, and MSS if none of the markers examined showed instability (30).

### *Data analysis*

The distribution of *APC*, K-*ras* and *p53* mutations, p53 overexpression, and MSI in the colon carcinomas was determined. Differences in (tumor) characteristics between 'never smoked' and 'ever smoked' cases and between MSI-H and MSS tumors were assessed using *t*-tests for continuous and Chi-Square tests for categorical variables; *P* values <0.05 were considered significant. Case-case comparisons were conducted to evaluate heterogeneity in risk factors for the different tumor subsets. In addition, case-control comparisons, separately comparing cases with and cases without specific alterations with the population-based controls, were conducted to estimate the relative risk of developing carcinomas respectively with and without this particular status. The risk factors evaluated are: cigarette smoking status (never, ever), number of cigarettes usually smoked per day (never, <15, ≥15), total years of smoking (never, ≤30, >30), and years since first started smoking (never, ≤35, >35). Odds ratios (OR) and the corresponding 95% confidence intervals (95% CI) were calculated using multiple logistic regression models. 'Never' was always the referent category. All analyses were adjusted for age, sex, total energy

and alcohol intake. Additional adjustment for Dukes' stage, tumor location, body mass index, and the consumption of vegetables, fruit and red meat did not change the estimates importantly (*i.e.*, not more than 10%). All analyses were performed with the use of the SAS® statistical software package (SAS version 6.12, SAS Institute Inc. Cary, NC, USA).

## Results

Characteristics of the cases in this study population, categorized by cigarette smoking status, are given in Table 5.1. There were significantly more women among the never smokers. Total energy and alcohol intake were higher among the ever smokers whereas age and body mass index did not differ between the two groups. Current smokers smoked more cigarettes per day and had smoked for more years than ex-smokers (data not shown).

Tumor characteristics, categorized by cigarette smoking status of the cases, are described in Table 5.2. Although Dukes' stage CD and proximal tumors (cecum, ascending colon, hepatic flexure and transverse colon) were both more common among the never smokers, the difference with ever smokers was not statistically significant. There were significantly more carcinomas with truncating *APC* mutations among the never smokers. P53 overexpression was observed at a higher frequency in the carcinomas of never smokers, whereas K-*ras* mutations, and especially codon 12 transversion mutations, were more common in the carcinomas of ever smokers than of never smokers (both not statistically significant). Transversion mutations, G→T in particular, were, overall, more common among ever smokers. Tumor MSI status did not differ between never and ever smokers. Regarding MSI analysis of the tumors for which no matching normal DNA was available (*n*=22), in 10 of these tumors both *BAT25* and *BAT26* were unstable while in the other 12 tumors both markers were stable. Fifty-five percent of all p53 overexpression-positive tumors exhibited a *p53* mutation. Regarding the genetic alterations examined,

frequencies observed among women were comparable with those observed among men (data not shown).

**Table 5.1** Characteristics of the colon cancer cases by cigarette smoking status

| | All cases (*n*=176) | Never smokers (*n*=55) | Ever smokers (*n*=121) |
|---|---|---|---|
| Age (in years, mean ± SD) | 61.7 ± 10.3 | 62.6 ± 9.9 | 61.3 ± 10.2 |
| Sex (% women) | 47.2 | 69.1 | 37.2[a] |
| Body mass index (kg/m$^2$, mean ± SD) | 25.9 ± 4.4 | 25.0 ± 4.4 | 26.3 ± 4.4 |
| *Smoking variables* (%) | | | |
| Current smokers | - | - | 26.4 |
| ≥15 cigarettes/day | - | - | 51.2 |
| ≥25 cigarettes/day | - | - | 32.3 |
| >30 years of smoking | - | - | 49.2 |
| >35 years since first smoked | - | - | 72.2 |
| *Dietary factors* (mean ± SD) | | | |
| Total energy intake (kJ/day) | 10373.7 ± 3241.6 | 9491.4 ± 2767.7 | 10774.8 ± 3370.2[a] |
| Vegetables (g/day) | 170.5 ± 81.7 | 153.8 ± 74.9 | 178.5 ± 83.7 |
| Fruit (g/day) | 213.5 ± 155.7 | 242.6 ± 166.8 | 200.2 ± 149.3 |
| Red meat (g/day) | 79.7 ± 35.4 | 73.9 ± 34.9 | 82.3 ± 35.5 |
| Alcohol[b] (g/day) | 17.1 ± 25.4 | 9.8 ± 17.6 | 20.4 ± 27.6[a] |

[a] Never smokers versus ever smokers $P<0.05$, *t*-test for continuous variables and Chi-square test for categorical variables. [b] Adjusted for total energy intake by regression analysis, for women and men separately.

MSI-H tumors exhibited a significantly lower frequency of *APC*, K-*ras* and *p53* mutations, and were less often p53 overexpression-positive than MSS tumors (MSI-H tumors: 21% *APC*, 21% K-*ras*, 8% *p53*, 15% p53 overexpression-positive; MSS tumors: 38% *APC*, 41% K-*ras*, 36% *p53*, 51% p53 overexpression-positive. Not in table). They were also significantly

**Table 5.2** Tumor characteristics, categorized by cigarette smoking status of the cases

| | All cases (*n*=176) | Never smokers (*n*=55) | Ever smokers (*n*=121) |
|---|---|---|---|
| Dukes' stage (% CD) | 36.8 | 44.4 | 33.6 |
| Tumor location (% proximal) | 45.7 | 54.2 | 42.2 |
| *Mutation distribution* [*n* (%)] | | | |
| *APC* | | | |
| Tumors with mutation | 60 (34.1) | 25 (45.5) | 35 (28.9)[a] |
| Transversion | 8 (13.3) [4x G→T] | 1 (4.0) [0x G→T] | 7 (20.0) |
| Transitions | 9 (15.0) [all C→T] | 3 (12.0) | 6 (17.1) |
| Insertions/deletions | 43 (71.7) | 21 (84.0) | 22 (62.9) |
| K-*ras* | | | |
| Tumors with mutation(s)[b] | 64 (36.4) | 16 (29.1) | 48 (39.7) |
| Codon 12 mutations | 53 (81.5) | 13 (81.3) | 40 (81.6) |
| Transversions | 30 (56.6) [21x G→T] | 6 (46.2) [5x G→T] | 24 (60.0) |
| Transitions | 23 (43.4) [all G→A] | 7 (53.8) | 16 (40.0) |
| Codon 13 mutations | 12 (18.5) | 3 (18.7) | 9 (18.4) |
| Transversions | 1 (8.3) [G→T] | 0 (0) | 1 (11.1) |
| Transitions | 11 (91.7) [all G→A] | 3 (100) | 8 (88.9) |
| *p53* | | | |
| Overexpression-positive | 78 (44.3) | 29 (52.7) | 49 (40.5) |
| Tumors with mutation | 54 (30.7) | 16 (29.1) | 38 (31.4) |
| Transversions | 13 (24.1) [5x G→T] | 2 (12.5) [0x G→T] | 11 (28.9) |
| Transitions | 35 (64.8) [20x G→A] | 13 (81.3) [7x G→A] | 22 (57.9) |
| Insertions/deletions | 6 (11.1) | 1 (6.3) | 5 (13.2) |
| MSI[c] | | | |
| MSI-H | 39 (22.2) | 13 (23.6) | 26 (21.5) |
| MSI-L | 20 (11.4) | 7 (12.7) | 13 (10.7) |
| MSS | 117 (66.5) | 35 (63.6) | 82 (67.8) |

[a] Chi-square test, never smokers versus ever smokers $P<0.05$. [b] One tumor exhibited a missense mutation in codon 12 as well as in codon 13. [c] MSI-H: ≥2 markers unstable; MSI-L: 1 marker unstable; MSS: 0 markers unstable.

more often located in the proximal part of the colon. In most (54%) MSI-H tumors, none of the examined genes was mutated and p53 was not overexpressed (data not shown).

Ever smoking was not associated with increased overall colon cancer risk in this study population (Table 5.3). Table 5.3 also shows the adjusted ORs and 95% CIs of the case-case and case-control comparisons for cigarette smoking status and the occurrence of the various genetic alterations examined in this study. No significant associations were observed between cigarette smoking status and tumor *APC*-mutation status. Of the different other cigarette smoking variables evaluated (*i.e.*, usual number of cigarettes smoked per day, total years of smoking, and years since first started), only first starting smoking 35 or less years ago was significantly different associated with $APC^+$ tumors compared to $APC^-$ tumors ($APC^+$ versus $APC^-$, OR (95% CI): 0.2 (0.1-0.7), not in table). In the case-control comparisons, first starting smoking 35 or less years ago was found significantly, inversely, associated with $APC^+$ tumors only ($APC^+$ versus controls, OR (95% CI): 0.2 (0.1-0.8); $APC^-$ versus controls, OR (95% CI): 1.2 (0.6-2.3), not in table). No associations were observed between first starting smoking more than 35 years ago and tumor *APC*-mutation status.

Ever smoking, though not significantly, seems more notably, positively, associated with tumors that exhibit K-*ras* mutations, especially K-*ras* transversion mutations, than with tumors without K-*ras* mutations (Table 5.3). The other smoking variables evaluated were, again not significantly, also positively associated with tumors with K-*ras* mutations, more pronounced with K-*ras* transversion mutations, and negatively with tumors without K-*ras* mutations (data not shown).

Ever smoking was significantly different associated with p53 overexpression-positive ($p53^{pos}$) tumors compared to p53 overexpression-negative ($p53^{neg}$) tumors (Table 5.3). The case-control comparisons showed that ever smoking was inversely associated with $p53^{pos}$ tumors and

**Table 5.3** Cigarette smoking status and mutations in sporadic colon carcinomas: case-case and case-control comparisons

| | Odds ratios (95% confidence intervals)[a] | |
|---|---|---|
| | Never smoked | Ever smoked |
| Overall | | |
| No. cases/controls | 55/79 | 121/170 |
| Cases versus controls | 1.0 | 1.0 (0.6-1.5) |
| *APC*[b] | | |
| No. $APC^+$/$APC^-$/controls | 25/30/79 | 35/86/170 |
| $APC^+$ versus $APC^-$ | 1.0 | 0.6 (0.3-1.2) |
| $APC^+$ versus controls | 1.0 | 0.7 (0.4-1.4) |
| $APC^-$ versus controls | 1.0 | 1.2 (0.7-2.1) |
| K-*ras*[c] | | |
| No. K-$ras^+$/K-$ras^-$/controls | 16/39/79 | 48/73/170 |
| K-$ras^+$ versus K-$ras^-$ | 1.0 | 1.7 (0.8-3.4) |
| K-$ras^+$ versus controls | 1.0 | 1.4 (0.7-2.8) |
| K-$ras^-$ versus controls | 1.0 | 0.8 (0.5-1.4) |
| No. K-$ras^{transv}$/ K-$ras^{trans}$/K-$ras^-$/controls | 6/10/39/79 | 25/23/73/170 |
| K-$ras^{transv}$ versus K-$ras^-$ | 1.0 | 2.2 (0.8-6.2) |
| K-$ras^{transv}$ versus controls | 1.0 | 2.2 (0.7-6.3) |
| K-$ras^{trans}$ versus K-$ras^-$ | 1.0 | 1.4 (0.6-3.4) |
| K-$ras^{trans}$ versus controls | 1.0 | 1.0 (0.4-2.5) |
| *p53* (mutations)[d] | | |
| No. $p53^+$/$p53^-$/controls | 16/39/79 | 38/83/170 |
| $p53^+$ versus $p53^-$ | 1.0 | 1.1 (0.5-2.2) |
| $p53^+$ versus controls | 1.0 | 0.9 (0.4-1.9) |
| $p53^-$ versus controls | 1.0 | 1.0 (0.6-1.7) |
| Transversion mutations[e] | | |
| No. $transv^+$/$transv^-$/controls | 8/47/79 | 38/83/170 |
| $transv^+$ versus $transv^-$ | 1.0 | 2.5 (1.0-5.9) |
| $transv^+$ versus controls | 1.0 | 2.2 (0.9-5.6) |
| $transv^-$ versus controls | 1.0 | 0.8 (0.5-1.3) |
| Transition mutations[f] | | |
| No. $trans^+$/$trans^-$/controls | 21/34/79 | 46/75/170 |
| $trans^+$ versus $trans^-$ | 1.0 | 1.0 (0.5-2.0) |

| *Table 5.3 cont.* | Never smoked | Ever smoked |
|---|---|---|
| $trans^+$ versus controls | 1.0 | 0.9 (0.5-1.7) |
| $trans^-$ versus controls | 1.0 | 1.0 (0.6-1.8) |
| p53 (overexpression)[g] | | |
| No. $p53^{pos}/p53^{neg}$/controls | 29/26/79 | 49/72/170 |
| $p53^{pos}$ versus $p53^{neg}$ | 1.0 | 0.4 (0.2-0.9) |
| $p53^{pos}$ versus controls | 1.0 | 0.5 (0.3-1.0) |
| $p53^{neg}$ versus controls | 1.0 | 1.5 (0.9-2.8) |
| MSI[h] | | |
| No. MSI-H/MSS/controls | 13/35/79 | 26/82/170 |
| MSI-H versus MSS | 1.0 | 0.8 (0.3-1.7) |
| MSI-H versus controls | 1.0 | 0.8 (0.3-1.8) |
| MSS versus controls | 1.0 | 1.1 (0.6-1.8) |

[a] Adjusted for age, sex, total energy and alcohol intake. [b] $APC^+$, tumors with truncating *APC* mutation; $APC^-$, tumors without truncating *APC* mutation. [c] K-$ras^+$, tumors with mutation in K-*ras*; K-$ras^-$, tumors without mutation in K-*ras*; K-$ras^{transv}$, tumors with transversion mutation in K-*ras*. K-$ras^{trans}$, tumors with transition mutation in K-*ras.* [d] $p53^+$, tumors with mutation in *p53*; $p53^-$, tumors without mutation in *p53*. [e] $transv^+$, tumors with transversion mutations in *APC*, K-*ras*, or *p53*; $transv^-$, tumors without transversion mutations in one of these genes. [f] $trans^+$, tumors with transition mutations in *APC*, K-*ras*, or *p53*; $trans^-$, tumors without transition mutations in one of these genes. [g] $p53^{pos}$, tumors in which ≥20% of the cells displayed nuclear positivity; $p53^{neg}$, tumors in which <20% of the cells displayed nuclear positivity. [h] MSI-H, tumors in which two or more markers showed instability; MSS, tumors with no instable markers.

positively, not significantly, associated with $p53^{neg}$ tumors. Evaluation of the other cigarette smoking variables provided additional support that, in this population, cigarette smoking is inversely associated with $p53^{pos}$ tumors. Smoking 15 or more cigarettes per day, smoking more than 30 years, and first starting smoking more than 35 years ago were significantly different associated with $p53^{pos}$ tumors compared to $p53^{neg}$ tumors ($p53^{pos}$ versus $p53^{neg}$, OR (95% CI): 0.4 (0.2-1.0), 0.3 (0.1-0.7), 0.4 (0.2-1.0), respectively. Not in table). Additionally, all were inversely associated with $p53^{pos}$ tumors and positively with $p53^{neg}$ tumors when compared to the population-based controls ($p53^{pos}$ versus controls, OR (95% CI): 0.5 (0.2-1.0), 0.4 (0.2-0.9), 0.6

(0.3-1.3), respectively; $p53^{neg}$ versus controls, OR (95% CI): 1.4 (0.7-2.8), 1.6 (0.8-3.1), 1.7 (0.9-3.2), respectively. Not in table). Interestingly, no clear associations were observed between cigarette smoking and tumor *p53*-mutation status.

To evaluate whether cigarette smoking was specifically associated with the presence of transversion mutations, we additionally assessed the associations between smoking and carcinomas with transversion mutations in *APC*, K-*ras*, or *p53* ($transv^{+}$ tumors). The majority of the $transv^{+}$ tumors harbored, at least, a transversion mutation in K-*ras*. Ever smoking was significantly different associated with tumors with transversion mutations compared to tumors without transversion mutations (Table 5.3). The case-control comparisons showed that ever smoking was positively, not significantly, associated with tumors with transversion mutations and negatively, again not significantly, with tumors without transversion mutations. Similar patterns were observed for the other smoking variables evaluated (data not shown). No clear associations were observed between smoking and tumors with transition mutations in *APC*, K-*ras* or *p53* (Table 5.3).

Regarding MSI, no clear associations were observed between the smoking variables examined and MSI status (Table 5.3). In addition, no clear associations were observed when the analyses were repeated with the subset MSI-L/MSS instead of MSS (data not shown).

## Discussion

In this study, associations between cigarette smoking and various genetic alterations in sporadic colon carcinomas were assessed to evaluate the hypothesis that cigarette smoking is primarily linked to a specific colon tumor subset(s). Cigarette smoking was significantly different related to $p53^{pos}$ tumors than to $p53^{neg}$ tumors. Consistent inverse associations were observed with $p53^{pos}$ tumors while positive associations were found with $p53^{neg}$ tumors. Smoking was also

significantly different related to tumors with transversion mutations in *APC*, K-*ras*, or *p53* than to tumors without transversion mutations in one of these genes. Positive associations were observed with tumors with transversion mutations but not with tumors without transversion mutations. In addition, smoking seemed to especially increase the risk of developing tumors with K-*ras* mutations, K-*ras* transversion mutations in particular. No clear associations were observed with MSI status.

Truncating *APC* mutations were identified in 34.1%, K-*ras* mutations in 36.4 %, *p53* mutations in 30.7%, and p53 overexpression in 44.3% of the carcinomas in this study. Twenty-two percent of the carcinomas were MSI-H and the occurrence of MSI was significantly, inversely related to the presence of genetic alterations in the *APC*, K-*ras* and *p53* genes, and to p53 overexpression. Microdissection was performed and the observed frequencies and characteristics of the mutations identified were consistent with those, in comparable populations, previously reported by others (*APC* database: http://perso.curie.fr/Thierry.Soussi/APC/html; IARC *p53* database: http://www.iarc.fr/p53/index.html; 4-6, 13, 19, 20, 25, 26). However, it remains possible that, due to contaminating normal DNA, alterations were missed eventually resulting in misclassifications which in turn may have attenuated some of our results.

In this study, the MSI status of 22 tumors was determined using *BAT25* and *BAT26* tumor results only as for these tumors matching normal DNA was not available. The markers were either both unstable or both stable. Recently, polymorphisms have been identified in *BAT25* as well as in *BAT26* disputing the earlier suggested quasimonomorphic allelic profile of these two loci and warranting caution with the interpretation of MSI data based on *BAT25* and *BAT26* tumor results only (31, 32). However, the polymorphisms appear to be population dependent and to occur significantly more frequent in African Americans than in Caucasians (31, 32). In the latter they truly seem to be uncommon, and being polymorphic at both loci will most likely be even more uncommon. Therefore, we do not expect that our decision to use

*BAT25* and *BAT26* tumor results only, when matching normal DNA was not available, has resulted in extensive misclassification or lead to serious misinterpretation of our data.

As in any retrospective study, an important concern is the possibility of information and selection bias. The smoking habits of our controls were comparable to those of the general Dutch population at the time of interview (33). Since the cases are unaware of the molecular profile of their tumors, systematic errors in recall are less likely to bias results from case-case comparisons. Recall of (smoking) habits, however, can also be influenced by tumor stage or treatments. Our cases were relatively healthy, that is, the frequency of Dukes' A and B tumors among the cases was relatively high, 63%, compared to the 51% reported by the Dutch Cancer Registry (34). Adjusting the case-case comparisons for Dukes' stage did not change the estimates significantly. A long time lag between smoking exposure and occurrence of colon carcinomas has previously been suggested as a possible explanation for smoking being a risk factor for adenomas but not for colon cancer (35, 36). In our study population, 72% of the ever smokers among the cases first started smoking 35 or more years ago.

This is, to our knowledge, the first study that has evaluated associations between cigarette smoking and alterations in the *APC* gene in sporadic colon carcinomas. If anything, our data suggest a slight inverse association between the two. It seems that most sporadic colon cancers related to smoking are not initiated via alterations in the *APC* gene. This is not entirely unexpected considering that the frequency of *APC* mutations observed in sporadic adenomas is similar to that in sporadic carcinomas (4) and that APC appears to play a role in later stages of tumor development as well (37, 38).

A few studies have previously looked at cigarette smoking and other genetic alterations in colon tumors. In a large population-based case-control study on sporadic colon cancer, Slattery *et al.* (6) observed a slight increased risk of K-ras$^-$ tumors when smoking 20 cigarettes or more per day (K-ras$^-$ versus controls, OR: 1.3, 95% CI: 1.1-1.6). However, the risk of K-ras$^+$

tumors increased as well (K-ras$^{+}$ versus controls, OR: 1.2, 95% CI: 0.9-1.5). Additionally, smoking over 20 cigarettes per day was associated with an increased risk of overall colon cancer in their study population (39). In line with our results for K-*ras*, Martinez *et al*. (40) reported a slight positive, but non-significant, association with K-*ras* mutations in 0.5cm or larger sporadic colorectal adenomas for current versus never smokers.

In the study population of Slattery *et al*., cigarette smoking was found positively associated with microsatellite unstable tumors (20). Yang *et al*. (21), who purposely enriched their study for MSI-H cases, also reported a positive association between cigarette smoking and MSI-H tumors. We observed no clear associations between smoking and MSI-H tumors and, hence, did not confirm their results. Both Slattery *et al*. and Yang *et al*. did not use the Bethesda reference panel (30) to assess MSI which may explain the difference in results. Additionally, it is possible that we observed no associations due to the size of our study population and/or because the frequency of heavy smokers among our smokers was lower. Similar to our results, Slattery *et al*. reported an inverse relation between K-*ras* mutations and MSI (13, 20).

We observed no clear associations with *p53* mutations but our results for p53 overexpression suggest that most colon cancers related to cigarette smoking develop through a p53 overexpression-negative pathway (which is not necessarily also *p53* mutation-negative, see below). Consistent with our findings for p53 overexpression, Freedman *et al.* (19) observed an increased risk of p53 overexpression-negative colon tumors for current and former smokers. They didn't evaluate *p53* mutations. Although p53 overexpression is often used as an indicator of *p53* mutations, not all *p53* mutations (*e.g.*, nonsense and frameshift mutations) result in the accumulation of inactive p53 protein and can be detected with immunohistochemical analysis (41). In our population for instance, 20% of the *p53* mutations resulted in p53 overexpression-negative tumors. Under normal circumstances, wildtype p53 is activated and accumulates in response to DNA-damage and various other types of stress, resulting in either growth arrest or

apoptosis (42). Cancer cells need to somehow circumvent this checkpoint to be able to proliferate. A possible explanation for the observed association with a p53 overexpression-negative pathway, is that cigarette smoking can somehow (*e.g.*, by preventing post-translational modifications to occur) suppress the induction or stabilization of wildtype p53 (resulting in overexpression-negative cells), allowing cells to proliferate in conditions where cells with intact p53 function are eliminated.

Interestingly, we observed a positive association between cigarette smoking and tumors with transversion mutations in *APC*, K-*ras*, or *p53*. This suggests that cigarette smoking is particularly involved in the production of transversion mutations in colon cells. However, it should be noted that genetic alterations in tumors not only represent the interactions of carcinogens with DNA repair processes but also reflect the, possibly tissue-specific, selection of those mutations that provide pre-malignant and malignant cells with a clonal growth advantage. Consistent with our results, transversion mutations in *p53* and K-*ras* are also commonly found in smoking-related lung cancers (15-18). Additionally, Conway *et al.* (43) reported recently that *p53* transversion mutations, and especially G→T transversions, were significantly more prevalent in breast tumors from current smokers than in breast tumors from never smokers.

To conclude, our data suggest that smoking-related colon cancers develop through a p53 overexpression-negative pathway and that cigarette smoking particularly results in colon tumor cells with transversion mutations. Regarding the latter, it appears that cigarette smoking especially results in colon tumors with K-*ras* transversion mutations. This may be due to hypersensitivity of codons 12 and 13 of K-*ras* for exposure to tobacco smoke carcinogens or to a higher selective advantage for colon tumor formation exerted by these mutations in K-*ras* than in one of the other genes examined. Our results, if confirmed in other studies, provide support

for the hypothesis that cigarette smoking is primarily associated with specific colon tumor subgroups.

## Acknowledgements

We thank Winny van Geloof and Hanneke Braam for excellent technical assistance, and the Pathology Departments of the following hospitals for providing tumor tissue: Canisius-Wilhelmina Hospital, Nijmegen; UMC Utrecht, Utrecht; St Antonius Hospital, Nieuwegein; and Eemland Hospital, Amersfoort. The initial case-control study was conducted in collaboration with regional hospitals and cancer registries, and with financial support from the Dutch Dairy Foundation on Nutrition and Health. The current study was funded by the Dutch Cancer Society (grants: LUW 96-1374 and LUW 98-1687).

## References

1. Giovannucci, E. (2001) An updated review of the epidemiological evidence that cigarette smoking increases risk of colorectal cancer. *Cancer Epidemiol. Biomark. Prev.*, **10**, 725-731.
2. Vogelstein, B. Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M., and Bos, J.L. (1988) Genetic alterations during colorectal-tumor development. *N. Engl. J. Med.*, **319**, 525-532.
3. Fearon, E.R., and Vogelstein, B. (1990) A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759-767.
4. Powell, S.M., Zilz, N., Beazer-Barclay, Y., Bryan, T.M., Hamilton, S.R., Thibodeau, S.N., Vogelstein, B., and Kinzler, K.W. (1992) *APC* mutations occur early during colorectal tumorigenesis. *Nature*, **359**, 235-237.
5. Bos, J.L., Fearon, E.R., Hamilton, S.R., Verlaan-de Vries, M., Van Boom, J.H., Van der Eb, J.A., and Vogelstein, B. (1987) Prevalence of ras *gene* mutations in human colorectal cancers. *Nature*, **327**, 293-297.
6. Slattery, M.L., Anderson, K., Curtin, K., Ma, K-N., Schaffer, D., Edwards, S., and Samowitz, W. (2001) Lifestyle factors and Ki-*ras* mutations in colon cancer tumors. *Mut. Res.*, **483**, 73-81.
7. Kikuchi-Yanoshita, R., Konishi, M., Ito, S., Seki, M., Tanaka, K., Maeda, Y., Lino, H., Fukayama, M., Koike, M, and Mori, T. (1992) Genetic changes of both *p53* alleles associated with the conversion from colorectal adenoma to early carcinoma in familial adenomatous polyposis and non-familial adenomatous polyposis patients. *Cancer Res.*, **52**, 3965-3971.
8. Aaltonen, L.A., Peltomäki, P., Leach, F., Sistonen, P., Pylkkänen, L., Mecklin, J-P., Järvinen, H., Powell, S.M., Jen, J., Hamilton, S.R., Petersen, G.M., Kinzler, K.W., *et al*. (1993) Clues to the pathogenesis of familial colorectal cancer. *Science*, **260**, 812-816.

9. Thibodeau, S.N., Bren, G., and Schaid, D. (1993) Microsatellite instability in cancer of the proximal colon. *Science*, **260**, 816-819.
10. Ionov, Y., Peinado, M.A., Malkhosyan, S., Shibata, D., and Perucho, M. (1993) Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature*, **363**, 558-561.
11. Aaltonen, L.A., Peltomäki, P., Mecklin, J-P., Järvinen, H., Jass, J.R., Green, J.S., Lynch, H.T., Watson, P., Tallqvist, G., Juhola, M., Sistonen, P., Hamilton, S.R., *et al*. (1994) Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. *Cancer Res*., **54**, 1645-1648.
12. Salahshor, S., Kressner, U., Pahlman, L., Glimelius, B., Lindmark, G., and Lindblom, A. (1999) Colorectal cancer with and without microsatellite instability involves different genes. *Genes Chromosomes Cancer*, **26**, 247-252.
13. Samowitz, W.S., Holden, J.A., Curtin, K., Edwards, S.L., Walker, A.R., Lin, H.A., Robertson, M.A., Nichols, M.F., Gruenthal, K.M., Lynch, B.J., Leppert, M.F., and Slattery, M.L. (2001) Inverse relationship between microsatellite instability and K-*ras* and *p53* gene alterations in colon cancer. *Am. J. Pathology*, **158**, 1517-1524.
14. Bardelli, A., Cahill, D.P., Lederer, G., Speicher, M.R., Kinzler, K.W., Vogelstein, B., and Lengauer, C. (2001) Carcinogen-specific induction of genetic instability. *Proc. Natl. Acad. Sci. USA*, **98**, 5770-5775.
15. Mills, N.E., Fishman, C.L., Rom, W.N., Dubin, N., and Jacobson, D.R. (1995) Increased prevalence of K-*ras* oncogene mutations in lung adenocarcinoma. *Cancer Res*., **55**, 1444-1447.
16. Ahrendt, S.A., Decker, P.A., Alawi, E.A., Zhu, Y., Sanchez-Cespedes, M., Yang, S.C., Haasler, G.B., Kajdacsy-Balla, A., Demeure, M.J., and Sidransky, D. (2001) Cigarette smoking is strongly associated with mutation of the K-*ras* gene in patients with primary adenocarcinoma of the lung. *Cancer*, **92**, 1525-1530.
17. Hainaut, P., and Pfeifer, G.P. (2001) Patterns of *p53* G→T transversions in lung cancer reflect the primary mutagenic signature of DNA-damage by tobacco smoke. *Carcinogenesis*, **22**, 367-374.
18. Hainaut, P., Olivier, M., and Pfeifer, G.P. (2001) *TP53* mutation spectrum in lung cancers and mutagenic signature of components of tobacco smoke: lessons from the IARC *TP53* mutation database. *Mutagenesis*, **16**, 551-553.
19. Freedman, A.N., Michalek, A.M., Marshall, J.R., Mettlin, C.J., Petrelli, N.J., Zhang, Z-F, Black, J.D., Satschidanand, S., and Asirwatham, J.E. (1996) The relationship between smoking exposure and p53 overexpression in colorectal cancer. *Br. J. Cancer*, **73**, 902-908.

20. Slattery, M.L., Curtin, K., Anderson, K., Ma, K-N, Ballard, L., Edwards, S., Schaffer, D., Potter, J., Leppert, M., and Samowitz, W.S. (2000) Associations between cigarette smoking, lifestyle factors, and microsatellite instability in colon tumors. *J. Natl. Cancer Inst.*, **92**, 1831-1836.
21. Yang, P., Cunningham, J.M., Halling, K.C., Lesnick, T.G., Burgart, L.J., Wiegert, E.M., Christensen, E.R., Lindor, N.M., Katzmann, J.A., and Thibodeau, S.N. (2000) Higher risk of mismatch repair-deficient colorectal cancer in alpha(1)-antitrypsin deficiency carriers and cigarette smokers. *Mol. Genet. Metab.*, **71**, 639-645.
22. Kampman, E., Van ’t Veer, P., Hiddink, G.J., Van Aken-Schneijder, P., Kok, F.J., and Hermus, R.J.J. (1994) Fermented dairy products, dietary calcium and colon cancer: a case-control study in the Netherlands. *Int. J. Cancer*, **59**, 170-176.
23. Kampman, E., Verhoeven, D., Sloots, L., and Van ’t Veer, P. (1995) Vegetable and animal products as determinants of colon cancer risk in Dutch men and women. *Cancer Causes and Control*, **6**, 225-234.
24. Voskuil, D.W., Kampman, E., Van Kraats, A.A., Balder, H.F., Van Muijen, G.N.P., Goldbohm, R.A., and Van ’t Veer, P. (1999) P53 overexpression and *p53* mutations in colon carcinomas: relation to dietary risk factors. *Int. J. Cancer*, **81**, 675-681.
25. Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T., and Nakamura, Y. (1992) Somatic mutations of the *APC* gene in colorectal tumors: mutation cluster region in the *APC* gene. *Hum. Mol. Genet.*, **1**, 229-233.
26. Miyaki, M., Konishi, M., Kikuchi-Yanoshita, R., Enomoto, M., Igari, T., Tanaka, K., Muraoka, M., Takahashi, H., Amada, Y., Fukayama, M., Maeda, Y., Iwama, T., *et al*. (1994) Characteristics of somatic mutation of the adenomatous polyposis coli gene in colorectal tumors. *Cancer Res.*, **54**, 3011-3020.
27. Rowan, A.J., Lamlum, H., Ilyas, M., Straub, J., Papadopoulou, A., Bicknell, D., Bodmer, W.F., and Tomlinson, I.P.M. (2000) *APC* mutations in sporadic colorectal tumors: A mutational “hotspot” and interdependence of the “two hits”. *Proc. Natl. Acad. Sci. USA*, **97**, 3352-3357.
28. Kampman, E., Voskuil, D.W., Van Kraats, A.A., Balder, H.F., Van Muijen, G.N.P., Goldbohm, R.A., and Van ’t Veer, P. (2000) Animal products and K-*ras* codon 12 and 13 mutations in colon carcinomas. *Carcinogenesis*, **21**, 307-309.
29. Greenblatt, M.S., Bennett, W.P., Hollstein, M., and Harris, C.C. (1994) Mutations in the *p53* tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res.*, **54**, 4855-4878.
30. Boland, C.R., Thibodeau, S.N., Hamilton, S.R., Sidransky, D., Eshleman, J.R., Burt, R.W., Meltzer, S.J., Rodrigues-Bigas, M.A., Fodde, F., Ranzani, G.N., and Srivastava, S. (1998) A National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res.*, **58**, 5248-5257.

31. Samowitz, W.S., Slattery, M.L., Potter, J.D., and Leppert, M.F. (1999) BAT-26 and BAT-40 instability in colorectal adenomas and carcinomas and germline polymorphisms. *Am. J. Pathology*, **154**, 1637-1641.
32. Pyatt, R., Chadwick, R.B., Johnson, C.K., Adebamowo, C., De la Chapelle, A., and Prior, T.W. (1999) Polymorphic variation and the BAT-25 and BAT-26 loci in individuals of African origin. Implications for microsatellite instability testing. *Am. J. Pathology*, **155**, 349-353.
33. Nicholaides-Bouman, A, Wald, N., Forey, B., and Lee, P. (eds.). *International smoking statistics.* Oxford University Press, London, 1993.
34. Coebergh, J.W.W., Van der Heijden, L.H., and Janssen-Heijnen, M.L.G. (1995) *Cancer incidence and survival in the southeast of the Netherlands*. Comprehensive Cancer Centre South, Eindhoven.
35. Giovanucci, E., Rimm, E.B., Stampfer, M.J., Colditz, G.A., Ascherio, A., Kearny, J., and Willett, W.C. (1994) A prospective study of cigarette smoking and risk of colorectal adenoma and colorectal cancer in U.S. men. *J. Natl. Cancer Inst*., **86**, 183-191.
36. Giovanucci, E., Colditz, G.A., Stampfer, M.J., Hunter, D., Rosner, B.A., Willett, W.C., and Speizer, F.E. (1994) A prospective study of cigarette smoking and risk of colorectal adenoma and colorectal cancer in U.S. women. *J. Natl. Cancer Inst*., **86**,192-199.
37. Fodde, R., Kuipers, J., Rosenberg, C., Smits, R., Kielman, M., Gaspar, C., Van Est, J.H., Breukel, C., Wiegant, J., Giles, R.H., and Clevers, H. (2001) Mutations in the *APC* tumour suppressor gene cause chromosomal instability. *Nature Cell Biology*, **3**, 433-438.
38. Kaplan, K.B., Burds, A.A., Swedlow, J.R., Bekir, S.S., Sorger, P.K., and Näthke, I.S. (2001) A role for the adenomatous polyposis coli protein in chromosome segregation. *Nature Cell Biology*, **3**, 429-432.
39. Slattery, M.L., Potter, J.D., Friedman, G.D., Ma, K-N, and Edwards, S. (1997) Tobacco use and colon cancer. *Int. J. Cancer*, **70**, 259-264.
40. Martinez, M.E., Maltzman, T., Marshall, J.R., Einsphar, J., Reid, M.E., Sampliner, R., Ahnen, D.J., Hamilton, S.R., and Alberts, D.S. (1999) Risk factors for Ki-*ras* protooncogene mutation in sporadic colorectal adenomas. *Cancer Res*., **59**, 5181-5185.
41. Soussi, T., and Béroud, C. (2001) Assessing *TP53* status in human tumours to evaluate clinical outcome. *Nature Reviews*, **1**, 233-240.
42. Pluquet, O., and Hainaut, P. (2001) Genotoxic and non-genotoxic pathways of p53 induction. *Cancer Lett*., **174**, 1-15.
43. Conway, K., Edmiston, S.N., Cui, L., Drouin, S.S., Pang, J., He, M., Tse, C-K., Gerardts, J., Dressler, L., Liu, E.T., Millikan, R., and Newman, B. (2002) Prevalence and spectrum of *p53* mutations associated with smoking in breast cancer. *Cancer Res*., **62**, 1987-1995.

# | Chapter 6

# Environmental factors, truncating *APC* mutations, and HNPCC-associated colorectal tumors

Brenda Diergaarde

Hanneke Braam

Hans F. Vasen

Fokko M. Nagengast

Goos N.P. van Muijen

Frans J. Kok

Ellen Kampman

***Submitted for publication***

## Abstract

Individuals with hereditary nonpolyposis colorectal cancer (HNPCC) are at increased risk for colorectal cancer. To gain insight into the effects of environmental factors on colorectal tumor risk in individuals with HNPCC, we examined associations between dietary factors previously reported to be related to sporadic colorectal cancer risk, cigarette smoking, and HNPCC-associated colorectal tumors in a Dutch case-control study (145 cases; 103 tumor-free controls; all study participants were known or suspected carriers of a germline mutation in one of the DNA mismatch repair genes). We additionally assessed associations between the various environmental factors and occurrence of truncating *APC* mutations in HNPCC-associated polyps in a subset of the study population. Fruit consumption was inversely associated with ever developing HNPCC-associated colorectal tumors [OR (95% CI) for highest versus lowest tertile, 0.4 (0.2-0.9)]; a borderline significant inverse association was observed for dietary fiber intake [0.5 (0.2-1.0)]. Cigarette smoking and alcohol intake seemed to increase the risk of HNPCC-associated colorectal tumors. Truncating *APC* mutations were detected in 30 (37.5%) of the 80 available HNPCC-associated polyps; frameshift mutations were most common (73.3%). None of the evaluated environmental factors was distinctively associated with a specific *APC* status of the polyps. Our data suggest that fruit consumption and dietary fiber intake may decrease the risk of colorectal tumors in individuals with HNPCC, whereas cigarette smoking and possibly alcohol intake may increase the risk of HNPCC-associated colorectal tumors. The observed associations, if confirmed by other studies, support the hypothesis that also HNPCC-associated outcomes may be modified by environmental factors.

## Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC) is one of the most common cancer predisposition syndromes in humans. It is characterized by an autosomal dominant mode of inheritance and an early onset of carcinogenesis (mean age at diagnosis of colorectal cancer, ~45 years). Colorectal tumors are the predominant form of cancer in HNPCC but extracolonic cancers, endometrial, ovarian and stomach cancer in particular, are also often observed (reviewed in Refs. 1 and 2). Germline mutations in DNA mismatch repair (MMR) genes are responsible for HNPCC; most (~90%) have been detected in *hMLH1* (3) and *hMSH2* (4, 5) [see

mutation database at the website of the International Collaborative Group on HNPCC: www.nfdht.nl].

HNPCC is thought to account for 1-5% of all colorectal cases (6-9), and mutation carriers may have a lifetime risk of developing colorectal cancer of 60-80% (10). HNPCC-associated colorectal tumors are often located in the proximal part of the colon, have a more favorable prognosis than sporadic colorectal cancer, and most exhibit microsatellite instability (MSI) (1, 2). Colorectal polyps in individuals with HNPCC progress into cancer more rapidly, within 2-3 years instead of the 10-15 years in the general population, but don't seem to occur with obviously increased frequency. Several studies have reported the occurrence of inactivating mutations in the adenomatous polyposis coli (*APC*) tumor suppressor gene in HNPCC-associated colorectal tumors (11-14). This suggests that, like in sporadic colorectal carcinogenesis and familial adenomatous polyposis (15, 16), loss of APC function may be an important initiating event in a subset of HNPCC-associated colorectal tumors.

The incomplete penetrance of disease among mutation carriers and the observation that the clinical expression of HNPCC seems to vary between regions and to have changed over time suggest that environmental factors may play a role in HNPCC-associated carcinogenesis (1, 10, 17). It is possible that dietary and lifestyle factors known to be relevant in sporadic colorectal carcinogenesis [*e.g.*, red meat and vegetable intake, and cigarette smoking (18, 19)] also play a role in HNPCC-associated colorectal cancer; however, epidemiological data supporting this hypothesis are currently limited. To our knowledge, only one study has previously examined associations between environmental factors and HNPCC-associated colorectal tumors in humans, and this study looked at meat consumption and meat preparation only (20). It has been shown, however, that MMR-deficient cells are more resistant to the cytotoxic effects of various DNA-damaging agents and display a greater increase in induced mutations than MMR-

proficient cells (21-23). The same response to DNA-damaging agents has been observed in MMR-deficient mice (24).

The possibility that a (simple) change in diet and/or lifestyle may result in a decrease in colorectal tumor risk in individuals with HNPCC warrants further investigation. Especially since even individuals with HNPCC who are participating in an intensive colorectal surveillance program still seem to have a substantial risk of developing colorectal cancer (25). Therefore, in this study, we examined associations between dietary factors previously reported to be associated with sporadic colorectal cancer risk, cigarette smoking, and development of HNPCC-associated colorectal tumors. *APC* mutation analysis was performed on polyp tissue from a subset of the study population and, to further explore the relationship between environmental factors and HNPCC-associated colorectal tumors, we additionally assessed associations between the various dietary factors, cigarette smoking, and the occurrence of truncating *APC* mutations in HNPCC-associated colorectal polyps.

## Material and methods

### *Study population*

Participants for this case-control study on environmental factors and HNPCC-associated colorectal tumors were recruited in the Netherlands between April 1999 and April 2002. All participants belonged to a family fulfilling the Amsterdam criteria for HNPCC (see Table 1.1 in Chapter 1; 26, 27) and were known or suspected carriers of a germline mutation in one of the MMR genes. In addition to this, all participants were Dutch speaking, Caucasian, between 18 and 75 years old, mentally competent to participate, had no known ulcerative colitis, Crohn's disease, or serious disabling morbidity, and had undergone at least one complete colonoscopy (*i.e.*, reaching the cecum). Proven non-carriers and individuals who had undergone a total colectomy were not eligible. In the Netherlands, surveillance recommendations for known and suspected 'HNPCC-ers' currently include screening for colorectal tumors by complete colonoscopy at an interval of two years or less, starting at 20-25 years of age (www.nfdht.nl; 28). Potential study participants were either registered at The Netherlands Foundation for the Detection of Hereditary Tumors (Leiden, The Netherlands), which keeps a registry for (Dutch)

families with HNPCC, or undergoing 'HNPCC' surveillance colonoscopies at the Department of Gastroenterology of UMC St Radboud (Nijmegen, The Netherlands). They were identified by review of randomly selected medical records at the two participating sites. After approval of their medical specialist, potential participants were informed about the study by mail. Overall, 84% (*n*=312) of the subjects contacted agreed to participate and provided written informed consent. Dietary and lifestyle questionnaires were mailed to those who had agreed to participate. Medical records were checked for information on undergone colonoscopies, history of colorectal cancer and polyps and other relevant medical history. Case-control status was determined after written informed consent was obtained. If a colorectal polyp(s) had been detected during the last colonoscopy or during a colonoscopy performed maximally 5 years earlier, we tried to obtain the polyp tissue from the pathology departments of the hospitals where the study participants were under surveillance. Thirty-two (10%) of those who had agreed to participate did not meet the eligibility criteria retrospectively and were therefore excluded from the analyses. In addition, 32 (10%) did not return the questionnaires, leaving a final number of 248 participants (belonging to 113 different 'HNPCC' families) with dietary and lifestyle information. Cases (*n*=145) were women and men ever diagnosed with colorectal cancer or polyps. In total, 119 (82%) cases had at least one polyp diagnosed during their last colonoscopy or during a colonoscopy performed maximally 5 years earlier. But, due to various reasons (administrative, tissue had been used up by others, tissue had never been stored, etc.), polyp tissue from 43 (36%) of those cases was not available to us. Cases whose polyp tissue we did obtain were comparable with cases whose polyp tissue was not available to us (data not shown). The 119 cases diagnosed with a polyp during their last colonoscopy or during a colonoscopy performed maximally 5 years earlier differed significantly from the other 26 cases only in vegetable consumption; mean vegetable intake was lower in the latter group (data not shown). Controls (*n*=103) were women and men never diagnosed with colorectal cancer or any kind of colorectal polyp. The average time between enrollment and last colonoscopy was 2.1 (± 1.9) years for cases and 1.9 (± 2.1) years for controls ($P$=0.51). The study protocol was reviewed and approved by the Medical Ethical committees of UMC St Radboud and Wageningen University.

### *Data collection*

Information on lifestyle factors including smoking habits, medical history, reproductive history, physical activity and family history of colorectal cancer was collected using a self-administered, structured questionnaire. Regarding smoking habits, information on cigarette smoking status (current, former, never), number of cigarettes usually smoked per day, total number of years smoked and, if applicable, age at which participant stopped smoking was obtained. Medical records were checked for information on history of colorectal cancer and polyps and other relevant medical history. Usual dietary intake was assessed with a validated, semi-quantitative food frequency questionnaire that was originally developed

for the Dutch cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC) [for details see Ocké *et al*. (29, 30)]. Briefly, this self-administered questionnaire contained questions on the average consumption frequency during one year for 79 main food items. Referent period for the current study was the year preceding inclusion in the study. For 21 foods, the questionnaire contained photographs of 2-4 different sized portions. For most other items the consumption frequency was asked in numbers of specified units (*e.g*., slices or glasses); for a few foods a standard portion size was assumed. Frequencies per day and portion sizes were multiplied to obtain grams per day for each food item. In total, the average daily consumption of 178 foods was estimated with the information obtained by the questionnaire. Energy and nutrient intakes were calculated using an adapted version of the computerized Dutch food composition table. When questionnaires were returned incomplete, study staff followed up with the participants by phone. Information on completeness of colonoscopy (*i.e*., whether cecum was reached or not), location (proximal: cecum through transverse colon; distal: splenic flexure through sigmoid; rectum: first 15cm from anal opening; colon: exact location unknown), size, number, and type of colorectal tumors detected was obtained from endoscopy and pathology reports. The study pathologist re-evaluated the histology of all obtained colorectal polyps.

### *DNA extraction*

DNA was extracted from formalin-fixed, paraffin-embedded tissue (10-12 10µm thick sections) using the Puregene$^{TM}$ DNA isolation kit (Gentra Systems, Minneapolis, MN). Polyp tissue was available from 76 cases and also from 11 participants who had agreed to participate and fulfilled all eligibility criteria but did not return the dietary and lifestyle questionnaires. Regarding individuals for whom tissue was available from more than one colorectal polyp (all detected during the same colonoscopy), the largest polyp in size was selected for mutation analysis. Microdissection was performed, guided by a hematoxylin and eosin-stained 4µm section, and only those areas containing >60% tumor cells were used. Isolated tissue was incubated overnight at 55ºC in 500µl cell lysis solution containing 0.5mg/ml proteinase K (Roche Diagnostics, Mannheim, Germany), followed by 72 hours at 37ºC. Proteins were removed with the protein precipitation solution according to the manufacturer’s protocol. DNA was precipitated with 500µl 100% isopropanol at 4ºC for 30 minutes. The pellet was washed with 70% ethanol, air-dried, and subsequently the DNA was rehydrated in 15µl DNA hydration solution.

### *APC mutation detection*

Codons 1286 to 1585 of the *APC* gene [this area includes the mutation cluster region (MCR) in which most somatic inactivating *APC* mutations have been detected (31, 32)] were analyzed for truncating mutations (*i.e*., nonsense and frameshift mutations) by direct-sequencing. The region was divided into five overlapping fragments (codons 1286-1358, 1337-1404, 1387-1455, 1437-1526, and 1509-1585,

respectively), which were separately amplified in two consecutive PCRs as described previously (33). PCR products were checked on an ethidium bromide stained 2% agarose gel and purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Subsequently, the PCR products were sequenced using ABI PRISM® BigDye™ Terminators v3.0 (Applied Biosystems, Foster City, CA) and cycle sequencing with AmpliTaq® DNA polymerase, FS (Applied Biosystems, Foster City, CA). Sequencing was performed in both directions using the same primers as in the second PCR. Samples were analyzed on an ABI PRISM® 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Mutation analysis started in all samples with fragment 1, and only if no truncating mutations were detected was fragment 2 screened for mutations, and so on. We focused on truncating mutations because these mutations indisputably result in function loss of the APC protein whereas the biological significance of missense mutations in *APC* is uncertain. We were unable to perform mutation analysis on 7 polyps (5 from cases and two from participants who did not return their questionnaires) due to bad quality DNA and/or insufficient amounts of DNA. Polyps were classified as $APC^+$ (with truncating mutation in *APC*) or $APC^-$ (without truncating mutation in *APC* and all five fragments completely analyzed for mutations).

### *Statistical analyses*

Energy-adjusted nutrient intake was computed with the residual method for women and men separately (34). To convey the sense of an actual nutrient intake, we added the mean nutrient intake to each residual (34). Differences in characteristics between groups were assessed using *t*-tests for continuous variables and Chi-square tests or Fisher's exact tests for categorical variables. *P* values <0.05 were considered significant. All significance tests were two-sided. The categorization of the dietary factors in tertiles was based on the distribution of intake in the control population. The interquartile ranges ($Q_3$-$Q_1$), used to quantify associations on a continuous scale, were also based on the distribution of intake in the control population. Cases were compared with the never-affected controls to estimate the relative risk of developing colorectal tumors. Case-control comparisons, separately comparing the $APC^+$-polyp cases and $APC^-$-polyp cases with the controls, were conducted to estimate the relative risk of developing HNPCC-associated polyps with and without truncating *APC* mutation, respectively. To evaluate heterogeneity in environmental risk factors for the two different polyp subsets, $APC^+$-polyp cases were also compared with $APC^-$-polyp cases. Odds ratios (OR) and the corresponding 95% confidence intervals (95% CI) were calculated using multiple logistic regression models. For continuous risk factors, linear trend was assessed using the tertile medians as continuous variables in multiple logistic regression models. All analyses were adjusted for age (continuously), sex, total energy intake (continuously), carrier status (known carrier, suspected carrier), and cigarette smoking status (never, ever, unknown). Analyses of cigarette smoking were also adjusted for alcohol intake (continuously). Additional adjustment for

polyp location (proximal, distal, rectum, colon), polyp size (<5mm, ≥5mm), polyp number (single, multiple), polyp type (hyperplastic, tubular, tubulovilleus), body mass index (continuously), and other dietary factors (continuously) did not change the estimates by more than 10%. All analyses were performed using the SAS® statistical software package (SAS version 8.2 SAS Institute Inc. Cary, NC).

## Results

Characteristics of the study population are presented in Table 6.1. In total, the study population consisted of 73 known carriers [*hMLH1*, 49 (67%); *hMSH2*, 22 (30%); gene not disclosed to us, 2 (3%)]. The frequency of known carriers did not differ significantly between cases and controls. Of the cases with unknown carrier status ($n$=100), 26 (26%) had been diagnosed with at least one adenomatous polyp before 40 years of age and 14 (14%) had been diagnosed with colorectal cancer before 50 years of age. Age at last colonoscopy and body mass index were significantly higher among the cases than among the never-affected controls. The case group also contained significantly more ever smokers than the control group. Regarding dietary factors, no marked differences were observed between cases and controls except for alcohol intake, which was significantly higher among cases than among controls.

Age was strongly, positively associated with ever been diagnosed with colorectal tumors: OR (95% CI) for ≥45 years at last colonoscopy versus ≤35 years at last colonoscopy, 6.4 (3.0-13.9), and $P_{trend}<0.05$ (adjusted for sex, total energy intake, carrier status, and cigarette smoking; not in tables). No significant association with colorectal tumors was observed for body mass index: OR (95% CI) for >25.5kg/m$^2$ versus <23.2kg/m$^2$, 0.9 (0.4-1.8), and $P_{trend}=0.83$ (adjusted for age at last colonoscopy, sex, total energy intake, carrier status, and cigarette smoking; not in tables).

**Table 6.1** Characteristics of the study population

| | Controls ($n$=103) | Cases ($n$=145) |
|---|---|---|
| Known carrier [$n$ (%)] | 28 (27.2) | 45 (31.0) |
| Sex [women, $n$ (%)] | 57 (55.3) | 80 (55.2) |
| Age at last colonoscopy (in years, mean ± SD) | 41.1 ± 10.9 | 50.0 ± 10.9[a] |
| Age at last colonoscopy (in years, range) | 23-69 | 24-75 |
| Body mass index (kg/m$^2$, mean ± SD) | 24.3 ± 2.9 | 25.1 ± 3.0 [a] |
| Total energy intake (kJ/day, mean ± SD) | 9813.5 ± 3945.7 | 9190.8 ± 2550.0 |
| Ever smoked[b] [$n$ (%)] | 53 (53.0) | 99 (71.2) [a] |
| *Food groups* (g/day; mean ± SD) | | |
| Total vegetables & fruit | 295.9 ± 134.1 | 298.0 ± 147.8 |
| Vegetables | 112.6 ± 40.6 | 113.6 ± 47.0 |
| Fruit | 183.3 ± 121.8 | 184.4 ± 131.7 |
| Cereals | 59.5 ± 43.5 | 52.3 ± 40.7 |
| Total meat | 93.6 ± 45.1 | 96.3 ± 47.8 |
| Red meat | 58.4 ± 29.6 | 56.3 ± 30.8 |
| Poultry | 11.8 ± 9.2 | 11.5 ± 10.5 |
| Fish | 10.0 ± 10.0 | 10.8 ± 8.5 |
| Dairy products | 410.8 ± 293.3 | 414.3 ± 279.7 |
| *Nutrients*[c] (g/day; mean ± SD) | | |
| Alcohol | 9.8 ± 10.9 | 14.0 ± 15.2 [a] |
| Fat | 90.4 ± 17.0 | 88.2 ± 16.3 |
| Protein | 83.7 ± 22.8 | 82.6 ± 16.1 |
| Carbohydrates | 258.4 ± 48.8 | 257.5± 48.3 |
| Dietary fiber | 25.4 ± 6.0 | 24.9 ± 6.2 |
| Calcium (mg/day) | 1121.5 ± 393.1 | 1130.8 ± 363.4 |
| Vitamin C (mg/day) | 113.5 ± 64.6 | 108.7 ± 46.5 |
| ß-Carotene (µg/day) | 1491.1 ± 644.3 | 1420. 4 ± 478.8 |
| Folate (µg/day) | 200.5 ± 46.7 | 203.9 ± 43.5 |

[a] $P$<0.05; $t$-tests for continuous and Chi-square tests for categorical variables. [b] Information on smoking status was not available for 9 participants (6 cases; 3 controls). [c] Adjusted for total energy intake by the residual method.

**Table 6.2** Dietary factors and HNPCC-associated colorectal tumors

| | Odds ratios (95% confidence intervals)[a] | | | | |
|---|---|---|---|---|---|
| | T1 | T2 | T3 | $P_{trend}$[b] | continuous[c] |
| *Total energy intake* (kJ/day) | ≤7915.7 | 7915.7-10770.8 | ≥10770.8 | | /4266.2 |
| No. Cases/Controls | 54/35 | 53/33 | 38/35 | | 145/103 |
| Cases versus Controls | 1.0 | 1.1 (0.6-2.2) | 0.8 (0.4-1.7) | 0.51 | 0.9 (0.5-1.3) |
| FOOD GROUPS | | | | | |
| *Total vegetables & fruit* (g/day) | ≤235.6 | 235.6-352.9 | ≥352.9 | | /178.6 |
| No. Cases/Controls | 57/35 | 45/33 | 43/35 | | 145/103 |
| Cases versus Controls | 1.0 | 0.8 (0.4-1.5) | 0.5 (0.3-1.1) | 0.07 | 0.9 (0.6-1.3) |
| *Vegetables* (g/day) | ≤97.0 | 97.0-121.8 | ≥121.8 | | /45.3 |
| No. Cases/Controls | 60/35 | 29/33 | 56/35 | | 145/103 |
| Cases versus Controls | 1.0 | 0.5 (0.2-1.0) | 1.2 (0.6-2.4) | 0.62 | 1.1 (0.8-1.4) |
| *Fruit* (g/day) | ≤122.8 | 122.8-242.5 | ≥242.5 | | /168.1 |
| No. Cases/Controls | 56/35 | 47/33 | 42/35 | | 145/103 |
| Cases versus Controls | 1.0 | 0.7 (0.3-1.3) | 0.4 (0.2-0.9) | 0.03 | 0.9 (0.6-1.3) |
| *Cereals* (g/day) | ≤34.0 | 34.0-73.6 | ≥73.6 | | /58.2 |
| No. Cases/Controls | 59/35 | 44/33 | 42/35 | | 145/103 |
| Cases versus Controls | 1.0 | 1.3 (0.6-2.6) | 1.4 (0.6-2.8) | 0.43 | 1.1 (0.7-1.6) |
| *Total meat* (g/day) | ≤73.3 | 73.3-114.8 | ≥114.8 | | /67.9 |
| No. Cases/Controls | 53/35 | 48/33 | 44/35 | | 145/103 |
| Cases versus Controls | 1.0 | 0.9 (0.5-1.8) | 1.0 (0.5-2.1) | 0.90 | 1.1 (0.7-1.7) |
| *Red meat* (g/day) | ≤46.2 | 46.2-71.4 | ≥71.4 | | /44.7 |
| No. Cases/Controls | 59/35 | 44/33 | 42/35 | | 145/103 |
| Cases versus Controls | 1.0 | 0.8 (0.4-1.5) | 0.8 (0.4-1.6) | 0.43 | 0.9 (0.6-1.4) |
| *Poultry* (g/day) | ≤7.8 | 7.8-13.1 | ≥13.1 | | /9.5 |
| No. Cases/Controls | 56/35 | 43/33 | 46/35 | | 145/103 |
| Cases versus Controls | 1.0 | 0.8 (0.4-1.6) | 0.8 (0.4-1.5) | 0.45 | 1.0 (0.7-1.3) |
| *Fish* (g/day) | ≤4.0 | 4.0-11.9 | ≥11.9 | | /12.5 |
| No. Cases/Controls | 39/35 | 47/33 | 59/35 | | 145/103 |
| Cases versus Controls | 1.0 | 1.0 (0.5-1.9) | 1.2 (0.6-2.4) | 0.57 | 1.0 (0.7-1.5) |
| *Dairy products* (g/day) | ≤245.7 | 245.7-457.3 | ≥457.3 | | /374.0 |
| No. Cases/Controls | 43/35 | 49/33 | 53/35 | | 145/103 |
| Cases versus Controls | 1.0 | 1.2 (0.6-2.5) | 1.6 (0.8-3.4) | 0.20 | 1.1 (0.8-1.7) |

| *Table 6.2 cont.* | T1 | T2 | T3 | $P_{trend}$[b] | Continuous[c] |
|---|---|---|---|---|---|
| NUTRIENTS[d] | | | | | |
| *Alcohol* (g/day) | ≤2.6 | 2.6-12.8 | ≥12.8 | | /14.3 |
| No. Cases/Controls | 40/35 | 50/33 | 55/35 | | 145/103 |
| Cases versus Controls | 1.0 | 1.2 (0.6-2.4) | 1.0 (0.5-2.0) | 0.85 | 1.2 (0.9-1.7) |
| *Fat* (g/day) | ≤83.0 | 83.0-98.3 | ≥98.3 | | /25.1 |
| No. Cases/Controls | 66/35 | 37/33 | 42/35 | | 145/103 |
| Cases versus Controls | 1.0 | 0.5 (0.3-1.1) | 0.5 (0.2-1.3) | 0.12 | 0.7 (0.4-1.2) |
| *Protein* (g/day) | ≤72.0 | 72.0-89.6 | ≥89.6 | | /30.0 |
| No. Cases/Controls | 39/35 | 61/33 | 45/35 | | 145/103 |
| Cases versus Controls | 1.0 | 1.5 (0.7-3.0) | 1.1 (0.5-2.7) | 0.86 | 0.9 (0.5-1.6) |
| *Carbohydrates* (g/day) | ≤228.8 | 228.8-279.1 | ≥279.1 | | /71.7 |
| No. Cases/Controls | 44/35 | 53/33 | 48/35 | | 145/103 |
| Cases versus Controls | 1.0 | 1.4 (0.6-3.0) | 1.4 (0.5-4.2) | 0.51 | 1.1 (0.6-2.1) |
| *Dietary Fiber* (g/day) | ≤22.6 | 22.6-27.5 | ≥27.5 | | /7.5 |
| No. Cases/Controls | 61/35 | 39/33 | 45/35 | | 145/103 |
| Cases versus Controls | 1.0 | 0.6 (0.3-1.2) | 0.5 (0.2-1.0) | 0.06 | 0.7 (0.5-1.1) |
| *Calcium* (mg/day) | ≤972.1 | 972.1-1214.9 | ≥1214.9 | | /365.0 |
| No. Cases/Controls | 56/35 | 41/33 | 48/35 | | 145/103 |
| Cases versus Controls | 1.0 | 0.6 (0.3-1.2) | 0.8 (0.4-1.6) | 0.61 | 1.0 (0.8-1.3) |
| *Vitamin C* (mg/day) | ≤84.3 | 84.3-131.4 | ≥131.4 | | /66.5 |
| No. Cases/Controls | 49/35 | 48/33 | 48/35 | | 145/103 |
| Cases versus Controls | 1.0 | 0.9 (0.4-1.8) | 0.8 (0.4-1.6) | 0.56 | 0.9 (0.6-1.3) |
| *β-Carotene* (μg/day) | ≤1249.1 | 1249.1-1579.3 | ≥1579.3 | | /522.0 |
| No. Cases/Controls | 56/35 | 38/33 | 51/35 | | 145/103 |
| Cases versus Controls | 1.0 | 0.8 (0.4-1.6) | 1.0 (0.5-2.0) | 0.98 | 0.9 (0.7-1.1) |
| *Folate* (μg/day) | ≤182.7 | 182.7-212.9 | ≥212.9 | | /53.0 |
| No. Cases/Controls | 53/35 | 40/33 | 52/35 | | 145/103 |
| Cases versus Controls | 1.0 | 1.0 (0.5-2.0) | 0.9 (0.4-1.9) | 0.71 | 1.2 (0.8-1.7) |

[a] Adjusted for age at last colonoscopy, sex, total energy intake, carrier status, and cigarette smoking. [b] Trend was assessed using the median values of the tertiles as continuous variables. [c] Per interquartile range ($Q_3$-$Q_1$). [d] Adjusted for total energy intake by the residual method.

Results of case-control comparisons conducted to evaluate associations between dietary factors and risk of developing HNPCC-associated colorectal tumors are presented in Table 6.2. Consumption of fruits was significantly inversely associated with ever been diagnosed with colorectal tumors. A borderline significant inverse association was observed with dietary fiber intake. None of the other examined dietary factors was statistically significantly associated with ever been diagnosed with colorectal tumors (Table 6.2).

**Table 6.3** Cigarette smoking and HNPCC-associated colorectal tumors

| | Controls *n* (%) | Cases *n* (%) | OR (95% CI)[a] |
|---|---|---|---|
| *Smoking status*[b] | | | |
| Never | 47 (47.0) | 40 (28.8) | 1.0 |
| Ever | 53 (53.0) | 99 (71.2) | 1.5 (0.8-2.8) |
| Former | 36 (36.0) | 67 (48.2) | 1.1 (0.6-2.3) |
| Current | 17 (17.0) | 32 (23.0) | 2.4 (1.1-5.3) |
| $P_{trend}$ | | | 0.05 |
| *No. of cigarettes usually smoked/day*[c] | | | |
| Never smokers | 47 (47.0) | 40 (29.4) | 1.0 |
| <15 | 25 (25.0) | 56 (41.1) | 2.0 (1.0-3.9) |
| ≥15 | 28 (28.0) | 40 (29.4) | 1.0 (0.5-2.2) |
| $P_{trend}$ | | | 0.80 |
| *Total years of smoking*[d] | | | |
| Never smokers | 47 (48.5) | 40 (29.4) | 1.0 |
| <20 | 34 (35.1) | 47 (34.5) | 1.5 (0.8-2.9) |
| ≥20 | 16 (16.5) | 49 (36.0) | 1.7 (0.7-3.8) |
| $P_{trend}$ | | | 0.18 |

[a] Adjusted for age at last colonoscopy, sex, total energy intake, carrier status, and alcohol intake. [b] Information on smoking status was not available for 9 participants (6 cases; 3 controls). [c] Information on number of cigarettes smoked/day was not available for 3 smokers (all cases). [d] Information on total years of smoking was not available for 6 smokers (3 cases; 3 controls).

Current cigarette smoking and smoking less than 15 cigarettes per day were significantly associated with increased risk of colorectal tumors (Table 6.3). However, no significant association was observed for smoking 15 or more cigarettes per day. Although not statistically significant, total years of smoking seemed positively associated with risk of developing colorectal tumors. Additional adjustment for red meat intake did not change the observed estimates significantly (data not shown).

We were able to perform *APC* mutation analysis on 80 polyps (71 polyps from cases; 9 polyps from study participants who did not return their questionnaires but were otherwise eligible). Polyp characteristics categorized by *APC* status are presented in Table 6.4.

**Table 6.4** Polyp characteristics and occurrence of *APC* mutations[a]

| *n* (%) | All polyps (*n*=80) | $APC^+$ (*n*=30; 37.5%) | $APC^-$ (*n*=50; 62.5%) | $P$[b] |
|---|---|---|---|---|
| *Histological type* | | | | <0.05 |
| Hyperplastic | 20 (25.0) | 1 (3.3) | 19 (38.0) | |
| Tubular | 44 (55.0) | 19 (63.3) | 25 (50.0) | |
| Tubulovillous | 16 (20.0) | 10 (33.3) | 6 (12.0) | |
| *Location* | | | | 0.45 |
| Proximal | 30 (37.5) | 11 (36.7) | 19 (38.0) | |
| Distal | 22 (27.5) | 6 (20.0) | 16 (32.0) | |
| Rectum | 17 (21.3) | 9 (30.0) | 8 (16.0) | |
| Colon[c] | 11 (13.8) | 4 (13.3) | 7 (14.0) | |
| *Size* (in mm) | | | | 0.64 |
| <5 | 43 (53.8) | 17 (56.7) | 26 (52.0) | |
| ≥5 | 22 (27.5) | 9 (30.0) | 13 (26.0) | |
| unknown | 15 (18.8) | 4 (13.3) | 11 (22.0) | |
| *APC mutation type* | | | | |
| Frameshift | 22 (27.5) | 22 (73.3) | n.a.[d] | |
| Nonsense | 8 (10.0) | 8 (26.7) | n.a. | |

[a] $APC^+$: polyps with truncating *APC* mutation; $APC^-$: polyps without truncating *APC* mutation. [b] $APC^+$ versus $APC^-$, Fisher's exact test. [c] Exact location unknown. [d] n.a., not applicable.

Truncating *APC* mutations were detected in 30 (37.5%) of the 80 polyps. Frameshift mutations were observed most often (*n*=22, 73.3% of all truncating *APC* mutations detected); nonsense mutations were identified in 8 polyps (2 transversions, 6 transitions). Individuals with an $APC^+$ polyp were comparable to those with an $APC^-$ polyp with regard to age at polyp diagnosis, sex, and carrier status (data not shown). Four (25%) of the hyperplastic polyps were from known carriers. Overall, most polyps were tubular adenomas; most were located in the proximal colon; and the majority was smaller than 5 mm. Hyperplastic polyps were more common in the $APC^-$ group; tubulovillous adenomas were more common in the $APC^+$ group. $APC^+$ polyps were more often located in the rectum than $APC^-$ polyps.

**Table 6.5** Dietary factors and truncating *APC* mutations in HNPCC-associated colorectal polyps: case-control and case-case comparisons.

| | Odds ratios (95% confidence intervals)[a] | | | |
|---|---|---|---|---|
| | All polyp cases vs. controls[b] 71/103 | $APC^+$ vs. controls 27/103 | $APC^-$ vs. controls 44/103 | $APC^+$ vs. $APC^-$ 27/44 |
| *Total energy intake*, per 4266.2kJ/day | 0.6 (0.3-1.2) | 0.5 (0.2-1.3) | 0.8 (0.4-1.5) | 0.3 (0.1-1.2) |
| FOOD GROUPS | | | | |
| *Total vegetables & fruit*, per 179.6g/day | 0.9 (0.6-1.5) | 0.7 (0.3-1.5) | 1.0 (0.6-1.7) | 0.8 (0.4-1.7) |
| *Vegetables*, per 45.3g/day | 1.1 (0.8-1.6) | 1.2 (0.7-2.1) | 1.1 (0.7-1.7) | 1.4 (0.8-2.3) |
| *Fruit*, per 168.1g/day | 0.9 (0.6-1.4) | 0.6 (0.3-1.4) | 1.0 (0.6-1.7) | 0.7 (0.3-1.5) |
| *Cereals*, per 58.2g/day | 1.1 (0.7-1.7) | 0.8 (0.3-1.8) | 1.3 (0.8-2.1) | 0.5 (0.2-1.2) |
| *Total meat*, per 67.9g/day | 1.1 (0.6-1.9) | 1.1 (0.5-2.5) | 1.1 (0.6-2.1) | 0.7 (0.3-1.9) |
| *Red meat*, per 44.7g/day | 0.9 (0.5-1.6) | 1.2 (0.5-2.5) | 0.8 (0.4-1.6) | 1.0 (0.4-2.7) |
| *Poultry*, per 9.5g/day | 1.0 (0.7-1.3) | 0.8 (0.5-1.4) | 1.0 (0.7-1.4) | 0.9 (0.6-1.4) |
| *Fish*, per 12.5g/day | 1.0 (0.7-1.6) | 0.7 (0.3-1.4) | 1.2 (0.8-2.0) | 0.5 (0.2-1.2) |
| *Dairy products*, per 374.0g/day | 1.2 (0.7-2.0) | 0.7 (0.3-1.8) | 1.4 (0.8-2.4) | 0.4 (0.1-1.1) |
| NUTRIENTS[c] | | | | |
| *Alcohol*, per 14.3g/day | 1.6 (1.1-2.5) | 1.2 (0.7-2.3) | 1.9 (1.2-3.0) | 0.8 (0.4-1.5) |
| *Fat*, per 25.1g/day | 0.6 (0.3-1.1) | 1.0 (0.4-2.7) | 0.4 (0.2-1.0) | 1.9 (0.6-5.7) |

| ***Table 6.5 cont.*** | All polyp cases vs. controls[b] 71/103 | APC$^+$ vs. controls 27/103 | APC$^-$ vs. controls 44/103 | APC$^+$ vs. APC$^-$ 27/44 |
|---|---|---|---|---|
| *Protein*, per 30.0g/day | 0.9 (0.4-2.1) | 0.7 (0.2-2.3) | 1.0 (0.5-2.4) | 0.4 (0.1-2.2) |
| *Carbohydrates*, per 71.7g/day | 1.0 (0.4-2.1) | 0.9 (0.3-2.9) | 1.0 (0.4-2.3) | 0.9 (0.3-3.0) |
| *Dietary fiber*, per 7.5g/day | 0.8 (0.5-1.4) | 0.9 (0.4-2.1) | 0.7 (0.4-1.4) | 1.3 (0.6-3.2) |
| *Calcium*, per 365.0mg/day | 1.0 (0.7-1.5) | 0.8 (0.5-1.5) | 1.1 (0.7-1.6) | 0.7 (0.4-1.4) |
| *Vitamin C*, per 66.5mg/day | 0.9 (0.6-1.6) | 0.7 (0.3-1.5) | 1.1 (0.6-1.8) | 0.7 (0.3-1.7) |
| *β-Carotene*, per 522.0μg/day | 0.8 (0.6-1.2) | 0.9 (0.6-1.5) | 0.8 (0.5-1.2) | 1.5 (0.9-2.7) |
| *Folate*, per 53.0μg/day | 1.3 (0.8-2.1) | 0.8 (0.4-1.8) | 1.6 (0.9-2.6) | 0.6 (0.3-1.6) |

[a] Adjusted for age (*i.e.*, cases: age at diagnosis polyp; controls: age at last colonoscopy), sex, total energy intake, carrier status, and cigarette smoking. [b] All polyp cases: all APC$^+$-polyp cases plus all APC$^-$-polyp cases. [c] Adjusted for total energy intake by the residual method.

Case-control and case-case comparisons were conducted to evaluate associations (quantified on a continuous scale) between dietary factors and the occurrence of truncating *APC* mutations in HNPCC-associated polyps (Table 6.5). Results of 'all polyp cases versus control' comparisons were generally comparable to the results observed for the total study population (see Table 6.2). Alcohol intake seemed to increase the risk of APC$^+$ polyps as well as APC$^-$ polyps, most noticeably and statistically significantly for APC$^-$ polyps. A borderline significant inverse association was observed between fat intake and APC$^-$ polyps. No statistically significant associations were observed for the other evaluated dietary factors. Additionally, no clear associations were observed between the evaluated smoking variables (*i.e.*, smoking status, number of cigarettes usually smoked per day, and total years of smoking) and the occurrence of truncating *APC* mutations in HNPCC-associated polyps (data not shown).

## Discussion

In this study, we evaluated associations between various environmental factors reported previously to be associated with sporadic colorectal cancer risk and the occurrence of colorectal tumors in known or suspected carriers of a germline mutation in one of the MMR genes. As anticipated, age was strongly, positively associated with ever been diagnosed with colorectal tumors in our study population. Fruit consumption lowered the risk of ever developing HNPCC-associated colorectal tumors and a borderline significant inverse association was observed for dietary fiber intake. Cigarette smoking and alcohol consumption seemed to increase the risk of HNPCC-associated colorectal tumors. None of the evaluated environmental risk factors was distinctively associated with a specific *APC* status of the colorectal polyps.

The primary function of the MMR system is the correction of base-base mismatches and insertion/deletion loops that arise during DNA replication, but MMR proteins also appear to be involved in DNA-damage signaling and apoptosis (35, 36). Thus, loss of MMR may provide cells with both an increased mutation rate and a selective growth advantage (36). MSI, *i.e.*, the presence of alterations in the length of simple, repetitive microsatellite sequences, is the hallmark of MMR-deficiency. Most HNPCC-associated colorectal tumors, but also approximately 10-20% of the sporadic colon carcinomas, exhibit MSI.

Individuals with HNPCC are at increased risk for colorectal cancer because they have inherited a germline mutation in one of the MMR genes. In contrast to the general population, only one allele (*i.e.*, the other, ‘normal’, allele) needs to be inactivated in order for the MMR system to become defective. Environmental factors may influence colorectal cancer risk in individuals with HNPCC by, for instance, (a) being involved in the actual production or prevention of molecular alterations in the normal allele, resulting in deficient MMR, and/or in

other cancer-related genes in already MMR-deficient cells; or (b) creating an environment that favors the proliferation of certain cells and not others.

In this study, fruit consumption and dietary fiber intake were both found to be inversely associated with ever developing HNPCC-associated colorectal tumors. Fruits contain many potentially anticarcinogenic substances (*e.g.*, antioxidants), which may influence the occurrence of DNA damage and thus the production of mutations. Results from a study in MMR-deficient cells suggest that dietary antioxidants may decrease the risk of colorectal tumor formation in individuals with HNPCC by removing the selective pressure for MMR-deficiency and the accompanying increases in mutation rate due to reactive oxygen species (23).

Since 1971 when Burkitt reported the association of high dietary fiber intake with a low incidence of colon cancer in Africa (37), many studies investigated the role of dietary fiber in colorectal carcinogenesis, but results have been somewhat inconsistent (18, 19). However, recent large studies reported a strong inverse association between dietary fiber intake and sporadic colorectal adenomas (38) and sporadic colorectal carcinomas (39). Several mechanisms have been proposed to explain the protective effect of dietary fiber, including dilution of potential carcinogens, reduction of transit time, and the production of short-chain fatty acids, acetate, propionate, and butyrate (40). Our results suggest that high intake of dietary fiber may also lower the risk of colorectal cancer in individuals with HNPCC.

Cigarette smoking and alcohol intake seemed to increase the risk of ever developing HNPCC-associated colorectal tumors in our study population. However, the positive association with alcohol intake was statistically significant only in the study subset with cases for which polyp DNA was available. Like Voskuil *et al*. (20), who investigated associations between meat consumption and HNPCC-associated colorectal adenomas, no clear associations were observed with meat intake. In line with our results, Slattery *et al*. (41) reported a positive association

between cigarette smoking and MSI-positive sporadic colon carcinomas. Alcohol intake has also been found to be positively associated with MSI-positive sporadic colon carcinomas (42, 43).

Direct-sequencing in both directions was used to screen codons 1286 through 1585 of the *APC* gene for truncating mutations. Most colorectal tumors in which *APC* is mutated exhibit at least one truncating mutation in the MCR (44). Therefore, we do not expect that our decision to concentrate on truncating mutations in this region has resulted in extensive misclassification. Truncating *APC* mutations were identified in 37.5% of the polyps; frameshift mutations were most common. This is in line with findings reported by previous studies (11-14) and supports the idea that loss of APC function is also an important early event in a subset of HNPCC-associated colorectal tumors.

Our study population consisted of 73 known carriers (67% *hMLH1*, 30% *hMSH2*, and 3% gene not disclosed to us) and 175 suspected carriers. All suspected carriers belonged to a family fulfilling the Amsterdam criteria, which, compared to other existing clinical criteria for HNPCC, have the highest specificity for the presence of germline mutations in *hMLH1* or *hMSH2* (45). Additionally, 40% of the 'suspected carrier' cases had been diagnosed with colorectal tumors at an early age (*i.e.*, adenomatous polyps <40 years or colorectal cancer <50 years), which in addition to fulfillment of the Amsterdam criteria is a strong predictive factor for the presence of mutations in *hMLH1* or *hMSH2* (46). Still, a possible limitation of this study is that there may be individuals who do not have a germline mutation in one of the MMR genes among the suspected carriers.

Our study population was relatively small, and it is possible that some associations were not detected due to insufficient power. It should also be noted that multiple comparisons might lead to chance findings. Regarding truncating *APC* mutations, although microdissection was performed, it remains possible that, due to contaminating normal tissue, mutations were missed

eventually resulting in misclassification (*i.e.*, polyps with truncating *APC* mutation in the APC$^{-}$ group) and attenuation of our results.

To conclude, our data suggest that fruit consumption and dietary fiber intake may decrease the risk of colorectal tumors in individuals known or suspected of carrying an inherited mutation in one of the MMR genes. Cigarette smoking and possibly alcohol intake may increase the risk of HNPCC-associated colorectal tumors. The observed associations, if confirmed by other studies, support the hypothesis that also HNPCC-associated outcomes may be modified by environmental factors. This is intriguing and calls for further investigation. Enhanced knowledge of the effects of environmental factors on colorectal cancer risk in individuals with HNPCC may eventually result in the development of evidence-based dietary and/or lifestyle intervention strategies that, in combination with regular screening by colonoscopy, effectively lower the risk of colorectal cancer in this high-risk population.

## Acknowledgements

We thank Mary Velthuizen (The Netherlands Foundation for the Detection of Hereditary Tumors, Leiden) for assistance with participant recruitment; Maria van Vugt for assistance with data collection and participant recruitment; Dr. Marga Ocké [National Institute of Public Health and the Environment (RIVM), Bilthoven] for calculation of dietary intake; and, Dr. Han van Krieken (UMC St Radboud, Nijmegen) for re-evaluation of polyp histology. The medical specialists of the study participants and the numerous pathology departments that provided tumor material are gratefully acknowledged for their collaboration. We are indebted to all study participants for their cooperation and enthusiasm. This study was supported by the Dutch Cancer Society (grant LUW 98-1687).

## References

1. Marra, G., and Boland, C.R. (1995) Hereditary nonpolyposis colorectal cancer: the syndrome, the genes, and historical perspectives. *J. Natl. Cancer Inst.*, **87**, 1114-1125.
2. Lynch, H.T., and De la Chapelle, A. (2003) Hereditary colorectal cancer. *N. Engl. J. Med.*, **348**, 919-932.

3. Papadopoulos, N., Nicolaides, N.C., Wei, Y.F., Ruben, S.M., Carter, K.C., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., Adams, M.D., Venter, J.C., Hamilton, S.R., *et al*. (1994) Mutation of a mutL homolog in hereditary colon cancer. *Science*, **263**, 1625-1629.
4. Fishel, R., Lescoe, M.K., Rao, M.R., Copeland, N.G., Jenkins, N.A., Garber, J., Kane, M., and Kolodner, R. (1993) The human mutator gene homolog *MSH2* and its association with hereditary nonpolyposis colon cancer. *Cell*, **75**, 1027-1038.
5. Leach, F.S., Nicolaides, N.C., Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomaki, P., Sistonen, P., Aaltonen, L.A., Nystrom-Lahti, M., Guan, X.Y., Zhang, J., *et al*. (1993) Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. *Cell*, **75**, 1215-1225.
6. Rodriguez-Bigas, M.A., Boland, C.R., Hamilton, S.R, Henson, D.E., Jass, J.R., Meera Khan, P., Lynch, H., Perucho, M., Smyrk, T., Sobin, L., and Srivastava, S.A. (1997) A National Cancer Institute work shop on hereditary nonpolyposis colorectal cancer syndrome: meeting highlights and Bethesda guidelines. *J. Natl. Cancer Inst*., **89**, 1758-1762.
7. Aaltonen, L.A., Salovaara, R., Kristo, P., Canzian, F., Hemminki, A., Peltomaki, P., Chadwick, R.B., Kaariainen, H., Eskelinen, M., Jarvinen, H., Mecklin, J-P, and De la Chapelle, A. (1998) Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. *N. Engl. J. Med*., **338**, 1481-1487.
8. Samowitz, W., Curtin, K., Lin, H.H., Robertson, M.A., Schaffer, D., Nichols, M., Gruenthal, K., Leppert, M.F., and Slattery, M.L. (2001) The colon cancer burden of genetically defined hereditary nonpolyposis colon cancer. *Gastroenterology*, **121**, 830-838.
9. Cunningham, J.M., Kim, C-Y, Christensen, E.R. Tester, D.J., Parc, Y., Burgart, L.J., Halling, K.C., McDonnell, S.K., Schaid, D.J., Walsh-Vockley, C. Kubly, V., Nelson, H., *et al*. (2001) The frequency of hereditary defective mismatch repair in a prospective series of unselected colorectal carcinomas. *Am. J. Hum. Genet*., **69**, 780-790.
10. Mitchell, R.J., Farrington, S.M., Dunlop, M.G., and Campbell, H. (2002) Mismatch repair genes *hMLH1* and *hMSH2* and colorectal cancer: a HuGE review. *Am. J. Epidemiol*., **156**, 885-902.
11. Huang, J., Papadopoulos, N., McKinley, A.J., Farrington, S.M., Curtis, L.J., Wyllie, A.H., Zheng, S., Willson, J.K.V., Markowitz, S.D., Morin, P., Kinzler, K.W., Vogelstein, B., and Dunlop, M.G. (1996) *APC* mutations in colorectal tumors with mismatch repair deficiency. *Proc. Natl. Acad. Sci. USA*, **93**, 9049-9054.
12. Miyaki, M., Iijima, T., Kimura, J., Yasuno, M., Mori, T., Hayashi, Y., Koike, M., Shitara, N., Iwama, T., and Kuroki, T. (1999) Frequent mutation of *ß-Catenin* and *APC* genes in primary colorectal tumors from patients with hereditary nonpolyposis colorectal cancer. *Cancer Res*., **59**, 4506-4509.
13. Akiyama, Y., Nagasaki, H., Yagi, K.O., Nomizu, T., and Yuasa, Y. (2000) *ß-Catenin* and adenomatous polyposis coli (*APC*) mutations in adenomas from hereditary nonpolyposis colorectal cancer patients. *Cancer Lett*., **157**, 185-191.

14. Huang, J., Zheng, S., Jin, S-H, and Zhang, S-Z. (2004) Somatic mutations of *APC* gene in carcinomas from hereditary nonpolyposis colorectal cancer patients. *World J. Gastroenterol*., 10, 834-836.
15. Fearon, E.R., and Vogelstein, B. (1990) A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759-767.
16. Powell, S.M., Zilz, N., Beazer-Barclay, Y., Bryan, T.M., Hamilton, S.R., Thibodeau, S.N., Vogelstein, B., and Kinzler, K.W. (1992) *APC* mutations occur early during colorectal tumorigenesis. *Nature*, **359**, 235-237.
17. Park, J-G., Park, Y.J., Wijnen, J.T., and Vasen, H.F.A. (1999) Gene-environment interaction in hereditary nonpolyposis colorectal cancer with implications for diagnosis and genetic testing. *Int. J. Cancer*, **82**, 516-519.
18. World Cancer Research Fund (WCRF) Panel (Potter J.D., Chair). (1997) *Food, nutrition and the prevention of cancer: a global perspective*. WCRF/American Institute for Cancer Research, Washington, DC.
19. Potter, J.D. (1999) Colorectal cancer: molecules and populations. *J. Natl. Cancer Inst*., **91**, 916-932.
20. Voskuil, D.W., Kampman, E., Grubben, M.J.A.L., Kok, F.J., Nagengast, F.M., Vasen, H.F.A., and Van 't Veer, P. (2002) Meat consumption and meat preparation in relation to colorectal adenomas among sporadic and HNPCC family patients in the Netherlands. *Eur. J. Cancer*, **38**, 2300-2308.
21. Glaab, W.E., and Skopek, T.R. (1999) Cytotoxic and mutagenic response of mismatch repair-defective human cancer cells exposed to a food-associated heterocyclic amine. *Carcinogenesis*, **20**, 391-394.
22. Glaab, W.E., Kort, K.L., and Skopek, T.R. (2000) Specificity of mutations induced by the food-associated heterocyclic amine 2-amino-1-methyl-6-phenylimidazo-[4,5-b]-pyridine in colon cancer cell lines defective in mismatch repair. *Cancer Res*., **60**, 4921-4925.
23. Glaab, W.E., Hill, R.B., and Skopek, T.R. (2001) Suppression of spontaneous and hydrogen peroxide-induced mutagenesis by the antioxidant ascorbate in mismatch repair-deficient human colon cancer cells. *Carcinogenesis*, **22**, 1709-1713.
24. Wei, K., Kucherlapati, R., and Edelmann, W. (2002) Mouse models for human DNA mismatch-repair gene defects. *Trends Mol. Med*., **8**, 346-352.
25. De Vos tot Nederveen Cappel, W.H., Nagengast, F.M., Griffioen, G., Menko, F.H., Taal, B., Kleibeuker, J.H., and Vasen H.F. (2002) Surveillance for hereditary nonpolyposis colorectal cancer. *Dis. Colon Rectum*, **45**, 1588-1594.
26. Vasen, H.F., Mecklin, J-P., Meera Khan, P., and Lynch, H.T. (1991) The International Collaborative group on Hereditary Nonpolyposis Colorectal Cancer (ICG-HNPCC). *Dis. Colon. Rectum*, **34**, 424-425.

27. Vasen, H.F., Watson, P., Mecklin, J-P., and Lynch, H.T. (1999) New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. *Gastroenterology*, **116**, 1453-1456.
28. Menko, F.H., Griffioen, G., Wijnen, J. Th., Tops, C.M.J., Fodde, R., and Vasen, H.F.A. (1999) Genetica van darmkanker. I. Non-polyposis- en polyposisvormen van erfelijke darmkanker [Genetics of colorectal cancer. I. Non-polyposis and polyposis forms of hereditary colorectal cancer]. *Ned. Tijdschr. Geneeskd*., **143**, 1201-1206.
29. Ocké, M.C, Bueno de Mesquita, H.B., Goddijn, H.E., Jansen, A., Pols, M.A., Van Staveren, W.A., and Kromhout, D. (1997) The Dutch EPIC food frequency questionnaire. I.. Description of the questionnaire, and relative validity and reproducibility for food groups. *Int. J. Epidemiol*., **26** (Suppl. 1), S37-S48.
30. Ocké, M.C, Bueno de Mesquita, H.B., Pols, M.A., Smit, H.A., Van Staveren, W.A., and Kromhout, D. (1997) The Dutch EPIC food frequency questionnaire. II. Relative validity and reproducibility for nutrients. *Int. J. Epidemiol*., **26** (Suppl. 1), S49-S58.
31. Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T., and Nakamura, Y. (1992) Somatic mutations of the *APC* gene in colorectal tumors: mutation cluster region in the *APC* gene. *Hum. Mol. Genet*., **1**, 229-233.
32. Miyaki, M., Konishi, M., Kikuchi-Yanoshita, R., Enomoto, M., Igari, T., Tanaka, K., Muraoka, M., Takahashi, H., Amada, Y., Fukayama, M., Maeda, Y., Iwama, T., *et al.* (1994) Characteristics of somatic mutation of the adenomatous polyposis coli gene in colorectal tumors. *Cancer Res*., **54**, 3011-3020.
33. Diergaarde, B., Van Geloof, W.L., Van Muijen, G.N.P., Kok, J.F., and Kampman, E. (2003) Dietary factors and the occurrence of truncating *APC* mutations in sporadic colon carcinomas: a Dutch population-based study. *Carcinogenesis*, **24**, 283-290
34. Willett, W.C., Howe, G.R., and Kushi, L.H. (1997) Adjustment for total energy intake in epidemiologic studies. *Am J. Clin. Nutr*., **65 (**suppl), 1220S-1228S.
35. Buermeyer, A.B., Deschênes, S.M., Baker, S.M., and Liskay, R.M. (1999) Mammalian DNA mismatch repair. *Annu. Rev. Genet*., **33**, 533-564.
36. Fishel, R. (2001) The selection for mismatch repair defects in hereditary nonpolyposis colorectal cancer: revising the mutator hypothesis. *Cancer Res*., **61**, 7369-7374.
37. Burkitt, D.P (1971) Epidemiology of cancer of the colon and rectum. *Cancer*, **28**, 3-13.
38. Peters, U., Sinha, R., Chatterjee, N., Subar, A.F., Ziegler, R.G., Kulldorff, M., Bresalier, R., Weissfeld, J.L., Flood, A., Schatzkin, A., and Hayes, R.B. for the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial Project Team (2003) Dietary fibre and colorectal adenoma in a colorectal cancer early detection programme. *Lancet*, **361**, 1491-1495.

39. Bingham, S.A., Day, N.E., Luben, R., Ferrari, P., Slimani, N., Norat, T., Clavel-Chapelon, F., Kesse, E., Nieters, A., Boeing, H., Tjønneland, A., Overvad, K., *et al*. (2003) Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study. *Lancet*, **361**, 1496-1501.
40. Bingham, S.A. (1990) Mechanisms and experimental evidence relating dietary fibre and starch to protection against large bowel cancer. *Proc. Nutr. Soc*., **49**, 153-171.
41. Slattery, M.L., Curtin, K., Anderson, K., Ma, K-N, Ballard, L., Edwards, S., Schaffer, D., Potter, J., Leppert, M., and Samowitz, W.S. (2000) Associations between cigarette smoking, lifestyle factors, and microsatellite instability in colon tumors. *J. Natl. Cancer Inst*., **92**, 1831-1836.
42. Slattery, M.L., Anderson, K., Curtin, K., Ma, K-N, Schaffer, D., and Samowitz, W. (2001) Dietary intake and microsatellite instability in colon tumors. *Int. J. Cancer*, **93**, 601-607.
43. Diergaarde, B., Braam, H., Van Muijen, G.N.P., Ligtenberg, M.J.L., Kok, F.J., and Kampman, E. (2003) Dietary factors and microsatellite instability in sporadic colon carcinomas. *Cancer Epidemiol., Biomarkers & Prev*., **12**, 1130-1136.
44. Rowan, A.J., Lamlum, H., Ilyas, M., Wheeler, J., Papadopoulou, A., Bicknell, D., Bodmer, W.F., and Tomlinson, I.P.M. (2000) APC mutations in sporadic colorectal polyps: A mutational "hotspot" and interdependence of the "two hit". *Proc. Natl. Acad. Sci. USA*, 97, 3352-3357.
45. Syngal, S., Fox, E.A., Eng, C., Kolodner, R.D., and Garber, J.E. (2000) Sensitivity and specificity of clinical criteria for hereditary non-polyposis colorectal cancer associated mutations in *MSH2* and *MLH1*. *J. Med. Genet*., **37**, 641-645.
46. Wijnen, J.T., Vasen, H.F.A., Khan, P.M., Zwinderman, A.H., van der Klift, H., Mulder, A., Tops, C., Moller, P., and Fodde, R. (1998) Clinical findings with implications for genetic testing in families with clustering of colorectal cancer. *N. Engl. J. Med*., **339**, 511-518.

# Chapter 7

# General discussion

The studies described in this thesis examined associations between environmental risk factors and molecular alterations that drive colorectal carcinogenesis. In this final chapter, we will summarize the main findings of the five different studies, and discuss methodological considerations, public health implications and future research directions.

## Main findings

The overall aim of the studies described in this thesis was to gain further insight into the relationship(s) between environmental factors previously reported to be associated with colorectal cancer risk and molecular alterations known to be involved in colorectal carcinogenesis. We examined associations between: (a) dietary factors and the occurrence of truncating *APC* mutations in sporadic colorectal adenomas (Chapter 2) and sporadic colon carcinomas (Chapter 3); (b) dietary factors and MSI in sporadic colon carcinomas (Chapter 4); (c) cigarette smoking and *APC*, K-*ras* and *p53* alterations and MSI in sporadic colon carcinomas (Chapter 5); and, (d) dietary factors, cigarette smoking, and HNPCC-associated colorectal tumors (Chapter 6).

Red meat consumption and fat intake increased the risk of colorectal adenomas without truncating *APC* mutation ($APC^-$) in particular, while carbohydrates seemed to especially decrease the risk of $APC^-$ adenomas. Most other evaluated dietary factors were not specifically associated with adenomas with truncating *APC* mutation ($APC^+$) or $APC^-$ adenomas, but seemed to influence the development of these two distinct early stages in colorectal carcinogenesis equally. This suggests that they are probably not specifically involved in the initiation of truncating mutations in the *APC* gene (Chapter 2).

Consumption of vegetables lowered the risk of $APC^+$ carcinomas as well as $APC^-$ carcinomas, but the protective effect was significantly higher for $APC^-$ carcinomas. Alcohol intake was associated with an increased risk of $APC^-$ carcinomas only. Interestingly, red meat, fish and fat seemed to especially increase the risk of $APC^+$ carcinomas (Chapter 3). Red meat consumption was also found to be associated with an increased risk of MSI-L/MSS carcinomas in particular, whereas alcohol intake seemed to increase the risk of MSI-H carcinomas. Fruit consumption seemed to especially decrease the risk of MSI-H carcinomas exhibiting epigenetically silenced *hMLH1* (Chapter 4).

Smoking-related colon carcinomas seemed to develop through a p53 overexpression-negative pathway, and cigarette smoking was found to be positively associated with colon carcinomas exhibiting transversion mutations in particular. Regarding the latter, our results suggested that cigarette smoking especially results in colon tumors with K-*ras* transversion mutations. No clear associations were observed with MSI status of the carcinomas. Our results support the hypothesis that cigarette smoking is primarily associated with specific colon tumor subgroups (Chapter 5).

Fruit consumption and dietary fiber intake lowered the risk of ever developing colorectal tumors in individuals with HNPCC. Cigarette smoking and possibly alcohol consumption seemed to increase the risk of HNPCC-associated colorectal tumors. These results support the hypothesis that environmental factors play a role in HNPCC-associated colorectal carcinogenesis. Truncating *APC* mutations were detected in 37.5% of the HNPCC-associated polyps, but none of the evaluated environmental risk factors was distinctively associated with a specific *APC* status of the polyps (Chapter 6).

Overall, our results, if confirmed by other studies, support the hypothesis that environmental factors previously reported to be associated with colorectal cancer risk exert their effect on this risk by affecting the occurrence of specific molecular alterations that drive

colorectal carcinogenesis. In addition, it seems that environmental factors may affect progression into later stages differently depending on the advantage for tumor formation exerted by the molecular alterations already present.

## Methodological considerations

Many of the methodological strengths and limitations of the different studies described in this thesis have already been addressed in the previous chapters. In this section, we will continue the discussion of methodological issues related to the studies performed and to molecular epidemiological studies of colorectal cancer in general. We will in particular focus on issues related to the assessment of outcome and exposure. For an extensive general discussion of epidemiological methods, including case-control studies, please see Rothman and Greenland (1) or Koepsell and Weiss (2).

### ***Molecular alterations: detection and significance***

Errors in the detection of molecular alterations may result in misclassification. In addition, to allow comparison with other studies and avoid inappropriate interpretations, it is necessary to provide as much detail as feasible about the alterations that are being evaluated. *How* alterations are measured and *what* is measured influences the validity of a study.

In the studies described in this thesis, we focused on molecular alterations that are considered to be important and are often observed in colorectal carcinogenesis. That is, we evaluated the occurrence of (markers of) functional alterations in colorectal tumors. Established methods were used to detect the alterations, and we don’t expect that extensive misclassification due to ‘missed’ mutations has occurred. Overall, frequencies of the alterations evaluated were in line with those reported by others (see Chapters 2-6).

Colorectal polyps and colon carcinomas were screened for truncating mutations in the MCR of the *APC* gene (Chapters 2, 3, 5 and 6). Tumors were classified as $APC^+$ if a truncating mutation in *APC* was detected; as $APC^-$ if no truncating mutations were detected in *APC* and codons 1286 to 1585 were completely analyzed for mutations. Although we only screened a relatively small part of the large *APC* gene for mutations, we do not expect that this has resulted in extensive misclassification (*i.e.*, tumors with truncating mutations in the $APC^-$ group) because most colorectal tumors in which *APC* is mutated exhibit at least one truncating mutation in the MCR (3). We focused on truncating mutations because these mutations indisputably result in function loss of the APC protein; all truncating mutations in the region analyzed result in the complete absence of the C-terminal portion of the APC protein which harbors functional domains (*e.g.*, domains involved in β-catenin regulation and microtubule association) critical for APC's tumor suppressing function (4). Missense mutations in *APC* do occur (see Chapter 3 and Ref. 5), but their biological significance is uncertain. It should be noted that hypermethylation of the *APC* promoter region may also result in inactivation of APC (6, 7). This is much less common than function loss through truncating mutations and probably involves other mechanisms, but it is possible that in a few of the tumors in the $APC^-$ group APC is non-functional due to this. However, these tumors were not misclassified because they do not exhibit a truncating *APC* mutation.

Regarding the K-*ras* gene, codons 12 and 13 were screened for alterations and only mutations resulting in amino acid change, and, thus, in constitutively active ras protein, were taken into account (Chapter 5). That is, the alterations examined are biologically significant. In addition, because most (~90%) activating K-*ras* mutations have been detected in codons 12 and 13 (8), we expect that the number of functional alterations missed by only screening these codons is low.

We examined sporadic colon carcinomas for mutations in exons 5-8 of the *p53* gene as well as for overexpression of the p53 protein (Chapter 5). We focused on exons 5-8 because most *p53* mutations appear to occur in this region of the gene (9). However, by not screening the whole gene we may have missed functional mutations in other exons. Carcinomas were defined as $p53^+$ if they exhibited a mutation that resulted in amino acid change or truncation of the protein; as $p53^-$ if no missense or truncating mutations were detected and all 4 codons were completely analyzed for mutations. Overexpression of p53 can be relatively easily determined by immunohistochemistry and is, possibly because of that, often used as an indicator of *p53* mutations (*e.g.*, 10, 11). However, not all *p53* mutations result in the accumulation of inactive p53 protein and can be detected with immunohistochemical analysis (12, Chapter 5). Moreover, overexpression of p53 may, for instance, also be the result of alterations in proteins that modify the induction or stabilization of p53. Thus, results obtained from immunohistochemistry of p53 overexpression are not necessarily the same as those obtained from sequencing the *p53* gene (see Chapter 5).

We used the criteria and the five 'reference' panel markers (*i.e.*, *BAT25*, *BAT26*, *D5S346*, *D2S123*, and *D17S250*) recommended by a special workshop on MSI in colorectal cancer sponsored by the US National Cancer Institute (13) for our analyses of MSI (Chapters 4 and 5). 'Normal' DNA was isolated from tumor-free colon tissue but may, for instance, also be isolated from blood if that is available. Tumors were classified as MSI-H if two or more markers showed instability, MSI-L if one marker showed instability, and MSS if none of the markers examined showed instability (13). The distinction between MSI-L and MSS groups is highly dependent on the number and type of microsatellites analyzed. We analyzed five markers, which may be insufficient to conclude with certainty whether or not an apparently stable tumor would demonstrate MSI if more markers were used. Therefore, MSI-L and MSS groups were combined when we assessed associations between diet and the occurrence of MSI (Chapter 4); the MSI-H

group was compared with the MSS group as well as with the MSI-L/MSS group when investigating cigarette smoking (Chapter 5). When publishing results involving MSI, extensive information about the markers and definition of MSI used should be provided to enable comparison of results.

Expression of hMLH1 and hMSH2 was determined in all sporadic colon carcinomas by immunohistochemistry (Chapter 4). In addition to being an alternative marker for defective MMR, immunohistochemistry of hMLH1 and hMSH2 provides information on the specific MMR gene that is involved. MSI-H as determined with the reference panel had a 100% sensitivity for identifying colon carcinomas exhibiting hMLH1 or hMSH2 loss of expression. However, hMLH1 and hMSH2 were both present in 8 of the MSI-H tumors (Chapter 4), and results obtained from using immunohistochemistry of hMLH1 and hMSH2 as marker of defective MMR are not necessarily the same as results obtained from using occurrence of MSI.

***Molecular alterations and colorectal tumors***

Evaluation of associations between putative environmental risk factors and the presence of somatic changes in cancer-related genes in colorectal tumors can potentially provide further clues to the relationships between dietary and lifestyle factors and the molecular alterations that drive colorectal carcinogenesis. But, which colorectal tumors should be screened for alterations?

Thus far, most epidemiological studies used sporadic colorectal carcinomas to investigate the relationship between environmental factors and the occurrence of specific molecular alterations (*e.g.*, 10, 11, 14-20, Chapters 3-5), possibly because, compared to colorectal polyps, molecular alterations in carcinomas are easier to study. Carcinomas are usually larger (more DNA available) and individuals usually only get one once (and that is the one that is screened for alterations). Colorectal polyps, on the other hand, are usually small, and individuals are often diagnosed with multiple polyps during a colonoscopy and often develop

polyps more than once in their life. Moreover, while carcinomas are almost always sent to pathology departments for histological examination and their tissue is usually stored for long periods of time, colorectal polyps, small polyps in particular, are, if detected and retrieved, not always kept and stored. However, because colorectal polyps are an early stage in colorectal carcinogenesis, examining the relationship between environmental exposure and molecular alterations in polyps may provide insight into how, and which, environmental factors are involved in the production of early occurring functional mutations.

The study described in Chapter 2 is, to our knowledge, the first study that has evaluated associations between dietary factors and the occurrence of *APC* mutations in sporadic colorectal adenomas. Formalin-fixed, paraffin-embedded polyp tissue was obtained from the pathology departments of the collaborating hospitals. The study pathologist re-evaluated the histology of all obtained polyps, and mutation analysis was performed on adenomas only. Regarding individuals for whom tissue was available from more than one histologically confirmed adenomatous polyp, the largest adenoma obtained was selected for mutation analysis. We did this for practical reasons, the larger the polyp the more polyp-DNA is available for mutation analysis, but also because we were particularly interested in polyps with potential for progression, and larger polyps are more likely to progress to invasive carcinoma (21). In addition, larger polyps are more likely to be retrieved and stored than smaller polyps. However, because we choose this approach, we don't have information about the mutational status of the other, smaller adenomas, and there may be individuals with multiple adenomas in which the largest polyp has no truncating *APC* mutation but one of the smaller polyps does. We used the same approach in the HNPCC study, although in that study we also screened hyperplastic polyps for truncating *APC* mutations (Chapter 6).

Martinez *et al*. (22) used a different approach in a study on environmental risk factors and K-*ras* mutations in colorectal polyps. They decided to analyze all adenomas ≥ 0.5cm for

mutations in codons 12 and 13 of K-*ras* and defined individuals as K-*ras*-positive if one or more adenomas exhibited a K-*ras* mutation. However, it is unclear whether they were able to obtain tissue from all resected polyps ≥ 0.5cm. Additionally, they were unable to sequence 12% of the adenomas they did obtain. Thus, despite analyzing all adenomas ≥ 0.5cm available, some of the individuals defined as K-*ras*-negative may still have adenomas with K-*ras* mutations. In addition, it is currently unclear what the strengths and limitations are of grouping individuals with multiple polyps which all exhibit the alteration that is being evaluated together with individuals in which only one of the polyps screened exhibits the alteration.

Studies on molecular alterations in colon carcinomas, including our own, point to the existence of different molecular pathways in colorectal carcinogenesis, which seem to reflect different environmental exposures (10, 11, 14-20, Chapters 3-5). As noted earlier, environmental factors may influence the occurrence of somatic molecular alterations in colon carcinomas directly, *i.e.*, by being involved in the actual production or prevention of these alterations, and/or indirectly, *i.e.*, by being involved in the promotion or evasion of progression into later stages and eventually into carcinomas. However, epidemiological studies with molecular alterations in carcinomas as end points do not answer the question of exactly *how* environmental factors are involved in the development of carcinomas with specific alterations. That is, one can't conclude from these studies that, for instance, "meat produces frameshift mutations", but will have to phrase it like: "meat enhances the development of carcinomas with frameshift mutations".

When interpreting results it should also be noted that molecular alterations in tumors not only represent the interactions of carcinogens with DNA repair processes but often also reflect the, possibly tissue-specific, selection of those mutations that provide pre-malignant and malignant cells with a clonal growth advantage. Moreover, due to practical reasons it is usually impossible to obtain all targeted tumor-tissue samples and, subsequently, for instance, to extract

'useable' DNA from these samples. This may affect the validity of the study (*i.e.*, result in selection bias), and attempts should be made to determine if the study population differs from the targeted population.

To conclude, in the United States most professional organizations currently advise that average-risk persons >50 years of age should be regularly screened for colorectal cancer. In the Netherlands, like in most other European countries, routine screening (*i.e.*, screening without having complaints) for colorectal tumors is not (yet) recommended for the general population. This may result in differences in study populations and in the type, size and number of colorectal polyps and cancers detected, which should be acknowledged when comparing results from US studies with results from European studies.

***Assessment of environmental exposure***

As discussed above, the interpretation of data from studies on environmental risk factors and molecular alterations in colorectal tumors depends on *how* the molecular alterations are measured, *what* alteration is measured, and *where* the alterations are located. But, obviously, the interpretation of data from these studies depends on the methods used to measure environmental exposures as well. Measurement of dietary intake and other lifestyle factors is complex. The assessment of molecular alterations is generally more accurate than the assessment of environmental exposures and less heavily dependent on study design. For a general discussion of methods of dietary assessment and issues related to these different methods see Willett (23).

Overall, quality of the assessment of environmental exposure in our studies is comparable to that in other studies. In the sporadic adenoma study (Chapter 2) and in the HNPCC study (Chapter 6), usual dietary intake was assessed with a validated, semi-quantitative food frequency questionnaire that was originally developed for the Dutch cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC). The reproducibility and relative

validity of this questionnaire for food group and nutrient intakes are regarded as sufficiently high (median Spearman correlation coefficient of 0.6 between intakes based on the questionnaire and those based on 24-hour recalls), and the questionnaire is considered adequate for ranking Dutch individuals according to intake of most food groups and nutrients, although the relative validity for some food groups (*e.g.*, vegetables and fish) and nutrients (*e.g.*, β-carotene) was low (*i.e.*, correlation coefficient <0.4) (24, 25). Information on lifestyle factors, including smoking habits, was collected using a self-administered, structured questionnaire. Regarding smoking, information on cigarette smoking status (current, former, never), number of cigarettes usually smoked per day, total number of years smoked and, if applicable, age at which participant stopped smoking was obtained. The frequency of ever smokers in the sporadic adenoma study population was comparable to the frequencies observed in a random sample of the general population and in the group of invited subjects who decided not to participate in the study (26). The frequency of current smokers in the HNPCC study population was lower than the frequency observed in the general Dutch population during the time of enrollment (Centraal Bureau Voor de Statistiek, 2004; www.cbs.nl). The lower rate might be due to underreporting, although smoking misclassification rates are usually low (27). Alternatively, it is also possible that our HNPCC study participants, because they are aware of their higher risk for cancer, are more health conscious and truly smoke less often.

In the case-control study of sporadic colon carcinomas (Chapters 3-5), usual dietary intake was assessed with an interview-based questionnaire. The questionnaire covered the complete dietary pattern and was an extended version of a validated questionnaire used in a case-control study of breast cancer (28, 29). Frequently used household utensils and cups were weighed to enable estimation of portion sizes. As noted, the questionnaire was not self-administered but interview-based, which is thought to result in a higher degree of internal

consistency in the reported food pattern, and, thus, in a more precise measurement of intake (30). Information on current and previous smoking habits was also obtained during the interview. The smoking habits of our controls were comparable to those of the general Dutch population at the time of interview (31).

Even when questionnaires are perfect, study participants may not always answer the questions correctly (*i.e.*, correctly in the sense that their answers reflect their true exposure). Cases and controls are, by definition, individuals who differ in respect to their disease experience, and this may affect recall. In all our studies (Chapters 2-6), participants were asked to recall their diets from the past and, therefore, differential recall, which may have resulted in differential exposure misclassification, is possible. The bias caused by differential misclassification can either exaggerate or underestimate an effect (1). As discussed previously, one of the advantages of the conducted case-case comparisons is that the cases are unaware of the mutational status of their tumors. Consequently, systematic errors in dietary recall are less likely to bias results from case-case comparisons.

## Public health implications

By themselves, the studies described in this thesis have no immediate public health implications. However, they do add to the current knowledge of colorectal carcinogenesis and, in particular, provide clues to the relationships between dietary factors, cigarette smoking, and molecular alterations in colorectal carcinogenesis. Eventually, enhanced insight into this may prove useful for the development of effective colorectal cancer prevention strategies.

Molecular epidemiological studies on somatic mutations in colorectal tumors, including the studies described in this thesis, have also provided information about the distribution of specific molecular alterations in colorectal tumors in different patient populations (5, 10, 14-17,

19, 20, 22, 32, 33, Chapters 2-6). The pattern of molecular alterations observed in colorectal tumors is heterogeneous, and multiple molecular pathways to colorectal cancer appear to exist (32-35, Chapter 5). Recognition of this may, when more is known about how the presence of specific alterations in a tumor affect prognosis and response to treatment, result in the development of new methods of diagnosis (*e.g.*, molecular profiling), and more effective treatment methods (*i.e.*, treatment methods that are tailor-made to fit a specific molecular pattern) (36, 37, 38).

Additionally, because colorectal cancer cells are shed into the stool, knowledge about the distribution of mutations that cause colorectal cancer is also useful for the development of reliable molecular genetic tests for the early detection of colorectal cancer in stool samples (39). The use of specific molecular alterations provides a theoretical advantage over the use of conventional markers (*e.g.*, fecal occult blood tests) for early colorectal cancer detection because the molecular alterations reflect a qualitative rather than a quantitative difference between normal and neoplastic states (39). Moreover, if the tests prove to be reliable (*i.e.*, have a high sensitivity and specificity), the fact that they are noninvasive will probably make them more easily accepted by the different target populations.

To conclude, an unexpected negative consequence of effective colorectal cancer screening strategies might be the loss of evidence of a family history of colorectal cancer. The purpose of colorectal cancer screening is to detect adenomatous polyps, which are considered to be the precursor lesions of most colorectal carcinomas. When, subsequently, the detected polyps are removed, colorectal cancer can be prevented. As a result, individuals, who otherwise maybe would have developed colorectal cancer, have (only) a personal history of adenomatous polyp(s). Because the detection of polyps is usually not as dramatic as the detection of cancer, people may not discuss their polyps with family members. However, adenomatous polyps are a risk factor for colorectal cancer, and the detection of adenomatous polyps in multiple family

members does indicate that the family might have a higher than average risk for colorectal cancer, even if no colorectal cancer has occurred. Therefore, efforts are needed to help doctors and patients to communicate to family members the importance of a family history of adenomatous polyps.

## Future directions

Although much progress has been made in the prevention and treatment of colorectal cancer, the problem is far from solved: worldwide, still approximately 945,000 new cases are diagnosed and about 490,000 people die of the disease each year (40). In this section, we will briefly discuss several research directions that together may help to improve colorectal cancer prevention and treatment strategies.

***The role of environmental and genetic factors in colorectal carcinogenesis***

Colorectal cancer arises through complex interactions between multiple genetic and environmental factors. Understanding how, and which, genetic and environmental factors interact to produce colorectal cancer is critical to the development of useful prevention and treatment strategies. The studies described in this thesis examined associations between environmental risk factors and specific molecular alterations in colorectal tumors but did not take genetic variation into account [*i.e.*, common polymorphic variants; we did examine the role of environmental factors in individuals with HNPCC (see Chapter 6)]. However, common genetic variants likely contribute to colorectal cancer risk as well. Well-designed molecular epidemiological studies that evaluate environmental exposure as well as genetic variation are necessary, and now that the human genome is sequenced they are also possible, to further

elucidate the etiology of colorectal cancer (41-43). Important issues related to these studies include:

- **Polymorphism choice**. What genes and variants should be studied? In general, genetic variants that are most likely to affect phenotypic functions and ultimately contribute to disease (44). Evolution-based approaches may help to prioritize the genetic variants (45), but, ultimately, knowledge about the functional significance of the variant is necessary for correct interpretation of the results. To start with, functional variants of genes involved in metabolic pathways, DNA repair mechanisms, methylation, and histone modification seem promising.
- **End points**. End points need to be well defined, and etiological heterogeneity should be addressed by evaluating, for instance, histopathological data and/or the occurrence of specific patterns of somatic molecular alterations. A recently proposed two-stage regression model (46) might be useful for the efficient and systematical analysis of epidemiological data with multivariate disease classification information.
- **Assessment of genetic variants and somatic molecular alterations**. To avoid misclassification, methods should be precise and accurate. For large studies, high throughput methods will need to be used.
- **Assessment of exposure**. Correct assessment of diet is still a problem for epidemiological studies due to, among other things, real within-person variation, changes over time in both behavior and food supply, recall bias, and mismatches between foods analyzed for dietary tables and foods actually consumed (23). The use of biomarkers of exposure in addition to food frequency questionnaires may improve measurements of dietary intake. For biomarkers to be useful, the methods to determine them should be highly specific and sensitive. In addition, they should be able to measure the exposure marker in easily accessible biological samples, such as blood and urine.

- **Study size and population**. To examine the complex relationships between environmental and genetic factors in colorectal carcinogenesis, large study populations with sufficient genetic and exposure variation are necessary to ensure adequate statistical power to detect small effect sizes and to study appropriate interactions within relevant strata (43, 44).
- **False-positive findings**. Multiple comparisons might lead to chance findings. Consideration of the false positive report probability (*i.e.*, the probability of no true association between a genetic variant and disease given a statistically significant finding) might help to protect against over-interpreting statistically significant findings that are not likely to signify a true association (47).

***Early detection***

The earlier colorectal cancer is detected, the better the chances of treatment and survival. Thus, successful early detection methods for colorectal cancer are likely to reduce morbidity and mortality due to this disease. Well-designed molecular epidemiological studies are necessary to develop and evaluate biomarkers for early detection (48). These studies, should, among other things, address the specificity of the marker. That is, they should evaluate how well the marker distinguishes neoplasia from non-neoplasia, but also how well the marker distinguishes lesions with potential for progression into carcinomas from lesions without this potential.

***Social and behavioral research***

Although understanding the etiology of colorectal cancer is very important, by itself it is not enough to prevent or treat cancer. Advances in cancer research need to be translated into effective, socially and ethically acceptable intervention strategies in order to improve health and prevent cancer. Social and behavioral research plays a key role in the development and evaluation of these strategies and should not be neglected.

For instance, a better understanding of the interactions between environmental and genetic factors in colorectal carcinogenesis is expected to bring opportunities for tailored prevention that will maximize the likelihood of staying well (49). However, it is currently unclear how to use personalized risk information optimally (50). Moreover, there is considerable uncertainty regarding the potential for this information to motivate behavioral change and improve health (50, 51). Therefore, in addition to examining the analytical and clinical validity of the colorectal cancer risk tests before implementing them (52), it will be necessary to investigate how individuals process and understand risk-related information, how best to communicate risk information, how that risk information influences health strategies and behaviors, and how these, subsequently, affect health outcomes and costs (50, 53).

## References


1. Rothman, K.J., and Greenland, S. (1998) *Modern Epidemiology. Second edition*. Philadelphia, PA: Lippincott-Raven Publishers.
2. Koepsell, T.D., and Weiss, N.S. (2003) *Epidemiologic methods. Studying the occurrence of illness*. New York, NY: Oxford University Press.
3. Rowan, A.J., Lamlum, H., Ilyas, M., Wheeler, J., Papadopoulou, A., Bicknell, D., Bodmer, W.F., and Tomlinson, I.P.M. (2000) *APC* mutations in sporadic colorectal tumors: A mutational "hotspot" and interdependence of the "two hits". *Proc. Natl. Acad. Sci. USA*, **97**, 3352-3357.
4. Fodde, R., Smits, R., and Clevers, H. (2001) *APC*, signal transduction and genetic instability in colorectal cancer. Nature Reviews, 1, 55-67
5. Lüchtenborg, M., Weijenberg, M.P., Roemen, G.M.J.M., De Bruïne, A.P., Van den Brandt, P. A., Lentjes, M.H.F.M., Brink, M., Van Engeland, M., Goldbohm, A., and De Goeij, A.F.P.M. (2004) *APC* mutations in sporadic colorectal carcinomas from The Netherlands Cohort Study. *Carcinogenesis*, **25**, 1219-12226.
6. Hiltunen, M.O., Alhonen, L., Koistinaho, J., Myohanen, S., Paakkonen, M., Marin, S., Kosma, V.M., and Janne, J. (1997) Hypermethylation of the *APC* (adenomatous polyposis coli) gene promoter region in human colorectal carcinoma. *Int. J. Cancer*, **70**, 664-648.

7. Esteller, M., Sparks, A., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M.A., Gonzalez, S., Tarfa, G., Sidransky, D., Meltzer, S.J., Baylin, S.B., and Herman, J.G. (2000) Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. *Cancer Res.,* **60**, 4366-4371.
8. Bos, J.L., Fearon, E.R., Hamilton, S.R., Verlaan-de Vries, M., Van Boom, J.H., Van der Eb, J.A., and Vogelstein, B. (1987) Prevalence of ras *gene* mutations in human colorectal cancers. *Nature*, **327**, 293-297.
9. Greenblatt, M.S., Bennett, W.P., Hollstein, M., and Harris, C.C. (1994) Mutations in the *p53* tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res*., **54**, 4855-4878.
10. Freedman, A.N., Michalek, A.M., Marshall, J.R., Mettlin, C.J., Petrelli, N.J., Zhang, Z-F, Black, J.D., Satschidanand, S., and Asirwatham, J.E. (1996) The relationship between smoking exposure and p53 overexpression in colorectal cancer. *Br. J. Cancer*, **73**, 902-908.
11. Freedman, A.N., Michalek, A.M., Marshall, J.R., Mettlin, C.J., Petrelli, N.J., Black, J.D., Zhang, Z-F, Satschidanand, S., and Asirwatham, J.E. (1996) Familial and nutritional risk factors for p53 overexpression in colorectal cancer. *Cancer Epidemiol. Biomark. Prev*., **5**, 285-291.
12. Soussi, T., and Béroud, C. (2001) Assessing *TP53* status in human tumours to evaluate clinical outcome. *Nature Reviews*, **1**, 233-240.
13. Boland, C.R., Thibodeau, S.N., Hamilton, S.R., Sidransky, D., Eshleman, J.R., Burt, R.W., Meltzer, S.J., Rodrigues-Bigas, M.A., Fodde, F., Ranzani, G.N., and Srivastava, S. (1998) A National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res*., **58**, 5248-5257.
14. Voskuil, D.W., Kampman, E., Van Kraats, A.A., Balder, H.F., Van Muijen, G.N.P., Goldbohm, R.A., and Van ’t Veer, P. (1999) P53 overexpression and *p53* mutations in colon carcinomas: relation to dietary risk factors. *Int. J. Cancer*, **81**, 675-681.
15. Kampman, E., Voskuil, D.W., Van Kraats, A.A., Balder, H.F., Van Muijen, G.N.P., Goldbohm, R.A., and Van ‘t Veer, P. (2000) Animal products and K-*ras* codon 12 and 13 mutations in colon carcinomas. *Carcinogenesis*, **21**, 307-309.
16. Slattery, M.L., Curtin, K., Anderson, K., Ma, K-N, Edwards, S., Leppert, M., Potter, J., Schaffer, D., and Samowitz, W.S. (2000) Associations between dietary intake and Ki-*ras* mutations in colon tumors: a population-based study. *Cancer Res*., **60**, 6935-6941.
17. Slattery, M.L., Curtin, K., Anderson, K., Ma, K-N, Ballard, L., Edwards, S., Schaffer, D., Potter, J., Leppert, M., and Samowitz, W.S. (2000) Associations between cigarette smoking, lifestyle factors, and microsatellite instability in colon tumors. *J. Natl. Cancer Inst*., **92**, 1831-1836.
18. Slattery, M.L., Anderson, K., Curtin, K., Ma, K-N, Schaffer, D., and Samowitz, W. (2001) Dietary intake and microsatellite instability in colon tumors. *Int. J. Cancer*, **93**, 601-607.

19. Wu, A.H., Shibata, D., Yu, M. C., Lai, M-Y, and Ross, R.K. (2001) Dietary heterocyclic amines and microsatellite instability in colon adenocarcinomas. *Carcinogenesis*, **22**, 1681-1684.
20. Slattery, M.L., Curtin, K., Ma, K-N, Edwards, S., Schaffer, D., Anderson, K., and Samowitz, W.S. (2002) Diet, activity, and lifestyle associations with *p53* mutations in colon tumors. *Cancer Epidemiol. Biomark. Prev.*, **11**, 541-548.
21. Winaver, S.J., Zauber, A.G., Ho, M.N., O'Brien, M.J., Gottlieb, L.S., Sternberg, S.S., Waye, J.D., Schapiro, M., Bond, J.H., Panish, J.F., Ackroyd, F., Shike, M., *et al*. (1993) Prevention of colorectal cancers by colonoscopic polypectomy. The National Polyp Study Workgroup. *N. Engl. J. Med.*, **329**, 1977-1981.
22. Martinez, M.E., Maltzman, T., Marshall, J.R., Einspahr, J., Reid, M.E., Sampliner, R., Ahnen, D.J., Hamilton, S.R., and Alberts D.S. (1999) Risk factors for Ki-*ras* protooncogene mutation in sporadic colorectal adenomas. *Cancer Res.*, **59**, 5181-5185.
23. Willett, W. (1998) *Nutritional Epidemiology. Second edition*. New York, NY: Oxford University Press.
24. Ocké, M.C, Bueno de Mesquita, H.B., Goddijn, H.E., Jansen, A., Pols, M.A., Van Staveren, W.A., and Kromhout, D. (1997) The Dutch EPIC food frequency questionnaire. I. Description of the questionnaire, and relative validity and reproducibility for food groups. *Int. J. Epidemiol.*, **26** (Suppl. 1), S37-S48.
25. Ocké, M.C, Bueno de Mesquita, H.B., Pols, M.A., Smit, H.A., Van Staveren, W.A., and Kromhout, D. (1997) The Dutch EPIC food frequency questionnaire. II. Relative validity and reproducibility for nutrients. *Int. J. Epidemiol.*, **26** (Suppl. 1), S49-S58.
26. Tiemersma, E.W. (2002) *Meat, smoking, alcohol, and colorectal tumors: the role of genetic susceptibility.* Thesis Wageningen University.
27. Wells, A.J., English, P.B., Posner, S.F., Wagenknecht, L.E., and Perez-Stable, E.J. (1998) Misclassification rates for current smokers misclassified as nonsmokers. *Am. J. Public Health*, **88**, 1503-1509.
28. Kampman, E. (1994) *Do fermented dairy products and calcium protect against colorectal cancer? Contributions from epidemiology*. Thesis Maastricht.
29. Van 't Veer, P., Kok, F.J., Brants, H.A., Ockhuizen, T., Sturmans, F., and Hermus, R.J. (1990) Dietary fat and the risk of breast cancer. *Int. J. Epidemiol.*, **19**, 12-18.
30. Friedenreich, C.M., Slimani, N., and Riboli, E. (1992) Measurement of past diet: review of previous and proposed methods. *Epidemiol. Rev.*, **14**, 177-196.
31. Nicholaides-Bouman, A, Wald, N., Forey, B., and Lee, P. (eds.). *International smoking statistics.* Oxford University Press, London, 1993.

32. Smith, G., Carey, F.A., Beattie, J., Wilkie, M.J.V., Lightfoot, T.J., Coxhead, J., Garner, R.C., Steele, R.J.C., and Wolf, C.R. (2002) Mutations in *APC*, Kirsten-*ras*, and *p53* – alternative genetic pathways to colorectal cancer. *Proc. Natl. Acad. Sci. USA*, **99**, 9433-9438.
33. Salahshor, S., Kressner, U., Pahlman, L., Glimelius, B., Lindmark, G., and Lindblom, A. (1999) Colorectal cancer with and without microsatellite instability involves different genes. *Genes Chromosomes Cancer*, **26**, 247-252.
34. Olschwang, S., Hamelin, R., Laurent-Puig. P., Thuille, B., De Rycke, Y., Li, Y.J., Muzeau, F., Girodet, J., Salmon, R.J., and Thomas, G. (1997) Alternative genetic pathways in colorectal carcinogenesis. *Proc. Natl. Acad. Sci. USA*, **94**, 12122-12127.
35. Samowitz, W.S., Holden, J.A., Curtin, K., Edwards, S.L., Walker, A.R., Lin, H.A., Robertson, M.A., Nichols, M.F., Gruenthal, K.M., Lynch, B.J., Leppert, M.F., and Slattery, M.L. (2001) Inverse relationship between microsatellite instability and K-*ras* and *p53* gene alterations in colon cancer. *Am. J. Pathology*, **158**, 1517-1524.
36. Ishikawa, T., Fujita, T., Suzuki, Y., Okabe, S., Yuasa, Y., Iwai, T., Kawakami, Y. (2003) Tumor-specific immunological recognition of frameshift-mutated peptides in colon cancer with microsatellite instability. *Cancer Res.*, **63**, 5564-5572.
37. Risques, R.A., Moreno, V., Ribas, M., Marcuello, E., Capella, G., Peinado, M.A.. (2003) Genetic pathways and genome-wide determinants of clinical outcome in colorectal cancer. *Cancer Res*., **63**, 7206-7214.
38. Bertucci, F., Salas, S., Eysteries, S., Nasser, V., Finetti, P., Ginestier, C., Charafe-Jauffret, E., Loriod, B., Bachelart, L., Montfort, J., Victorero, G., Viret, F., *et al*. (2004) Gene expression profiling of colon cancer by DNA microarrays and correlation with histoclinical parameters. *Oncogene*, **23**, 1377-1391.
39. Dong, S.M., Traverso, G., Johnson, C., Geng, L., Favis, R., Boynton, K., Hibi, K., Goodman, S.N., D'Allesio, M., Paty, P., Hamiltion, S.R., Sidransky, D., *et al*. (2001) Detecting colorectal cancer in stool with the use of multiple genetic targets. *J. Natl. Cancer Inst*., **93**, 858-865.
40. Ferlay, J., Bray, F., Posani, P., and Parkin, D.M. (2001) *GLOBOCAN 2000: Cancer incidence, mortality and prevalence worldwide, version 1*. IARC CancerBase No. 5. Lyon (France): IARC Press.
41. Collins, F.S. (2004) The case for a US prospective cohort study of genes and environment. *Nature*, **429**, 475-477.
42. Bingham, S., and Riboli, E. (2004) Diet and cancer – the European Prospective Investigation into Cancer and Nutrition. *Nature Reviews Cancer*, **4**, 206-215.
43. Potter, J.D. (2004) Towards the last cohort. *Cancer Epidemiol. Biomark. Prev*., **13**, 895-897.

44. Rebbeck, T.R., Ambrosone, C.B., Bell, D.A., Chanock, S.J., Hayes, R.B., Kadlubar, F.F., and Thomas, D. (2004) SNPs, haplotypes, and cancer: applications in molecular epidemiology. *Cancer Epidemiol. Biomark. Prev.*, **13**, 681-687.
45. Zhu, Y., Spitz, M.R., Amos, C.I., Lin, J., Schabath, M.B., and Wu, X. (2004) An evolutionary perspective on single-nucleotide polymorphism screening in molecular cancer epidemiology. *Cancer Res.*, **64**, 2251-2257.
46. Chatterjee, N. (2004) A two-stage regression model for epidemiological studies with multivariate disease classification data. *J. Am. Statistical Ass.*, **99**, 127-138.
47. Wacholder, S., Chanock, S., Garcia-Closas, M., El ghormli, L., and Rothman, N. (2004) Assessing the probability that a positive report is false: an approach for molecular epidemiology studies. *J. Natl. Cancer Inst.*, **96**, 434-442.
48. Pepe, M.S., Etzioni, R., Feng, Z., Potter, J.D., Thompson, M.L., Thornquist, M., Winget, M., and Yasui, Y. (2001) Phases of biomarker development for early detection of cancer. *J. Natl. Cancer Inst.*, **93**, 1054-1061.
49. Collins, F.S. (1999) Medical and Societal Consequences of the Human Genome Project *N. Engl. J. Med.*, **341**, 28-37.
50. Miller, S.M., Bowen, D.J., Campbell, M.K., Diefenbach, M.A., Gritz, E.R., Jacobsen, P.B., Stefanek, M., Fang, C.Y., Lazovich, D., Sherman , K.A., and Wang, C. (2004) Current research promises and challenges in behavioral oncology: report from the American Society of Preventive Oncology Annual Meeting, 2002. *Cancer Epidemiol. Biomark. Prev.*, **13**, 171-180.
51. Marteau, T.M., and Lerman, C. (2001) Genetic risk and behavioural change. *BMJ*, **322**, 1056-1059.
52. Haga, S.B., Khoury, M.J., and Burke, W. (2003) Genomic profiling to promote a healthy lifestyle: not ready for prime time. *Nature Genetics*, **34**, 347-350.
53. Collins, F.S., Green, E.D., Guttmacher, A.E., and Guyer, M.S., on behalf of the US National Human Genome research Institute. (2003) A vision for the future of genomics research. *Nature*, **422**, 835-847.

# Summary

Colorectal cancer is one of the most common malignancies in the Western world. The etiology of colorectal cancer is complex and involves environmental as well as genetic factors. Known risk factors include a positive family history, age, high intake of red meat, alcohol and fat, and, possibly, cigarette smoking. Inverse associations have been observed with vegetable consumption, dietary fiber, calcium, use of non-steroidal anti-inflammatory drugs, physical activity, and, less consistently, fruit intake.

Most colorectal carcinomas seem to arise through a series of well-defined histopathological stages, the so-called adenoma-carcinoma sequence, as a result of the accumulation of genetic and/or epigenetic alterations in genes involved in the regulation of key cellular processes. For the studies described in this thesis we mostly focused on functional alterations in the adenomatous polyposis coli (*APC*) gene, and microsatellite instability (MSI) and the DNA mismatch repair (MMR) genes. Inactivating mutations in the *APC* tumor suppressor gene are thought to be key initiating events in sporadic as well as familial colorectal carcinogenesis and can be detected in many colorectal adenomas and carcinomas. MSI is a hallmark of MMR deficiency that in turn appears to be primarily due to inherited and/or acquired alterations in the MMR genes *hMLH1* and *hMSH2*. Approximately 10-20% of the sporadic colorectal carcinomas and most colorectal tumors associated with the hereditary nonpolyposis colorectal cancer (HNPCC) syndrome are characterized by MSI.

The relationships between environmental factors reported to be associated with colorectal cancer risk and the molecular alterations that drive colorectal carcinogenesis, *e.g.*, inactivating mutations in *APC*, are not (yet) clear. Environmental factors may well exert their effect on colorectal cancer risk by affecting the occurrence of specific molecular alterations, and the different molecular pathways to colorectal cancer may reflect different environmental

exposures. The overall aim of the studies described in this thesis was to gain further insight into the relationships between dietary and lifestyle risk factors and the molecular alterations known to play important roles in colorectal carcinogenesis.

**Dietary factors and truncating *APC* mutations in sporadic colorectal tumors**

Associations between dietary factors and *APC* mutations in colorectal tumors had, to our knowledge, not been examined previously. We used data from a case-control study of sporadic colorectal polyps (278 cases; 414 polyp-free controls) to evaluate associations between dietary factors and truncating *APC* mutations in sporadic adenomas (Chapter 2). Adenomas are an early stage in colorectal carcinogenesis and examining the relationship between diet and the occurrence of truncating *APC* mutations in adenomas may provide further insight into how, and which, dietary factors are involved in the production of these specific, early occurring mutations. Fifty-eight percent of the adenomas included in the study exhibited a truncating *APC* mutation ($APC^+$). Polyp size was positively associated with the occurrence of *APC* mutations. High intake of red meat and high fat intake seemed to increase the risk of polyps without truncating *APC* mutation ($APC^-$) in particular [OR (95% CI) for highest *vs*. lowest tertile, $APC^+$ *vs*. controls: red meat, 1.0 (0.6-1.6); fat, 1.1 (0.6-1.9). $APC^-$ *vs*. controls: red meat, 1.8 (1.0-3.1); fat, 1.9 (1.0-3.7). $APC^+$ *vs*. $APC^-$: red meat, 0.5 (0.3-1.0); fat, 0.5 (0.2-1.2)], while high intake of carbohydrates seemed to especially decrease the risk of $APC^-$ polyps [$APC^+$ *vs*. controls: 0.8 (0.5-1.4); $APC^-$ *vs*. controls: 0.5 (0.3-0.9); $APC^+$ *vs*. $APC^-$: 1.7 (0.9-3.4)]. Most evaluated dietary factors were, however, not specifically associated with $APC^+$ polyps or $APC^-$ polyps but seemed to affect both pathways equally, if at all. None of the evaluated dietary factors was found specifically associated with a particular type of truncating *APC* mutation.

Data from a population-based case-control study on sporadic colon cancer (184 cases; 259 controls) were used to assess associations between dietary factors and the occurrence of

truncating *APC* mutations in carcinomas (Chapter 3). Truncating *APC* mutations were detected in 34% of the tumors. Consumption of vegetables lowered the risk of $APC^+$ carcinomas as well as $APC^-$ carcinomas, although the protective effect of vegetables appeared to be less influential in the $APC^+$ subset [$APC^+$ *vs*. controls: 0.6 (0.3-1.3); $APC^-$ *vs*. controls: 0.3 (0.2-0.5); $APC^+$ *vs*. $APC^-$: 2.3 (1.0-5.3)]. Alcohol intake was associated with an increased risk of $APC^-$ carcinomas only [$APC^+$ *vs*. controls: 0.5 (0.3-1.1); $APC^-$ *vs*. controls: 1.7 (1.0-3.0); $APC^+$ *vs*. $APC^-$: 0.3 (0.2-0.7)], whereas meat, fish and fat seemed to especially increase the risk of $APC^+$ carcinomas.

**Diet and microsatellite instability in sporadic colon carcinomas**

Thus far, few epidemiological studies have examined associations between diet and MSI and knowledge about the role of dietary factors in MSI-related sporadic colon carcinogenesis is limited. We used the population-based case-control study of sporadic colon carcinomas mentioned above to evaluate associations between dietary factors and MSI, hMLH1 expression and *hMLH1* hypermethylation (Chapter 4). Twenty-two percent of the tumors were MSI-H. Most MSI-H tumors, 65%, had an *hMLH1*-associated etiology (that is, hMLH1 was absent); *hMLH1* was inactivated by promoter hypermethylation in 71% of the tumors in which hMLH1 was absent. Red meat consumption was found to be associated with an increased risk of MSI-L/MSS carcinomas in particular [MSI-H *vs*. controls: 0.5 (0.2-1.2); MSI-L/MSS *vs*. controls: 1.5 (0.9-2.6); MSI-H *vs*. MSI-L/MSS: 0.3 (0.1-0.9)], whereas high alcohol intake seemed to increase the risk of MSI-H carcinomas [MSI-H *vs*. controls: 1.9 (0.8-4.7)]. Fruit consumption seemed to especially decrease the risk of MSI-H carcinomas exhibiting epigenetically silenced *hMLH1*.

**Cigarette smoking and genetic alterations in sporadic colon carcinomas**

To evaluate the hypothesis that smoking is primarily linked to a specific colon tumor subgroup(s), we also assessed associations between cigarette smoking and the occurrence of mutations in the *APC*, K-*ras* and *p53* genes, p53 overexpression and MSI in the population-

based case-control study of sporadic colon carcinomas (Chapter 5). Our results suggested that smoking-related colon cancers develop through a p53 overexpression-negative pathway and that cigarette smoking particularly results in colon tumor cells with transversion mutations. Regarding the latter, cigarette smoking seemed to especially result in colon tumors with K-*ras* transversion mutations. No clear associations were observed with MSI. Our results provide support for the hypothesis that cigarette smoking is primarily associated with specific colon tumor subgroups.

**Environmental factors and HNPCC-associated colorectal tumors**

Individuals with HNPCC are at increased risk for colorectal cancer. To gain insight into the effects of environmental factors on colorectal tumor risk in individuals with HNPCC, we examined associations between dietary factors, cigarette smoking, and HNPCC-associated colorectal tumors in a case-control study (145 cases; 103 never-affected controls) (Chapter 6). All study participants were known or suspected (Amsterdam criteria) carriers of a germline mutation in one of the MMR genes. Fruit consumption and dietary fiber intake lowered the risk of ever developing colorectal tumors in individuals with HNPCC [fruit: 0.4 (0.2-0.9); dietary fiber: 0.5 (0.2-1.0)]. Cigarette smoking, and possibly alcohol consumption, seemed to increase the risk of HNPCC-associated colorectal tumors. These results support the hypothesis that also HNPCC-associated outcomes may be modified by environmental factors. Truncating *APC* mutations were detected in 38% of the 80 available HNPCC-associated polyps. None of the evaluated environmental factors was distinctively associated with a specific *APC* status of the polyps.

**Discussion**

In *Chapter 7*, the main findings of the studies described in this thesis are summarized, and methodological considerations, public health implications and future research directions are

discussed. Overall, our results, if confirmed by other studies, support the hypothesis that environmental factors previously reported to be associated with colorectal cancer risk exert their effect on this risk by affecting the occurrence of specific molecular alterations that drive colorectal carcinogenesis.

Regarding methodological considerations, as discussed in *Chapter 7*, the interpretation of data from studies on environmental factors and molecular alterations in colorectal tumors depends on *how* the molecular alterations are measured (*i.e.*, methods used), *what* alteration is measured (Is it a functional change or not?), *where* the alterations are located (Which colorectal tumors are screened for alterations?), and, obviously, also on the methods used to measure environmental exposures.

Understanding how, and which, environmental and genetic factors interact to produce colorectal cancer is critical to the development of useful prevention and treatment strategies. Well-designed molecular epidemiology studies that evaluate environmental exposure as well as genetic variation are necessary to further elucidate the etiology of colorectal cancer. Well-designed molecular epidemiology studies are also necessary to develop and evaluate biomarkers for early detection. However, although understanding the etiology of colorectal cancer is very important, by itself it is not enough to prevent or treat cancer. Advances in cancer research need to be translated into effective, socially and ethically acceptable intervention strategies in order to improve health and prevent cancer. Social and behavioral research plays a key role in the development and evaluation of these strategies and should not be neglected.

# Samenvatting

Dikke darmkanker is een van de meest voorkomende kankersoorten in de Westerse wereld. In Nederland worden per jaar gemiddeld 8500 nieuwe gevallen vastgesteld en overlijden jaarlijks ongeveer 4000 personen aan deze kankersoort. Zowel omgevingsfactoren (voeding en leefstijl) als genetische factoren spelen een belangrijke rol in de ontwikkeling van dikke darmkanker. Een positieve familiegeschiedenis, leeftijd, hoge inname van rood vlees, alcohol en vet, en het roken van sigaretten lijken het risico op dikke darmkanker te verhogen. De consumptie van groente, fruit, vezels en calcium, het regelmatig gebruiken van aspirines, en regelmatig bewegen zijn daarentegen geassocieerd met een lager risico op dikke darmkanker.

De meeste dikke darmtumoren ontwikkelen zich via relatief eenvoudig van elkaar te onderscheiden opeenvolgende stadia, de zogenaamde adenoma-carcinoma reeks. In eerste instantie gaat normaal darmepitheel zich abnormaal vaak delen waardoor een kleine goedaardige uitstulping, een poliep (colorectaal adenoom), ontstaat. Deze poliep kan vervolgens uitgroeien tot een kwaadaardige tumor (colorectaal carcinoom), dit duurt meestal 10-15 jaar. De overgang van het ene stadium naar het volgende blijkt samen te hangen met het optreden van veranderingen, mutaties, in specifieke genen. Normaal gesproken zorgen de eiwitten waarvoor deze genen coderen er, onder andere, voor dat de cel niet ongecontroleerd door blijft groeien en delen. Door de mutaties werkt deze controle echter niet meer met als gevolg dat kanker kan ontstaan.

De studies beschreven in dit proefschrift richten zich met name op mutaties in het zogenaamde adenomateuze polyposis coli (*APC*) gen, en microsatelliet instabiliteit (MSI) en de DNA-mismatch-repair (MMR) genen. Mutaties in het *APC* gen spelen een sleutelrol in de initiatie van zowel sporadische als erfelijke dikke darmkanker. Ze kunnen worden aangetroffen in vele dikke darmpoliepen en carcinomen. Het APC eiwit is betrokken bij een groot aantal

cellulaire processen zoals celadhesie en gecontroleerde celdood. MMR genen coderen voor eiwitten die betrokken zijn bij de herkenning en reparatie van fouten gemaakt tijdens DNA replicatie. Door een mutatie in een of meer van de MMR genen (de meeste mutaties zijn gevonden in *hMLH1* en *hMSH2*) functioneert dit herstelmechanisme niet of minder goed wat zich onder meer uit in het frequent voorkomen van veranderingen in korte repeterende stukjes DNA (microsatellieten) in het genoom. Dit verschijnsel wordt MSI genoemd. Ongeveer 10-20% van de sporadische colorectale carcinomen en de meeste darmtumoren geassocieerd met het Hereditair Non-Polyposis Colorectaal Carcinoom (HNPCC) syndroom worden gekenmerkt door MSI.

De relatie tussen de omgevingsfactoren waarvan bekend is dat ze het risico op dikke darmkanker beïnvloeden en de moleculaire veranderingen die een rol spelen in de ontwikkeling van dikke darmkanker, zoals bijvoorbeeld mutaties in het *APC* gen, zijn (nog) niet duidelijk. Het is mogelijk dat omgevingsfactoren een effect hebben op het risico op dikke darmkanker doordat ze direct of indirect het voorkomen van specifieke moleculaire veranderingen beïnvloeden. De verschillende moleculaire veranderingen die in dikke darmtumoren worden gevonden weerspiegelen dan verschillen in blootstelling aan bepaalde omgevingsfactoren. Doel van de studies beschreven in dit proefschrift was om meer inzicht te verwerven in de relaties tussen voedings- en leefstijlfactoren en de moleculaire veranderingen waarvan bekend is dat ze een belangrijke rol spelen in de ontwikkeling van dikke darmkanker.

**Voeding en *APC* mutaties in sporadische dikke darmtumoren**

Associaties tussen voedingsfactoren en het voorkomen van *APC* mutaties in dikke darmtumoren waren niet eerder onderzocht. Wij hebben gegevens gebruikt van een patiënt-controle studie naar sporadische dikke darmpoliepen (278 patiënten met minimaal 1 adenomateuze poliep; 414 controles zonder poliep) om associaties te onderzoeken tussen voedingsfactoren en *APC*

mutaties in sporadische adenomen (Hoofdstuk 2). Adenomen zijn een vroeg stadium in de ontwikkeling van dikke darmkanker en het onderzoeken van de relatie tussen voeding en het voorkomen van *APC* mutaties in adenomen leidt mogelijk tot meer inzicht in hoe, en welke, voedingsfactoren betrokken zijn in de produktie van deze belangrijke mutaties. In 58% van de adenomateuze poliepen in onze studie werd een functionele mutatie (d.w.z. een mutatie die leidt tot een verandering in de werking van het APC eiwit) in het *APC* gen gevonden ($APC^+$). De grootte van de poliepen was positief geassocieerd met het voorkomen van *APC* mutaties. Hoge innamen van rood vlees en vet bleken met name het risico op poliepen zonder *APC* mutatie ($APC^-$) te verhogen [OR (95% CI) voor hoogste *vs*. laagste tertiel, $APC^+$ *vs*. controles: rood vlees, 1.0 (0.6-1.6); vet, 1.1 (0.6-1.9); $APC^-$ *vs*. controles: rood vlees, 1.8 (1.0-3.1); vet, 1.9 (1.0-3.7); $APC^+$ *vs*. $APC^-$: rood vlees, 0.5 (0.3-1.0); vet, 0.5 (0.2-1.2)] terwijl de consumptie van koolhydraten het risico op $APC^-$ poliepen juist leek te verlagen [$APC^+$ *vs*. controles: 0.8 (0.5-1.4); $APC^-$ *vs*. controles: 0.5 (0.3-0.9); $APC^+$ *vs*. $APC^-$: 1.7 (0.9-3.4)]. De meeste voedingsfactoren waren echter niet specifiek geassocieerd met $APC^+$ poliepen of $APC^-$ poliepen maar leken beide pathways gelijk te beïnvloeden. Geen van de onderzochte voedingsfactoren was specifiek geassocieerd met een bepaald type *APC* mutatie.

Gegevens van een patiënt-controle studie naar sporadische dikke darmkanker (184 patiënten; 259 controles) zijn gebruikt voor het evalueren van associaties tussen voedingsfactoren en het voorkomen van *APC* mutaties in colon carcinomen (Hoofdstuk 3). Functionele *APC* mutaties werden gevonden in 34% van de carcinomen. Consumptie van groente verlaagde het risico op zowel $APC^+$ carcinomen als $APC^-$ carcinomen. Echter, het beschermende effect van het eten van groenten was minder groot in de $APC^+$ subgroep [$APC^+$ *vs*. controles: 0.6 (0.3-1.3); $APC^-$ *vs*. controles: 0.3 (0.2-0.5); $APC^+$ *vs*. $APC^-$: 2.3 (1.0-5.3)]. De inname van alcohol was alleen geassocieerd met een verhoogd risico op $APC^-$ carcinomen [$APC^+$ *vs*. controles: 0.5 (0.3-1.1); $APC^-$ *vs*. controls: 1.7 (1.0-3.0); $APC^+$ *vs*. $APC^-$: 0.3 (0.2-

0.7)] terwijl consumptie van vlees, vis en vet met name het risico op $APC^+$ carcinomen leek te verhogen.

**Voeding en microsatelliet instabiliteit in sporadische dikke darmkanker**

Tot op heden hebben slechts een klein aantal epidemiologische studies onderzoek gedaan naar associaties tussen voeding en MSI, en kennis over de rol van voedingsfactoren in MSI-gerelateerde ontwikkeling van dikke darmkanker is beperkt. Wij gebruikten de hierboven genoemde patiënt-controle studie naar sporadische dikke darmkanker voor het evalueren van associaties tussen voedingsfactoren en MSI, expressie van het hMLH1 eiwit en hypermethylatie van de promotor regio van het *hMLH1* gen (Hoofdstuk 4). Tweeëntwintig procent van de carcinomen in onze studie werden gekenmerkt door de aanwezigheid van MSI, ze waren MSI-H. In 65% van de MSI-H carcinomen was het hMLH1 eiwit niet aanwezig. In 71% van de carcinomen waarin het hMLH1 eiwit niet aanwezig was, was *hMLH1* geïnactiveerd via hypermethylatie van de promoter regio. De consumptie van rood vlees was met name geassocieerd met een verhoogd risico op carcinomen zonder MSI (MSI-L/MSS carcinomen) [MSI-H *vs*. controles: 0.5 (0.2-1.2); MSI-L/MSS *vs*. controles: 1.5 (0.9-2.6); MSI-H *vs*. MSI-L/MSS: 0.3 (0.1-0.9)]. Hoge inname van alcohol leek daarentegen het risico op MSI-H carcinomen te verhogen [MSI-H *vs*. controles: 1.9 (0.8-4.7)]. De consumptie van fruit leek met name het risico te verlagen op MSI-H carcinomen waarin *hMLH1* was geïnactiveerd via hypermethylatie van de promoter regio.

**Roken en genetische veranderingen in sporadische dikke darmkanker**

De eerder genoemde patiënt-controle studie naar sporadische dikke darmkanker hebben we ook gebruikt om de hypothese dat het roken van sigaretten hoofdzakelijk resulteert in bepaalde colon carcinoom subgroepen te evalueren. We bepaalden associaties tussen het roken van sigaretten en het voorkomen van: (1) functionele veranderingen in het *APC* gen, het K-*ras* gen en het *p53*

gen; (2) overexpressie van het p53 eiwit; en (3) MSI in sporadische colon carcinomen (Hoofdstuk 5). Onze resultaten suggereren dat roken-gerelateerde dikke darmtumoren zich ontwikkelen via een p53 overexpressie-negatieve pathway, en dat het roken van sigaretten met name resulteert in dikke darmtumorcellen met zogenaamde transversie mutaties. Wat betreft deze transversie mutaties, roken bleek met name geassocieerd te zijn met het voorkomen van transversie mutaties in het K-*ras* gen. Er werden geen duidelijke associaties gevonden met het voorkomen van MSI. Onze resultaten ondersteunen de hypothese dat het roken van sigaretten met name geassocieerd is met specifieke dikke darmkanker subgroepen.

**Omgevingsfactoren en HNPCC-geassocieerde dikke darmtumoren**

Personen met HNPCC, een van de meest voorkomende erfelijke dikke darmkankersyndromen, hebben een hoger risico op het ontwikkelen van dikke darmkanker dan de algemene populatie. Om inzicht te verwerven in de effecten van omgevingsfactoren op het risico op dikke darmtumoren in personen met HNPCC hebben we associaties onderzocht tussen voedingsfactoren, het roken van sigaretten, en HNPCC-geassocieerde dikke darmtumoren in een patiënt-controle studie (145 patiënten; 103 controles bij wie nooit een darmtumor was geconstateerd) (Hoofdstuk 6). Deelnemers aan ons onderzoek waren drager van een erfelijke mutatie in een van de MMR genen of verdacht van het hebben van een erfelijke mutatie in een van deze genen (Amsterdam criteria). Consumptie van fruit en inname van vezels verlaagden het risico op het ooit ontwikkelen van dikke darmtumoren in personen met HNPCC [fruit: 0.4 (0.2-0.9); vezels: 0.5 (0.2-1.0)]. Het roken van sigaretten, en mogelijk de consumptie van alcohol, leek het risico op HNPCC-geassocieerde dikke darmtumoren te verhogen. Onze resultaten ondersteunen de hypothese dat ook HNPCC-geassocieerde uitkomsten beïnvloed kunnen worden door omgevingsfactoren. Functionele mutaties in het *APC* gen werden gevonden in 38%

van de 80 beschikbare HNPCC-geassocieerde dikke darmpoliepen. Geen van de onderzochte omgevingsfactoren was onderscheidend geassocieerd met $APC^+$ poliepen of $APC^-$ poliepen.

**Discussie**

In *Hoofdstuk 7* zijn de resultaten van de studies beschreven in dit proefschrift samengevat, en worden methodologische aandachtspunten, implicaties voor de volksgezondheid en toekomstige onderzoeksrichtingen besproken. Onze resulaten, indien bevestigt door andere studies, ondersteunen de hypothese dat omgevingsfactoren een effect hebben op het risico op dikke darmkanker doordat ze het voorkomen van specifieke moleculaire veranderingen die een rol spelen in de ontwikkeling van dikke darmkanker beïnvloeden.

Wat betreft de methodologische aandachtspunten, zoals besproken in *Hoofdstuk 7*, de interpretatie van resultaten uit onderzoek naar associaties tussen omgevingsfactoren en moleculaire veranderingen in dikke darmtumoren is afhankelijk van *hoe* de moleculaire veranderingen zijn bepaald (d.w.z. welke methoden zijn er gebruikt), *wat* er is bepaald (Is het een functionele verandering of niet?), *waar* de veranderingen zijn gelocaliseerd (Welke dikke darmtumoren zijn gescreend voor mutaties?), en natuurlijk ook van de methoden die zijn gebruikt om de blootstelling aan omgevingsfactoren te bepalen.

Inzicht in hoe, en welke, omgevings- en genetische factoren een rol spelen in de ontwikkeling van dikke darmkanker is van groot belang voor het ontwikkelen van bruikbare preventie- en behandelingsmethoden. Moleculair epidemiologische studies die zowel blootstelling aan omgevingsfactoren als genetische variatie meten zijn noodzakelijk voor het verwerven van meer inzicht in de etiologie van dikke darmkanker. Moleculair epidemiologische studies zijn ook noodzakelijk voor het ontwikkelen en evalueren van biomarkers die kunnen worden gebruikt voor de vroege detectie van dikke darmkanker. Echter, alhoewel inzicht in de etiologie van dikke darmkanker van groot belang is, op zichzelf is het niet voldoende voor de

preventie of het behandelen van kanker. Voor het verbeteren van de gezondheid en het voorkomen van kanker dienen resultaten uit kankeronderzoek te worden vertaald in effectieve, sociaal en ethisch accepteerbare interventiemethoden. Sociaal-wetenschappelijk onderzoek speelt een sleutelrol in de ontwikkeling en evaluatie van deze methoden en mag niet worden verwaarloosd.

# Dankwoord/Acknowledgements |

Een proefschrift maak je niet alleen. Velen hebben op de één of andere manier bijgedragen aan de totstandkoming van dit boekje. Iedereen heel hartelijk bedankt hiervoor. Er zijn een aantal personen die ik hier graag in het bijzonder wil noemen.

Allereerst de deelnemers aan de in dit proefschrift beschreven studies. Heel erg bedankt voor uw medewerking. Zonder u was het onderzoek niet mogelijk geweest.

Mijn promotor, Frans Kok, ben ik dankbaar voor de vrijheid die ik van hem kreeg om het op mijn manier te doen. Dank voor het vertrouwen dat je altijd in mij had, en ook voor je steun en hulp op de momenten dat het nodig was. Weet dat ik dat zeer heb gewaardeerd. Zeer veel dank ben ik verschuldigd aan mijn twee co-promotoren, Ellen Kampman en Goos van Muijen. Ellen, ik wist niks van epidemiologie toen ik aan dit project begon en heb de afgelopen jaren veel van je geleerd (zie dit boekje...). Dank voor je vertrouwen en hulp, en voor het blijven vragen of ik al wat voor je te lezen had. Fijn was het dat ik af en toe bij je kon blijven logeren. Dat waren leuke avonden, vaak met goede gesprekken en wijn. Goos, jij zorgde er voor dat op het lab in Nijmegen alles goed verliep, regelde blokjes, dacht mee met het project, en was altijd behulpzaam. Ik vond het prettig om met jullie samen te werken.

Maria van Vugt, ik had mij geen betere onderzoeksassistent en kamergenoot kunnen wensen. Wat heb jij veel werk verzet! Tussen het werven van deelnemers, opwerken van bloedjes en invoeren van vragenlijsten zorgde je er bovendien ook nog eens voor dat ik niet vergat te lunchen en op zijn tijd een kopje thee dronk. Zonder jou hulp zou dit proefschrift er niet zijn geweest. Heel erg bedankt. Ik ben blij dat we ook nu nog steeds contact hebben.

Winny van Geloof en Hanneke Braam, de analisten die in het lab in Nijmegen alle analyses uitvoerden en vele tumorblokjes en DNA monsters door hun handen hebben zien gaan. Heel

hartelijk bedankt voor de fijne samenwerking. Marjolijn Ligtenberg dank ik voor haar bijdragen aan het MSI artikel en de prettige samenwerking. Han van Krieken heeft vele poliepen en carcinomen voor ons bekeken en beoordeeld. Heel hartelijk bedankt hiervoor. Alle anderen uit het lab in Nijmegen, dank voor de gezelligheid en gastvrijheid. Ook al was ik er niet heel vaak, jullie zorgden er voor dat ik mij altijd welkom voelde.

Hans Vasen en Mary Velthuizen van de Stichting Opsporing Erfelijke Tumoren (STOET) te Leiden dank ik voor hun hulp met het werven van deelnemers voor de HNPCC-studie. Zonder de STOET zou het mij niet zijn gelukt om zoveel deelnemers te werven.

Fokko Nagengast ben ik dankbaar voor zijn hulp met het werven van deelnemers voor de verschillende studies en de prettige samenwerking. Marga Ocké dank ik voor het omrekenen van de voedselfrequentievragenlijsten. Fijn dat je dat altijd zo snel kon doen.

Dorien Voskuil, Edine Tiemersma, Petra Wark, Maureen van den Donk, Mariken Tijhuis en alle andere leden van de Wageningse ‘kankergroep’, bedankt voor de vele discussies, het werven van deelnemers, het verzamelen en invoeren van gegevens, en jullie hulp met allerhande andere zaken.

Alle collega’s van de afdeling Humane Voeding, en met name de aio’s, heel erg bedankt voor jullie belangstelling en steun, en natuurlijk voor de gezelligheid tijdens en na werktijd.

Riccardo Fodde, dank dat ik altijd welkom was in jou lab. Je steun en vertrouwen waren erg belangrijk voor mij en ik heb heel veel van je geleerd. De Fodde groep, Ron, Peter, Claudia, Shantie, Cor, Ada, Marjan, Juul, Patrick, Heleen, Leon, Lia, Marjan, Joana en alle anderen, heel hartelijk bedankt voor de vele discussies, de goede sfeer, de vele borrels en etentjes, en alle andere leuke dingen die we samen deden.

Annemarie Mol, bedankt voor alles. Ik mis onze wandelingen en het zo maar langs kunnen komen. Gelukkig lukt het ons om via telefoon en e-mail met elkaar in contact te blijven.

Vilan van de Loo, dank voor de vele (telefoon)gesprekken. Wat hebben we inmiddels veel met elkaar gedeeld. Ik denk dat ik het bij dit ene boekje laat, van jou hand verwacht ik echter nog vele boeken.

Mijn paranimfen, Marleen Manders en Rosalie Rutten. Lieve dames, eindelijk is het dan zo ver, wat fijn dat jullie naast mij willen staan op 'de dag'. Wie had vantevoren kunnen bedenken dat we er met z'n zessen zouden staan! Heel erg gefeliciteerd! Dank voor al jullie steun en hulp, met name toen ik al in Seattle woonde. In Wageningen was jullie kamer een van mijn meest favoriete plekken, het was er altijd gezellig en bovendien was er ook altijd wel iets lekkers te eten. Rosalie, dank ook dat ik indien nodig altijd bij jou en Pankaj kon blijven logeren. Dat was zeker in tijden van stress heel erg prettig. Ik hoop dat we nog lang met elkaar in contact zullen blijven.

Andrea Detter, thank you for reading most chapters and improving my English. Linda Massey, thanks so much for your help with the cover and for converting the Word files into PDF files.

Isa, Pierre, Nadine, Esther, Sanne, Deanna, Kelly, Victoria, Catherine, Heleen, Kees, Ellen, Deb & Linda, Charlotte, Kerry, Menna, and all of my other old and new friends, thanks so much for all your support. My life would not be the same without you.

Lieve papa en mama, jullie steun en goede zorgen zijn voor mij de afgelopen jaren van onmisbare waarde geweest. Heel erg bedankt voor alles. And last but definitely not least, Tonja Ayala. I am so glad that we met and am looking forward to spending the rest of my life with you.

# About the author

Brenda Bernardine Martine Elisabeth Diergaarde was born on 14 August 1968 in Leiden, the Netherlands. After completing secondary school (Scholengemeenschap De Vlietschans, Leiden) in 1987, she attended Leiden University to study chemistry. She specialized in biochemistry and obtained her MSc degree in 1993. After her graduation, her interest in the social implications of biomedical research inspired her to get involved in science and technology studies. She participated in several workshops of the Graduate School Science, Technology and Modern Culture, and, in 1995, was awarded a fellowship from the Netherlands Organization for Scientific Research to study standardization processes in the production and use of genetic maps and the implications for notions of diversity. After this, she participated in two more 'science studies' projects which both focused on implications of the application of genetic knowledge and technologies in (colon and breast) cancer practices. Although she thoroughly enjoyed the research, she did miss 'the lab' and when she was offered the opportunity to do a PhD in molecular epidemiology, she decided to take it. Brenda started her PhD project, of which the results are described in this thesis, at the Division of Human Nutrition and Epidemiology at Wageningen University (Wageningen, the Netherlands) in October 1998. She participated in several courses on nutrition, epidemiology, and the gastrointestinal tract within the framework of the educational program of the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition, and Health Sciences). In addition, in 1999, she attended the Molecular Epidemiology course organized by the International Agency for Research on Cancer (IARC) in Torino, Italy, and the 4$^{th}$ Gaslini-IARC-Menarini course on cancer genetics in Sestri Levante, Italy. She attended the Erasmus Summer Programme (Rotterdam, the Netherlands) in 2000, course on regression analyses by Stanley Lemeshow, and participated in a course on design of case-control studies by Kenneth Rothman in 2001 (Bilthoven, the Netherlands). Brenda was awarded a three-year Dutch Cancer Society Training Fellowship in 2003, and currently works as an affiliate fellow in the Cancer Prevention Program at the Fred Hutchinson Cancer Research Center in Seattle, WA, US. She is also an affiliate fellow in the Biobehavioral Cancer Prevention and Control Program at the University of Washington (Seattle, WA).

The research described in this thesis was financially supported by the Dutch Cancer Society (grants LUW 96-1374 and LUW 98-1687).

Financial support by the Dutch Cancer Society and Wageningen University for the publication of this thesis is gratefully acknowledged.

Cover: Linda A. Massey

Printing: Grafisch Bedrijf Ponsen & Looijen B.V., Wageningen, The Netherlands

© 2004 B.B.M.E. Diergaarde