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MEAT, FAT, SMOKING AND GENETIC ALTERATIONS

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Margreet Luchtenborg

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**A MOLECULAR EPIDEMIOLOGICAL STUDY
ON COLORECTAL CANCER:
MEAT, FAT, SMOKING AND GENETIC ALTERATIONS**

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. mr. G.P.M.F. Mols,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op vrijdag 1 juli 2005 om 12:00 uur

door

Margreet Lüchtenborg

PROMOTORES

Prof. dr. ir. P.A. van den Brandt
Prof. dr. ir. P. van 't Veer (Wageningen Universiteit)

CO-PROMOTORES

Dr. ir. M.P. Weijnenberg
Dr. A.F.P.M. de Goeij

BEOORDELINGSCOMMISSIE

Prof. dr. A. Bäst (voorzitter)
Prof. dr. M.J.A.P. Daemen
Dr. H.J. van Kranen (Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven)
Prof. dr. ir. F.E. van Leeuwen (Nederlands Kanker Instituut, Amsterdam)
Prof. dr. F.C.S. Ramaekers

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Voor papa

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CHAPTER 1

INT

BACKGROUND

The burden of colorectal cancer is high, with approximately one million new cases worldwide each year.¹ Colorectal cancer is one of the most common causes of morbidity and mortality among European and North American populations. Approximately one half of these populations will develop a colorectal adenoma by the age of 70. A fraction of these adenomas will progress to cancer, equivalent to a lifetime cancer risk in the general population of about 5%. In the Netherlands, where the studies described in this thesis were carried out, approximately 8,500 new colorectal cancer cases are diagnosed each year and around 40% of these cases eventually result in death.² Colorectal cancer is the third most common cancer (after lung and prostate cancer) in men and the second most common cancer (after breast cancer) in women in the Netherlands.²

Colorectal cancer is a multi-factorial disease. As with other types of cancer, the risk of colorectal cancer increases with advancing age. A positive family history of colorectal cancer gives rise to a two-fold increase in the risk of developing colorectal cancer,^{3, 4} suggesting a hereditary component. However, less than 10% of colorectal cancers are attributable to familial cancer syndromes, the two most well known being hereditary non-polyposis colorectal cancer (HNPCC), and familial adenomatous polyposis (FAP). The overall majority of colorectal cancers seem to arise in a non-hereditary, sporadic fashion.

World-wide, the age-standardised incidence of colon cancer varies 20-fold with the highest rates observed in more developed countries,¹ which indicates that ethnic background or lifestyle factors may play a role in the aetiology of colorectal cancer. Migrant studies have shown that incidence rates in groups moving from a low-risk to a high-risk area adapt to the incidence rate of the high-risk area within one or two generations,⁵ suggesting that the development of a colorectal tumour can, for a large part, be ascribed to environmental factors, of which diet is a likely candidate.

The epithelium of the colon and rectum is continuously exposed to dietary and other environmental factors that may bear carcinogenic potential and exposure to these factors may eventually result in the formation of a tumour. In addition, systemic exposure to these potential carcinogens may affect the colon and rectum. Factors that possibly increase the risk of colorectal tumour development include a high intake of fat, meat, alcohol, and cigarette smoking, whereas vegetables, fibre and possibly fruit may decrease the risk of colorectal cancer.⁶ However, evidence is weak and sometimes inconsistent. In part, this may be due to the fact that the various molecular alterations underlying the carcinogenic process have not been taken into account in most studies to date.

In the following paragraphs, the molecular alterations contributing to colorectal cancer and the risk factors thought to play a role in the aetiology of colorectal cancer are described, which will lead to the rationale and the aims of the studies described in this thesis.

MOLECULAR ALTERATIONS CONTRIBUTING TO COLORECTAL CANCER

The normal adult colon contains approximately 10^7 crypts. Four to six stem cells at the bottom of each crypt replenish the whole crypt.⁷ The progeny of stem cells migrates up the crypt and continues to divide until it reaches its midportion. Subsequently, the migrating epithelial cells stop dividing and instead differentiate into mature cells. When the differentiated cells reach the top of the crypt, they undergo apoptosis and are engulfed by stromal cells or are shed directly into the lumen. This journey from the base of the crypt to its apex takes around three to six days. Normally, the birth rate of the colonic epithelial cell precisely equals the rate of cellular loss from the crypt apex. If the birth/loss ratio increases, this results in hyperproliferation, which may eventually progress to a neoplasm.

It is widely believed that most adenomas develop from normal stem cells at the bases of the colorectal crypts that acquire a mutation.⁸ Although aberrant crypt foci have been recognised as putative pre-neoplastic lesions that may not develop through the adenoma stage,⁹ the large majority of colorectal tumours progresses through a series of histopathological stages ranging from dysplastic crypts through small benign adenomas and ultimately resulting in malignant tumours with metastatic properties. This chain of events takes five to 40 years.

Underlying this process are genetic alterations. A first somatic mutation is thought to cause a clonal expansion that initiates the neoplastic process.¹⁰ Successive somatic mutations result in additional rounds of clonal expansion. The progression of a dysplastic crypt into a malignant tumour is generally believed to be the result of a series of genetic changes that involves activation of oncogenes and inactivation of tumour suppressor genes that control cell growth, differentiation and apoptosis.¹⁰

An elegant model describing colorectal tumourigenesis as proceeding through a series of genetic alterations in key genes, such as adenomatous polyposis coli (APC), Kirsten-ras (*K-ras*), deleted in colorectal cancer (DCC), and tumour protein 53 (*TP53*) was proposed by Fearon and Vogelstein in 1990.¹¹ Although it is generally acknowledged that mutations in APC, *K-ras* and *TP53* are the cornerstones of the pathway leading to colorectal cancer, a recent report has shown that colorectal cancer is much more heterogeneous and that most tumours do not show simultaneous occurrence of mutations in all of those three genes and that colorectal cancer is probably a multi-pathway disease.¹² In addition, epi-genetic silencing through methylation of tumour suppressor and DNA repair genes are thought to contribute to the development of a carcinoma. The accumulation of (epi-)genetic alterations is probably more important than the sequential order of events.¹³

Genetic instability is seen in most types of cancer.¹⁴ It is as yet under debate whether genetic instability preceeds the carcinogenic process and leads to aberrations in genes that subsequently result in tumour progression or whether mutations in certain genes may cause genetic instability.¹⁵ Two distinct types of

genetic instability are found in colorectal cancer: chromosomal and microsatellite instability.¹⁶ Chromosomal instability results in gains or losses of entire chromosomes or parts of them and gives rise to aneuploid tumours and occurs in the majority of cancers. Approximately 10 to 15% of colorectal cancers displays microsatellite instability, represented by diploid cells acquiring high mutation rates, and was found to be associated with defective mismatch repair.¹⁷ These tumours are less likely to harbour mutations in genes associated with chromosomally unstable and generally aneuploid tumours, such as *APC*, *K-ras* and *TP53*.¹⁸⁻²⁶

Although numerous molecular alterations have been identified in colorectal carcinogenesis, within the framework of this molecular epidemiological study on environmental factors and colorectal cancer, we have studied mutations in the genes that are thought to be important in the early stages of carcinogenesis (*APC*, *CTNNB1*, *K-ras*) and human Mut-L homologue 1 (hMLH1) deficiency that probably represents a separate pathway. Additionally, we have considered genes (*GSTM1* and *GSTT1*) that are involved in carcinogen metabolism and detoxification and may determine individual susceptibility to the exposure to potential carcinogens. These genetic aspects will be briefly described in the following paragraphs.

APC

The familial adenomatous polyposis coli syndrome is marked by a germline mutation in the *APC* gene that predisposes individuals to developing large numbers of (benign) adenomas that eventually may develop into (malignant) carcinomas.²⁷ Inactivating mutations in the *APC* gene can result in adenoma formation.²⁸ Mutations in the gate-keeping *APC* gene are considered an early event in colorectal carcinogenesis and it is estimated that up to 80% of sporadic colorectal tumours have somatic mutations in the *APC* gene.²⁹ Whereas germline mutations in FAP are confined to the 5' half of the *APC* gene, somatic mutations are clustered in the central region of the open reading frame, spanning codons 1,286 to 1,513 of exon 15.^{30, 31} Although this mutation cluster region represents only 8% of the 8,535 bp *APC* gene, 68 to 77% of somatic mutations in *APC* are found in this region.^{32, 33}

The tumour suppressing *APC* gene is located on chromosome 5q21²⁷ and encodes a large protein (312 kDa) that is involved in many cellular processes.³⁴ Central to *APC*'s tumour suppressive function is its down-regulatory capacity of β -catenin, a key component of the Wnt signalling transduction pathway. This pathway plays an important role in both embryonic development and tumorigenesis. In the central region of *APC*, seven motifs of 20 amino acids were identified, which not only bind β -catenin but also facilitate downregulation of β -catenin through interactions with Axin and GSK3 β .^{35, 36} Through inactivating mutations in the central and N-terminal part of the *APC* gene, which often lead to complete or partial loss of function of this region, β -catenin can accumulate in the cell and translocate to the cell nucleus where it serves as a co-activator of Tcf/Lef proteins that activate transcription of Wnt target genes, which a.o. leads to enhanced proliferation.³⁷⁻³⁹

CTNNB1

Nuclear β -catenin levels can also be upregulated directly through mutations in the *CTNNB1* gene encoding β -catenin. Indeed, in a small proportion of tumours that lack inactivating mutations in the *APC* gene, mutations in exon 3 of the *CTNNB1* gene encoding β -catenin, were observed.⁴⁰ These mutations are generally found at any of four phosphorylation sites (codons 33, 37, 41 and 45, located in exon 3) that play a crucial role in the down-regulation of β -catenin.⁴¹

K-RAS

A second step occurring early in colorectal cancer progression is the mutational activation of the *K-ras* oncogene, which is associated with the transitory state of an early adenoma into an intermediate adenoma. Ten to 15% of adenomas smaller than 1 cm and 30 to 60% of larger adenomas and colorectal carcinomas harbour such a mutation.^{11, 42} The *K-ras* gene is located on chromosome 12p and encodes a 21 kDa protein that transduces extracellular signals via multiple downstream effectors to the nucleus. The hallmark of Ras activation is a transactivation switch between the inactive state, in which it is bound to GDP and the active state, in which conversion to GTP occurs. In the activated state, Ras can transduce mitogenic signals. Mutations in codons 12 and 13 of exon 1 and codon 61 of exon 2 of the *K-ras* gene result in prolonged activation of the Ras protein that stimulates proliferation of the cell. Mutations in codons 12 and 13 are more frequently observed (90 to 95%) than mutations in codon 61 (approximately 5%).⁴²

HMLH1

The majority of colorectal tumours are marked by chromosomal instability and this is associated with mutations in a number of proto-oncogenes and tumour suppressor genes, whereas approximately 10 to 15% of sporadic colorectal tumours display microsatellite instability.^{43, 44} Microsatellite instability is the result of defective mismatch repair. Hereditary non-polyposis colorectal cancer or Lynch syndrome is a relatively common cancer syndrome that is marked by a genetic defect in the mismatch repair mechanism, predisposes patients to developing colorectal cancer, but also endometrial, ovarian, and gastric cancer.^{45, 46} The majority of germline mutations occur in the hMLH1 and human Mut-S homologue 2 (hMSH2) mismatch repair genes.⁴⁷ In sporadic colorectal cancer, the hMLH1 and hMSH2 genes are usually silenced due to hypermethylation of the promotor regions of these genes resulting in suppressed expression of the protein.⁴⁸⁻⁵⁰ Approximately 90% of microsatellite instable tumours show absence of hMLH1 expression.⁵¹

GENETIC SUSCEPTIBILITY

Most carcinogens require metabolic activation before being able to cause DNA damage. Genetic polymorphisms in genes involved in the metabolism of dietary factors and other environmental exposures, leading to increased or decreased activity of the encoded enzymes, may render individuals more or less susceptible to developing colorectal cancer. In humans, in the metabolism of certain carcinogens, two modification steps are required, the first of which is the activation by the phase I enzymes. Some of the intermediate metabolites formed by this process can give rise to adduct formation with proteins and DNA, thus causing damage to cells. After the phase I reactions, conjugating reactions

by phase II enzymes render these metabolites water soluble, thus facilitating their excretion and this process may inactivate or further activate the metabolites. Variation in enzyme activity with regard to the detoxification or activation of carcinogens may lead to variation in susceptibility to cancer by environmental exposure.

GSTM1 AND GSTT1

Glutathione S-transferases (GSTs) are phase II enzymes that may directly inactivate heterocyclic amines and mainly PAH metabolites, but may also play an indirect role through induction of CYP1A2. Polymorphisms in the *GSTM1* and *GSTT1* genes consist of presence or absence of the alleles, thus giving rise to present or absent GST activity. Since GSTs are primarily involved in the metabolism of polycyclic aromatic hydrocarbons (PAH), differential susceptibility to cigarette smoke may be ascribed to polymorphism in the GST genes. Previous reports on the interaction between tobacco smoking and *GSTM1* and/or *GSTT1*, however, have not shown a clear relationship between smoking and genotype.⁵²⁻⁵⁶

ENVIRONMENTAL RISK FACTORS FOR COLORECTAL CANCER

Factors that possibly increase the risk of colorectal tumour development include a high intake of fat, meat, alcohol, and cigarette smoking, whereas vegetables and fibre may decrease risk of colorectal cancer.⁶ In the studies described in the present thesis, we have focussed on three risk factors that previously have been associated with specific genetic aberrations, i.e. meat and fat consumption, and cigarette smoking.

MEAT

With regard to meat consumption as a risk factor for colorectal cancer, the preparation of as well as the animal fat and haem iron content of meat have all been proposed as possible explanations. Nineteen heterocyclic aromatic amines have been isolated from cooked meat.⁵⁷ These compounds are formed via condensation of creatine with the amino acid pool when meat is cooked at temperatures ranging from 150°C to 250°C e.g., by frying, broiling or barbecuing.⁵⁸ The most abundant heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) has been shown to produce colon tumours in male F344 rats and mammary tumours in female F344 rats.⁵⁹ Barbecuing meat can result in the formation of PAH. PAH have been shown to specifically induce G→T transversions.^{60, 61}

N-Nitrosamines and their precursors are compounds that can be formed endogenously,⁶² but have also been detected in foods with added nitrates and nitrites, such as salt-preserved fish and meat, and in foods processed by smoking or direct-fire drying.⁶³ Through their alkylating action, they can be carcinogenic. N-nitroso compounds can induce G→A transitions that have been observed in codons 12 and 13 of the *K-ras* gene in human colonic tissue.⁶²

Population based case-control studies have shown that red meat was associated with an increased risk of *TP53* mutated cancers,⁶⁴ an increased risk of colorectal adenomas without a truncating *APC* mutation,⁶⁵ but colon carcinomas with a truncating mutation in the *APC* gene.⁶⁶ Finally, a high intake of red meat was inversely associated with microsatellite instable tumours.⁶⁷

DIETARY FAT

Dietary fats may exert direct effects on colorectal tumourigenesis by augmenting bile acid production and subsequently increased colonic cell proliferation and ultimately increased risk of endogenous mutations.⁶⁸ Systemic effects include the effect of specific types of fat on membrane characteristics of cells, thereby influencing risk of insulin resistance and survival of tumour cells, the protective effect of n-3 fatty acids by decreasing prostaglandin E2 production, and the effect of essential polyunsaturated fatty acids on inflammatory responses.⁶⁸ Previously, there has been emphasis on the effect of the quantity of total fat in the diet, however, it seems that fat quality in combination with quantity is more important in predisposing to colorectal cancer. For example, n-3 polyunsaturated fatty acids seem to have protective properties at different stages of colorectal carcinogenesis,⁶⁹ whereas n-6 PUFA may increase risk of colorectal cancer. A plausible explanation for the potentially carcinogenic properties of the n-6 polyunsaturated fatty acids lies in the peroxidation products of malondialdehyde and 4-hydroxynonenal that can form adducts with DNA and induce G→T and G→A transitions.⁷⁰⁻⁷⁴

Whereas Bautista *et al*⁷⁵ observed a decreased risk of tumours with wild type *K-ras* with high intake of total fat and monounsaturated fatty acids, Slattery *et al*⁷⁶ found associations between dietary fat, saturated fat and monounsaturated fatty acids and tumours harbouring specific G→T mutations in the *K-ras* gene only. Brink *et al*⁷⁷ observed an increased risk of polyunsaturated fatty acids and linoleic acid and colon tumours harbouring a *K-ras* mutation, whereas total fat and monounsaturated fatty acids seemed to decrease risk of tumours with specific G→C and G→T mutations in the *K-ras* gene in colon tumours. With regard to mutations in the *APC* gene, in a recent case-control study, high intake of fat seemed to increase the risk of adenomas without a truncating *APC* mutation.⁶⁵ However, in colon carcinomas, positive associations were observed for fat with tumours that harboured a truncating *APC* mutation.⁶⁶

CIGARETTE SMOKING

Carcinogenic compounds present in tobacco smoke may cause irreversible damage to the colorectal mucosa.⁷⁸ The burning of tobacco produces numerous genotoxic compounds, including PAH, heterocyclic aromatic amines (HCA), and N-nitrosamines.⁷⁹ The colon and rectum may become exposed to these carcinogens directly, by ingestion of tobacco smoke or indirectly, through contact with blood or bile that may contain potential carcinogens.⁷⁸ In rodent studies benzo(a)pyrene was found to induce G→T transversions and N-nitrosamines to induce G:C→A:T transitions in *ras* oncogenes,⁸⁰ both of which are important constituents of cigarette smoke.

Cigarette smoking was found to be associated with microsatellite instable colorectal tumors in three case-control studies,^{26, 81, 82} whereas another case-control study could not confirm this.⁸³

RATIONALE AND AIMS OF THIS THESIS

Environmental factors may have a direct effect on carcinogenesis through induction of DNA damage, resulting in mutations that potentially affect the function of encoded proteins or may drive the selection of tumours harbouring specific mutations. A few molecular epidemiological studies have included the mutation status of the tumour (*K-ras* oncogene, *APC* and *TP53* tumour suppressor genes, and microsatellite instability) and have observed differences in associations between certain exposures and colon and rectal tumours with and without such aberrations. However, apart from one cross-sectional and one small case-control study, these studies were performed within the framework of one large case-control study in the United States, and a case-control study in the Netherlands. The only studies in a cohort study setting were on the associations between dietary factors and *K-ras* mutations in colorectal cancer and these have previously been carried out within the cohort study that was also the setting of the studies described in this thesis.^{77, 84, 85}

The aim of the studies described in this thesis was two-fold. Firstly, we sought to describe the genetic alterations present in incident colorectal tumours from our cohort study and secondly, we assessed associations between proposed risk factors and colorectal tumours, according to genetic alterations present.

All studies were performed in the population-based, prospective Netherlands Cohort Study on diet and cancer that was initiated in September 1986.⁸⁶ At baseline, 58,279 men and 62,573 women aged between 55 and 69 years, who originated from a random sample from 204 municipal population registries throughout the Netherlands, completed a self-administered food frequency and lifestyle questionnaire. After baseline exposure assessment, a subcohort of 1,688 male and 1,812 female subjects was randomly drawn from the cohort and the vital status of these subjects was followed-up biennially to determine accumulated person years in the subcohort. Tumour material was obtained from the majority of the 929 colorectal cancer patients that were identified within the cohort after 7.3 years of follow-up (and excluding the first 2.3 years), which enabled us to study several molecular alterations that are thought to be important to colorectal cancer development in a large group of patients.

In the first study, we have investigated the occurrence of mutations in the *APC* gene of colorectal tumours from patients identified within the cohort and investigated whether certain mutations were related to patient or tumour characteristics (**chapter 2**). The mutation status of the *APC* tumour suppressor, and *CTNNB1* and *K-ras* oncogenes as well as the *hMLH1* expression status of the tumour were included in a second study, in which potential differences between different groups of tumours according to their mutation status were investigated (**chapter 3**). **Chapters 4** through **6** describe the studies on the associations between possible risk factors, consumption of meat and fish (**chapter 4**), dietary fat (**chapter 5**) and of cigarette smoking (**chapter 6**) and colon and rectal cancer with and without specific molecular alterations. The last study also took into account the genetic susceptibility as determined by polymorphisms in the *GSTM1* and *GSTT1* genes, to investigate whether imputed

differences in GST activity may alter the risk of cigarette smoking on colorectal cancer. Finally, the results and their implications are discussed in **chapter 7**.

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C H A P T E R 2

APC MUTATIONS IN SPORADIC COLORECTAL CARCINOMAS FROM THE NETHERLANDS COHORT STUDY

MARGREET LÜCHTENBORG
MATTY P. WEIJENBERG
GUIDO MJM ROEMEN
ADRIAAN P. DE BRUIJNE
PIET A. VAN DEN BRANDT
MARJOLEIN HFM LENTJES
MIRIAN BRINK
MANON VAN ENGELAND
R. ALEXANDRA GOLDBOHN
ANTON FPM DE GOEIJ

ABSTRACT

The adenomatous polyposis coli (APC) gene is considered to be a gatekeeper in colorectal tumourigenesis. Inactivating mutations in APC have been reported in 34 to 70% of sporadic colorectal cancer patients, the majority of which occur in the mutation cluster region.

In this study, tumour tissue from 665 incident colorectal cancer patients, who originate from 120,852 men and women (55-69 years of age at baseline) participating in the Netherlands Cohort Study, was evaluated for the occurrence and type of APC mutations with regard to age at diagnosis, sex, family history of colorectal cancer, Dukes' stage, tumour differentiation and sub-localisation. Mutation analysis of the mutation cluster region, which spans codons 1,286-1,513, was performed on archival adenocarcinoma samples using macrodissection, nested PCR and direct sequencing of purified PCR fragments.

A large number of genetic aberrations ($n=978$), including point mutations ($n=833$), deletions ($n=126$) and insertions ($n=19$) was detected in APC's mutation cluster region, in 72% of patients (479/665). In particular, we observed a large number of missense mutations, more than previously reported. This may indicate involvement in colorectal carcinogenesis, although their significance for APC functions is unclear. Truncating mutations were found in 37% of patients (248/665). Patients with rectosigmoid and rectum tumours relatively more frequently harboured C→T nonsense mutations and truncating frameshift mutations as compared to patients with proximal and distal colon tumours ($p=0.009$ and $p=0.045$, respectively).

Differences in occurrence of truncating mutations with regard to tumour sub-localisation suggest a different aetiology of tumourigenesis in colon and rectum.

INTRODUCTION

It is well established that sporadic colorectal cancer arises through a gradual series of histological changes, which are accompanied by specific genetic alterations involving tumour suppressor genes (e.g., *APC*, *DCC*, *SMAD4*, *TP53*) and oncogenes (e.g., *K-ras*).^{1, 2} Adenomatous polyposis coli (*APC*) gene mutation is considered to be an early event in colorectal carcinogenesis.

Linkage analysis of families with the autosomal dominant inherited disease familial adenomatous polyposis coli (FAP) has led to mapping of the *APC* gene to 5q21.³ This large gene with 21 exons, was subsequently cloned, identified and characterised.⁴ Mutations in both *APC* alleles were also found in most sporadic colorectal tumours.⁵ Whereas germline mutations in FAP are confined to the 5' half of the *APC* gene, somatic mutations are clustered in the central region of the open reading frame, spanning codons 1,286 to 1,513 of exon 15.^{5, 6} Although this mutation cluster region represents only 8% of the 8,535 bp *APC* gene, 68 to 77% of somatic mutations in *APC* occur in this region.^{7, 8}

The *APC* protein consists of 2,843 amino acids, and comprises a number of functional domains, motifs and other signatures, as reviewed by Polakis.⁶ *APC* is capable of binding β -catenin through its 15 amino acids repeats, situated between amino acids 1,020 and 1,169.⁹ More importantly, in the central region of *APC*, seven motifs of 20 amino acids were identified, which not only bind β -catenin but also facilitate downregulation of β -catenin through interactions with Axin and GSK3 β .^{10, 11} Through inactivating mutations in the central part of the *APC* gene, which often lead to complete or partial loss of function of this region, β -catenin levels are upregulated and the Wnt signalling pathway is activated, which in turn may lead to enhanced proliferation.¹² In addition to its role in the Wnt pathway, *APC* has other functions. Through its binding of β -catenin *APC* is also linked to E-cadherin and α -catenin, which in turn bind actin and actin-associated proteins, thus taking part in intercellular adhesion.¹³ Additionally, *APC* associates with the microtubule cytoskeleton directly through its carboxyl terminus. There is also evidence that *APC* may play a role in the regulation of apoptosis.¹⁴

Frequencies and specific types of somatic mutations in the *APC* gene have been investigated in a number of studies. Somatic mutations in *APC* have been found in 34 to 70% of sporadic tumours in colorectal cancer patients.^{7, 8, 15-21} Higher prevalences of 75 to 95% have been found in studies on the occurrence of somatic *APC* mutations in colorectal cancer cell lines.²²⁻²⁴ The large majority of mutations observed in different studies would lead to a truncated and therefore inactivated *APC* protein, either by nonsense point mutations (30%) or by frameshift mutations (68%), as reviewed by Bérout.²⁵ Most reports, however, were based on limited numbers of (usually selected) patients. Studies on somatic *APC* mutations are often based on FAP patients and it has been shown that the occurrence of a second mutation in the *APC* gene is somehow dependent on the position of the germline mutation, thereby introducing a bias.²⁶ Examination of *APC* mutations in colorectal cell lines has indicated that there may also be an interdependence of the two hits in *APC* in sporadic colorectal cancer.²³ Furthermore, results from different reports are difficult to

compare as different methods of detecting mutations in APC have been used and different regions of the APC gene have been studied.

In the current report, the frequency and type of APC gene mutations are assessed in a large series of unselected, incident colorectal cancer patients from The Netherlands identified in a prospective cohort study. The occurrence and type of APC gene mutations in the mutation cluster region are reported with regard to age at diagnosis, sex, family history of colorectal cancer, Dukes' stage, tumour differentiation and sub-localisation.

MATERIALS AND METHODS

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STUDY POPULATION

A prospective cohort study on diet and cancer was initiated in the Netherlands in September 1986. The study design has been described in detail elsewhere.²⁷ Briefly, at baseline a total of 58,279 men and 62,573 women, between the ages of 55 and 69 years, completed a self-administered food frequency and lifestyle questionnaire. The study population originated from 204 municipal population registries throughout the country. Incident cancer cases are identified by monitoring of the entire cohort for cancer occurrence through annual record linkage to the Netherlands Cancer Registry (NCR), nine regional cancer registries throughout The Netherlands, and to the Pathologisch Anatomisch Landelijk Geautomatiseerd Archief (PALGA), a nationwide database of pathology reports.²⁸ The PALGA database was used to identify and locate tumour tissue in Dutch Pathology laboratories. The first 2.3 years of follow up were excluded due to incomplete nationwide coverage of PALGA. From 1989 until 1994, 819 incident cases with histologically confirmed colorectal cancer were identified. Colorectal cancer was classified according to site as follows, proximal colon: cecum through transverse colon (ICD-O codes 153.0, 153.1, 153.4, 153.5, 153.6); distal colon: splenic flexure through sigmoid colon (ICD-O codes 153.2, 153.3, 153.7); rectosigmoid (ICD-O code 154.0) and rectum (ICD-O code 154.1). Information about age at diagnosis, sex and family history of colorectal cancer (at baseline) was retrieved from the Netherlands Cohort Study on diet and cancer (NLCS) database. Information about tumour sub-localisation, Dukes' stage and differentiation was retrieved from the NCR database.

TISSUE SAMPLES

Tumour material of colorectal cancer patients was collected after approval by the Ethical Review Board of Maastricht University, PALGA and the NCR. Tissue samples from 819 colorectal cancer patients were retrieved from 54 pathology laboratories throughout the Netherlands. Tumour tissue specimen collection started in August 1999 and was completed in December of 2001. Forty-four tumour tissue samples (5%) could not be traced. Of 775 eligible tissue samples 737 (90%) contained sufficient tumour material as confirmed by a pathologist (A.d.B.) for molecular analyses.

DNA ISOLATION

DNA isolation was described in detail elsewhere.²⁹ Briefly, one 5 μ m section were taken from each paraffin-embedded tumour tissue block and stained with haematoxylin and eosin (HE) for histopathological examination. Five 20 μ m sections of tumour tissue were taken from each sample for DNA isolation. After deparaffination of the sections, tumour tissue was separated from the normal colonic epithelium using the HE section as a reference. Genomic DNA was extracted from macrodissected tumour tissue using proteinase K (Qiagen, St. Louis, MO, USA) and the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). DNA concentration and purity was measured in a spectrophotometer at 260 and 280 nm. DNA from the fresh, unfixed colorectal cancer cell lines and the 10 fresh-frozen tissue samples was extracted as described for paraffin-embedded sections.

APC MUTATION ANALYSIS

Since the majority of somatic mutations in APC occur within the mutation cluster region, we amplified the mutation cluster region as four overlapping fragments (codons 1,286-1,520) in a nested PCR strategy. Flank PCR was performed to generate two fragments A and B. Genomic DNA (250 ng) was added to 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.0 mM MgCl₂, 250 μ M dNTP (Pharmacia, Uppsala, Sweden), 200 nM of each primer (table 1), and 1U Platinum Taq (Invitrogen, Breda, the Netherlands) in a final volume of 50 μ l. Fragment A was used as starting material for the amplification of nested fragments S1 and S2, and fragment B was used for nested fragments S3 and S4. An alternative semi-nested PCR strategy was chosen when nested PCR amplification failed for one or more fragments originating from flank A or B. Alternative flank fragments F1 to F4 were then generated for each of the four nested fragments S1 to S4, respectively. The (semi-) nested PCR was performed in a final volume of 50 μ l, containing 5 μ l of a 1:100 dilution of the flank product, 50mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5-2.5 mM MgCl₂, 250 μ M dNTP, 200 nM of each primer (table 1), and 1 U Platinum Taq. The four (semi-) nested fragments were labelled with biotin at one of the two primers for subsequent direct sequencing as indicated in table 1. The cycles for both flank, nested and semi-nested PCR consisted of 3 min predenaturation at 94°C, 40 s at 94°C for denaturation, 1 min at 52°C to 57°C for annealing, 1 min at 72°C for elongation, and 10 min at 72°C for post-elongation. In each PCR one round of 35 cycles was performed using a Peltier Thermal Cycler-200 (MJ Research, Biozym, Landgraaf, The Netherlands) or a I-cycler (BioRad, Veenendaal, the Netherlands). In each PCR series positive (DNA from colorectal cancer cell lines) and negative controls (no DNA) were included. The length and concentration of the PCR products were checked by electrophoresis on 2% agarose gels and visualised with ethidium bromide.

Mutation analysis was performed by direct sequencing using the Autoload SPS Sequencing Kit according to the manufacturers instructions (Amersham Biosciences, Roosendaal, the Netherlands). In brief, the biotinylated PCR product was captured on a sequencing comb coated with streptavidin. After removal of the non-biotinylated strands by alkaline denaturation, the remaining immobilised strand was used as a template for dideoxy sequencing reactions with extended Cy5 labelled primers (table 1) and T7 DNA polymerase. The sequence profile was analysed on ALFexpress DNA Analysis System using ALFwin

Table 1. Fragment characteristics for the amplification and sequencing of the mutation cluster region of the APC gene

Fragment	Nucleotide position	Fragment size (bp)	Primer sequences
Flank A	3874 - 4229	356	sense: 5'-GAA ATA GGA TGT AAT CAG ACG-3' antisense: 5'-GA GCT GGC AAT CGA ACG ACT-3'
Flank B	4114 - 4624	511	sense: 5'-GCT CAG ACA CCC AAA AGT CC-3' antisense: 5'-C ATT CCC ATT GTC ATT TTC C-3'
Flank F1	3796 - 4092	297	sense: 5'-CAG ACT TAT TGT GTA GAA GA-3' antisense: 5'-CGC TCC TGA AGA AAA TTC AAC-3'
Flank F2	3982 - 4003	248	sense: 5'-GAA GTT CCA GCA GTG TCA CAG C-3' antisense: 5'-GA GCT GGC AAT CGA ACG ACT-3'
Flank F3	4114 - 4383	270	sense: 5'-GCT CAG ACA CCC AAA AGT CC-3' antisense: 5'-ATT TTT AGG TAC TTC TCG CTT G-3'
Flank F4	4328 - 4624	297	sense: 5'-AA ACA CCT CCA CCA CCT CC-3' antisense: 5'-C ATT CCC ATT GTC ATT TTC C-3'
Nested S1	3874 - 4092	219	sense: 5'-GAA ATA GGA TGT AAT CAG ACG-3' ^a antisense: 5'-CGC TCC TGA AGA AAA TTC AAC-3'
Nested S2	4026 - 4229	204	sequence primer: 5' (Cy5)- CGC TCC TGA AGA AAA TTC AAC AGC-3' sense: 5'-A CTG CAG GGT TCT AGT TTA TC-3' ^a antisense: 5'-GA GCT GGC AAT CGA ACG ACT-3'
Nested S3	4179 - 4383	205	sequence primer: 5' (Cy5)- GA GCT GGC AAT CGA ACG ACT CTC-3' sense: 5'-T ACT TCT GTC AGT TCA CTT GAT A-3' ^a antisense: 5'-ATT TTT AGG TAC TTC TCG CTT G-3'
Nested S4	4328 - 4594	267	sequence primer: 5' (Cy5)- ATT TTT AGG TAC TTC TCG CTT GGT TTG-3' sense: 5'-AA ACA CCT CCA CCA CCT CC-3' antisense: 5'-G CAT TAT TCT TAA TTC CAC ATC-3' ^a sequence primer: 5' (Cy5)- AA ACA CCT CCA CCA CCT CCT CA-3'

^a biotin labelled primer

software (Amersham Biosciences, Roosendaal, the Netherlands). Evaluation of the sequence patterns was performed by two independent observers, based on the criteria that an increase of at least 5% is observed for the mutant peak, as well as a decrease of at least 5% of the wild type peak, relative to the wild type pattern in the same sequence run. The reference sequence for human APC was derived from Genbank Accession Number M74088.1. Data entry was performed blindly, by two independent observers.

From 72 patients of the 737 patients whose tumour samples contained sufficient DNA, one or more fragments of the mutation cluster region could not be amplified and these patients were not included in this study. The 665 patients, from whom mutation cluster region mutation analysis was complete, were comparable with the 819 colorectal cancer patients initially recognised within the cohort with respect to mean age at diagnosis, sex, family history of colorectal cancer, Dukes' stage, tumour differentiation and sub-localisation.

VALIDITY AND REPRODUCIBILITY OF APC MUTATION ANALYSIS

In order to validate mutation analysis on paraffin-embedded tissue, 10 freshly obtained colorectal cancer specimens were each divided in two adjacent tissue blocks, one of which was fresh-frozen, and the other routinely fixed and embedded in paraffin. Two samples contained wild type APC, three samples harboured a mutation and five samples had a polymorphism. These control specimens were obtained from patients who did not participate in the NLCS. Six colorectal cancer cell lines, i.e. HT29, Colo205, CaCo2, SW480, HCT116 and LOVO (obtained from the American Type Culture Collection, Rockville, MD, USA) were used to check the specificity of mutation detection in the mutation

cluster region of APC. The detection limit of mutation analysis was determined by mixing homozygously mutated DNA isolated from CaCo2 cells (C→T substitution at codon 1,367) with increasing concentrations of the corresponding wild type DNA sequence prepared from SW480 cells. Reproducibility of mutation analysis was established by subjecting 72 fragments, derived from 54 NLCS adenocarcinoma specimens twice to the complete mutation analysis procedure, from flank PCR of genomic DNA to sequencing of the fragments S1-S4.

STATISTICAL ANALYSIS

The overall frequency of APC mutations as well as the type of mutation was computed for all 665 cases with respect to age at diagnosis, sex and family history of colorectal cancer. Since tumour sub-localisation was unknown for 11 patients, 654 patients could be analysed for different mutation types with regard to tumour sub-localisation. Differences in mean age at diagnosis were evaluated pairwise between patients with wild type APC and patients with APC mutations using the Mann-Whitney *U* test. The mean age at diagnosis of patients with an APC mutation, i.e. silent, missense or nonsense/frameshift mutations was compared to that of patients with wild type APC. Differences in the categorical variables sex and family history of colorectal cancer, Dukes' stage, tumour differentiation and sub-localisation between patients without and with APC mutations and type of mutation were evaluated using the χ^2 test. A *p*-value of 0.05 or less was considered to be statistically significant. All statistical procedures were performed with SPSS software (SPSS version 10.0, SPSS Chicago, IL, USA).

RESULTS

In the colorectal cancer cell lines HCT116, Colo205 and HT29, wild type sequences were confirmed for the mutation cluster region of APC. Direct sequencing of the mutation cluster region of APC revealed a homozygous C→T substitution at codon 1,367 in CaCo2 cells, a heterozygous C→T substitution at codon 1,338 in SW480 cells and a deletion of C at codon 1,430 in LOVO cells, as previously described.²³ The effect of tissue processing as assessed in 10 specimens, showed that the mutation status of the mutation cluster region of APC for the paraffin-embedded block was identical to fresh unfixed tissue. The lowest level of detection was 5% mutant DNA in a background of wild type DNA as found in three independent experiments. In 85% (61/72) of the fragments analysed for reproducibility assessment, the same APC mutation status was observed in duplicate experiments.

In 72% of tumours from colorectal cancer patients (479/665) a total of 978 mutations were observed in the mutation cluster region of the APC gene. The number of observed mutations per tumour ranged from one to eight mutations. In 215 tumours (45%) one mutation was found, in 137 tumours (29%) two mutations and in 127 tumours (27%) more than two mutations were observed. Of all mutations observed, 85% (833/978) were point mutations and 15% (145/978) were insertions and deletions (table 2). Point mutations were

Table 2. Number and type of mutations in the mutation cluster region of APC. All 978 mutations detected in tumours from 665 sporadic colorectal cancer patients are represented

Type of mutation	Mutation	Silent mutation ^a	Missense mutation ^b	Mutation leading to truncation ^c
Point mutation (total)	833	192 (24%)	509 (61%)	132 (16%)
Transition				
C→T	352	63 (18%)	208 (59%)	81 (23%)
G→A	286	62 (22%)	223 (77%)	1 (0%)
A→G	63	24 (38%)	39 (62%)	0 (0%)
T→C	29	20 (69%)	9 (31%)	0 (0%)
Transversion				
A→C	2	0 (0%)	2 (100%)	0 (0%)
T→G	5	0 (0%)	3 (60%)	2 (40%)
A→T	11	4 (36%)	1 (9%)	6 (55%)
T→A	20	16 (80%)	3 (15%)	1 (5%)
C→A	9	1 (11%)	4 (44%)	4 (44%)
G→T	38	1 (3%)	3 (8%)	34 (89%)
C→G	11	1 (9%)	7 (64%)	3 (27%)
G→C	7	0 (0%)	7 (100%)	0 (0%)
Insertion ^d	19			18 (90%)
Deletion ^e	126			126 (100%)

^a The frequency of silent mutations is calculated by dividing the number of specific mutations (e.g. C→T transitions) resulting in silent mutations by the total number of that specific type of mutation

^b The frequency of missense mutations is calculated by dividing the number of specific mutations (e.g. C→T transitions) resulting in missense mutations by the total number of that specific type of mutation

^c The frequency of nonsense and frameshift mutations is calculated by dividing the number of specific mutations (e.g. C→T transitions) resulting in nonsense mutations by the total number of that specific type of mutation

^d One insertion spanned 3 bp and would not result in frameshift or truncation

^e One deletion spanned 2 bp and would result in frameshift but not truncation

predominantly missense mutations (61%) and less frequently consisted of nonsense mutations (16%) or silent mutations (23%). Transitions were the most common type of point mutations (730/833), in large majority represented by C→T and G→A transitions ($n=352$ and $n=286$, respectively). Of C→T and G→A transitions 23% occurred at CpG dinucleotides. A polymorphism (G4497A), which does not result in an altered amino acid, was observed in 74% (493/665) of patients.

Eighty-seven percent of frameshift mutations (126/145) were deletions (table 2). The length of the frameshift ranged from 1 to 5 bp for insertions and 1 to 49 bp for deletions. Generally, frameshift mutations would lead to serious alterations in the amino acid sequence downstream of the mutation and also result in the introduction of a stop codon. However, one insertion spanned 3 bp and would not result in frameshift or truncation.

In the distribution of the truncating mutations several hot spots could be observed (figure 1, top panel). The frameshift mutations seem to cluster in the regions at codons 1,350-1,356, 1,411-1,419, 1,465, 1,485-1,495, and at codon 1,309, where an AAAAG deletion was detected in 26 tumours. A high frequency of nonsense point mutations was detected at codons 1,294, 1,306, 1,328, 1,367, 1,378, 1,406, 1,429 and 1,450. The large number of missense mutations was distributed rather evenly throughout the mutation cluster region of APC and did not show distinct hot spots (figure 1, bottom panel).

Figure 1. Distribution and frequency of 143 frameshift mutations and 132 nonsense mutations (top panel) and 509 missense mutations (bottom panel) observed in tumour material from 665 sporadic colorectal cancer patients. The X-axis schematically represents the codons of the APC mutation cluster region.

The 479 patients that harboured mutations were divided into different groups on basis of having a tumour with either a wild type or a mutant APC with various types of gene mutations (table 3). In total, 52% (248/479) of the patients with a mutation in the mutation cluster region of APC, which corresponds to 37% of all patients (248/665) harboured at least one nonsense or frameshift mutation that would result in a truncated APC protein. Of patients with a truncating mutation 59% (145/247) had more than one mutation leading to a change in the APC protein, i.e. another nonsense and frameshift mutation ($n=27$) or missense mutation ($n=118$). Forty per cent of the patients (191/479) with a mutation in the mutation cluster region, corresponding to 29% of all patients (191/665), harboured at least one missense mutation and no nonsense or frameshift mutations. Eighteen per cent of patients (84/479) had two or more missense mutations. Of all patients, 6% (40/665) had one or more silent mutations and no other type of mutation, and six of these patients had two silent mutations (table 3).

The mean age at diagnosis was lower for patients with mutation(s) in APC as compared to patients with wild type APC (67.66 ± 4.21 versus 68.75 ± 4.32 , respectively, $p=0.002$) (table 3). Detailed analysis of subgroups of mutations revealed that patients with silent mutations ($p=0.008$), missense mutations ($p=0.065$) and nonsense or frameshift mutations ($p=0.002$) were relatively younger when diagnosed with a colorectal tumour as compared to patients with wild type APC (table 3). No differences were observed in the occurrence of APC mutations with respect to sex and family history of colorectal cancer ($p=0.630$ and $p=0.364$, respectively) (table 3). No statistically significant differences

in the occurrence and type of APC mutations with respect to Dukes' stage and tumour differentiation were observed (data not shown).

No statistically significant overall difference between patients without and with any APC mutation was observed with respect to tumour sub-localisation. However, when considering specific types of mutations, patients with rectosigmoid and rectal tumours had a relatively higher frequency of truncating APC mutations as compared to patients with a proximal or distal colon tumour (53% and 45% versus 31% and 32%, respectively, $p=0.029$) (table 3).

Table 3. Characteristics of 665 colorectal cancer cases without an APC mutation or with at least one silent, missense or nonsense mutation

	Total	APC status Wild type ^b	Mutation ^c	Type of APC Silent mutation ^d	Missense mutation ^e	Truncating mutation ^f
Number of patients	665	186	479	40	191	248
Age at diagnosis mean, SD	67.96,4.26	68.75,4.32	67.66,4.21	66.85,4.21	67.97,4.31	67.55,4.12
<i>p</i> -Value ^g			0.002	0.01	0.07	0.002
Sex (% male)	371	101 (54)	270 (56)	16 (39)	112 (59)	142 (57)
<i>p</i> -Value			0.63	0.10	0.40	0.54
Family history of colorectal cancer (% yes)	64	21 (11)	43 (9)	4 (10)	16 (8)	23 (9)
<i>p</i> -Value ^g			0.36	0.81	0.34	0.49
Sub-localisation ^a	654	183	472	39	190	242
Proximal colon (%) ^h	223	71 (32)	152 (68)	15 (7)	68 (31)	69 (31)
Distal colon (%) ^h	206	56 (27)	150 (73)	12 (6)	72 (35)	66 (32)
Rectosigmoid (%) ^h	74	18 (24)	56 (76)	3 (4)	14 (19)	39 (53)
Rectum (%) ^h	151	38 (25)	113 (75)	9 (6)	36 (24)	68 (45)
<i>p</i> -Value ^g			0.42	0.97	0.45	0.03

^a Information on tumour sub-localisation was available for 654 patients

^b Patients without any mutations

^c Patients with at least one mutation

^d Patients with at least one silent mutation, but without any missense or nonsense or frameshift mutations

^e Patients with at least one missense mutation, but without any nonsense or frameshift mutations

^f Patients with at least one nonsense or frameshift mutation

^g *p*-Values were calculated for pairwise comparisons of distribution of sub-localisation for patients with an APC mutation, with a silent mutation, with a missense mutation or with a nonsense/frameshift mutation to patients with wild type APC

^h The frequency of patients with a silent, missense or nonsense/frameshift mutation per sub-localisation was calculated by dividing the number of patients per mutation status group (e.g., silent mutation) by the total number of colorectal cancer patients

Most mutations that would lead to a truncated APC protein are caused by C→T transitions, G→T transversions and frameshift mutations. Patients with proximal colon tumours have relatively less C→T nonsense mutations than patients with a distal colon, rectosigmoid and rectal tumour (7% versus 12%, 22% and 13%, respectively, $p=0.009$) (table 4). Patients with rectosigmoid and rectal tumours have a relatively higher frequency of truncating APC mutations as compared to patients with a proximal or distal colon tumour (23% and 30% versus 19% and 16%, respectively, $p=0.045$) (table 4). No statistically significant difference in the occurrence of G→T nonsense mutations was observed between patients with tumours at the different sub-localisations (table 4). With regard to distribution of all C:G→T:A transitions over the different sub-localisations, irrespective of their

putative effect on the APC protein, these seemed to occur relatively more frequently in distal colon, rectosigmoid and rectum as compared to the proximal colon, but this was not statistically significant (data not shown).

Table 4. Characterisation of most common types of truncating APC mutations according to sub-localisation of the tumour in 654 sporadic colorectal cancer patients

	Total patients	Wild type ^c	Nonsense mutation C→T transition ^{a,c}	G→T transversion ^{a,b,c}	Frameshift mutation ^{b,c}
Sub-localisation					
Proximal colon (%) ^d	223	71 (32)	15 (7)	12 (5)	42 (19)
Distal colon (%) ^d	206	56 (27)	25 (12)	7 (3)	33 (16)
Rectosigmoid (%) ^d	74	18 (24)	16 (22)	8 (11)	17 (23)
Rectum (%) ^d	151	38 (25)	19 (13)	6 (4)	46 (30)
p-Value ^e			0.01	0.12 ^f	0.05

^a For one patient a C→T transition and a G→T transversion was found. This patient was included in both groups

^b For 13 patients frameshift as well as C→T transitions were found. These patients were included in both groups

^c For 5 patients frameshift as well as G→T transversions were found. These patients were included in both groups

^d The frequency of patients with wild type APC, C→T transitions, G→T transversions or frameshift mutations per tumour sub-localization was calculated by dividing the number of patients per mutation group (e.g. C→T transition) by the total number of colorectal cancer patients

^e p-Values were calculated for pairwise comparisons of distribution of sub-localization for patients with at least one C→T transition (n=75), patients with at least one G→T transversion (n=33) or those with at least one frameshift mutation (n=137) to patients with wild type APC

^f p-Value could not be interpreted due to lack of sufficient patients in respective groups

DISCUSSION

The occurrence and type of somatic mutations in the mutation cluster region of the APC gene was evaluated in 665 tumour samples from an unselected, well-defined group of incident colorectal cancer patients from the Netherlands (age at diagnosis between 57 and 67 years). Seventy-two percent of patients (479/665) harboured a total of 978 mutations in the APC mutation cluster region. In particular, we observed a large number (n=508) of missense point mutations. Mutations that would lead to protein truncation were found in 37% of patients (248/665). Patients with rectosigmoid and rectum tumours relatively more frequently harboured C→T nonsense mutations and deletions and insertions that would lead to protein truncation as compared to patients with proximal and distal colon tumours.

In this study, DNA from archival tumour tissue was used. As this formalin-fixed, paraffin-embedded tissue contains highly fragmented genomic DNA, gene analysis can be difficult. The analysis of APC mutations is based on nested amplification and direct sequencing of purified PCR fragments, a highly sensitive and specific detection method. Using this method on archival tissue yields valid results, as shown in the identical mutations status of fresh tissue and paraffin-embedded tissue. Moreover, most mutations leading to truncation of the APC protein observed in this study were also reported in an APC database (<http://www.umd.necker.fr:2008/>).

Due to fragmentation of DNA from archival sources, a protein truncation test or similar methods are not feasible, as these techniques require fully intact DNA strands. In other studies, protein truncation test,⁷ in vitro synthesised protein / in vitro transcription and translation assay^{19, 24} were used, thereby selectively

identifying truncating mutations. Although these methods provide conclusive information on the functional aberrations in APC, i.e. truncating mutations, these do not yield information on the occurrence of other than truncating mutations. Indeed, the large number of mutations detected in our study is mainly due to the high prevalence of other than truncating mutations.

Techniques used for mutation screening such as single-strand conformational polymorphism,^{8, 16-18, 21, 30} RNase protection assay⁷ or denaturing gradient gel electrophoresis³¹ show differences in sensitivity and/or specificity of mutation detection. In particular, the interpretation of single-strand conformational polymorphism assays is difficult and its sensitivity is relatively low.³² Omission of a screening assay prior to sequence analysis may therefore partially explain the large number of mutations found in this study.

We observed multiple mutations in 40% of adenocarcinomas, ranging from two to eight mutations per tumour. Possibly, this reflects the occurrence of mutations in both alleles of the APC locus. Since APC is a tumour suppressor gene, more than one inactivating mutation would be necessary to result in loss of APC function in the cell. However, we are unable to distinguish different alleles in our analyses. Another explanation for multiple mutations within a tumour may be that it is a reflection of tumour heterogeneity. In a recent study of advanced colorectal tumours, heterogeneity in the mutational status of TP53 and K-ras within the same tumour was demonstrated.³³ We performed macrodissection on the samples that were analysed, as a result of which we may have included different subclones of a tumour that possibly harbour different mutations.

Another plausible cause of multiple mutations is a defect in the DNA repair systems. Inherited variants of the base excision repair gene MYH were found to give rise to G→T and C→A transversions in a family that is affected with multiple colorectal adenomas and carcinoma.³⁴ Recently, these inherited variants of MYH have also been observed in a population-based series of colorectal cancer patients,³⁵ suggesting that a proportion of the 46 patients that harboured a G→T or C→A transversions in our study may be due to inherited MYH mutations. Defects in the mismatch repair genes hMLH1 and hMSH2, resulting in mismatch repair deficiency is found in hereditary non-polyposis coli and is also found in approximately 15% of sporadic colorectal tumours. However, reported results are inconsistent as to whether mismatch repair deficiency is an alternative pathway in colorectal tumours with intact APC function^{36, 37} or that it independently gives rise to an increased frequency of APC mutations.³⁸⁻⁴⁰

The majority of observed mutations were point mutations, 61% of which give rise to substitution of one amino acid. The relevance of missense mutations in APC is not yet understood. It is unclear which of these changes would affect the structure and function of the APC protein and whether such an alteration would lead to selective advantage of tumour cells and tumour growth. Intriguingly, 52% of patients with truncating mutations also had one or more concurrent missense mutation(s). If the occurrence of these missense mutations is the reflection of a biallelic event, this may indicate involvement of missense APC mutations in colorectal tumourigenesis.

Twenty-three percent of the point mutations would not lead to protein truncation or an altered amino acid. In addition, a polymorphism (G4497A), which does not result in an altered amino acid was observed in 74% (493/665) of patients. This polymorphism has also been recently described in a series of adenoma samples from Dutch patients,³¹ which suggests that this polymorphism represents a wild type DNA sequence in APC in the Dutch population.

Considering all mutations that are detected in the mutation cluster region, only 28% (276/978) would lead to a truncated APC protein, half of which are represented by point mutations. This is in contrast with previous reports that up to 98% of mutations would lead to a truncated APC protein, and only one third of these are caused by point mutations.²⁵

Only 37% of patients (247/665) harboured mutations that would lead to a truncated APC protein. Considering the general idea that most colorectal tumours follow a molecular pathway involving APC, our observed frequency seems low. It is, however, in keeping with the frequency of 34% truncating mutations found in the mutation cluster region in another Dutch population-based case-control study.²¹ The mutation cluster region spans several 20 amino acid repeats, which form the β -catenin binding sites and therefore the mutation cluster region represents a functionally important domain of the APC gene. According to several reports 68 to 77% of the somatic mutations in APC are found in the mutation cluster region, which represents less than 10% of the APC coding region.^{5, 7, 8} We are unable to make any predictions of the occurrence of mutations in the complete APC gene, since our mutation analysis was restricted to the mutation cluster region of APC.

Other explanations for the relatively low frequency of truncating mutations of APC in the colorectal tumours observed here, are that these tumours may have arisen through other genetic or epi-genetic events. APC is a key player in the Wnt pathway. Inactivating mutations that cause loss of β -catenin binding and degradation sites on the APC protein, would lead to increased levels of intranuclear β -catenin.^{9, 22} However, it has also been shown that activating mutations in the *CTNNB1* gene can lead to stabilisation and accumulation of the β -catenin protein,^{41, 42} thereby circumventing the necessity for mutations in APC to activate the Wnt pathway. Hypermethylation of the promotor region of APC constitutes an alternative mechanism for gene inactivation. This was found to occur in 18% of primary sporadic colorectal carcinomas,⁴³ and in 39% of a sample of 122 colorectal cancer cases from our study, although these cases were selected on high and low methyl donor intake.⁴⁴

FAP tumours generally arise at a young age through a germline mutation in the APC gene. Our APC gene mutation analysis is based on a relatively large series of unselected, incident colorectal cancer patients, who were between 55 and 69 years of age at baseline and have developed a first colorectal tumour since. This strongly suggests that the mutations observed in this study are acquired and therefore of somatic nature. Moreover, no significant differences in family history of colorectal cancer between patients without and with APC mutations were observed, which also argues against germline mutations. The observation that there were no statistically significant differences in occurrence and type of APC mutations with respect to Dukes' stage and tumour differentiation support the

hypothesis that APC gene mutation is an early event in colorectal tumourigenesis.

38 The differences in the occurrence of specific types of mutations in colon versus rectosigmoid and rectum cancer shown here, suggests a different aetiology of tumourigenesis in the different sub-localisations and it may also reflect the exogenous origin of mutations. This is supported by a recent report of K-ras mutations in colorectal cancer tissue of patients from our study, in which mutations in K-ras were also more frequently observed in rectum tumours as compared to rectosigmoid and colon tumours.²⁹ The data with respect to the rectosigmoid have to be regarded with some caution as the rectosigmoid is a more clinically applied term rather than an anatomically defined transitional zone between colon and rectum.²⁹ When rectosigmoid tumours were excluded from the analyses, both frameshift and C→T mutations that would result in protein truncation occurred relatively more frequently in the rectum as compared to proximal and distal colon. However, the asymmetry of C→T nonsense mutations with regard to tumour sub-localisation was less pronounced.

In conclusion, a large number of mutations was found in the mutation cluster region of the APC gene, in particular, we observed many missense point mutations, more than previously reported. Missense mutations need further exploring with regard to their role in APC function and colorectal tumourigenesis. Patients with a rectosigmoid or rectum tumour relatively more frequently have a truncating C→T transition or frameshift mutation as compared to patients with a proximal or distal colon tumour. The pattern of APC mutations observed in the different sub-localisations of the colorectal tract suggests a different aetiology for tumour site within the large bowel.

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C H A P T E R 3

**MUTATIONS IN APC, CTNNB1,
AND K-RAS GENES
AND EXPRESSION OF HMLH1 IN
SPORADIC COLORECTAL CANCER**

MARGREET LÜCHTENBORG
MATTY P. WEIJENBERG
PETRA A. WARK
A. MERDAN SARITAS
GUIDO MJM. ROEMEN
GOOS NP. VAN MUIJEN
ADRIAAN P. DE BRUÏNE
PIET A. VAN DEN BRANDT
ANTON FPM. DE GOEIJ

ABSTRACT

In colorectal carcinogenesis several pathways have been identified. The majority of colorectal tumours are thought to be driven by a sequential accumulation of mutations in the *APC*, *K-ras* and *TP53* genes. A smaller proportion of cancers displays microsatellite instability.

In a group of 656 unselected incident colorectal cancer patients, the occurrence of mutations in the *APC*, *K-ras*, and *CTNNB1* genes as well as expression of hMLH1 was investigated. Additionally, tumours were divided in groups on the basis of molecular features and compared with respect to age at diagnosis, sex, family history of colorectal cancer, tumour sub-localisation, Dukes' stage and tumour differentiation.

Mutations at the phosphorylation sites in the *CTNNB1* gene were observed in tumours from only five of 464 patients. Tumours with truncating *APC* mutations and activating *K-ras* mutations in codons 12 and 13 occurred at similar frequencies (37% (245/656) and 36% (235/656), respectively). Seventeen percent of tumours harboured both an *APC* and a *K-ras* mutation (109/656). Nine percent of all tumours (58/656) lacked hMLH1 expression. Patients harbouring a tumour with absent hMLH1 expression were older, more often women, more often had proximal colon tumours that showed poorer differentiation when compared to patients harbouring tumours with an *APC* and/or *K-ras* mutation.

From data of a large, unselected group of sporadic colorectal cancer patients, we conclude that several pathways in colorectal cancer can be identified, but that the main differences in tumour and patient characteristics are found between groups of patients based on mismatch repair deficiency.

INTRODUCTION

Carcinogenesis is a multi-step process that involves the accumulation of numerous genetic and epigenetic changes in cells.¹ These genetic aberrations affect a limited number of identified pathways that are involved in (colorectal) carcinogenesis.² In colorectal cancer a number of specific genetic alterations have been identified. A genetic model for sporadic colorectal cancer has been proposed, which describes the sequential accumulation of specific genetic alterations in various pathways, involving tumour suppressor genes (e.g., *APC*, *SMAD4*, *TP53*) and oncogenes (e.g., *CTNNB1*, *K-ras*).^{3, 4} Two important molecular pathways that upon activation affect the early and intermediate stages of colorectal carcinogenesis are the Wnt and Ras signalling pathways.

Activation of the Wnt pathway plays a central role in the aetiology of most colorectal cancers and is often the result of mutations in the central domain of the adenomatous polyposis coli (*APC*) gene, that lead to partial or complete loss of this region and thereby to loss of the β -catenin regulating function.^{5, 6} Conversely, activating missense mutations at one of the phosphorylation sites in exon 3 of the *CTNNB1* gene, encoding the β -catenin protein, can render it stable as it can no longer be tagged for cellular degradation.⁷ Activation of the Ras pathway in cancer is marked by the loss of the intrinsic GTPase activity of the Ras protein, which can be ascribed to missense mutations in codons 12 and 13 of exon 1 of the Kirsten ras (*K-ras*) gene in 90% of cases.⁸

According to the paradigm for colorectal cancer development, mutations in the *APC*, *K-ras* and *TP53* genes are all thought to contribute to the aetiology of colorectal cancer.³ However, a recent study based on the analysis of *APC*, *K-ras* and *TP53* genes concluded that simultaneous occurrence of all three genetic alterations is rare and that multiple genetic pathways may be relevant to colorectal cancer.⁹

Genetic instability is seen in most types of cancer.¹⁰ Two distinct types of genetic instability appear to occur in colorectal cancer:¹¹ chromosomal and microsatellite instability. Chromosomal instability results in gains or losses of entire chromosomes or parts of them, and gives rise to aneuploid tumours and occurs in the majority of cancers. A smaller proportion of colorectal cancers displays microsatellite instability, represented by diploid cells acquiring high mutation rates, and was found to be associated with defective mismatch repair.¹² These tumours are less likely to harbour mutations in genes associated with chromosomally unstable and generally aneuploid tumours, such as *APC*, *K-ras* and *TP53*.¹³⁻²¹ suggesting that these tumours form a distinct group. Moreover, microsatellite instable tumours are found predominantly in the proximal colon,^{22, 23} are more likely to occur in patients with a positive family history of colorectal cancer,^{22, 23} are often less differentiated than microsatellite stable tumours,²³ and occur more frequently in women²⁴ and at older age.²⁵

Considering the aforementioned issues, there is a need for studies addressing the heterogeneity of affected genes involved in colorectal cancer development in large groups of patients. We have previously studied the occurrence of *APC* and *K-ras* mutations separately.^{26, 27} In the current study, in addition to investigating mutations in the *APC*, *CTNNB1* and *K-ras* genes as well

as mismatch repair deficiency and combinations of these aberrations, their relation with various tumour and patient characteristics were studied in a large, unselected group of incident colorectal cancer patients.

MATERIALS AND METHODS

STUDY POPULATION

46 The prospective Netherlands Cohort Study on diet and cancer was initiated in September 1986. The study design has been described in detail elsewhere.²⁸ At baseline, a total of 58,279 men and 62,573 women, between the ages of 55 and 69 years, completed a self-administered food frequency and lifestyle questionnaire. The study population originated from 204 municipal population registries throughout The Netherlands.

Incident cancer cases are identified by monitoring of the entire cohort for cancer occurrence through annual record linkage to the Netherlands Cancer Registry, i.e. nine regional cancer registries throughout The Netherlands, and to PALGA, a nationwide network and registry of histo- and cytopathology.²⁹ Together, the NCR and PALGA provide a near 100% coverage of the municipalities included in the NLCS. The first 2.3 years of follow up were excluded because of possible pre-clinical disease affecting exposure status and because of incomplete nationwide coverage of PALGA in some of the municipalities included in the NLCS in that period. From 1989 until 1994, 929 incident cases with histologically confirmed colorectal cancer were identified within the cohort, of whom 819 could also be linked to a PALGA report of the lesion.

The PALGA reports were used to identify and locate tumour tissue from eligible colorectal cancer patients in Dutch pathology laboratories. Colon and rectal cancer were classified according to site as follows, colon: cecum through sigmoid colon (ICD-O codes 153.0, 153.1, 153.2, 153.3, 153.4, 153.5, 153.6, 153.7), rectosigmoid (ICD-O code 154.0), and rectum (ICD-O code 154.1).

TISSUE SAMPLES

Tumour material of colorectal cancer patients was collected after approval by the Ethical Review Board of Maastricht University, PALGA and the NCR. Tissue samples from 819 colorectal cancer patients were localised in 54 pathology laboratories throughout The Netherlands. Forty-four (5%) tumour tissue samples could not be retrieved from the pathology archives. Of 775 available tissue samples, 737 (95%) contained sufficient tumour material for molecular analyses. Tissue sections were cut from each sample, which were used for DNA isolation and immunohistochemical analysis.

DNA ISOLATION

DNA isolation was described in detail elsewhere.²⁶ Briefly, a 4 μ m section, cut from each paraffin-embedded tumour tissue block, was stained with haematoxylin and eosin (HE) for histopathological examination by a pathologist. Five 20 μ m sections of tumour tissue were cut from each sample for DNA isolation. Tumour tissue was separated from normal colon epithelium using the HE section as a reference. Genomic DNA was extracted from

compare as different methods of detecting mutations in APC have been used and different regions of the APC gene have been studied.

In the current report, the frequency and type of APC gene mutations are assessed in a large series of unselected, incident colorectal cancer patients from The Netherlands identified in a prospective cohort study. The occurrence and type of APC gene mutations in the mutation cluster region are reported with regard to age at diagnosis, sex, family history of colorectal cancer, Dukes' stage, tumour differentiation and sub-localisation.

MATERIALS AND METHODS

STUDY POPULATION

A prospective cohort study on diet and cancer was initiated in the Netherlands in September 1986. The study design has been described in detail elsewhere.²⁷ Briefly, at baseline a total of 58,279 men and 62,573 women, between the ages of 55 and 69 years, completed a self-administered food frequency and lifestyle questionnaire. The study population originated from 204 municipal population registries throughout the country. Incident cancer cases are identified by monitoring of the entire cohort for cancer occurrence through annual record linkage to the Netherlands Cancer Registry (NCR), nine regional cancer registries throughout The Netherlands, and to the Pathologisch Anatomisch Landelijk Geautomatiseerd Archief (PALGA), a nationwide database of pathology reports.²⁸ The PALGA database was used to identify and locate tumour tissue in Dutch Pathology laboratories. The first 2.3 years of follow up were excluded due to incomplete nationwide coverage of PALGA. From 1989 until 1994, 819 incident cases with histologically confirmed colorectal cancer were identified. Colorectal cancer was classified according to site as follows, proximal colon: cecum through transverse colon (ICD-O codes 153.0, 153.1, 153.4, 153.5, 153.6); distal colon: splenic flexure through sigmoid colon (ICD-O codes 153.2, 153.3, 153.7); rectosigmoid (ICD-O code 154.0) and rectum (ICD-O code 154.1). Information about age at diagnosis, sex and family history of colorectal cancer (at baseline) was retrieved from the Netherlands Cohort Study on diet and cancer (NLCS) database. Information about tumour sub-localisation, Dukes' stage and differentiation was retrieved from the NCR database.

TISSUE SAMPLES

Tumour material of colorectal cancer patients was collected after approval by the Ethical Review Board of Maastricht University, PALGA and the NCR. Tissue samples from 819 colorectal cancer patients were retrieved from 54 pathology laboratories throughout the Netherlands. Tumour tissue specimen collection started in August 1999 and was completed in December of 2001. Forty-four tumour tissue samples (5%) could not be traced. Of 775 eligible tissue samples 737 (90%) contained sufficient tumour material as confirmed by a pathologist (A.d.B.) for molecular analyses.

Table 1. Description of patient, tumour and molecular characteristics of tumours harbouring a *CTNNB1* mutation from a total of 464 tumours analysed

Patient	Sex	Age at diagnosis (yr)	Sub-localisation	Dukes' stage	Tumour differentiation
1	M	65	Proximal colon	B	Moderate
2	M	67	Proximal colon	A	Moderate
3	M	69	Proximal colon	B	Good
4	M	61	Proximal colon	C	Moderate
5	F	69	Proximal colon	A	Undifferentiated
6 ^a	M	69	Distal colon	B	Moderate
7 ^a	F	65	Proximal colon	A	Moderate

(pH 8.0) and incubated with 10% normal horse serum for ten min at room temperature. Then, sections were incubated overnight at 4°C with mouse monoclonal antibodies against hMLH1 protein (clone G168-15, PharMingen, San Diego, CA) at a 1:100 dilution. Antibody binding was detected by incubating the sections at room temperature with the peroxidase-labelled DAKO Envision System (DAKO, Carpinteris, CA), using diaminobenzidine as a chromogen. Sections were counterstained with diluted haematoxylin.

Lesions were considered to lack hMLH1 protein expression when unequivocal absence of nuclear staining of the tumour epithelial cells was observed. Nuclear staining of normal epithelial and stromal cells or lymphocytes served as an internal positive control. Staining was scored independently by at least two observers and in case of discordant results discussed with a pathologist until consensus was reached. hMLH1 expression could be determined in 724 of 737 patients.

BAT-26

Analysis of the BAT-26 mononucleotide repeat was performed in tumour samples from 162 patients to determine the correlation between the microsatellite instability marker BAT-26 and hMLH1 expression including 48 of 61 tumours that lacked hMLH1 expression. The primer sequences and PCR conditions for the BAT-26 mononucleotide repeat were used as described previously.³¹

STATISTICAL ANALYSIS

In the statistical analysis data from 656 patients for whom information on APC and *K-ras* mutation status as well as hMLH1 expression was complete were included.

Characteristics of patients (age at diagnosis, sex, family history of colorectal cancer) and tumours (tumour sub-localisation, Dukes' stage and tumour differentiation) were compared between patients with and without an activating *K-ras* or a truncating APC mutation as well as patients harbouring tumours with and without hMLH1 expression, using Student's *t* test (age at diagnosis) and χ^2 tests (sex, family history of colorectal cancer, tumour sub-localisation, Dukes' stage and differentiation). Additionally, patient and tumour characteristics of tumours with an activating *K-ras* and/or a truncating APC mutation were compared to tumours lacking hMLH1 expression. All *p*-values are

Table 1. Continued

Codon	Mutation	Amino acid change	hMLH1 expression ^a	Truncating APC mutation ^b	K-ras mutation
37	C→T	Ser→Phe	Present	No	Yes
45	C→T	Ser→Phe	Absent	No	Yes
45	C→T	Ser→Phe	Absent	No	No
45	C→T	Ser→Phe	Present	No	Yes
45	C→T	Ser→Phe	Absent	No	Yes
22	G→A	Val→Ile	Present	No	Yes
29	C→A	Ser→Tyr	Present	No	No

^a Two patients (numbers 6 and 7) harboured a *CTNNB1* mutation that would not lead to loss of a known phosphorylation site

^b 411 Tumours studied were selected based on absence of a truncating APC mutation, 58 tumours were selected based on absence of hMLH1 expression

reported for a two-sided test; *p*-values of less than 0.05 were considered to be statistically significant.

RESULTS

464 Tumours without a truncating APC mutation and/or lacking hMLH1 expression, were analysed for mutations in exon 3 of the *CTNNB1* gene. Table 1 describes the patient, tumour, and molecular characteristics of seven colorectal tumours that harboured a mutation in *CTNNB1* exon 3. Five colorectal cancers with a *CTNNB1* mutation that would lead to loss of one of the Ser/Thr phosphorylation sites and subsequent stabilisation of the protein, all occurred at codons 37 and 45, all were C→T transitions, leading to Ser→Phe amino acid changes and all occurred in the proximal colon. All but one also had an activating mutation in the *K-ras* gene. Three of the five tumours showed hMLH1 deficiency. Two colorectal cancer patients harboured a mutation in the *CTNNB1* gene, that did not occur at the Ser/Thr phosphorylation sites, but would result in an amino acid alteration at codons 22 and 29, the effects of which are unknown. Because of the very low frequency of tumours harbouring a *CTNNB1* mutation, these mutations were not included in further analyses. Moreover, mutation analysis of the remaining samples was abandoned since this was deemed irrelevant.

Of all tumours for which the other molecular alterations, i.e. mutations in the APC and *K-ras* genes and hMLH1 expression, were successfully analysed, 103 colorectal tumours did not harbour a truncating or missense APC mutation, an activating *K-ras* mutation or showed lack of hMLH1 expression, as depicted in figure 1.

Truncating as well as missense APC mutations and activating *K-ras* mutations were relatively common. Truncating APC mutations alone and activating *K-ras* mutations in codons 12 and 13 only, occurred at similar frequencies (20% (130/656) and 18% (121/656), respectively). A combination of a truncating mutation in APC and an activating mutation in *K-ras* occurred less often than the sole occurrences of mutations in both genes. However, as shown in table 2,

Figure 1. Distribution of (combinations of) genetic aberrations in the APC and K-ras genes and hMLH1 expression in tumours from 656 sporadic colorectal cancer patients. The bars represent the different molecular alterations present in mutually exclusive groups of tumours.

the simultaneous occurrence of mutations in both genes occurred more frequently than expected on the basis of chance alone.

A χ^2 test for the occurrence of a truncating APC mutation and an activating K-ras mutation, revealed that the occurrence of these mutations was not independent ($p < 0.001$), but the correlation was weak (Cramér's $V = 0.138$). Finally, although 11 tumours that harboured a mutation in the APC or K-ras gene also lacked hMLH1 expression, hMLH1 deficiency occurred more frequently in tumours that did not harbour these mutations ($\chi^2 = 36.6$, $p < 0.001$)

Table 2. Crosstabulation of colorectal carcinomas with and without activating K-ras and truncating APC mutation(s)

Activating K-ras mutation		Truncating APC mutation		Total
		No	Yes	
No		258	136	421
	Expected ^a	263.8	157.2	
Yes		126	109	235
	Expected ^a	147.2	87.8	
Total		411	245	656

^a Expected based on chance alone

With respect to localisation in the colorectal tract, tumours of the rectosigmoid and rectum more frequently harboured truncating APC mutations when compared to colon tumours ($p = 0.001$), as shown in table 3. Rectosigmoid and rectal tumours have a relatively higher frequency of K-ras mutations in codons 12 and 13 when compared colon tumours ($p = 0.05$) (table 3). Tumours lacking hMLH1 expression occur almost exclusively in the proximal colon ($p < 0.001$) and relatively more frequently show poor differentiation or are undifferentiated ($p < 0.001$) when compared to tumours with hMLH1 expression (table 3).

Next, we compared the patient and tumour characteristics of tumours harbouring a truncating APC and/or an activating K-ras mutation to those of tumours without hMLH1 expression, and these results are presented in table 4. Patients with a hMLH1 deficient tumour were slightly older when diagnosed with colorectal cancer (69.3 ± 4.3 yr versus 67.8 ± 4.2 yr,

Table 3. Comparisons between colorectal cancer patients with or without a *K-ras* and *APC* gene mutation and with or without hMLH1 expression, according to patient and tumour characteristics

	Genetic aberration				hMLH1 expression	
	APC ^a		K-ras ^b		Present	Absent
	No truncating mutation	Truncating mutation	No codon 12/13 mutation	Codon 12/13 mutation		
Number of patients (%)	411 (63)	245 (37)	421 (64)	235 (36)	598 (91)	58 (9)
Age at diagnosis (yr, mean (SD))	68.2 (4.3)	67.5 (4.2)	67.7 (4.3)	68.3 (4.3)	67.9 (4.2)	68.7 (4.7)
p-value	0.07		0.08		0.18	
Sex (% male)	54	58	56	55	57	45
p-Value	0.45		0.84		0.08	
Family history of colorectal cancer (% yes)	10	9	11	7	10	9
p-Value	0.89		0.07		0.79	
Tumour sub-localisation ^c						
Proximal colon	151 (69%)	67 (31%)	141 (65%)	77 (35%)	169 (78%)	49 (12%)
Distal colon	138 (68%)	66 (32%)	144 (71%)	60 (29%)	199 (98%)	5 (2%)
Rectosigmoid	34 (47%)	39 (53%)	44 (60%)	29 (40%)	73 (100%)	0 (0%)
Rectum	83 (55%)	67 (45%)	85 (57%)	65 (43%)	146 (97%)	4 (3%)
p-Value	<0.001		0.05		<0.001	
Dukes' stage ^c						
A	94 (60%)	63 (40%)	103 (66%)	54 (34%)	142 (90%)	15 (10%)
B	147 (67%)	73 (33%)	142 (65%)	78 (35%)	192 (87%)	28 (13%)
C	97 (60%)	64 (40%)	107 (66%)	54 (34%)	151 (94%)	10 (6%)
D	39 (54%)	33 (46%)	40 (56%)	32 (44%)	69 (96%)	3 (4%)
p-Value	0.21		0.42		0.07	
Differentiation ^c						
Good	35 (56%)	28 (44%)	40 (63%)	23 (37%)	59 (94%)	4 (6%)
Moderate	250 (62%)	156 (38%)	258 (64%)	148 (34%)	380 (94%)	26 (6%)
Poor	66 (69%)	30 (31%)	68 (71%)	28 (29%)	82 (85%)	14 (15%)
Undifferentiated	6 (86%)	1 (14%)	5 (71%)	2 (29%)	4 (57%)	3 (43%)
p-Value	0.20		0.57		<0.001	

^a APC mutations: only mutations resulting in a stop codon^b K-ras mutations: only activating mutations in codon 12 or 13^c Tumour sublocalisation, Dukes's stage and differentiation gave rise to missing values

$p=0.03$), were relatively less frequently men (40% versus 58%, $p=0.02$) than patients with a tumour harbouring a truncating *APC* and/or activating *K-ras* mutation. Compared to tumours with an *APC* and/or *K-ras* mutation, tumours without hMLH1 expression occurred relatively more frequently in the proximal colon ($p<0.001$) and relatively more frequently showed poor differentiation or are undifferentiated ($p<0.001$).

When comparing tumours with a missense (but not a truncating) mutation in *APC* to tumours with a truncating mutation in the *APC* gene, missense mutations occurred relatively more frequently in the colon ($p=0.002$) less often also harboured an activating *K-ras* mutation ($p=0.004$), and more often also lacked hMLH1 expression ($p<0.001$). No differences were observed with regard to age at diagnosis, sex, Dukes' stage or tumour differentiation (data not shown).

Finally, to assess agreement between hMLH1 expression and microsatellite instability, for 162 tumours both hMLH1 expression and BAT-26 were analysed. All tumours that had normal BAT-26, also showed hMLH1 expression. Fourteen tumours with unstable BAT-26 also lacked hMLH1 expression, and two tumours

with unstable BAT-26, were found to express hMLH1, which demonstrates a high agreement between these molecular features of mismatch repair deficiency.

Table 4. Comparisons between patients with an APC and/or K-ras mutation and absent hMLH1 expression, according to patient and tumour characteristics

	APC ^a and/or K-ras ^b mutation		Absence of hMLH1		p-Value
Number of patients ^c	360		47		
Age at diagnosis (yr, mean (SD))	67.8	(4.2)	69.3	(4.3)	0.03
Sex (% male)	58		40		0.02
Family history of colorectal cancer (% yes)	8		11		0.60
Tumour sub-localisation					<0.001
Proximal colon	96	(70%)	41	(30%)	
Distal colon	107	(97%)	3	(3%)	
Rectosigmoid	51	(100%)	0	(0%)	
Rectum	100	(97%)	3	(3%)	
Dukes' stage					0.08
A	86	(90%)	10	(10%)	
B	117	(84%)	23	(16%)	
C	89	(91%)	9	(9%)	
D	47	(94%)	3	(6%)	
Differentiation					<0.001
Good	37	(92%)	3	(8%)	
Moderate	232	(92%)	20	(8%)	
Poor	43	(78%)	12	(22%)	
Undifferentiated	1	(33%)	2	(67%)	

^a APC mutations: only mutations resulting in a stop codon

^b K-ras mutations: only activating mutations in codon 12 or 13

^c Patients with both absent hMLH1 expression and a APC or K-ras mutation were excluded from the analysis

DISCUSSION

In this study, the occurrence of mutations in the APC, CTNNB1 and K-ras genes as well as expression of the hMLH1 protein in tumour tissue of 656 sporadic colorectal cancer cases was investigated. The occurrence of mutations in the CTNNB1, which codes for β -catenin, was rare: only five of 464 tumours analysed were found to have a mutation at one of the phosphorylation sites in exon 3. Truncating mutations in APC and activating mutations in K-ras appeared to occur at similar frequencies. Although tumours harbouring both mutations were relatively rare, mutations in APC and K-ras may occur co-dependently. Nine percent of all tumours (58/656) lacked hMLH1 expression, and in these tumours generally no APC or K-ras mutation was detected. Patients harbouring a tumour with absent hMLH1 expression were older, more often women, more often had proximal colon tumours that showed poorer differentiation when compared to patients who harboured a tumour with a truncating APC and/or activating K-ras mutation.

The selection of patients included in this study was based on the completeness of analyses of both APC and K-ras genes and hMLH1 expression and this led to a considerable reduction in the number of cases included in the analyses presented in this study. The largest reduction (72 cases) was due to incompleteness of the analysis of all four fragments comprising the APC mutation cluster region. However, characteristics of patients (age, sex, family history of colorectal cancer) and tumours (sub-localisation, Dukes' stage and differentiation) of the group under study are similar to the 737 patients for whom

tumour material was available and all 819 patients initially recognised within the cohort (data not shown). Moreover, the *K-ras* and hMLH1 data presented here, are similar to the data for *K-ras* and hMLH1 based on the complete groups (737 and 724 cases, respectively) (data not shown).

Mutations in exon 3 of the *CTNNB1* gene that would lead to loss of one of the phosphorylation sites were rare. Strikingly, all five of these mutations occurred in the proximal colon and three of these also had absent hMLH1 expression. This may indicate that these proximal colon tumours, which often also show mismatch repair deficiency, are more likely to harbour *CTNNB1* mutations. This was also found in a study of microsatellite instable colorectal tumours.³⁰ WAVE screening technique has not previously been used for formalin-fixed, paraffin-embedded tissue, and therefore it seems plausible that samples harbouring a *CTNNB1* mutation have escaped detection. However, all 58 hMLH1 deficient samples were analysed by direct sequencing without a prior screening step, and only three of these samples harboured a *CTNNB1* mutation.

The model describing the accumulation of genetic alterations of the *APC*, *K-ras*, *TP53* and *SMAD4* genes that drive the development of a carcinoma, has become generally accepted as a paradigm for the genetic basis of colorectal carcinogenesis.^{3, 32} The relatively low frequency of simultaneous occurrence of mutations in both *APC* and *K-ras* observed in this study seems to argue against this synergy. This contention is in accordance with observations from another cohort study, in which *APC*, *K-ras* and *TP53* gene mutations were studied in 109 tumours and these mutations were found to rarely occur together in the same tumour.⁹ However, the simultaneous occurrence of *APC* and *K-ras* mutations observed in our study occurs more frequently than expected based on chance alone and therefore mutations in the *APC* and *K-ras* genes do not seem to occur independently. When data on *APC* and *K-ras* mutations were derived from the study by Smith *et al.*,⁹ similar results, although not statistically significant, could be obtained.

Of the microsatellite instable tumours, approximately 90% show absence of hMLH1 expression.²⁴ In most sporadic colorectal cancers, the promoter region of hMLH1 is hypermethylated, resulting in absence of the protein.³³⁻³⁵ BAT-26 was previously shown to identify microsatellite instability in sporadic colorectal cancer³⁶ and results from our study showed a good agreement between unstable BAT-26 and absent hMLH1 expression.

Several studies have shown that microsatellite instability and mutations in *APC*, *TP53* and *K-ras* occur almost mutually exclusively,^{17, 18, 21, 37} suggesting that these characteristics represent separate pathways. However, others have observed aberrations in both pathways, but these studies have been performed in relatively small groups of HNPCC and sporadic colorectal cancer patients and this may have given rise to a relative overrepresentation of mutation detection in both pathways.^{19, 20, 38} Although in our study the simultaneous occurrence of hMLH1 deficiency as well as an *APC* or *K-ras* mutation was observed in a small number of tumours, the mutually exclusive occurrence of hMLH1 deficiency and mutations in *APC* and/or *K-ras* seemed to predominate.

Moreover, after exclusion of tumours displaying this overlap, a striking difference between occurrences of *APC* and/or *K-ras* mutations versus absence of hMLH1

expression was observed. The differences were most pronounced with regard to tumour sub-localisation and differentiation. hMLH1 deficient tumours occur almost exclusively in the proximal colon and are relatively more frequently poorly differentiated, which is in accordance with previous reports from other studies.²²⁻²⁵

In conclusion, this study shows that mutations in the *CTNNB1* gene seem to be of minor importance in sporadic colorectal cancer. Although APC and *K-ras* mutations are often observed separately in a tumour, these mutations appear to also occur in a co-dependent manner. Tumours that display mismatch repair deficiency, seem to form a distinct sub-group as they differ from tumours with APC and/or *K-ras* mutations with regard to age and sex of the patient, tumour sub-localisation and differentiation.

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C H A P T E R 4

**MEAT AND FISH CONSUMPTION,
APC GENE MUTATIONS
AND HMLH1 EXPRESSION
IN COLON AND RECTAL CANCER:
A PROSPECTIVE COHORT STUDY**

MARGREET LÜCHTENBORG
MATTY P. WEIJENBERG
ANTON FPM DE GOEIJ
PETRA A. WARK
MIRIAN BRINK
GUIDO MJM ROEMEN
MARJOLEIN HFM LENTJES
ADRIAAN P. DE BRUÏNE
R. ALEXANDRA GOLDBOHN
PIETER VAN 'T VEER
PIET A. VAN DEN BRANDT

ABSTRACT

The aim of this study was to investigate the associations between meat and fish consumption according to adenomatous polyposis coli (APC) mutation status and human mut-L homologue 1 (hMLH1) expression in colon and rectal cancer.

The associations were investigated in the Netherlands Cohort Study on diet and cancer, and included 434 colon and 154 rectal cancer patients on whom case-cohort analyses (subcohort $n=2,948$) were performed.

60 Total meat consumption was not associated with the endpoints studied. Meat product (i.e., processed meat) consumption showed a positive association with colon tumours harbouring a truncating APC mutation, whereas beef consumption was associated with an increased risk of colon tumours without a truncating APC mutation (incidence rate ratio (RR) for highest versus lowest quartile of intake 1.61, 95% confidence interval (CI) 0.96-2.71, p -trend=0.04 and 1.58, 95% CI 1.10-2.25, p -trend=0.01, respectively). Consumption of other meat (horsemeat, lamb, mutton, frankfurters and deep-fried meat rolls) was associated with an increased risk of rectal cancer without a truncating APC mutation (RR intake versus no intake 1.79, 95% CI 1.10-2.90). No associations were observed for meat consumption and tumours lacking hMLH1 expression.

Our data indicate that several types of meat may contribute differently to the aetiology of colon and rectal cancer, depending on APC mutation status but not hMLH1 expression of the tumour.

INTRODUCTION

Epidemiological studies have led to the current understanding that a significant proportion of colorectal cancer cases may be explained by dietary habits.¹ Although results from epidemiological studies on the risk of diet on colorectal cancer are inconsistent, meat consumption has been associated with an increased risk of colorectal cancer in several studies. According to the WCRF/AICR report on cancer prevention, evidence from previous studies shows that red meat (fresh meat excluding poultry) probably increases risk of colorectal cancer, and processed meat possibly increases risk.² Poultry consumption data are inconsistent and there may be no relationship with colorectal cancer risk.² Fish consumption possibly also has no relationship with the risk of colorectal cancer.² A recent meta-analysis of 34 case-control and 14 cohort studies, investigating the risk of meat consumption on colorectal cancer concluded that total meat consumption was not significantly associated with colorectal cancer, but high intake of red meat (beef, veal, pork, mutton, and lamb), and in particular processed meat, was associated with a moderate but significantly increased risk of colorectal cancer.³

Most epidemiological studies investigating the consumption of meat as a risk factor have not made a distinction between colon and rectal cancer, or included only colon cancer but not rectal cancer. Previous studies have suggested differences in aetiology between the subsites of the colorectal tract.⁴ The duration of exposure to and the composition of bowel content differ between colon and rectum. Moreover, molecular aspects of tumourigenesis also seem to differ between subsites of the large bowel.^{5, 6}

Most sporadic colorectal cancers are thought to arise through genetic pathways, which involve aberrations in a number of genes, as described by Fearon and Vogelstein.⁷ Inactivation of the adenomatous polyposis coli (APC) gene is thought to be a key event early in colorectal tumourigenesis. Somatic mutations in the mutation cluster region of APC have been found in 34 to 70% of sporadic tumours in colorectal cancer patients, which underscores the importance of APC gene mutation in sporadic colorectal carcinogenesis.⁸⁻¹⁶ The large majority of mutations observed in different studies would lead to a truncated and therefore inactivated APC protein. In our study, using a sensitive mutation detection technique, we found 72% of patients to have mutations in the APC gene, but only 37% of patients had a mutation that would lead to truncation of the APC protein.⁶ A considerable proportion of patients (29%) had a colorectal tumour that did not harbour truncating but missense mutations in the APC gene.⁶

In contrast with the majority of sporadic colorectal tumours being chromosomally unstable and harbouring mutations in APC, tumour protein 53 (TP53) and Kirsten ras (K-ras), a smaller subset (approximately 10 to 15% of tumours) exhibits loss of mismatch repair manifested by microsatellite instability. Studies have shown that microsatellite instability and mutations in APC, TP53 and K-ras occur almost mutually exclusively,¹⁷⁻¹⁹ suggesting that these characteristics represent separate pathways. In approximately 90% of

microsatellite instable tumours, absence of the mismatch repair enzyme human mut-L homologue 1 (hMLH1) expression was observed.²⁰

The animal fat and haem iron content of meat as well as meat preparation have been proposed as explanations for meat consumption as a risk factor for colorectal cancer. High temperature cooking of meat proteins generates heterocyclic aromatic amines (HCAs). The most abundant HCA in cooked meat, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), was shown to cause site-specific mutations in the APC gene in rats after induction of colon tumours with PhIP.²¹ N-Nitrosamines and their precursors are compounds that can be formed endogenously,²² but are also found in red and processed meat.²³ Through their alkylating action they can be carcinogenic. N-nitroso compounds could induce G→A transitions that have been observed in codons 12 and 13 of the K-ras gene in human colonic tissue.²² Similarly, meat consumption may be associated with specific mutations in the APC gene.

To our knowledge, only one study has addressed the issue of studying an association between dietary factors and truncating mutations of the APC gene in sporadic colon cancer in humans. In this case-control study, meat consumption was associated with colon carcinomas harbouring a truncating mutation in APC.¹⁶ In another report from that study, an inverse relation between red meat and microsatellite instability was found.²⁴

Here, we investigated the associations between fresh meat, meat products (i.e., processed meat) and fish consumption and the risk of colon and rectal tumours harbouring truncating mutations, missense mutations and specific types of point mutations in the mutation cluster region of the APC gene in cancer as well as colon tumours with absent hMLH1 expression in a large prospective cohort study in the Netherlands.

MATERIALS AND METHODS

STUDY POPULATION

The prospective Netherlands Cohort Study on diet and cancer (NLCS) was initiated in September 1986. The study design has been described in detail elsewhere.²⁵ Briefly, at baseline a total of 58,279 men and 62,573 women, between the ages of 55 and 69 years, completed a self-administered food frequency and lifestyle questionnaire. The study population originated from 204 municipal population registries throughout the country. For data processing and analysis, the case-cohort approach was used: the cases were enumerated for the entire cohort, while the person years at risk accumulating in the cohort were estimated from a random sample (subcohort). This subcohort of 3,500 men and women was randomly sampled from the cohort after baseline exposure measurement and was followed for migration and vital status by contacting the participants and the municipalities biennially to calculate accumulated person-time in the cohort.²⁶ 154 Cases with prevalent cancer other than non-melanoma skin cancer were excluded, resulting in a subcohort of 3,346 men and women. During the first 2.3 years of follow-up, 83 subcohort members deceased or were diagnosed with cancer other than non-melanoma skin

cancer, leaving 3,263 subcohort members for analysis. No subcohort members were lost to follow-up.

Incident cancer cases are identified by monitoring of the entire cohort for cancer occurrence through annual record linkage to the Netherlands Cancer Registry (NCR), nine regional cancer registries throughout the Netherlands, and to the Pathologisch Anatomisch Landelijk Geautomatiseerd Archief (PALGA), a nationwide network and registry of histo- and cytopathology reports.²⁶ The NCR and PALGA together provide a near 100% coverage of the municipalities included in the NLCS. The first 2.3 years of follow up were excluded because of possible pre-clinical disease affecting exposure status and because of incomplete nationwide coverage of PALGA in some of the municipalities included in the NLCS. From 1989 until 1994, 929 incident cases with histologically confirmed colorectal cancer were identified within the cohort, of whom 819 could also be linked to a PALGA report of the lesion. The PALGA reports were used to identify and locate tumour tissue from eligible colorectal cancer patients in Dutch pathology laboratories. Colon and rectal cancer were classified according to site as follows, colon: cecum through sigmoid colon (ICD-O codes 153.0, 153.1, 153.2, 153.3, 153.4, 153.5, 153.6, 153.7), rectosigmoid (ICD-O code 154.0), and rectum (ICD-O code 154.1).

TISSUE SAMPLES

Tumour material of colorectal cancer patients was collected after approval by the Ethical Review Board of University Maastricht, the NCR and PALGA. Tissue samples from 819 colorectal cancer patients were distributed among 54 pathology laboratories throughout the Netherlands. From 95% (775/819) of patients, tissue samples could be retrieved. Tissue samples from nine patients registered in one pathology laboratory could not be retrieved due to administrative inconsistencies. From 34 patients, tumour tissue blocks were not available in the archive of pathology laboratories. One patient had a gastric instead of a colorectal carcinoma. Of 776 tissue samples available, from 20 patients only normal mucosa was available, 10 tissue samples were revised as being a benign adenoma instead of a carcinoma by a pathologist (A.d.B.), from six patients, DNA yield was insufficient for molecular analyses, and from three patients the available tissue did not include malignant colorectal cancer tissue. Hence, tumour tissue from 737 colorectal cancer patients was available for APC mutation analysis and hMLH1 immunohistochemistry.

APC MUTATION ANALYSIS

The majority of somatic mutations in APC occurs within the mutation cluster region. Mutation analysis of this region (codons 1,286-1,520) was performed on archival adenocarcinoma specimens, using macrodissection to extract tumour DNA, followed by nested polymerase chain reaction (PCR) of the mutation cluster region in four overlapping DNA fragments and direct sequencing of purified fragments. This procedure has been described in detail elsewhere.⁶ In brief, in a first round of PCR, two overlapping fragments were generated, that served as templates for a second round of PCR to amplify four overlapping biotin-labelled PCR fragments that were subsequently used for direct sequencing. The sequence profile was analysed on ALFexpress DNA Analysis System using ALFwin software (Amersham Biosciences, Roosendaal, The

Netherlands). Evaluation of the sequence patterns and data entry was performed independently by two observers. The detection limit was 5% of mutated DNA. Duplicate experiments revealed good reproducibility (85%).

From 72 of the 737 patients, one or more fragments of the mutation cluster region could not be amplified and these patients were not included in this study. Since the rectosigmoid can be considered as a clinically applied term rather than an anatomically defined transitional zone between the colon and rectum, patients with a rectosigmoid tumour were excluded from data analysis. Moreover, the group of patients with a rectosigmoid tumour was too small for adequate stratified analysis. After exclusion of the 74 patients with a rectosigmoid tumour, 440 colon and 151 rectal cancer patients remained available.

hMLH1

Formalin-fixed, paraffin-embedded tissues were sectioned at 4 μ m and included tumour tissue with normal adjacent mucosa. Endogeneous peroxidase activity was blocked with 3% H₂O₂. Slides were submitted to microwave antigen retrieval in 1mM EDTA buffer (pH 8.0) and incubated with 10% normal horse serum for ten min at room temperature. Then, sections were incubated overnight at 4°C with mouse monoclonal antibodies against hMLH1 protein (clone G168-15, PharMingen, San Diego, CA) at a 1:100 dilution. Antibody binding was detected by incubating the sections at room temperature with the peroxidase-labelled DAKO Envision System (DAKO, Carpinteris, CA) using diaminobenzidine as a chromogen. Sections were counterstained with diluted haematoxyline.

Lesions were considered to lack hMLH1 protein expression when unequivocal absence of nuclear staining of the tumour epithelial cells was observed. Nuclear staining of normal epithelial and stromal cells or lymphocytes served as internal positive control. Staining profiles were scored independently by at least two observers and in case of discordant results discussed with a pathologist until consensus was reached. hMLH1 expression could be determined in 724 of 737 patients, for whom tumour DNA was available. With the exclusion of 84 patients with a rectosigmoid tumour, 468 colon and 172 rectal cancer cases were available for the analysis.

EXPOSURE ASSESSMENT

The dietary section of the questionnaire, a 150-item semi-quantitative food frequency questionnaire, concentrated on habitual intake of food and beverages during the year preceeding the start of the study. Questionnaire data were key-entered twice and processed for all incident cases in the cohort and for all subcohort members in a manner blinded with respect to case/subcohort status in order to minimise observer bias in coding and interpretation of the data.

Subjects whose dietary data were incomplete or inconsistent, were excluded from the analysis, leaving 484 colon cancer patients of whom 13 were also subcohort members and 160 rectal cancer patients of whom one was also a subcohort member, and 3,048 subcohort members. Criteria used for this selection were (a) 60 or more questionnaire items left blank and 35 items that

were eaten less than once per month and/or (b) one or more item blocks (groups of items, e.g. beverages) left blank.

The questionnaire contained 14 items on the consumption of meat with the hot meal (mainly fresh meat, including chicken), five items on the consumption of meat products, which are used as sandwich filling, and three items on fish consumption.²⁷ As for serving sizes, a question was included on the quantity of fresh meat usually purchased (per person, per meal). For meat products, the number of sandwiches filled with a product was asked and a standard serving size per sandwich filling of 15 g was used. For chicken and fish standard serving sizes were also used. Fresh meat is defined as meat that has not undergone any form of preservation, such as smoking, fermentation, and/or treatment with nitrate and/or nitrite salt ("curing"), which is consumed during the main (hot) meal. Meat products are defined as meat items that have undergone preservation as described above. The questionnaire was validated against a nine-day diet record.²⁸ The Spearman correlation coefficients for meat, meat products and fish were 0.46, 0.54 and 0.53, respectively.

Quartiles of intake of fresh meat, beef, pork, minced meat and meat products were computed separately for men and women based on the distribution of intake in subcohort members. For liver and other meat eaten with main meal, categories of intake were a non-user and a user group (>0 g/day). For poultry, categories of intake were classified into a non-user and three user categories (0-13.2, 13.2-22.8 and ≥ 22.8 g/day). For fish, categories of intake were a non-user group and three user groups (0-10, 10-20 and ≥ 20 g/day).

STATISTICAL ANALYSIS

The data analysis was conducted separately for overall colon and rectal cancer, colon and rectal cancer with or without a truncating APC mutation, described here as APC+ and APC- tumours, and colon cancer lacking hMLH1 expression. In addition, in colon and rectal cancer without a truncating APC mutation, separate analyses were conducted for tumours harbouring a missense APC mutation and tumours that did not have such a mutation. Finally, tumours with a specific C:G $\rightarrow$ T:A or G:C $\rightarrow$ T:A point mutation, irrespective of their putative effect were analysed. Truncating APC mutations lead to introduction of a stop codon and would result in a truncated and therefore inactive APC protein. Missense mutations lead to alteration of a single amino acid at the protein level. In the data analysis, associations with missense mutations in APC were studied in patients with at least one missense mutation, but without a truncating mutation.

Mean values of the intake of fresh meat, beef, pork, minced meat, liver, other meat, poultry, meat products, fish (all g/day), and possible confounding variables including age at baseline (years), intake of vegetable (g/day), fruit (g/day), alcohol (g/day), fat (g/day), dietary fibre (g/day), calcium (mg/day), energy (kJ/day), and body mass index BMI (kg/m²), as well as distributions of the variables sex, family history of colorectal cancer (yes/no), smoking status (never/ex/current smoker) and physical activity in leisure time (<30 , 30-60, 60-90, >90 min/day) were evaluated for subcohort members, colon and rectal cancer patients with or without a truncating APC mutation and colon cancer patients lacking hMLH1 expression. Differences in distribution of the continuous variables

Figure 1. Flow diagram of subcohort members and colorectal cancer patients on whom the analyses were based.

^a Netherlands Cancer Registry

^b Pathologisch Anatomisch Landelijk Geautomatiseerd Archief

^c Adenomatous polyposis coli

^d Mutation cluster region

^e human mut-L homologue 1

^f Patients with rectosigmoid tumours were not included in the analysis

between patients with or without truncating nonsense or frameshift mutations in the mutation cluster region of the APC gene were tested with the Student's *t* test or the Mann-Whitney *U* test if the variables were not normally distributed. The distributions of the categorical variables between patients with and without truncating APC mutations were compared with the χ^2 test.

Incidence rate ratios (RR) and corresponding 95% confidence interval (CI) for colon and rectal cancer patients according to quartiles or categories of intake of fresh meat, meat products and fish intake and one standard deviation increment of intake were estimated using Cox proportional hazards regression models. In addition, associations were estimated for specific molecular endpoints of the tumours.

Standard errors were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling from the cohort.²⁹ The proportional hazards assumption was tested using the scaled Schoenfeld residuals.³⁰ Tests for dose response trends over the different quartiles and categories of meat and fish intake were estimated by fitting the ordinal exposure variables as continuous variables using the Wald test.

Age at baseline, sex, intake of vegetables, fruit, alcohol, fat, dietary fibre, calcium, body mass index, smoking status, family history of colorectal cancer, energy intake, and physical activity in leisure time were considered as potential confounders. Those variables that were found to statistically significantly ($p < 0.05$) contribute to the multivariate model for colon and/or rectal cancer (age at baseline, sex, body mass index, family history of colorectal cancer, and smoking status), as well as energy intake, were included as covariates in the multivariate analyses. None of the above-mentioned variables influenced the risk estimates by more than 10%.

RESULTS

The flow diagram of patients and subcohort members available for the analysis, is depicted in figure 1. 434 Colon and 154 rectal cancer patients from whom tumour material was available were included in the analysis of meat and fish consumption as a risk factor for colon and rectal cancer. Of the colon tumours, 220 were localised in the proximal colon, 204 in the distal colon and from 10 localisation was unspecified. With regard to Dukes stage, 77 colon tumours were graded Dukes' A, 154 Dukes' B, 112 Dukes' C, 59 Dukes' D, and 32 were unspecified, 59 rectal tumours were graded Dukes' A, 32 Dukes' B, 40 Dukes' C, and 11 Dukes' D, and 12 were unspecified.

Of the 401 colon and 130 rectal cancer patients, for whom APC mutation analysis was complete, 35% (188/535) had one or more mutations in the mutation cluster region of the APC gene that would lead to introduction of a stop codon and hence to a truncated and therefore inactive APC protein. In addition to these nonsense and frameshift mutations, a large number of silent and missense mutations were observed. When considering all mutations, regardless of their putative effect, 81% (431/535) of patients harboured at least one mutation in the APC gene.

Table 1. Meat intake and other (dietary) characteristics of the study population at baseline

	Subcohort	Colon cancer hMLH1 present	hMLH1 absent	p-Value ^a
	n=2,948	n=374	n=54	
Meat variables				
Total fresh meat (g/day, mean, SD)	99.6, 42.1	99.2, 37.8	98.3, 36.2	0.93
Beef (g/day, mean, SD)	25.5, 22.4	28.2, 22.8	26.8, 20.8	0.66
Pork (g/day, mean, SD)	38.6, 30.3	3.6.6, 29.5	37.7, 33.7	0.79
Minced meat (g/day, mean, SD)	18.3, 17.1	17.2, 14.0	18.1, 15.8	0.69
Liver (g/day, mean, SD)	2.0, 4.5	1.9, 4.1	2.0, 5.0	0.83
Other meat (g/day, mean, SD)	2.5, 6.1	3.1, 7.9	1.8, 4.9	0.18
Poultry (g/day, mean, SD)	13.7, 15.5	13.3, 15.3	12.9, 16.1	0.52
Meat products (g/day, mean, SD)	13.2, 15	14.1, 15.8	12.8, 14.3	0.55
Fish (g/day, mean, SD)	12.7, 15.1	12.3, 14.8	10.1, 12.6	0.42
Potential confounding factors				
Age (yr, mean, SD)	61.3, 4.2	63.0, 4.0	62.8, 4.5	0.91
Sex (% Male)	48	56	41	0.04
Family history of colorectal cancer (% yes)	6	13	9	0.49
Fibre (g/day, mean, SD)	27.0, 8.2	27.5, 8.1	25.2, 7.1	0.05
Alcohol (g/day, mean, SD) ^b	10.1, 14.1	11.0, 14.8	10.7, 17.0	0.49
Fruit (g/day, mean, SD)	177.0, 118.0	178.8, 122.2	160.5, 137.7	0.31
Vegetable (g/day, mean, SD)	193.2, 82.2	191.3, 82.3	185.4, 73.8	0.62
Fat (g/day, mean, SD)	83.9, 27.6	84.2, 26.3	79.7, 24.5	0.24
Energy intake (kJ/day, mean, SD)	8022, 2161	8080, 2059	7505, 1718	0.07
Body mass index (kg/m ² , mean, SD)	25.1, 3.1	25.6, 3.2	25.6, 3.5	0.98
Smoking status (%)				0.12
Never	37	37	33	
Ex smoker	35	45	37	
Current smoker	28	18	30	
Physical activity (%) ^c				0.20
< 30 min/day	21	21	22	
30-60 min/day	31	33	22	
60-90 min/day	21	21	19	
>90 min/day	27	25	37	

Multiple mutations in the APC gene were observed in tumours from 44% (236/535) of patients. These figures are similar to the results that were obtained for the all patients, including rectosigmoid cancer patients that were analysed for the occurrence of APC mutations.⁴

In 428 colon and 150 rectal cancer patients hMLH1 expression was determined. Of the colon tumours 54 were hMLH1 deficient, 49 of which were located in the proximal colon. Two rectal tumours lacked hMLH1 expression. Therefore, associations between meat and fish consumption and hMLH1 deficiency were investigated for colon cancer only.

The baseline characteristics of the patients and subcohort members are presented in table 1. Colon and rectal cancer patients were older, more often men, more often had a family history of colorectal cancer and were more often ex-smokers as compared to the subcohort (data not shown). Rectal cancer

Table 1. Continued

Colon cancer APC-	APC+	p-Value ^a	Rectal cancer APC-	APC+	p-Value ^a
n=274	n=127		n=73	n=57	
99.9, 37.0	98.7, 38.8	0.77	96.7, 35.3	93.5, 39.4	0.62
29.4, 22.7	25.7, 21.3	0.13	26.7, 21.4	22.2, 25.0	0.27
36.4, 30.4	37.6, 31.1	0.73	31.7, 22.6	38.8, 33.5	0.18
18.4, 14.9	15.6, 12.8	0.07	19.3, 17.8	18.6, 15.9	0.80
1.5, 3.7	2.6, 4.5	0.01	2.3, 4.7	1.6, 3.3	0.90
2.3, 4.8	3.8, 10.5	0.82	2.9, 4.7	2.5, 4.3	0.46
12.4, 14.6	14.5, 16.9	0.19	15.2, 15.9	10.9, 10.2	0.30
12.7, 13.2	17.3, 20.0	0.01	14.0, 12.9	13.8, 12.8	0.94
12.2, 14.5	12.2, 15.1	0.83	13.2, 12.6	10.7, 11.3	0.28
63.1, 4.1	62.7, 4.0	0.34	62.2, 4.4	62.6, 3.5	0.62
52	56	0.49	64	67	0.79
11	11	0.98	10	11	0.86
26.7, 7.9	27.9, 8.1	0.16	28.1, 7.0	28.2, 9.0	0.94
11.0, 14.9	11.3, 16.5	0.89	12.2, 14.9	14.5, 18.1	0.53
169.7, 121.9	187.5, 132.2	0.19	197.4, 155.1	169.0, 122.2	0.26
187.9, 80.7	192.7, 85.2	0.59	186.3, 68.5	205.0, 118.3	0.29
82.2, 25.0	86.8, 28.6	0.11	86.3, 25.0	87.6, 23.4	0.75
7845, 1899	8335, 2306	0.03	8433, 1924	8449, 1616	0.96
25.5, 3.3	25.8, 3.1	0.31	25.3, 3.1	25.1, 2.8	0.69
		0.88			0.59
36	38		26	33	
46	43		47	39	
18	19		27	28	
		0.31			0.43
19	26		17	26	
33	29		28	32	
22	19		24	18	
26	27		32	25	

^a p-Values are based on comparing colon cancer patients with and without hMLH1 expression and colon and rectal cancer patients without a truncating APC mutation to patients with a truncating APC mutation.

^b For alcohol intake, figures are based on 2,862 subcohort members, 478 and 400 colon cancer patients for hMLH1 and APC, respectively, and 128 rectal cancer patients.

^c For physical activity, figures are based on 2,915 subcohort members, 429 and 399 colon cancer patients for hMLH1 and APC, respectively, and 129 rectal cancer patients.

patients had a higher daily intake of alcohol than subcohort members. Patients with colon cancer lacking hMLH1 expression had a lower intake of than those expressing hMLH1 (25.2 ± 7.1 versus 27.5 ± 8.1 g/day, $p=0.05$). Colon cancer patients with a tumour harbouring a truncating APC mutation (APC+), had a higher intake of liver (2.6 ± 4.5 versus 1.5 ± 3.7 g/day, $p=0.01$) and meat products (17.3 ± 20.0 versus 12.7 ± 13.2 g/day, $p=0.01$) than colon cancer patients without a truncating APC mutation (APC-). The energy intake of colon cancer patients with an APC+ tumour was also higher than patients with an APC- tumour ($8,335 \pm 2,306$ versus $7,845 \pm 1,899$ kJ, $p=0.03$). No significant differences between rectal cancer patients with or without a truncating APC mutation were observed.

Table 2. Adjusted^a incidence rate ratios (RR) and 95% confidence intervals (CI) for colon (n=434) and rectal (n=154) cancer patients according to quartiles and categories of intake of fresh meat, types of fresh meat, meat products, and fish

		Median intake (g/day)		Person years	Colon cancer			Rectal cancer		
		Men	Women		Cases (n)	RR	95% CI	Cases (n)	RR	95% CI
Total fresh meat	1	61	50.7	3533	103	1.00		40	1.00	
	2	91	80.3	3606	109	1.04	0.78-1.40	45	1.11	0.71-1.72
	3	110.7	103.4	3561	121	1.11	0.83-1.49	39	0.93	0.59-1.47
	4	150.8	139.2	3572	101	0.96	0.71-1.31	30	0.72	0.44-1.19
	p-trend					0.92			0.14	
Beef	1 SD ^b					0.96	0.87-1.06		0.89	0.76-1.05
	1	4.1	3	3561	94	1.00		37	1.00	
	2	16.1	14	3640	95	0.98	0.72-1.33	48	1.24	0.80-1.96
	3	30	25.5	3557	111	1.09	0.81-1.47	31	0.79	0.48-1.31
	4	51.4	46.9	3514	134	1.29	0.96-1.73	38	0.95	0.59-1.54
Pork	p-trend					0.06			0.42	
	1 SD ^b					1.06	0.97-1.17		0.94	0.78-1.14
	1	7.9	5	3497	118	1.00		43	1.00	
	2	28	22.5	3564	116	0.98	0.74-1.30	44	1.01	0.65-1.56
	3	44.3	40.1	3569	108	0.90	0.67-1.20	36	0.81	0.51-1.28
Minced meat	4	76	66.2	3642	92	0.77	0.57-1.04	31	0.70	0.44-1.13
	p-trend					0.07			0.09	
	1 SD ^b					0.93	0.84-1.04		0.87	0.73-1.03
	1	3.2	0	3525	100	1.00		33	1.00	
	2	11.5	9.6	3605	116	1.11	0.83-1.49	36	1.06	0.65-1.72
Liver	3	21	18.1	3605	125	1.26	0.94-1.69	52	1.60	1.01-2.52
	4	37.8	32.9	3536	93	0.93	0.68-1.27	33	1.01	0.61-1.66
	p-trend					0.88			0.50	
	1 SD ^b					0.95	0.88-1.04		1.06	0.93-1.21
	1	0	0	9247	282	1.00		96	1.00	
Other meat	2	4.1	3.7	5025	152	1.04	0.84-1.29	58	1.11	0.79-1.57
	1	0	0	10098	302	1.00		100	1.00	
	2	6.6	7	4173	132	1.16	0.92-1.45	54	1.34	0.94-1.90
	3	13.2	13.2	3534	97	0.81	0.60-1.08	33	0.94	0.57-1.54
	4	22.8	22.8	3978	122	0.87	0.66-1.15	45	1.12	0.70-1.79
Poultry	p-trend					0.33			0.96	
	1 SD ^b					0.95	0.87-1.05		0.98	0.83-1.14
	1	1	0	3444	105	1.00		42	1.00	
	2	7.4	4.3	3541	92	0.89	0.65-1.21	29	0.68	0.41-1.11
	3	15.8	10.5	3682	117	1.05	0.78-1.42	38	0.84	0.53-1.34
Meat products	4	33.3	22.4	3606	120	1.17	0.86-1.59	45	1.04	0.64-1.68
	p-trend					0.20			0.71	
	1 SD ^b					1.05	0.94-1.17		0.97	0.83-1.14
	1	0	0	4089	121	1.00		44	1.00	
	2	4.6	4.6	3140	117	1.30	0.97-1.72	31	0.89	0.55-1.43
Fish	3	14.8	15.5	4318	109	0.82	0.62-1.09	48	0.97	0.63-1.48
	4	30.5	28.2	2725	87	1.03	0.76-1.40	31	0.94	0.59-1.52
	p-trend					0.40			0.89	
	1 SD ^b					0.93	0.84-1.04		0.93	0.81-1.07

^a Adjusted for age, sex, family history of colorectal cancer, smoking status, body mass index, and energy intake

^b For 1 standard deviation increment of the meat and fish variables in the subcohort, see table 1

Associations between the consumption of several types of meat, meat products and fish with the risk of colon or rectal cancer are presented in table 2. RR and 95% CI for colon and rectal cancer were adjusted for age and sex only as well as for age, sex, smoking, BMI, energy intake and family history of colorectal cancer. The age and sex adjusted RR and the multivariate RR were similar (data not shown). A high consumption of beef seemed associated with an increased risk of colon cancer (RR for highest versus lowest quartile of intake 1.29, 95% CI 0.96-1.73, *p*-trend 0.06). However, pork consumption seemed associated with a decreased risk of both colon cancer (RR for highest versus lowest quartile of intake 0.77, 95% CI 0.57-1.04, *p*-trend 0.07) and rectal cancer (RR for highest versus lowest quartile of intake 0.70, 95% CI 0.44-1.13, *p*-trend 0.09). Total fresh meat consumption was not associated with the overall risk of colon or rectal cancer.

Associations of several types of meat, meat products and fish with tumours without truncating APC mutations (APC-) and tumours with a truncating APC mutation (APC+) of colon and rectum are shown in table 3. Total fresh meat consumption was not associated with risk of APC+ or APC- colon tumours. A high consumption of beef was associated with an increased risk of colon tumours without an APC nonsense or frameshift mutation (RR for highest versus lowest quartile of intake 1.58, 95% CI 1.10-2.25, *p*-trend 0.01). Because the APC-colon tumours consisted of tumours that harboured a missense mutation (*n*=130) and tumours that did not harbour such a mutation (*n*=144), these groups were analysed separately. The risk estimates for both groups were similar (RR for highest versus lowest quartile of intake 1.64, 95% CI 0.99-2.71, *p*-trend=0.03 and 1.52, 95% CI 0.95-2.45, *p*-trend=0.09, respectively). The risk of an APC+ colon cancer was increased by high meat product consumption (RR for highest versus lowest quartile of intake 1.61, 95% CI 0.96-2.71, *p*-trend = 0.04).

For rectal cancer, consumption of total fresh meat and beef seemed to be associated with a decreased risk of APC+ tumours (RR for highest versus lowest quartile of intake 0.48 and 0.53, 95% CI 0.20-1.12 and 0.24-1.19, respectively, *p*-trend 0.05 for both). Consumption of other meat showed a statistically significant increased risk of APC- rectal tumours (RR for intake versus no intake 1.79, 95% CI 1.10-2.90). When incidence risk ratios were calculated for tumours that harboured a missense mutation (*n*=32) and tumours that did not harbour such a mutation (*n*=41), these were similar, but did not reach statistical significance (RR for intake versus no intake 1.99, 95% CI 0.95-4.16 and 1.64, 95% CI 0.88-3.08, respectively).

When considering colon and rectal tumours that harboured a C→T or G→A mutation, irrespective of its putative effect, no clear associations were observed for any of the meat variables and 214 colon cancer patients with such a mutation (data not shown). Other meat consumption was associated with an increased risk of rectal cancer with a C→T or G→A mutation (*n*=70) (RR for intake versus no intake 1.75, 95% CI 1.06-2.89), but is similar to the risk observed for rectal cancer without a truncating APC mutation. No associations were observed for any of the meat variables with colon tumours lacking hMLH1 expression (table 4).

Table 3. Adjusted^a incidence rate ratios (RR) and 95% confidence interval (CI) for colon and rectal cancer patients without and with a truncating APC gene mutations (APC- and APC+) according to quartiles and categories intake of fresh meat, types of fresh meat, meat products, and fish

		Median intake (g/day)		Person years ^b	Colon cancer APC-		
		Men	Women		Cases (n)	RR	95% CI
Total fresh meat	1	61	50.7	3533	61	1.00	
	2	91	80.3	3606	71	1.17	0.81-1.68
	3	110.7	103.4	3561	75	1.20	0.84-1.73
	4	150.8	139.2	3572	67	1.14	0.78-1.66
	p-trend					0.48	
Beef	1 SD ^c					1.01	0.90-1.14
	1	4.1	3	3561	56	1.00	
	2	16.1	14	3640	56	0.99	0.67-1.46
	3	30	25.5	3557	66	1.10	0.76-1.61
	4	51.4	46.9	3514	96	1.58	1.10-2.25
Pork	p-trend					0.01	
	1 SD ^c					1.13	1.01-1.25
	1	7.9	5	3497	75	1.00	
	2	28	22.5	3564	78	1.05	0.75-1.47
	3	44.3	40.1	3569	60	0.81	0.56-1.16
Minced meat	4	76	66.2	3642	61	0.84	0.58-1.20
	p-trend					0.17	
	1 SD ^c					0.95	0.83-1.08
	1	3.2	0	3525	58	1.00	
	2	11.5	9.6	3605	70	1.17	0.81-1.70
Liver	3	21	18.1	3605	81	1.45	1.01-2.09
	4	37.8	32.9	3536	65	1.15	0.78-1.68
	p-trend					0.29	
	1 SD ^c					1.02	0.93-1.12
	1	0	0	9247	187	1.00	
Other meat	2	4.1	3.7	5025	87	0.92	0.70-1.21
	1	0	0	10098	188	1.00	
	2	6.6	7	4173	86	1.25	0.95-1.64
	1	0	0	3297	72	1.00	
	2	5.3	5.3	3464	71	0.97	0.68-1.37
Poultry	3	13.2	13.2	3534	59	0.79	0.55-1.14
	4	22.8	22.8	3978	72	0.83	0.58-1.18
	p-trend					0.19	
	1 SD ^c					0.90	0.78-1.04
	1	1	0	3444	71	1.00	
Meat products	2	7.4	4.3	3541	62	0.90	0.62-1.30
	3	15.8	10.5	3682	71	0.97	0.68-1.39
	4	33.3	22.4	3606	70	1.07	0.73-1.56
	p-trend					0.66	
	1 SD ^c					0.99	0.68-1.14
Fish	1	0	0	4089	73	1.00	
	2	4.6	4.6	3140	77	1.42	1.00-2.01
	3	14.8	15.5	4318	68	0.86	0.60-1.22
	4	30.5	28.2	2725	56	1.13	0.78-1.64
	p-trend					0.77	
	1 SD ^c					0.95	0.83-1.08

Table 3. Continued

Colon cancer APC+			Rectal cancer APC			APC+		
Cases (n)	RR	95% CI	Cases (n)	RR	95% CI	Cases (n)	RR	95% CI
33	1.00		18	1.00		16	1.00	
32	0.93	0.56-1.53	22	1.18	0.63-2.23	19	1.16	0.59-2.30
33	0.89	0.54-1.47	18	0.93	0.48-1.81	14	0.84	0.41-1.73
29	0.76	0.45-1.28	15	0.76	0.38-1.54	8	0.48	0.20-1.12
	0.30			0.33			0.05	
	0.91	0.76-1.08		0.85	0.70-1.05		0.80	0.61-1.04
32	1.00		15	1.00		17	1.00	
27	0.79	0.47-1.33	21	1.34	0.67-2.68	19	1.06	0.54-2.07
37	1.05	0.64-1.71	15	0.95	0.45-2.00	11	0.61	0.28-1.33
31	0.84	0.50-1.42	22	1.37	0.69-2.74	10	0.53	0.24-1.19
	0.79			0.58			0.05	
	0.95	0.79-1.13		1.00	0.80-1.24		0.79	0.53-1.16
34	1.00		20	1.00		16	1.00	
33	0.94	0.57-1.54	21	1.02	0.55-1.89	17	1.05	0.53-2.10
34	0.93	0.57-1.54	20	0.94	0.50-1.78	11	0.67	0.31-1.46
26	0.69	0.40-1.19	12	0.56	0.27-1.16	13	0.80	0.39-1.67
	0.20			0.11			0.36	
	0.93	0.77-1.14		0.73	0.57-0.93		0.99	0.74-1.31
33	1.00		16	1.00		12	1.00	
36	1.01	0.62-1.65	14	0.83	0.40-1.72	16	1.30	0.61-2.78
37	1.05	0.65-1.72	32	1.97	1.06-3.64	14	1.19	0.54-2.61
21	0.59	0.33-1.03	11	0.67	0.31-1.44	15	1.27	0.58-2.79
	0.08			0.92			0.64	
	0.83	0.70-0.98		1.02	0.85-1.22		0.99	0.81-1.22
73	1.00		46	1.00		34	1.00	
54	1.34	0.95-2.00	27	1.07	0.65-1.76	23	1.23	0.72-2.12
88	1.00		42	1.00		37	1.00	
39	1.11	0.74-1.65	31	1.79	1.10-2.90	20	1.34	0.76-2.36
34	1.00		15	1.00		12	1.00	
23	0.65	0.38-1.12	18	1.15	0.57-2.32	17	1.39	0.65-2.96
30	0.82	0.50-1.35	16	0.98	0.48-2.02	15	1.17	0.54-2.54
40	0.94	0.59-1.50	24	1.29	0.66-2.50	13	0.90	0.40-2.01
	0.95			0.55			0.64	
	1.06	0.89-1.25		1.08	0.88-1.32		0.79	0.59-1.05
26	1.00		20	1.00		15	1.00	
23	0.87	0.49-1.56	12	0.57	0.27-1.19	12	0.79	0.36-1.74
33	1.15	0.67-1.97	19	0.85	0.44-1.65	14	0.89	0.41-1.92
45	1.61	0.96-2.71	22	1.02	0.52-1.99	16	1.03	0.47-2.27
	0.04			0.73			0.88	
	1.20	1.01-1.42		0.97	0.78-1.20		0.96	0.76-1.22
37	1.00		19	1.00		19	1.00	
32	1.15	0.70-1.90	14	0.91	0.45-1.86	11	0.73	0.34-1.56
33	0.80	0.49-1.32	24	1.10	0.60-2.03	19	0.90	0.47-1.73
25	0.92	0.54-1.56	16	1.10	0.56-2.18	8	0.56	0.24-1.30
	0.44			0.67			0.27	
	0.92	0.76-1.12		0.99	0.82-1.18		0.83	0.63-1.07

^a Adjusted for age, sex, family history of colorectal cancer, smoking status, body mass index, and energy intake

^b Person years at risk are estimated from the subcohort

^c For 1 standard deviation increment of the meat and fish variables in the subcohort, see table 1

DISCUSSION

74 Although colorectal cancer has often been studied as a single endpoint in epidemiologic studies, several differences between colon and rectal cancer have been described, such as differences in aetiology of tumour formation.^{4,31} It has also been suggested that the molecular profile of tumours varies along the colorectal tract. We previously found asymmetry with respect to APC mutation status, more truncating APC mutations were observed in rectal than in colon cancer.⁶ In the present study, we evaluated the associations between meat, meat products, and fish intake and the risk of colon and rectal cancer with and without mutations in the mutation cluster region of the APC gene and the expression of the mismatch repair enzyme hMLH1.

For colon cancer, 401 patients could be included in this study, which gave rise to reasonable power to perform sub-group analyses for APC mutation status of the tumours. The limited number of 130 rectal cancer patients included in this study provided limited power, especially when estimating risk of rectal cancer with or without an APC mutation. It should be noted that patients with tumours in the rectosigmoid were excluded from the analyses, to make a clear distinction between colon and rectum tumours. Moreover, because we have run several analyses to estimate risk ratios for different subsets of colon and rectal tumours based on APC mutation status, the associations observed in this study need to be treated with some caution and would need confirmation in a larger study. The loss of patients initially recognised within the cohort, due to irretrievable tumour material, incomplete analysis of the APC gene and incompleteness of the food frequency questionnaire, may have influenced the observed results. However, risk estimates for meat and fish consumption for the group under study here, were similar to the results obtained for all 819 patients originally recognised within the cohort (data not shown).

Previous studies have not always taken into account possible differences between sub-localisations along the colorectal tract. Risk factors for colon and rectal cancer seem to differ, although in a recent study by Wei *et al.*⁴ the risks associated with servings of beef, pork or lamb or processed meats were not found to differ for colon and rectum. We found some differences between colon and rectal cancer. The risk of colon cancer seemed to be increased with high intake of beef, whereas for rectal cancer total fresh meat consumption was found to be protective.

We observed no significant association between meat or fish consumption and overall risk of colon cancer, although there seemed to be a positive association with beef consumption. The associations seemed stronger for rectal than for colon cancer. A recent meta-analysis including 34 case-control and 14 cohort studies also found no risk of colorectal cancer associated with total meat intake, but a moderately increased risk associated with red meat and particularly processed meat consumption.³ In a recent systematic review of meat consumption and colorectal cancer risk in prospective cohort studies, a positive association was found between all meat, red meat and in particular processed meat consumption and colorectal cancer risk.³² The positive association between all meat consumption and colorectal cancer risk in the

Table 4. Adjusted^a incidence rate ratios (RR) and 95% CI for colon cancer patients lacking hMLH1 expression (n=54) according to quartiles and categories intake of fresh meat, types of fresh meat, meat products, and fish

		Median intake(g/day)		Personyears	Colon cancer Absent hMLH1		
		Men	Women		Cases (n)	RR	95% CI
Total fresh meat	1	61	50.7	3533	14	1.00	
	2	91	80.3	3606	12	0.83	0.38-1.83
	3	110.7	103.4	3561	16	1.11	0.53-2.33
	4	150.8	139.2	3572	12	0.87	0.39-1.96
	p-trend					0.95	
Beef	1 SD ^b					1.01	0.78-1.31
	1	4.1	3	3561	10	1.00	
	2	16.1	14	3640	14	1.42	0.62-3.24
	3	30	25.5	3557	15	1.45	0.64-3.27
	4	51.4	46.9	3514	15	1.46	0.65-3.26
Pork	p-trend					0.37	
	1 SD ^b					1.06	0.83-1.35
	1	7.9	5	3497	17	1.00	
	2	28	22.5	3564	14	0.82	0.40-1.71
	3	44.3	40.1	3569	12	0.69	0.32-1.48
Minced meat	4	76	66.2	3642	11	0.64	0.29-1.41
	p-trend					0.67	
	1 SD ^b					1.00	0.74-1.37
	1	3.2	0	3525	10	1.00	
	2	11.5	9.6	3605	17	1.70	0.77-3.73
Liver	3	21	18.1	3605	15	1.61	0.71-3.66
	4	37.8	32.9	3536	12	1.29	0.54-3.10
	p-trend					0.63	
	1 SD ^b					1.02	0.83-1.27
	1	0	0	9247	36	1.00	
Other meat	2	4.1	3.7	5025	18	0.99	0.55-1.80
	1	0	0	10098	42	1.00	
	2	6.6	7	4173	12	0.75	0.38-1.51
	Poultry						
	1	0	0	3297	16	1.00	
Meat Products	2	5.3	5.3	3464	13	0.78	0.38-1.64
	3	13.2	13.2	3534	11	0.67	0.31-1.44
	4	22.8	22.8	3978	14	0.72	0.34-1.50
	p-trend					0.34	
	1 SD ^b					0.93	0.69-1.27
Fish	1	1	0	3444	15	1.00	
	2	7.4	4.3	3541	13	0.86	0.41-1.83
	3	15.8	10.5	3682	12	0.75	0.34-1.66
	4	33.3	22.4	3606	14	1.01	0.47-2.17
	p-trend					0.92	
Fish	1 SD ^b					1.07	0.79-1.43
	1	0	0	4089	15	1.00	
	2	4.6	4.6	3140	17	1.56	0.76-1.14
	3	14.8	15.5	4318	15	0.94	1.02-1.17
	4	30.5	28.2	2725	7	0.73	0.73-2.53
Fish	p-trend					0.37	
	1 SD ^b					0.81	0.58-1.14

^a Adjusted for age, sex, family history of colorectal cancer, smoking status, body mass index, and energy intake
^b For 1 standard deviation increment of the meat and fish variables in the subcohort, see table 1

latter study may be due to inclusion of processed meat in the analysis of the "all meat" variable and differs from the "total fresh meat" variable used in our study in that respect, and residual confounding may be present in the systemic review.

Whereas we found beef consumption to be positively associated with risk of colon cancer, pork consumption was inversely associated with colon cancer risk. Confounding of the associations of consumption of beef or pork by pork or beef, respectively, seems unlikely, as mutually adjusting the analysis for beef and pork consumption did not influence risk estimates. An explanation of the observed difference in risk may lie in the differences in preparation and haem iron content of beef and pork.

76 The differences between colon and rectal cancer are more complex as these also involve a variety of genetic aberrations. Microsatellite instability is most frequently observed in the proximal colon, whereas mutations in the APC and K-ras gene occur more frequently in the distal colon and rectum.¹⁷⁻¹⁹ When taking into account the APC gene mutation status, we found specific associations between meat variables and colon and rectal cancer. Differences in associations between colon and rectal cancer and meat consumption may be explained by APC gene mutation status rather than by differences in exposure of the colon or rectum to meat. Consumption of meat products, i.e., processed meat, significantly increased risk of APC+ colon tumours. Beef intake was associated with an increased risk of APC- colon cancer. Total meat intake and in particular beef intake appears to be associated with a decreased risk of APC+ rectal cancer. No associations for poultry or fish were observed. In a case-control study of dietary intake and risk of colon cancer with or without truncating APC mutations, moderate positive associations for colon tumours that harbour a truncating APC mutation were found with red meat and fish consumption.¹⁶ The discrepancy between the results from the case-control study and from our study may be attributable to a different exposure assessment of meat consumption in the case control study (e.g., red meat); alternatively, information bias may have played a role while the limited number of subjects in the case-control study (184 cases, 259 controls) may have resulted in chance findings.

N-nitroso compounds found in processed meats could induce G→A transitions that have been observed in codons 12 and 13 of the K-ras gene in human colonic tissue.²² In one of our previous studies, meat product consumption was found to be associated with G→A transitions in the K-ras gene, but only in rectal cancer.³³ We found a positive association for meat product consumption with APC+ colon tumours. Since a G→A transition may occur on the non-coding strand, this would give rise to detection of a C→T transition of the coding strand of the APC gene and vice versa. However, when we analysed tumours with a specific C:G→T:A mutation that leads to introduction of a stop codon, no significant association with meat products was found. This may be due to the limited group size. Moreover, we observed a large proportion of tumours to have multiple mutations, which further complicates analysis on specific types of mutations.

Consumption of other meat was associated with an increased risk of colon and rectal cancer. Other meat consisted of other meat eaten with the main course, i.e., horsemeat, lamb, mutton, frankfurters and deep-fried meat rolls. The association was most pronounced in rectal tumours, and in both colon and rectal tumours without a truncating APC mutation. Although consumption of other meat was also positively associated with rectal tumours with C:G→T:A transitions, irrespective of their putative effect, the observed associations were similar to rectal cancer without a truncating APC mutation, and may be due to chance. Although the intake of other meat is low, it seems that consumption of other meat is associated with an increased risk of colon and rectal cancer without a truncating APC mutation.

Beef consumption was associated with an increased risk of APC- colon tumours. In previous analyses on beef and K-ras mutation status within the same cohort, beef was also positively associated with K-ras- colon tumours.³³ A proportion of the APC- tumours harbour missense APC mutations. Analysis of tumours without a truncating mutation, but with a missense mutation and tumours that harboured neither of these mutations yielded similar results. When tumours with missense mutations and those without show similar incidence risk ratios, but different from tumours harbouring a truncating mutation This suggest tumours with missense mutations may not be similar to tumours with impaired APC function. Possibly, the risk of beef consumption involves a pathway that does not involve inactivation of APC or activation of K-ras.

It has been suggested that the majority of colorectal cancer arises through the genetic pathway as described by Fearon and Vogelstein,⁷ which involves inactivation of the APC protein, generally followed by activation of the K-ras protein. Tumours that harbour mutations in the APC gene are generally chromosomally unstable tumours.³⁴ A proportion of colorectal tumours may develop through different (epi-)genetic aberrations. Microsatellite instability is such an alternative pathway and tumours showing microsatellite instability occur almost exclusively in the proximal colon. In a case-control study that addressed associations between dietary factors and microsatellite instability based on 40 microsatellite instable patients, an inverse association of red meat with microsatellite instable tumours was observed.²⁴ In 54 patients with hMLH1 deficient tumours in our study, no association with meat consumption was observed. This may indicate that the differences in observed associations may be due to the limited number of patients in both studies.

To our knowledge, this study is the first to have investigated the associations between meat, meat products and fish consumption and the occurrence of different types of APC mutations as well as expression of hMLH1 in a prospective cohort. Although most associations between meat, meat products and fish consumption are weak, our data suggest that several types of fresh meat and meat products seem to contribute differently to the aetiology of colon and rectal cancer, depending on the APC mutation status of the tumour, but not its hMLH1 expression status.

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CHAPTER 5

DIETARY FAT AND RISK OF COLORECTAL CANCER WITH ABERRANT HMLH1 EXPRESSION, APC OR K-RAS GENES

MATTY P. WEIJENBERG
MARGREET LÜCHTENBORG
ANTON FPM DE GOEIJ
MIRIAN BRINK
GOOS NP VAN MUIJEN
ADRIAAN P. DE BRUÏNE
R. ALEXANDRA GOLDBOHN
PIET A. VAN DEN BRANDT

ABSTRACT

Sporadic colorectal cancers arise through pathways characterised by specific genetic aberrations.

We investigated baseline fat intake and the risk of colon and rectal tumours lacking expression of the hMLH1 repair gene and with or without mutations in the mutation cluster region of the APC tumour suppressor gene and in codons 12 and 13 of the *K-ras* oncogene in the Netherlands Cohort Study on diet and cancer ($n=120,852$). After 7.3 years of follow-up, excluding the first 2.3 years, adjusted incidence rate ratios (RR) and 95% confidence intervals (CI) were computed, using 2,948 subcohort members (55-69 years at baseline) and 401 colon and 130 rectal cancer patients.

Total, saturated and monounsaturated fat were not associated with the risk of colon or rectal cancer or the different molecular subgroups, nor were associations observed between polyunsaturated fat and risk of rectal cancer. Linoleic acid was significantly associated with increased risk of colon tumours with a *K-ras* mutation and no additional truncating APC mutation or lack of hMLH1 expression (RR=6.74, 95% CI 2.36-19.51 for the highest versus the lowest quartile of intake, p -trend<0.001). Linoleic acid intake was neither associated with risk of colon tumours without any of the gene defects, with tumours only lacking hMLH1 expression, nor with tumours only harbouring an APC mutation.

Our data suggest that linoleic acid intake is associated with colon tumours with an aberrant *K-ras* gene, but an intact APC gene and hMLH1 expression, suggesting a unique aetiology of tumours with specific genetic aberrations.

INTRODUCTION

Although dietary fat has been implicated in the aetiology of colorectal cancer,¹ results from epidemiological studies are inconsistent²⁻³ and often do not support an association, as observed recently in the Women's Health Study.⁴ Fortunately, current efficient molecular techniques to detect DNA alterations on a large scale offer possibilities of studying molecular endpoints in colorectal cancer, characterised by acquired (epi)genetic defects in tumour DNA.⁵ This approach may improve our ability to observe associations between dietary factors and cancer that may otherwise remain undetected.

A multistep model linking sporadic colorectal carcinogenesis to molecular aberrations has been proposed and is still considered valid.⁶⁻⁸ Currently, three classes of genes are thought to be involved, i.e. tumour suppressor genes, oncogenes, and DNA repair genes, that operate in multiple genetic pathways leading to colorectal cancer. In most types of cancer, including colon and rectum cancer, two distinct forms of genomic instability have been identified,^{9, 10} microsatellite instability, resulting in varying sizes of short tandem repeats in the genome, and chromosomal instability, characterized by partial or complete loss of chromosomes and genes. Ten to 15% of sporadic colon carcinomas are characterised by microsatellite instability, predominantly due to promoter methylation of the human Mut-L homologue 1 (hMLH1) DNA mismatch repair gene, which prevents expression of the enzyme.¹¹ Up to 90 percent of colon and rectum carcinomas are chromosomally instable^{10, 12} and are associated with mutations in the adenomatous polyposis coli (APC) and tumour protein 53 (TP53) tumour suppressor genes and in the Kirsten ras (K-ras) oncogene.¹³ However, simultaneous occurrence of mutations in these three genes is rare, which suggests that (even in this group of chromosomally instable tumours) different genetic pathways to colorectal cancer exist.¹⁴ Mutations in the APC gene are found to occur relatively early in colorectal tumorigenesis and are observed in up to 80% of both adenomas and carcinomas.^{8, 15} Mutations in the K-ras gene are observed in approximately 10 to 20% of small adenomas and 40 to 50% of larger adenomas and carcinomas suggestive of it being an important event in the progression of adenoma to carcinoma.¹⁵ Mutations in the TP53 gene are postulated to affect relatively late stages of colorectal carcinogenesis.¹⁵

Several epidemiological studies on the relation between dietary factors and molecular features of colon and rectum cancer have been published previously. Two of three studies on diet and hMLH1 expression in colon carcinomas do not report on dietary fat intake^{16, 17} and one study reports no association between total fat intake and colon tumours either with or without hMLH1 expression, and does not report on specific types of fat.¹⁸ Regarding truncating APC mutations, one study shows an association between total fat intake, and especially unsaturated fat intake, and increased risk of colon carcinomas with a truncating APC mutation,¹⁹ whereas in another study an association between total fat intake and increased risk of colon adenomas

* Lüchtenborg M, Weijenberg MP, Wark PA, et al. Mutations in APC, CTNNB1 and K-ras genes and expression of hMLH1 in sporadic colorectal cancer, see chapter 3

without a truncating APC mutation was observed.²⁰ The studies on fat intake and risk of tumours with or without mutations in the *K-ras* gene have shown conflicting results. No associations were observed for total fat intake and colon or rectal tumours with or without mutations in the *K-ras* gene,²¹⁻²⁴ except for a positive association of intake of total, saturated and mono-unsaturated fat with colon tumours harbouring specific G→T mutations at the second position in codon 12.²⁵ A case-control study in Spain showed an inverse association between monounsaturated fat intake and risk of colon cancer with wild type *K-ras*.²¹ We previously reported a significant association between the intake of linoleic acid and increased risk of colon carcinomas with a mutated *K-ras* gene.²⁴

84 Breivik and Gaudernack⁹ proposed that the type of genetic instability in cancer cells reflects the selection pressures exerted by specific carcinogens. Bardelli *et al.*²⁶ subsequently tested this hypothesis in immortalised, genetically stable human cells and concluded that exposure to specific carcinogens can indeed select for tumour cells with distinct forms of genetic instability. In particular, cells resistant to the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) exhibited microsatellite instability associated with mismatch repair defects, whereas cells resistant to the bulky-adduct-forming agent 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) exhibited chromosomal instability. In line with this reasoning, DNA adducts derived from fat metabolism could also be associated with colorectal tumours exhibiting chromosomal instability. This is supported by the observations that malondialdehyde (MDA), generated during lipid peroxidation and arachidonic acid metabolism, can form DNA adducts and induce G→T transversions and G→A transitions in DNA.^{27, 28} In addition, higher levels of MDA-DNA adducts have been observed in colorectal tissue of adenoma patients than in tissue of controls.²⁹ MDA levels are modulated by dietary factors, i.e. n-6 polyunsaturated fatty acids presumably increase MDA levels, whereas dietary antioxidants and n-3 polyunsaturated fatty acids seemingly decrease MDA levels.²⁹

These observations and hypotheses prompted us to investigate the associations between the intake of total fat and different types of fat and the risk of colon and rectal tumours lacking hMLH1 expression, with and without APC and *K-ras* gene mutations within the Netherlands Cohort Study on diet and cancer (NLCS).

MATERIALS AND METHODS

STUDY POPULATION

The prospective NLCS was initiated in The Netherlands in September 1986. The study design has been described in detail elsewhere.³⁰ Briefly, at baseline a total of 58,279 men and 62,573 women, between the ages of 55 and 69 years, completed a self-administered food frequency and lifestyle questionnaire. The study population originated from 204 municipal population registries throughout the country. Incident cancer cases are identified by monitoring of the entire cohort for cancer occurrence through annual record linkage to the

Netherlands Cancer Registry (NCR), consisting of nine regional cancer registries throughout the Netherlands, and to the Pathologisch Anatomisch Landelijk Geautomatiseerd Archief (PALGA), a nationwide network and registry of histo- and cytopathology.³¹ The NCR and PALGA together provide a near 100% coverage of the municipalities included in the NLCS.

Accumulation of person-time in the cohort was estimated through biennial vital status follow-up of a subcohort of 3,500 men and women who were randomly selected after baseline exposure measurement.³¹ Cases with prevalent cancer other than non-melanoma skin cancer were excluded from the subcohort, which left 3,346 men and women for analysis. No subcohort members were lost to follow-up.

The first 2.3 years of follow up were excluded because of possible pre-clinical disease affecting exposure status and because of incomplete nationwide coverage of PALGA alone (i.e., not in combination with the NCR) in some of the municipalities included in the NLCS. Within this period, 83 subcohort members deceased or were diagnosed with cancer other than non-melanoma skin cancer, leaving 3,263 subcohort members for analysis. From 1989 until 1994, 929 incident cases with histologically confirmed colorectal cancer were identified within the cohort, of whom 819 could also be linked to a PALGA report of the lesion. The PALGA reports were used to identify and locate tumour tissues from eligible colorectal cancer patients in Dutch pathology laboratories. Colon and rectal cancer were classified according to site as follows, colon: cecum through sigmoid colon (ICD-O codes 153.0, 153.1, 153.2, 153.3, 153.4, 153.5, 153.6, 153.7), rectosigmoid (ICD-O code 154.0), and rectum (ICD-O code 154.1).

TISSUE SAMPLES

Tumour material of colorectal cancer patients was collected after approval by the Ethical Review Board of University Maastricht, the NCR and PALGA. Archival, paraffin embedded tissue samples from 819 colorectal cancer patients were distributed among 54 pathology laboratories throughout The Netherlands. The tissue specimen collection started in August 1999 and was completed in December of 2001. For five percent of patients, tissue samples could not be retrieved (44/819), nine of which were due to administrative inconsistencies in one pathology laboratory, and 34 of which were due to the unavailability of the paraffin blocks in the archives of pathology laboratories. One patient turned out to have a gastric instead of a colorectal tumour. Of 775 tissue samples available, only normal mucosa was available from 20 patients, 10 tissue samples were revised as being a benign adenoma instead of a carcinoma by a pathologist, from six patients the DNA yield was insufficient for molecular analyses, and from three patients the available tissue did not include malignant colorectal cancer tissue but cancer tissue from another site. Hence, tumour tissue from 737 colorectal cancer patients was available for hMLH1 expression analysis and for APC and K-ras mutation analysis.

Since the rectosigmoid can be considered as a clinically applied term rather than an anatomically defined transitional zone between the colon and rectum, 85 patients with a rectosigmoid tumour were excluded from data analysis. Moreover, the group of patients with a rectosigmoid tumour was too small for adequate stratified analysis.

HMLH1 EXPRESSION ANALYSIS

Formalin-fixed, paraffin-embedded tissues were sectioned at 4 μ m and contained tumour tissue and normal adjacent mucosa. Endogeneous peroxidase activity was blocked with 3% hydrogen peroxide. Slides were submitted to microwave antigen retrieval in 1mM EDTA buffer (pH 8.0) and incubated with 10% normal horse serum for ten minutes at room temperature. Then, sections were incubated overnight at 4°C with mouse monoclonal antibodies against hMLH1 protein (clone G168-15, PharMingen, San Diego, CA) at a 1:100 dilution. Antibody binding was detected by incubating the sections at room temperature with the peroxidase-labelled DAKO Envision System (DAKO, Carpinteris, CA) and using diaminebenzidine as a chromogen. Sections were counterstained with diluted haematoxylin.

Lesions were considered to lack hMLH1 protein expression when unequivocal absence of nuclear staining of the tumour epithelial cells was observed. Nuclear staining of normal epithelial and stromal cells or lymphocytes served as internal positive control. Staining profiles were scored independently by at least two observers and in case of discordant results discussed with a pathologist until consensus was reached. hMLH1 expression status could be determined in 724 of 737 patients.

APC MUTATION ANALYSIS

The majority of somatic mutations in APC occur within the mutation cluster region. Mutation analysis of the mutation cluster region (codons 1,286-1,520), was performed on archival adenocarcinoma specimens, using macrodissection followed by extraction of tumour DNA. Then nested PCR was used to amplify the mutation cluster region in four overlapping DNA fragments and the purified fragments were sequenced. This procedure has been described in detail elsewhere.³² In brief, in a first round of PCR, two overlapping fragments were generated, that served as templates for a second round of PCR to amplify four overlapping biotin-labelled PCR fragments that were subsequently used for direct sequencing. The sequence profile was analysed on ALFexpress DNA Analysis System using ALFwin software (Amersham Biosciences, Roosendaal, The Netherlands). Evaluation of the sequence patterns and data entry were independently performed by two observers. The detection limit was 5% mutated DNA. Duplicate experiments revealed good reproducibility (85%).

From 72 of the 737 patients, one or more fragments of the APC gene mutation cluster region could not be amplified and these patients were not included in this study.

K-RAS MUTATION ANALYSIS

Mutation analysis of the exon1 fragment of the *K-ras* oncogene, spanning codons 8-29, was performed on archival colorectal adenocarcinoma specimens of 737 patients, using nested PCR, followed by direct sequencing of purified fragments.³³ The detection limit was 5% mutated DNA and duplicate experiments revealed good reproducibility (88%).³³

EXPOSURE ASSESSMENT

The dietary section of the questionnaire consisted of a 150-item semi-quantitative food frequency questionnaire, which concentrated on habitual

consumption of food and beverages during the year preceding the start of the study. Mean individual nutrient intakes per day were computed using the computerized Dutch food composition table of 1986³⁴ by cumulating the multiplied frequencies and portion sizes of all food items with their tabulated nutrient contents. The questionnaire was validated against a nine-day diet record.³⁵ Crude and energy-sex-adjusted (in parentheses) correlation coefficients were 0.72 (0.52) for total fat, 0.73 (0.58) for saturated fat and 0.73 (0.75) for polyunsaturated fat.³⁵ For energy intake the correlation coefficient was 0.74. On average, the questionnaire covered 91% of the energy intake assessed by the record intake. Questionnaire data were key-entered twice and processed for all incident cases in the cohort and for all subcohort members in a manner blinded with respect to case/subcohort status. This was done in order to minimise observer bias in coding and interpretation of the data.

For 7% of all subjects (either cases or subcohort members), dietary data were incomplete or inconsistent, and they were excluded from the analyses. Questionnaires were considered incomplete when either: (a) more than 60 items were left blank and less than 35 items were eaten at least once a month; or (b) one or more item blocks (groups of items, e.g., beverages) were left blank. Additional details are given elsewhere.³⁵ This resulted in the availability of 3,048 subcohort members, 441 colon cancer cases for whom hMLH1 expression status was known, and 414 colon and 136 rectal cancer cases for whom APC and *K-ras* mutation status was known. No data-analyses were conducted for lack of hMLH1 expression in rectal cancer cases since there were only two such cases in the cohort.

Intake of specific fatty acids was based on a food composition database with specific fatty acids derived from the TRANSFAIR study.³⁶ For this database, the hundred foods that contributed most to fat intake in the Dutch dietary pattern were sampled and analysed as methyl esters of the fatty acids present in the foods. In the database, total fat includes triglycerides and other lipids such as phospholipids and sterols. The percentage of triglycerides in total fat is assumed to be 93 % on average, but varies across food sources. Daily intakes of total fat (g/day), saturated fat (g/day), monounsaturated fat (g/day), polyunsaturated fat (g/day), and linoleic acid (C18:2, C9, 12) (g/day) and linolenic acid (C18:3, C9, 12, 15) (g/day) as the main constituents of polyunsaturated fat, were used as exposure variables. Linoleic and linolenic acid were used as the most abundant sources of n-6 polyunsaturated fatty acids and n-3 polyunsaturated fatty acids in the diet. In all analyses, the values for fat intake variables are adjusted for energy intake by the residual method.³⁷ For data analyses, quartiles of the intake of fat and different types of fats were computed based on the distribution among subcohort members. Daily intake of dietary fibre (g/day), alcohol (g/day), fruit (g/day), vegetables (g/day) and total energy (kJ/day) and age at baseline (years), sex (men/women), body mass index (kg/m²), non-occupational physical activity (<30 min/day, 30-60 min/day, 60-90 min/day, >90 min/day), family history of colorectal cancer (yes/no) and smoking status (never/ex/current) were regarded as potential confounders.

STATISTICAL ANALYSIS

Data analyses were based on study participants for whom data on fat intake and confounding variables were complete, i.e., 2,948 subcohort members, 428 colon cancer patients for whom hMLH1 expression status was known, and 401 colon cancer and 130 rectal cancer patients for whom APC and K-ras mutation status was known (figure 1).

Data analyses were conducted separately for overall colon and rectal cancer, colon cancer lacking hMLH1 expression, colon and rectal cancer with or without a truncating APC mutation, described here as APC+ and APC- tumours, respectively. Truncating APC mutations lead to the introduction of a stop codon and result in a truncated and therefore inactive APC protein. The analyses with truncating APC mutations were also conducted separately for the most common point mutations that result in the introduction of a stop codon, i.e. C:G→T:A or G:C→T:A point mutations. As indicated previously, associations between fat intake and K-ras mutated tumours have been described in this population previously, and a positive association between the intake of linoleic acid and K-ras mutated colon tumours was observed.²⁴

Since tumours may harbour multiple mutations, it is difficult to assess whether observed associations are specific for tumours with a particular gene defect. We therefore conducted additional analyses when (borderline) significant associations were observed. In these, subgroups of tumours were analysed separately, based on either the absence of either of the three gene defects, or on defects in a single gene, i.e. either only lack of hMLH1 expression, only a truncating APC mutation or only an activating K-ras mutations. Activating K-ras mutations are defined as mutations in codons 12 and 13 of the K-ras gene that would lead to an altered amino acid.

Mean values of the intake of fat variables (g/day), and possible confounding variables including age at baseline (years), dietary fiber (g/day), alcohol (g/day), intake of fruit (g/day), vegetable (g/day), energy (kJ/day), and BMI (kg/m²), as well as distributions of the variables sex, family history of colorectal cancer (yes/no), smoking status (never/ex/current smoker) and physical activity in leisure time (<30, 30-60, 60-90, >90 min/day) were evaluated for subcohort members, colon and rectal cancer patients with or without a truncating APC mutation and colon cancer patients lacking hMLH1 expression. Differences in mean values of the continuous variables between patients with or without truncating nonsense or frameshift mutations in the mutation cluster region of the APC gene, and between patients with and without hMLH1 expression, were tested using the Mann-Whitney *U* test since the variables were not normally distributed among cases. We used the χ^2 test to test for differences in the distributions of the categorical variables between patients with and without truncating APC mutations, and between patients with and without hMLH1 expression.

Cox proportional hazards regression models were used to estimate incidence rate ratios (RR) and corresponding 95% confidence intervals (CI) of colon and rectal cancer according to quartiles of intake of (types of) dietary fat and according to one standard deviation increment of fat intake. In addition, associations were estimated for specific molecular endpoints of the tumours.

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

^a Netherlands Cancer Registry

^b Pathologisch Anatomisch Landelijk Geautomatiseerd Archief

^c Patients with rectosigmoid tumours were not included in the analyses

^d Kirsten ras

^e Adenomatous polyposis coli

^f Mutation cluster region

^g human mut-L homologue 1

^h Patients with rectal tumours were not included in the analysis according to hMLH1 expression

Standard errors were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling from the cohort.^{38, 39} The proportional hazards assumption was tested using the scaled Schoenfeld residuals.⁴⁰ Tests for dose response trends over the different quartiles and categories of fat intake were estimated by fitting the ordinal exposure variables as continuous variables and evaluated using the Wald test.

Age at baseline, sex, intake of fiber, alcohol, fruits and vegetables, body mass index, smoking status, family history of colorectal cancer, energy intake, and physical activity in leisure time were considered as potential confounders. Those variables that were found to statistically significantly ($p < 0.05$) contribute to the multivariate model for colon and/or rectal cancer (age at baseline, sex, body mass index, family history of colorectal cancer, and smoking status), or to influence the RR by more than 10%, as well as energy intake, were included as covariates in the multivariate analyses.

Table 1 Baseline dietary intake and other characteristics of the subcohort and colon and rectum cancer patients

	Subcohort	Colon cancer (n=428)		p-Value ^b
		hMLH1 expression ^a	No hMLH1 expression ^a	
	n=2,948	n=374	n=54	
Sex (%men)	48	56	41	0.04
Age (yr, mean, SD)	61.3, 4.2	63.0, 4.0	62.8, 4.5	0.91
Fat variables (g/day, mean, SD)				
Total fat	83.8, 15.8	85.1, 14.7	84.1, 17.5	0.57
Saturated fat	33.2, 7.5	33.5, 6.6	33.3, 7.6	0.64
MUFA ^d	31.4, 7.0	31.9, 6.5	31.1, 7.5	0.45
PUFA ^e	17.3, 7.5	17.9, 7.5	17.4, 6.8	0.85
Linoleic acid	16.0, 7.5	16.7, 7.5	16.6, 7.0	0.76
Linolenic acid	1.3, 0.6	1.2, 0.5	1.3, 0.6	0.98
Other dietary factors				
Fibre (g/day, mean, SD)	27.0, 8.2	24.5, 8.1	25.2, 7.1	0.13
Alcohol (g/day, mean, SD) ^f	10.1, 14.1	11.0, 14.8	10.7, 17.0	0.49
Fruit (g/day, mean, SD)	177.0, 118.0	178.8, 122.1	160.5, 137.7	0.07
Vegetables (g/day, mean, SD)	193.8, 82.2	191.3, 82.3	185.4, 73.8	0.80
Energy (kJ/day, mean, SD)	8028, 2164	8080, 2059	7505, 1718	0.07
Other characteristics				
Body mass index (kg/m ² , mean, SD)	25.1, 3.1	25.6, 3.2	25.6, 3.5	0.57
Family history of colorectal cancer (% yes)	6	13	9	0.49
Smoker (%)				
Never	37	37	33	
Ex-smoker	35	45	37	
Current smoker	28	18	30	0.12
Physical activity (%)^f				
< 30 min/day	21	21	22	
30-60 min/day	32	33	22	
60-90 min/day	21	21	19	
>90 min/day	27	25	37	0.20

RESULTS

Lack of expression in hMLH1 was observed in 13% (54/428) of tumours from colon cancer patients (table 1). APC truncating mutations were observed in tumours of 32% of colon cancer patients (127/401) and 44% of rectal cancer patients (57/130) (table 1). C:G→T:A transitions or G:C→T:A transversions that would result in a stop codon were observed in 10% and 5% of colon cancer patients and 12% and 5% of rectal cancer patients, respectively. These figures are similar to the percentages reported for the total group of colon and rectal cancer patients for whom APC mutation status was available but for whom dietary intake data were not always complete.³²

Colon and rectal cancer patients were generally older and more frequently men than subcohort members (table 1). Patients with colon cancer lacking

Table 1. *Continued*

Colon cancer (n=401)			Rectal cancer (n=130)		
APC ^c	APC ⁺ ³	p-Value ^b	APC ^c	APC ^c	p-Value ^b
n=274	n=127		n=73	n=57	
52	56	0.49	64	67	0.79
63.1, 4.0	62.7, 4.0	0.34	62.2, 4.4	62.6, 3.5	0.63
85.2, 15.0	84.8, 14.9	0.80	85.1, 14.2	86.7, 14.8	0.52
33.3, 6.7	33.5, 7.0	0.92	33.0, 5.8	35.5, 7.7	0.05
31.8, 6.6	31.9, 6.6	0.98	31.8, 6.1	32.8, 6.2	0.37
18.2, 7.3	17.8, 7.6	0.44	18.6, 8.7	16.5, 6.9	0.27
17.0, 7.4	16.6, 7.7	0.36	17.5, 8.8	15.2, 7.2	0.15
1.2, 0.5	1.3, 0.5	0.57	1.3, 0.6	1.3, 0.6	0.79
26.7, 7.9	27.9, 8.1	0.20	28.1, 7.0	28.2, 9.0	0.78
11.0, 14.9	11.3, 16.5	0.89	12.2, 14.9	14.5, 18.1	0.53
169.7, 121.9	187.5, 132.2	0.11	197.4, 155.1	205.0, 118.3	0.21
187.9, 80.7	192.7, 85.2	0.71	186.3, 68.5	169.0, 122.2	0.69
7845, 1899	8335, 2306	0.09	8433, 1924	8449, 1616	0.92
25.5, 3.3	25.8, 3.1	0.30	25.3, 3.1	25.1, 2.8	0.92
11	11	0.98	10	11	0.86
36	38		26	33	
46	43		47	39	
18	19	0.88	27	28	0.59
19	26		17	26	
33	28		28	32	
22	18		24	18	
26	27	0.31	32	25	0.43

^a There were only two patients with rectal cancer without hMLH1 expression, these are not shown in this table.

^b p-Value for the difference between cancer patients with and without hMLH1 expression and patients with and without a truncating APC mutation.

^c APC⁻: cancer patients without a mutation in the mutation cluster region of the APC gene leading to a stop codon;

APC⁺: cancer patients with a mutation in the mutation cluster region of the APC gene leading to a stop codon.

^d Monounsaturated fat.

^e Polyunsaturated fat.

^f For alcohol intake and physical activity the mean levels in the subcohort are based on 2,862 and 2,915 subjects respectively. Four and six colon cancer cases had missing values for alcohol intake and physical activity respectively. Two and one rectal cancer case had missing values for alcohol intake and physical activity respectively.

Table 2 Adjusted^a incidence rate ratios (RR) and 95% confidence intervals (CI) for colon cancer overall, without human mut-L homologue 1 (hMLH1) expression, and without and with a truncating adenomatous polyposis coli (APC) mutation according to quartiles of intake of (types of) dietary fat

Dietary fat intake	Median intake			Colon cancer Overall		
		Men	Women	Person years ^b	Cases (n)	RR 95% CI
Total fat	1	78.0	63.0	3549	110	1.00
	2	90.2	71.4	3556	125	1.09 (0.82-1.44)
	3	98.5	77.6	3579	89	0.75 (0.55-1.01)
	4	108.8	85.3	3588	110	0.96 (0.72-1.28)
	p-trend					0.29
Saturated fat	1 SD ^c					1.00 (0.88-1.13)
	1	28.9	23.9	3567	99	1.00
	2	33.7	27.8	3550	120	1.20 (0.89-1.61)
	3	38.3	30.9	3583	115	1.11 (0.82-1.49)
	4	45.8	36.6	3571	100	0.94 (0.69-1.27)
MUFA ^d	p-trend					0.54
	1 SD ^c					0.97 (0.87-1.08)
	1	28.2	22.4	3546	98	1.00
	2	33.2	26.0	3546	122	1.20 (0.89-1.61)
	3	36.9	28.9	3580	113	1.12 (0.83-1.52)
PUFA ^e	4	42.5	33.1	3600	101	0.99 (0.73-1.34)
	p-trend					0.79
	1 SD ^c					0.99 (0.88-1.12)
	1	11.6	8.8	3507	91	1.00
	2	16.0	12.4	3562	118	1.37 (1.02-1.86)
Linoleic acid	3	20.9	16.2	3618	113	1.24 (0.91-1.68)
	4	29.3	22.5	3580	112	1.21 (0.89-1.63)
	p-trend					0.38
	1 SD ^c					1.03 (0.94-1.14)
	1	10.0	7.5	3509	86	1.00
Linolenic acid	2	14.8	11.2	3586	122	1.49 (1.10-2.02)
	3	19.5	14.9	3599	112	1.32 (0.97-1.79)
	4	28.0	21.2	3574	114	1.30 (0.96-1.77)
	p-trend					0.20
	1 SD ^c					1.06 (0.96-1.17)
Linolenic acid	1	0.8	0.6	3518	107	1.00
	2	1.2	0.9	3574	104	0.95 (0.70-1.30)
	3	1.5	1.2	3571	117	1.10 (0.82-1.48)
	4	2.0	1.6	3604	106	1.01 (0.76-1.36)
	p-trend					0.68
	1 SD ^c					0.98 (0.89-1.09)

hMLH1 expression were significantly less often men than patients with expression of the gene [41 versus 56%]. There were no striking differences in dietary fat intake between patients and subcohort members or between patients with and without hMLH1 expression or APC mutations in their tumours. Only patients with rectal cancer that had a truncating APC mutation appeared to have a higher intake of saturated fat than rectal cancer patients without a truncating APC mutation ($p=0.05$). For the other food intake variables, only fruit intake appeared to be lower among the colon cancer patients without hMLH1 expression in their tumour as compared to those whose tumours presented expression of the gene ($p=0.07$). Total energy intake appeared to be lower

Table 2. Continued

Colon cancer No hMLH1 expression			No truncating APC mutation			Truncating APC mutation		
Cases (n)	RR	95% CI	Cases (n)	RR	95% CI	Cases (n)	RR	95% CI
11	1.00		61	1.00		40	1.00	
17	1.47	(0.69-3.14)	87	1.34	(0.94-1.90)	32	0.80	(0.49-1.28)
11	0.94	(0.40-2.17)	55	0.82	(0.56-1.21)	24	0.57	(0.33-0.96)
15	1.30	(0.59-2.86)	71	1.11	(0.77-1.59)	31	0.74	(0.46-1.20)
	0.82			0.71			0.13	
	1.21	(0.82-1.77)		1.04	(0.89-1.22)		0.93	(0.76-1.14)
9	1.00		59	1.00		33	1.00	
14	1.48	(0.63-3.49)	84	1.38	(0.97-1.97)	30	0.94	(0.55-1.61)
19	1.93	(0.86-4.32)	73	1.15	(0.79-1.66)	33	1.01	(0.60-1.68)
12	1.25	(0.52-3.00)	58	0.90	(0.62-1.32)	31	0.89	(0.53-1.48)
	0.48			0.35			0.72	
	1.12	(0.82-1.51)		0.95	(0.83-1.09)		0.95	(0.79-1.14)
11	1.00		56	1.00		31	1.00	
14	1.15	(0.52-2.53)	79	1.33	(0.92-1.91)	36	1.18	(0.72-1.95)
14	1.15	(0.52-2.54)	76	1.30	(0.89-1.89)	31	1.03	(0.60-1.77)
15	1.25	(0.58-2.71)	63	1.08	(0.74-1.57)	29	0.90	(0.54-1.52)
	0.59			0.80			0.57	
	1.04	(0.73-1.46)		1.01	(0.87-1.17)		0.98	(0.81-1.18)
8	1.00		49	1.00		32	1.00	
16	2.03	(0.86-4.81)	73	1.55	(1.06-2.28)	33	1.13	(0.68-1.89)
16	2.00	(0.84-4.76)	78	1.57	(1.07-2.31)	30	0.96	(0.57-1.62)
14	1.75	(0.72-4.24)	74	1.47	(1.01-2.16)	32	0.98	(0.59-1.63)
	0.26			0.06			0.79	
	1.07	(0.82-1.38)		1.09	(0.97-1.23)		1.00	(0.84-1.19)
8	1.00		49	1.00		26	1.00	
13	1.66	(0.69-3.98)	71	1.50	(1.02-2.21)	40	1.65	(0.99-2.76)
17	2.14	(0.91-5.00)	82	1.68	(1.15-2.45)	25	1.00	(0.57-1.76)
16	2.02	(0.86-4.76)	72	1.44	(0.99-2.11)	36	1.35	(0.80-2.28)
	0.08			0.05			0.65	
	1.15	(0.90-1.48)		1.12	(0.99-1.26)		1.02	(0.86-1.21)
11	1.00		68	1.00		31	1.00	
17	1.32	(0.60-2.89)	72	0.99	(0.68-1.43)	24	0.84	(0.47-1.49)
11	0.91	(0.40-2.10)	62	0.89	(0.62-1.29)	43	1.48	(0.91-2.40)
15	1.32	(0.61-2.84)	72	1.08	(0.76-1.53)	29	0.97	(0.57-1.65)
	0.73			0.82			0.52	
	1.07	(0.79-1.44)		0.97	(0.85-1.10)		1.01	(0.86-1.19)

^a Adjusted for age, sex, body mass index, smoking, energy intake and family history of colorectal cancer

^b Person years at risk are estimated from the subcohort

^c For 1 standard deviation increment of the dietary fat variables in the subcohort, see table 1

^d Monounsaturated fat

^e Polyunsaturated fat

among colon cancer patients without hMLH1 expression in their tumours ($p=0.07$) in comparison with patients with colon cancer expressing the gene, and colon cancer patients with a truncating APC mutation appeared to have a higher intake of energy than patients without such a mutation ($p=0.09$). Body mass index, a family history of colorectal cancer, smoking and physical activity did not differ significantly between subgroups of patients or between patients and subcohort members.

Neither total fat nor the different types of fat appeared to be associated with overall colon cancer risk in this population (table 2). For different subgroups of colon cancer based on absence of hMLH1 expression or absence or presence

Table 3 Adjusted^a incidence rate ratios (RR) and 95% confidence intervals (CI) for rectal cancer overall, and without and with a truncating adenomatous polyposis coli (APC) mutation according to quartiles of intake of (types of) dietary fat

				Rectal cancer		
Dietary fat intake	Median intake (g/day)		Person years ^b	Overall	RR	95% CI
	Men	Women		Cases (n)		
Total fat						
1	78.0	63.0	3549	43	1.00	
2	90.2	71.4	3556	39	0.89	(0.56-1.39)
3	98.5	77.6	3579	33	0.74	(0.46-1.19)
4	108.8	85.3	3588	38	0.87	(0.55-1.36)
<i>p</i> -trend					0.42	
1 SD ^c					0.91	(0.76-1.09)
Saturated fat						
1	28.9	23.9	3567	43	1.00	
2	33.7	27.8	3550	34	0.80	(0.49-1.29)
3	38.3	30.9	3583	44	1.02	(0.66-1.59)
4	45.8	36.6	3571	33	0.74	(0.46-1.18)
<i>p</i> -trend					0.38	
1 SD ^c					0.95	(0.81-1.12)
MUFA ^d						
1	28.2	22.4	3546	44	1.00	
2	33.2	26.0	3546	31	0.70	(0.44-1.13)
3	36.9	28.9	3580	40	0.91	(0.58-1.44)
4	42.5	33.1	3600	39	0.88	(0.56-1.37)
<i>p</i> -trend					0.82	
1 SD ^c					0.94	(0.78-1.12)
PUFA ^e						
1	11.6	8.8	3507	45	1.00	
2	16.0	12.4	3562	32	0.74	(0.46-1.18)
3	20.9	16.2	3618	39	0.86	(0.55-1.34)
4	29.3	22.5	3580	38	0.82	(0.53-1.29)
<i>p</i> -trend					0.53	
1 SD ^c					0.98	(0.83-1.16)
Linoleic acid						
1	10.0	7.5	3509	39	1.00	
2	14.8	11.2	3586	39	1.03	(0.65-1.64)
3	19.5	14.9	3599	35	0.90	(0.56-1.44)
4	28.0	21.2	3574	41	1.03	(0.65-1.62)
<i>p</i> -trend					0.95	
1 SD ^c					0.99	(0.84-1.17)
Linolenic acid						
1	0.8	0.6	3518	42	1.00	
2	1.2	0.9	3574	38	0.92	(0.57-1.48)
3	1.5	1.2	3571	36	0.87	(0.55-1.38)
4	2.0	1.6	3604	38	0.92	(0.58-1.44)
<i>p</i> -trend					0.68	
1 SD ^c					0.95	(0.81-1.12)

of truncating APC mutations in their tumours, total fat intake and most of the specific types of fat intake variables were also not associated with increased or decreased risk. However, polyunsaturated fat intake, and especially linoleic acid intake, appeared to be associated with an increased risk of colon tumours without hMLH1 expression and with colon tumours without truncating APC mutations, but not with colon tumours that harboured a truncating APC

Table 3. Continued

Rectal cancer: No truncating APC mutation			Truncating APC mutation		
Cases (n)	RR	95% CI	Cases (n)	RR	95% CI
24	1.00		15	1.00	
17	0.70	(0.37-1.34)	12	0.79	(0.36-1.70)
16	0.65	(0.33-1.26)	14	0.91	(0.43-1.90)
15	0.60	(0.31-1.16)	16	1.06	(0.51-2.19)
	0.13			0.80	
	0.84	(0.65-1.08)		0.94	(0.69-1.28)
23	1.00		14	1.00	
18	0.81	(0.42-1.56)	12	0.87	(0.39-1.91)
21	0.92	(0.49-1.73)	12	0.86	(0.40-1.88)
11	0.46	(0.22-0.97)	19	1.30	(0.65-2.63)
	0.07			0.47	
	0.81	(0.65-1.02)		1.16	(0.89-1.50)
23	1.00		15	1.00	
18	0.79	(0.41-1.49)	9	0.62	(0.27-1.43)
15	0.67	(0.34-1.33)	20	1.36	(0.67-2.78)
17	0.72	(0.38-1.36)	13	0.88	(0.40-1.90)
	0.28			0.76	
	0.85	(0.67-1.08)		1.00	(0.76-1.33)
17	1.00		21	1.00	
19	1.17	(0.60-2.28)	10	0.49	(0.23-1.07)
16	0.94	(0.47-1.88)	16	0.76	(0.39-1.48)
21	1.20	(0.63-2.29)	10	0.47	(0.22-1.00)
	0.73			0.11	
	1.06	(0.84-1.34)		0.78	(0.60-1.03)
18	1.00		18	1.00	
17	0.98	(0.50-1.92)	14	0.80	(0.40-1.63)
16	0.90	(0.45-1.79)	15	0.84	(0.42-1.68)
22	1.19	(0.63-2.24)	10	0.54	(0.25-1.18)
	0.65			0.15	
	1.09	(0.86-1.38)		0.79	(0.60-1.04)
18	1.00		18	1.00	
20	1.16	(0.58-2.31)	10	0.55	(0.25-1.24)
15	0.87	(0.43-1.75)	13	0.72	(0.35-1.48)
20	1.13	(0.60-2.14)	16	0.90	(0.45-1.80)
	0.93			0.91	
	0.98	(0.77-1.24)		1.03	(0.81-1.31)

^a Adjusted for age, sex, body mass index, smoking, energy intake and family history of colorectal cancer

^b Person years at risk are estimated from the subcohort

^c For 1 standard deviation increment of the dietary fat variables in the subcohort, see table 1

^d Monounsaturated fat

^e Polyunsaturated fat

mutations (table 2). For colon tumours without hMLH1 expression, the RR according to the quartiles of linoleic acid intake were increased for all the categories of intake above the reference (lowest quartile of intake); 1.66 (95% CI 0.69-3.98), 2.14 (95% CI 0.91-5.00) and 2.02 (95% CI 0.86-4.76) for the second through the fourth quartiles, respectively. Although none of the RRs reached statistical significance, the test for linear trend was borderline significant

($p=0.08$). A similar trend was observed for the risk of colon tumours without truncating APC mutations. The RR over the quartiles of linoleic acid intake were 1.50 (95% CI 1.02-2.21), 1.68 (95% CI 1.15-2.45) and 1.44 (95% CI 0.99-2.11) respectively and the p -value for trend was 0.05 (table 2). Additional analyses for subgroups of colon tumours with specific truncating point mutations in APC did not show any associations with the intake of fat or different types of fat (data not shown).

For overall rectal cancer, associations with the intake of total fat or different types of fat were not observed (table 3). Moreover, after taking into account truncating APC mutations in rectal tumours, none of the fat intake variables were significantly associated with risk of rectal cancer. Only the intake of saturated fat appeared to be inversely associated with rectal tumours without APC truncating mutations. The RR for the highest versus the lowest quartile of intake was 0.46 (95% CI 0.22-0.97) and the p -value for trend was 0.07 (table 3). For rectal tumours with specific types of truncating APC mutations no associations were observed with any of the fat intake variables (data not shown).

Additional analyses were conducted to assess whether the observed associations of polyunsaturated fat intake, and especially linoleic acid intake, with the increased risk of colon tumours lacking hMLH1 expression and with colon tumours without truncating APC mutations, were observed because of an underlying association with colon tumours harbouring a *K-ras* mutation, as previously observed.²⁴ Intake of polyunsaturated fat or linoleic acid was not associated with the risk of colon cancer without truncating APC mutations

Table 4. Adjusted^a incidence rate ratios (RR) and 95% confidence intervals (CI) for colon cancer without any of the gene defects or with only a single gene defect, i.e. either lack of hMLH1 expression, a truncating APC gene mutation or an activating *K-ras* gene mutation, according to quartiles of intake of polyunsaturated fat (PUFA) and linoleic acid intake

Dietary fat intake	Person years ¹	Colon cancer No gene defects ^b			Only lack of hMLH1 expression ^c		
		Cases (n)	RR	95% CI	Cases (n)	RR	95% CI
Polyunsaturated fat							
1	3507	39	1.00		6	1.00	
2	3562	44	1.15	(0.73-1.81)	14	2.48	(0.94-6.54)
3	3618	45	1.10	(0.70-1.73)	11	1.89	(0.69-5.16)
4	3580	35	0.86	(0.54-1.38)	10	1.72	(0.61-4.81)
<i>p</i> -trend			0.51			0.48	
1 SD ^d			0.96	(0.82-1.13)		1.01	(0.72-1.40)
Linoleic acid							
1	3509	38	1.00		7	1.00	
2	3586	45	1.20	(0.77-1.90)	10	1.51	(0.58-0.93)
3	3599	45	1.15	(0.73-1.81)	14	2.08	(0.84-5.19)
4	3574	35	0.90	(0.56-1.44)	10	1.49	(0.56-3.97)
<i>p</i> -trend			0.62			0.30	
1 SD ^d			0.98	(0.84-1.15)		1.08	(0.79-1.49)

when cases who also had activating *K-ras* mutations or lack of hMLH1 expression in their tumour were excluded from the analyses (data not shown). Also, no associations were observed with risk of colon cancer without any of the gene defects, or with only lack of hMLH1 expression, or with only truncating APC mutations (table 4). With increasing intake of polyunsaturated fat and of linoleic acid, a strong increased risk of colon cancer with only activating *K-ras* mutations was observed (table 4) (p -trend <0.001 for both polyunsaturated fat and linoleic acid intake). The RRs for one standard deviation increase in intake were 1.40 (95% CI 1.17-1.68) and 1.41 (95% CI 1.18-1.69), respectively.

Likewise, additional analyses were conducted for saturated fat intake in relation to the decreased risk of rectal cancer without truncating APC mutations, also excluding individuals with a *K-ras* mutation and lack of hMLH1 expression. The association did not change substantially. Again, only the highest level of intake showed a significant reduced risk of cancer compared to the reference category (RR 0.40 95% CI 0.14-1.15, p -trend=0.09).

DISCUSSION

In this large prospective study, we observed that the intake of total, saturated and monounsaturated fat was not associated with the risk of colon cancer, rectal cancer, or the different molecular subgroups of cancer based on lack of hMLH1 expression or truncating mutations in the APC gene. For polyunsaturated fat intake and risk of rectal cancer no association was observed. However, a

Table 4. Continued

Colon cancer Only truncating APC mutations ^a			Only activating <i>K-ras</i> mutations ^a		
Cases (n)	RR	95% CI	Cases (n)	RR	95% CI
18	1.00		4	1.00	
21	1.31	(0.68-2.53)	14	3.76	(1.21-11.69)
15	0.86	(0.43-1.74)	19	5.00	(1.67-14.99)
13	0.70	(0.34-1.45)	28	6.74	(2.36-19.51)
	0.19			<0.001	
	0.90	(0.71-1.14)		1.40	(1.17-1.68)
14	1.00		4	1.00	
26	2.03	(1.03-3.99)	15	4.06	(1.32-12.46)
13	0.99	(0.46-2.13)	21	5.61	(1.88-16.68)
14	0.97	(0.46-2.06)	25	6.02	(2.09-17.41)
	0.38			<0.001	
	0.91	(0.73-1.14)		1.41	(1.18-1.69)

^a Adjusted for age, sex, body mass index, smoking, energy intake and family history of colorectal cancer

^b Colon cancer with hMLH1 expression but without truncating APC or activating *K-ras* gene mutations

^c Colon cancer lacking hMLH1 expression but without truncating APC or activating *K-ras* gene mutations

^d Colon cancer with a truncating APC gene mutation but with intact hMLH1 expression and without activating *K-ras* gene mutation

^e Colon cancer with an activating *K-ras* gene mutations but with intact hMLH1 expression and without truncating APC gene mutations

^f Person years at risk are estimated from the subcohort.

^g For 1 standard deviation of fat intake in the subcohort, see table 1

high intake of linoleic acid seemed associated with an increased risk of colon tumours lacking hMLH1 expression and an increased risk of colon tumours lacking truncating APC mutations. Since tumours may harbour multiple mutations, we investigated whether these associations reflected an underlying association of linoleic acid intake with *K-ras* mutations in the tumours. Indeed, the associations disappeared when patients with tumours also harbouring *K-ras* mutations, or truncating APC mutations or lacking hMLH1 expression respectively, were excluded from the analyses. Linoleic acid showed a strikingly strong association with increased risk of those colon tumours with only an activating *K-ras* mutation and no additional truncating APC mutation or lack of hMLH1 expression.

As reviewed in the introduction, none of the other epidemiological studies report on specific fatty acids and the risk of molecular surrogate endpoints for colon or rectal cancer.^{17-23, 25} Only two other studies report on various types of fat depending on saturation level, but the results are inconsistent across the studies^{19, 21, 25} including our study.²⁴

Diergaarde *et al.*¹⁹ found unsaturated fat intake to be associated with increased colon carcinomas with a truncating APC mutation. No distinction was made between mono- and polyunsaturated fats. In our study, we did not observe any association between various types of fat intake and risk of colon or rectal cancer with or without truncating APC mutations after patients also harbouring a *K-ras* mutation in their tumour were excluded from the analyses. We observed a possible inverse association between saturated fat intake and risk of rectal tumours without a truncating APC mutation. However, the association was weak, did not increase gradually according to the quartiles of intake and was only a result of the reduced risk in the highest category of intake. Furthermore, in absence of a biological explanation for this finding and considering the large number of associations investigated, the observation may best be attributed to chance.

Slattery *et al.*²⁵ found saturated and monounsaturated fats, but not polyunsaturated fat, to be associated with increased risk of colon tumours with specific *K-ras* mutations, i.e. a G→T transversion at codon 12. No distinction was made between n-6 and n-3 fatty acids. We observed an association between polyunsaturated fat intake, especially linoleic acid, and increased risk of colon tumours with a *K-ras* mutation, regardless of the type of mutations.²⁴

Finally, Bautista *et al.*²¹ observed an inverse association between monounsaturated fats, mostly derived from olive oil in the Spanish diet, and risk of colon cancer without *K-ras* mutations. Since olive oil was rarely consumed by this elderly Dutch population in the years preceding 1986 (the cohort baseline), this could explain the lack of association for this type of fat in our study.

Several factors hamper comparisons between our findings and those of other epidemiological studies and may in part explain observed inconsistencies in findings across studies. First, as discussed above, other studies did not report specifically on fatty acids and most of these did not include specific types of fat. Second, our study is the first large prospective cohort study incorporating molecular end-points for colon and rectal cancer. One of the other studies was a cross-sectional case-case comparison study,²² and the other studies were

case-control studies of varying size, with the Spanish and the two Dutch case-control studies on carcinomas and adenomas being fairly small (with 108, 184, 278 cases, respectively)^{17, 19-21, 23} and the US case-control study being of large size (with 1,510 or 1,428 cases for analyses with hMLH1 and K-ras respectively).^{18, 25} Third, apart from the different aberrations studied, various endpoints were considered. Two of the other studies focussed on adenomas instead of carcinomas,^{20, 22} and only two studies also incorporated rectal tumours but did not distinguish between the colon and the rectum.^{20, 21}

We have previously reported the association between linoleic acid intake and increased risk of colon tumours with K-ras mutations.²⁴ We now report that the association appears to be confined to those colon tumours with activating K-ras mutations and an otherwise intact APC gene and with hMLH1 expression. In addition, the association is strikingly strong, with RRs clearly increasing over the quartiles of linoleic acid intake. The activating K-ras mutations at codons 12 and 13 are predominantly G→T and G→A mutations³³ which could be a result of MDA-DNA adduct formation.^{27, 28} MDA-DNA adduct formation is associated with increased n-6 polyunsaturated fat intake²⁹, of which linoleic acid is the most abundant source. Therefore, even though chance cannot be ruled out and verification by others is warranted, the observation seems plausible.

Yet, there are still several issues that remain puzzling. First, why is the observed association for linoleic acid confined to colon cancer and not rectum cancer with only a K-ras mutation? The multistep model for molecular aberrations underlying colorectal carcinogenesis is likely to apply equally for both tumour subsites.^{7, 8, 15} However, lack of hMLH1 expression in our study is rare among rectum cancer patients and there is growing evidence for differences in the aetiology of colon and rectal tumours.³⁹

Second, why is the association with linoleic acid observed for K-ras and not for truncating APC gene mutations? It is unlikely that adduct formation selectively occurs in one gene but not in the other. However, K-ras is an oncogene requiring only one mutation for the gene to be activated, whereas APC is a tumour suppressor gene that requires an additional aberration in the second allele for loss of function to occur. This may be reflected in more than half of the patients with an APC mutation having multiple mutations in the mutation cluster region of the APC gene,³² which complicates the analyses.

This still does not satisfy our third query, i.e., why are the associations specifically confined to this subgroup of colon cancer patients whose tumours are characterized by activating K-ras mutations but not truncating APC mutations or lack of hMLH1 expression? It is highly speculative but plausible that the mutated K-ras gene had an important role in tumour formation or development in this specific group of patients, since in those cases, it apparently was the only important colorectal cancer gene to be mutated. Therefore, associations with mutagens could especially be expected with K-ras in this situation. On the other hand, in patients whose tumours also contained mutations in other key genes, the mutation in the K-ras gene may be a result of the mutator phenotype of the tumour rather than exposure to exogenous mutagens, and the mutation in the K-ras gene may not have been crucial to tumour formation. Therefore, Breivik and Gaudernack's hypothesis for distinct carcinogens to exert their effect on

two proposed types of genetic instability, i.e. microsatellite instability and chromosomal instability,⁹ may need to be extended to the potential effect of carcinogens on more specific genetic pathways to colorectal tumourigenesis, as for example the K-ras mutated pathway.

In conclusion, the data from this large prospective cohort study suggest that linoleic acid intake is strongly associated with colon tumours with an aberrant K-ras gene, but an intact APC gene and hMLH1 expression. Verification in other studies is warranted. Possibly, tumours revealing the involvement of distinct genetic pathways on the basis of specific genetic aberrations, may have a unique aetiology.

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CHAPTER 6

**CIGARETTE SMOKING AND
COLORECTAL CANCER:
APC MUTATIONS,
HMLH1 EXPRESSION
AND GSTM1 AND GSTT1
POLYMORPHISMS**

MARGREET LÜCHTENBORG
MATTY P WEIJENBERG
ELLEN KAMPMAN
GOOS N VAN MUIJEN
GUIDO MJM ROEMEN
MAURICE PA ZEEGERS
R ALEXANDRA GOLDBOHN
PIETER VAN 'T VEER
ANTON FPM DE GOEIJ
PIET A VAN DEN BRANDT

ABSTRACT

The contribution of cigarette smoking to sporadic colorectal cancer may differ according to molecular aspect of the tumour or according to glutathione S-transferase M1 (*GSTM1*) or glutathione S-transferase T1 (*GSTT1*) genotype.

In the prospective Netherlands Cohort Study on diet and cancer, adjusted incidence rate ratios for 1986-1993 were computed for overall colorectal cancer, tumours with and without adenomatous polyposis coli (*APC*) mutations, and tumours with and without human mut-L homologue 1 (*hMLH1*) expression, according to cigarette smoking characteristics (661 cases, 2,948 subcohort members). Case-only analyses were performed to estimate odds ratios for interaction between cigarette smoking and *GSTM1* and *GSTT1* genotypes.

In comparison with never smokers, a high smoking frequency increased the risk of colorectal cancer (for a five cigarette/day increment, incidence rate ratio (RR) 1.07, 95% confidence interval (CI) 1.03-1.12), and this association was stronger in 371 tumours without a truncating *APC* mutation (RR 1.11, 95% CI 1.05-1.17). Long-term smoking was associated with lack of *hMLH1* expression in 56 tumours (for a 10 year increment, RR 1.17, 95% CI 1.00-1.37). No statistically significant interactions between smoking and *GSTM1* or *GSTT1* genotype were observed.

These results indicate that cigarette smoking is associated with risk of colorectal cancer, and this association may depend on molecular characteristics of the tumour as defined by *APC* mutation and *hMLH1* expression status.

INTRODUCTION

Long-term smoking is a potential risk factor for developing colorectal cancer.^{1, 2} Long-term, heavy smoking has consistently been found to be associated with colorectal adenomas,³ whereas associations with colorectal carcinomas have been inconsistent.³ The observed association between cigarette smoking and both small and large adenomas led to the current assumption that tobacco smoking is involved in early tumorigenesis.

If cigarette smoking is an early event in tumorigenesis, long-term follow-up would show a positive association between smoking and colorectal cancer when the extensive lag time (over 30 years) for the whole carcinogenic process to take place, is taken into account.³ Indeed, Giovannucci *et al.*^{4, 5} found a two-fold increase in risk of colorectal cancer after 35 years of smoking. This was supported by a report from another long-term US cohort,⁶ but not by other reports.⁷⁻⁹

Most sporadic colorectal cancers are thought to arise through underlying genetic pathways, which involve aberrations in a number of genes, as described by Fearon and Vogelstein.¹⁰ Inactivation of the adenomatous polyposis coli (APC) tumour suppressor gene is thought to be a key event early in colorectal tumorigenesis. This is underscored by the finding of somatic mutations in the mutation cluster region of APC leading to a truncated and therefore inactive APC protein in the majority of sporadic colorectal adenomas and carcinomas.¹¹⁻¹³ We previously reported on the occurrence of a large number of mutations in the mutation cluster region of the APC gene, however, in just 37% of patients these would lead to a truncated APC protein.¹⁴ Since smoking is thought to be involved in the early stages of colorectal tumorigenesis, smoking may be associated with mutations in the APC gene. This is supported by results from rodent studies in which benzo(a)pyrene was found to induce G→T transversions and *N*-nitrosamines to induce G:C→A:T transitions in *ras* oncogenes,¹⁵ both of which are important constituents of cigarette smoke.

Colorectal cancer can be divided broadly into two subgroups based on genomic instability. Whereas the majority of sporadic colorectal tumours is chromosomally instable, a smaller subset (~10 to 15% of tumours) exhibits loss of mismatch repair manifested by microsatellite instability. In approximately 90% of microsatellite instable tumours, absence of human mut-L homologue 1 (hMLH1) expression was observed.¹⁶ Studies have shown that microsatellite instability and mutations in APC, TP53 and K-ras genes occur almost mutually exclusively,¹⁷⁻¹⁹ which suggests that these aberrations represent separate pathways. The risk of smoking may be associated with the specific subset of colorectal tumours displaying microsatellite instability. Cigarette smoking was found to be associated with microsatellite instable colorectal tumours in three case-control studies,²⁰⁻²² whereas another case-control study could not confirm this.²³

Persons with a homozygous deletion of the glutathione S-transferase *GSTM1* or *GSTT1* genes, have no enzyme expression and may therefore be at an increased risk of developing colorectal cancer when exposed to carcinogens from cigarette smoke. However, previous reports on the interaction between

tobacco smoking and *GSTM1* and/or *GSTT1* have not shown a relation between smoking and genotype.²⁴⁻²⁸ In the absence of data on *GSTM1* and *GSTT1* genotypes in controls, a case-only design provides an efficient means of estimating possible interactions with a greater precision than can be obtained from case-control studies.²⁹

Our aim of this analysis was to investigate associations between cigarette smoking and overall colorectal cancer in a prospective cohort study. In addition, we studied associations between smoking and (specific) mutations in the *APC* gene and absence of hMLH1 expression, which are thought to represent distinct mechanisms involved in colorectal carcinogenesis. We evaluated possible interactions between cigarette smoking and the *GSTM1* and *GSTT1* genotypes in a nested case-only design.

MATERIALS AND METHODS

STUDY POPULATION

The prospective Netherlands Cohort Study on diet and cancer (NLCS) was initiated in September 1986. The study design has been described in detail elsewhere.³⁰ At baseline, a total of 58,279 men and 62,573 women between the ages of 55 and 69 years completed a self-administered food frequency and lifestyle questionnaire. The study population was derived from 204 municipal population registries throughout the Netherlands.

Incident cancer cases are identified by monitoring of the entire cohort for cancer occurrence through annual record linkage to the Netherlands Cancer Registry (nine regional cancer registries throughout the Netherlands) and to Pathologisch Anatomisch Landelijk Geautomatiseerd Archief (PALGA, a nationwide network and registry of histo- and cytopathology).³¹ Together the NCR and PALGA provide nearly 100% coverage of the municipalities included in the NLCS.

Accumulation of person-time in the cohort was estimated through biennial vital status follow-up of a subcohort of 3,500 men and women who were randomly selected after baseline exposure measurement.³¹ Cases with prevalent cancer other than non-melanoma skin cancer were excluded from the subcohort, resulting in a subcohort of 3,346 men and women. No subcohort members were lost to follow-up.

The first 2.3 years of follow up were excluded because of incomplete nationwide coverage of PALGA in some of the municipalities included in the NLCS in that period. Within this period, 83 subcohort members deceased or were diagnosed with cancer other than non-melanoma skin cancer, leaving 3,263 subcohort members for analysis. From 1989 until 1994, 929 incident cases with histologically confirmed colorectal cancer were identified within the cohort, of which 819 could also be linked to a PALGA report of the lesion (figure 1). The PALGA reports were used to identify and locate tumour tissue from eligible colorectal cancer patients in Dutch pathology laboratories.

TISSUE SAMPLES AND DNA ISOLATION

Tumour material from colorectal cancer patients was collected after approval by the Ethical Review Board of Universiteit Maastricht, PALGA, and the NCR. Tissue samples from 819 colorectal cancer patients were localised in 54 pathology laboratories throughout The Netherlands. Forty-four (5%) tumour tissue samples could not be retrieved. Of 775 available tissue samples, 737 (95%) contained sufficient tumour material for molecular analyses (figure 1). DNA isolation was described in detail elsewhere.³² Genomic DNA was extracted from macrodissected tumour tissue using proteinase K. DNA concentration and purity were measured in a spectrophotometer at 260 nm and 280 nm.

APC MUTATION ANALYSIS

The majority of somatic mutations in APC occur in the mutation cluster region (codons 1,286-1,520). Analysis of specific mutations in the mutation cluster region was performed on archival adenocarcinoma DNA by amplification of the mutation cluster region in four overlapping DNA fragments in a nested polymerase chain reaction (PCR) followed by direct sequencing of purified fragments as previously described.¹⁴ The detection limit was 5% mutated DNA and duplicate experiments revealed good reproducibility (85%).¹⁴ From 72 of the 737 patients with a sufficient DNA yield, one or more fragments of the mutation cluster region could not be amplified and these patients were excluded from this study, leaving 665 patients (90%) available for subsequent analyses (figure 1).

HMLH1 IMMUNOHISTOCHEMISTRY

Formalin-fixed, paraffin-embedded tissues were sectioned at 4 µm and included tumour tissue with normal adjacent mucosa. Endogeneous peroxidase activity was blocked with 3% hydrogen peroxide. Slides were submitted to microwave antigen retrieval in 1mM EDTA buffer (pH 8.0) and incubated with 10% normal horse serum for ten min at room temperature. Sections were then incubated overnight at 4°C with mouse monoclonal antibodies against hMLH1 protein (clone G168-15, PharMingen, San Diego, CA) at a 1:100 dilution. Antibody binding was detected by incubating the sections at room temperature with the peroxidase-labelled DAKO Envision System (DAKO, Carpinteris, CA) and using diaminobenzidine as a chromogen. Sections were counterstained with haematoxylin.

Lesions were considered to lack hMLH1 protein expression when unequivocal absence of nuclear staining of the tumour epithelial cells was observed. Nuclear staining of normal epithelial and stromal cells or lymphocytes served as internal positive control. Staining profiles were scored independently by at least two observers and, in case of discordant results discussed with a pathologist until consensus was reached. hMLH1 expression could be determined in 724 of 737 patients, for whom tumour DNA was available.

GSTM1 AND GSTT1 POLYMORPHISMS ANALYSES

A semi-nested multiplex PCR was performed for simultaneous assessment of the presence or absence of *GSTM1* and *GSTT1* alleles. Flank PCR was performed to generate PCR products for *GSTM1* and *GSTT1* alleles and β-globin using DNA derived from formalin-fixed paraffin-embedded tumour tissue. In a 1:100 dilution, the flank PCR product served as a template for a second round of PCR.

In each PCR one round of 35 cycles was performed. In each PCR series positive (β -globin amplification) and negative (no DNA) controls were included. The absence or presence and length of PCR products of *GSTM1* and *GSTT1* alleles were checked by electrophoresis on 2% agarose gels and visualised with ethidium bromide. Reproducibility was shown to be 91% (42/46). Genotypes of *GSTM1* or *GSTT1* could not be determined in 79 patients from whom tumour DNA was available and these patients were excluded from this study.

EXPOSURE ASSESSMENT

In the questionnaire, tobacco smoking was addressed through questions on age at first and last exposure to smoking, smoking frequency, smoking duration and inhalation for cigarette, cigar and pipe smokers. The dietary section of the 150-item semi-quantitative food frequency questionnaire, concentrated on habitual intake of food and beverages as well as lifestyle factors during the year preceding the start of the study.

Questionnaire data were key-entered twice and processed for all incident cases in the cohort and for all subcohort members in a manner blinded with respect to case/subcohort status. Subjects, whose dietary data were incomplete or inconsistent (49 cases, two of whom were also subcohort members and 216 subcohort members), were excluded from the analyses. Criteria used for this selection were 60 or more questionnaire items left blank or 35 items that were eaten less than once per month and/or one or more item blocks (groups of items, e.g. beverages) left blank. Eventually, 685 colorectal cancer patients (348 men, 268 women) of whom 12 were also subcohort members, and 3,048 subcohort members (1,525 men, 1,598 women) were included in the analyses (figure 1).

STATISTICAL ANALYSIS

The data analyses were conducted separately for overall colorectal cancer, colorectal cancer with and without a truncating APC mutation, tumours with a specific C:G→T:A or G:C→T:A point mutation resulting in a stop codon, and colorectal cancer with and without hMLH1 expression.

Using the Mann-Whitney *U* test or Student's *t* test, we analysed differences in mean values of age at baseline (years), intake of alcohol (g/day), and body mass index (BMI; kg/m²) between all colorectal cancer patients and subcohort members, between patients with and without a truncating mutation in the mutation cluster region of the APC gene, and between patients with absent and present hMLH1 expression. The distributions of sex, family history of colorectal cancer (yes/no), and leisure time physical activity (<30, 30-60, 60-90, >90 min/day) between all colorectal cancer patients, patients with a truncating APC mutation, or patients with absent hMLH1 expression and subcohort members were tested using the χ^2 test.

Incident rate ratios (RR) and corresponding 95% confidence interval (CI) for overall colorectal cancer according to smoking frequency (cigarettes/day), smoking duration (years), age when starting smoking (years), time since smoking cessation (years), and corresponding continuous variables compared to life-long non-smokers were estimated using Cox proportional hazards regression models. In addition, associations were estimated for specific tumour molecular endpoints.

Figure 1. Flow diagram of subcohort members and colorectal cancer patients on whom the analyses were based.

^a Netherlands Cancer Registry

^b Pathologisch Anatomisch Landelijk Geautomatiseerd Archief

^c Adenomatous polyposis coli

^d Mutation cluster region

^e human mut-L homologue 1

^f Glutathione S-transferase M1; T1

Standard errors were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling from the cohort.³³ The proportional hazards assumption was tested using the scaled Schoenfeld residuals.³⁴ Tests for dose-response trends over the different categories of smoking variables were estimated by fitting the ordinal exposure variables as continuous terms and evaluated using the Wald test. For estimation of the *p*-value for trend over the categories of age when starting smoking and years since smoking cessation life-long and the corresponding continuous analyses non-smokers were excluded.

Age at baseline (years), sex, family history of colorectal cancer (yes/no), intake of vegetables (g/day), fruit (g/day), alcohol (g/day), coffee (cups/day), meat (g/day), fat (g/day), dietary fibre (g/day), calcium (mg/day), energy (kJ/day), and BMI (kg/m²), and physical activity in leisure time (<30, 30-60, 60-90, >90 min/day) were considered as potential confounders. Those variables that were found to statistically significantly (*p*<0.05) contribute to the multivariate model of overall colorectal cancer (age at baseline, sex, BMI, family history of colorectal cancer), by means of the Wald test, or showed a 10 percent or more influence on the RR for colorectal cancer were included as covariates in the multivariate analyses. Adjustment for BMI gave rise to missing values in 100 subcohort members, 13 colon cancer cases and six rectal cancer cases (figure 1).

In order to estimate interaction of the *GSTM1* and *GSTT1* genotypes, a case-only design was applied. Odds ratios (OR) for interaction between genotype at risk (*GSTM1* or *GSTT1* null genotype) and smoking variables were obtained using logistic regression modelling. Adjustment for family history of colorectal cancer did not alter assessed ORs.

All *p*-values reported are for a two-sided test; *p*-values of less than 0.05 were considered to be statistically significant.

RESULTS

The presented multivariate adjusted RRs for colorectal cancer according to smoking are based on 661 colorectal cancer patients and 2,948 subcohort members (figure 1). Smoking habits and general characteristics of the population under study are shown in table 1. Colorectal cancer patients were slightly older than subcohort members (62.8 vs. 61.3 year, *p*<0.001) and more often men (56% vs. 48%, *p*<0.001). When considering specific molecular features of the colorectal tumours of these patients, relatively more men were found to harbour a tumour with hMLH1 expression than without (57% vs. 43%, *p*=0.04).

Multivariate adjusted RR for overall colorectal cancer, tumours with and without a truncating APC mutation and tumours with and without hMLH1 expression according to the smoking variables are shown in table 2. Additional adjustment for family history of colorectal cancer and BMI resulted in similar risk estimates as compared to the age and sex adjusted RRs. Since smoking of cigars and pipe was rare (table 1), only data on cigarette smoking are presented.

Table 1. Distribution of subcohort members, colorectal cancer patients with and without a truncating mutation in the adenomatous polyposis coli gene (APC stop), and patients with absent and present human Mut-L homologue 1 (hMLH1) expression according to age, sex, family history of colorectal cancer, body mass index, alcohol intake, physical activity, and tobacco product ever smoked^a

	Subcohort		Colorectal cancer									
			Overall		APC stop		no APC stop		hMLH1 absent		hMLH1 present	
<i>n</i>	2,948		661		217		377		56		594	
Sublocalisation												
Colon	11		434		127		274		54		374	
Rectosigmoid	-		73		33		30		-		72	
Rectum	1		154		57		73		2		148	
Age (years, mean (SD))	61.3 (4.2)		62.8 (4.0) ^b		62.6 (4.2)		62.9 (4.1)		63.0 (4.5)		62.8 (4.0)	
Sex (% male)	48		56 ^b		59		54		43		57 ^d	
Family history of colorectal cancer (% yes)	6		11 ^b		11		10		9		11	
Body mass index (kg/m2, mean (SD))	25.1 (3.1)		25.5 (3.1) ^c		25.7 (3.1)		25.4 (3.2)		25.6 (3.4)		25.5 (3.1)	
Alcohol (g/day, mean (SD))	10.1 (14.1)		11.5 (15.0)		12.2 (16.4)		11.3 (14.8)		10.4 (16.8)		11.6 (14.8)	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Physical Activity												
< 30 min/day	608	21	136	21	56	26	63	17	13	25	122	21
30-60 min/day	920	31	201	31	63	29	119	32	10	19	185	31
60-90 min/day	598	21	142	22	42	20	85	23	9	17	129	22
>90 min/day	789	27	174	27	53	25	105	28	21	40	150	25
Tobacco product												
Never smokers	1,036	35	206	31	68	31	118	31	18	32	186	31
Cigarette	1,468	50	323	49	111	51	179	47	31	55	288	48
Cigar	32	1	4	0.6	-	-	2	0.5	-	-	4	0.7
Pipe	5	0.2	2	0.3	1	0.5	1	0.3	-	-	2	0.3
Cigarette and Pipe	63	2	12	2	4	2	6	2	-	-	12	2
Cigarette and Cigar	179	6	60	9	17	8	36	10	4	7	53	9
Cigar and Pipe	14	0.4	5	0.8	2	0.9	3	0.8	-	-	5	0.8
All three products	149	5	49	7	14	6	32	8	3	5	44	7

^a Numbers are based on subcohort members and patients without data missing on family history of colorectal cancer or body mass index

^b $p < 0.001$, ^c $p < 0.005$; for comparisons between the colorectal cancer overall group and subcohort, patients that were also subcohort members were excluded from the subcohort group. ^d $p = 0.04$; for comparison between patients without and with hMLH1 expression.

Ex-smokers were found to be at increased risk of overall colorectal cancer in comparison with never, smokers (RR 1.30, 95% CI 1.03, 1.65), whereas current smokers were not (RR 0.91, 95% CI 0.71, 1.18). A high frequency of cigarette smoking was associated with a moderately increased risk of colorectal cancer (RR 1.07, 95% CI 1.03, 1.12 per five cigarettes/day increment). The Pearson correlation coefficient between smoking frequency and smoking duration was weak ($r = 0.22$). Additional adjustment for smoking duration slightly increased the estimated RR (RR 1.08, 95% CI 1.03, 1.14 per five cigarettes/day increment). Long-term smoking was not associated with overall colorectal cancer risk (RR 1.02, 95% CI 0.96, 1.07 for 10 years increment of smoking duration). These results are based on patients from whom tumour DNA was available. Analysis of the patients initially recognised within the cohort yielded similar results to those presented here (data not shown).

Tests for interactions between sex and any of the smoking characteristics used in this study were not statistically significant ($p = 0.16$ and $p = 0.47$ for smoking frequency and duration, respectively). Therefore, results are presented for men

Table 2. Adjusted^a incidence rate ratios (RR) and corresponding 95% confidence intervals (CI) for colorectal cancer, colorectal cancer with and without a truncating adenomatous polyposis coli (APC) gene mutation and colorectal cancer with absent and present human Mut-L homologue 1 (hMLH1) expression according to cigarette smoking features in categorical and continuous analyses

Cigarette smoking features	Colorectal cancer				APC stop		
	Person years ^b	Cases (n) ^c	RR	95% CI	Cases (n) ^c	RR	95% CI
Never tobacco smoker	5,088	206	1.00 (reference)		68	1.00 (reference)	
Status							
Ex	4,992	298	1.30	1.03-1.65	90	1.05	0.73-1.49
Current	3,937	146	0.91	0.71-1.18	56	0.91	0.62-1.35
Frequency (cigarettes/day)							
<5	1,096	47	1.02	0.71-1.46	18	1.11	0.64-1.91
5-<10	1,353	50	0.91	0.59-1.30	16	0.82	0.46-1.46
10-<15	1,705	84	1.10	0.80-1.52	27	0.95	0.57-1.61
15-<20	1,278	61	1.16	0.82-1.64	18	0.91	0.52-1.60
20-<25	1,593	76	1.15	0.83-1.59	30	1.18	0.74-1.90
25+	1,385	95	1.59	1.16-2.17	26	1.10	0.66-1.82
p-trend			0.01			0.60	
Increment, 5 cigarettes/day			1.07	1.03-1.12		1.01	0.95-1.08
Duration (years)							
<10	447	17	1.02	0.59-1.78	7	1.20	0.53-2.74
10-<20	1,151	53	1.16	0.81-1.64	12	0.70	0.37-1.33
20-<30	1,827	92	1.15	0.85-1.55	29	0.98	0.61-1.58
30-<40	2,311	128	1.32	1.00-1.73	43	1.18	0.78-1.80
40-<50	2,640	109	0.90	0.67-1.20	41	0.89	0.57-1.39
50+	370	38	1.45	0.93-2.28	11	1.15	0.56-2.37
p-trend			0.49			0.88	
Increment, 10 years			1.02	0.96-1.07		1.01	0.92-1.10
Age at first exposure (years)							
<15	1,428	71	1.14	0.81-1.62	23	1.03	0.60-1.78
15-<17	1,955	116	1.41	1.04-1.92	45	1.53	0.97-2.44
17-<19	2,203	103	1.11	0.83-1.50	36	1.09	0.68-1.75
19-<21	1,294	65	1.26	0.90-1.77	17	0.94	0.54-1.66
21-<25	735	36	1.17	0.78-1.77	11	1.05	0.54-2.06
25+	1,185	44	0.87	0.61-1.25	9	0.54	0.26-1.10
p-trend			0.32			0.13	
Increment, 1 year			0.99	0.97-1.01		0.98	0.94-1.02
Years since cessation							
<1	4,051	155	0.94	0.73-1.22	58	0.97	0.65-1.43
1-<10	1,605	101	1.39	1.03-1.86	29	1.06	0.66-1.70
10-<20	1,656	104	1.38	1.03-1.86	31	1.12	0.70-1.80
20-<30	1,113	65	1.25	0.88-1.77	23	1.20	0.70-2.07
30+	480	17	0.75	0.43-1.29	5	0.61	0.24-1.55
p-trend			0.27			0.91	
Increment, 10 years			0.86	0.75-0.99		0.95	0.76-1.19
Inhalation							
No	2,588	108	0.99	0.73-1.25	38	0.93	0.60-1.42
Yes	6,130	322	1.27	1.00-1.61	103	1.06	0.74-1.52
Filter usage							
Filter-tipped	2,409	104	1.14	0.88-1.50	32	1.03	0.66-1.59
Non filter-tipped	4,300	231	1.11	0.85-1.46	76	1.02	0.68-1.53

Table 2. Continued

Colorectal cancer no APC stop			hMLH1 absent			hMLH1 present		
Cases (n) ^a	RR	95% CI	Cases (n) ^a	RR	95% CI	Cases (n) ^a	RR	95% CI
118	1.00 (reference)		18	1.00 (reference)		186	1.00 (reference)	
180	1.49	1.10-2.00	20	1.48	0.72-3.05	270	1.26	0.98-1.60
73	0.85	0.61-1.18	18	1.79	0.90-3.54	127	0.85	0.65-1.11
26	1.02	0.64-1.63	3	0.94	0.27-3.24	43	1.00	0.69-1.46
27	0.89	0.56-1.42	4	1.18	0.40-3.52	45	0.87	0.60-1.27
51	1.25	0.85-1.84	9	2.18	0.89-5.30	73	1.01	0.72-1.41
33	1.18	0.76-1.84	7	2.53	1.02-6.30	53	1.06	0.74-1.53
36	1.03	0.67-1.58	5	1.47	0.51-4.23	69	1.09	0.78-1.53
63	2.02	1.38-2.96	7	2.41	0.92-6.31	86	1.50	1.08-1.53
	0.004			0.04			0.04	
	1.11	1.05-1.17		1.09	0.98-1.21		1.07	1.02-1.22
10	1.08	0.54-2.18	2	1.71	0.39-7.48	14	0.91	0.50-1.66
32	1.29	0.83-2.00	3	1.05	0.30-3.73	49	1.14	0.79-1.65
56	1.30	0.90-1.88	5	1.07	0.40-2.83	85	1.13	0.83-1.54
77	1.47	1.05-2.07	14	2.50	1.15-5.43	110	1.20	0.90-1.60
54	0.83	0.57-1.21	10	1.51	0.64-3.57	98	0.85	0.63-1.15
21	1.47	0.84-2.56	4	2.99	0.92-9.71	34	1.37	0.86-2.19
	0.61			0.05			0.86	
	1.01	0.94-1.08		1.17	1.00-1.37		1.00	0.95-1.06
37	1.09	0.69-1.71	3	0.94	0.25-3.50	67	1.14	0.79-1.64
60	1.33	0.89-1.97	9	2.02	0.82-5.01	104	1.34	0.97-1.85
61	1.19	0.83-1.72	11	1.98	0.83-4.74	90	1.04	0.76-1.42
39	1.36	0.89-2.07	6	1.84	0.74-4.58	59	1.23	0.86-1.75
22	1.28	0.76-2.13	3	1.47	0.41-5.20	32	1.13	0.73-1.74
30	1.04	0.68-1.60	6	1.45	0.57-3.69	36	0.79	0.53-1.16
	0.95			0.88			0.23	
	1.00	0.98-1.02		1.00	0.95-1.05		0.99	0.97-1.01
79	0.89	0.64-1.23	18	1.74	0.88-3.45	136	0.88	0.68-1.15
65	1.68	1.17-2.42	8	1.82	0.73-4.56	92	1.34	0.98-1.82
63	1.57	1.08-2.27	7	1.63	0.64-4.15	93	1.31	0.96-1.78
35	1.27	0.81-1.97	4	1.37	0.42-4.46	58	1.18	0.82-1.69
9	0.73	0.35-1.51	1	0.71	0.10-1.19	16	0.75	0.42-1.32
	0.18			0.38			0.25	
	0.79	0.66-0.93		0.75	0.49-1.15		0.87	0.75-1.00
59	0.96	0.68-1.36	10	1.35	0.62-2.95	95	0.90	0.86-1.20
185	1.40	1.02-1.89	26	1.94	0.94-3.99	290	1.20	0.94-1.54
63	1.22	0.88-1.71	6	0.88	0.35-2.22	98	1.17	0.89-1.54
128	1.10	0.78-1.55	22	2.30	0.90-5.89	201	1.00	0.76-1.32

^a RRs are adjusted for age (year), sex, family history of colorectal cancer (yes/no) and body mass index (kg/m²)^b Person years are estimated from the subcohort^c Missing values of smoking characteristics gave rise to diminished person-years and number of colorectal cancer patients for the different smoking variables

and women combined. When the association between smoking frequency and colorectal cancer was examined for men and women separately, no association was observed in women, but RRs for men were similar to the observed overall RRs.

When we considered APC mutation status, introduction of a stop codon does not appear to be associated with any of the smoking variables (table 2). Moreover, specific point mutations in APC that would lead to introduction of a stop codon and that can be expected to be associated with cigarette smoking, i.e. G:C→A:T transitions (65 patients) and G:C→T:A transversions (34 patients) were also not associated with any of the smoking variables, with two exceptions: The categorical variable for age at starting smoking was inversely associated with G:C→A:T mutations (p -trend=0.03), but no statistically significant association was observed for the continuous variable (data not shown). Smoking cessation was associated with a reduced risk of colorectal tumours harbouring a G:C→T:A mutation (RR 0.53, 95% CI 0.28, 0.99 per 10 years of smoking cessation). In contrast, tumours not harbouring a truncating APC mutation appeared to be associated with former smoking but not with current smoking status (RR 1.49, 95% CI 1.10, 2.00 and RR 0.85, 95% CI 0.61, 1.18 for ex-smokers and current smokers vs. never-smokers, respectively) and smoking frequency (RR 1.11, 95% CI 1.05, 1.17 per five cigarettes/day increment).

Absent hMLH1 expression appears to occur almost exclusively in patients without a truncating APC mutation. Of 56 patients with absent hMLH1 expression, only five also harboured a truncating APC mutation. When estimating associations for smoking variables in the limited group of patients with a tumour lacking hMLH1 expression, positive associations, though not statistically significant, were found for smoking status (RR 1.48, 95% CI 0.72, 3.05 and RR 1.79, 95% CI 0.90, 3.54 for ex-smokers and current smokers vs. never-smokers, respectively), smoking frequency (RR 1.09, 95% CI 0.98, 1.21 per five cigarettes/day increment), smoking duration (RR 1.17, 95% CI 1.00, 1.37 per 10 years increment), inhalation (inhalation vs. never smokers RR 2.18, 95% CI 1.03, 4.62) (table 3). When smoking frequency and smoking duration were simultaneously included in the analysis, risk ratios were smaller, and smoking duration was found to be more influential than smoking frequency (RR 1.03, 95% CI 0.91, 1.16 per five cigarettes/day increment and 1.15, 95% CI 0.97, 1.38 per 10 years increment of smoking).

Among patients with tumours that expressed hMLH1, a similar, statistically significant positive association with smoking frequency was observed (RR 1.07, 95% CI 1.02, 1.22 per five cigarettes/day increment), but no association with smoking duration was observed (RR 1.00, 95% CI 0.95, 1.06 per 10 years increment).

Table 3 presents the ORs for interaction between the *GSTM1* and *GSTT1* null genotypes and all smoking variables for overall colorectal cancer, as determined in the case-only design. A borderline-significant ($p=0.06$) interaction was observed between the *GSTM1* null genotype and smoking frequency (OR for interaction 1.07, 95% CI 1.00, 1.14 for *GSTM1* null and an increment of five cigarettes/day) but not with *GSTT1* null genotype and any of the smoking characteristics.

Table 3. Odds ratios for interaction (ORi) and corresponding 95% confidence intervals (CI) for smoking according to glutathione S-transferase *GSTM1* and *GSTT1* genotypes^a

	<i>GSTM1</i> null vs. <i>GSTM1</i> <i>GSTM1</i> null n=266 (45%) <i>GSTM1</i> n=320 (55%)		<i>GSTT1</i> null vs. <i>GSTT1</i> <i>GSTT1</i> null n=121 (21%) <i>GSTT1</i> n=465 (79%)	
	ORi	95% CI	ORi	95% CI
Smoking status				
Ex	1.18	0.81-1.72	0.78	0.50-1.23
Current	1.13	0.72-1.79	0.69	0.39-1.22
Frequency (5 cigarettes/day)	1.07	1.00-1.14	0.95	0.87-1.04
Duration (10 years)	1.00	0.91-1.09	0.95	0.87-1.05
Age at first exposure ^b	0.98	0.86-1.12	1.03	0.90-1.17
Years since cessation (10 years) ^b	1.00	0.77-1.29	1.01	0.98-1.04
Inhalation				
Yes	1.35	0.93-1.96	0.80	0.51-1.24
No	0.71	0.42-1.17	0.62	0.33-1.17
Filter usage				
Filter-tipped	1.47	0.90-2.42	0.68	0.36-1.26
Non filter-tipped	1.15	0.77-1.73	0.96	0.60-1.54

^a Results are based on subjects without missing values for family history of colorectal cancer and body mass index^b Lifelong non-smokers were excluded from the analysis

DISCUSSION

Among colorectal cancer patients from a large prospective cohort in the Netherlands, we investigated the associations between cigarette smoking characteristics and overall colorectal cancer, tumours without and with (specific) APC mutations and tumours with and without hMLH1 expression. Moreover, we examined interactions between smoking and *GSTM1* and *GSTT1* genotypes. Of the smoking characteristics examined in this study, smoking cigarettes, and mainly the number of cigarettes smoked per day (frequency), was found to be associated with an increased risk of colorectal cancer. This association was most pronounced in tumours without a truncating APC mutation. It was also observed in tumours lacking hMLH1 expression, but the latter were mainly associated with long-term smoking. No statistically significant interactions between the *GSTM1* or *GSTT1* null genotype and smoking frequency were observed.

In the questionnaire used in this study, questions retrospectively covered a period long enough to detect possible associations, as the suggested 30 to 45 years induction time³ was met in most cases. The relative importance of dose and duration of cigarette smoking remains somewhat in question, because these parameters are correlated. In this study, the correlation between the two characteristics was found to be rather low ($r=0.22$) and correcting smoking frequency for duration did not alter risk estimates much. For the association between cigarette smoking and overall colorectal cancer, frequency appeared to be more influential than smoking duration. This is accordance with previous studies that have also found that dose, or amount usually smoked per day, is an important predictor of risk.³⁵ It seems plausible that the absolute daily quantity of carcinogens from cigarettes delivered to the colorectal epithelium is important in producing mutations at a sufficiently high rate.

In accordance with a case-control study, where no association between cigarette smoking and truncating APC mutations was observed,²³ we did not observe an association between cigarette smoking and truncating mutations in the APC gene. The associations observed for specific point mutations leading to truncation may be chance findings and would need to be confirmed in another study.

Cigarette smoking appeared to be associated with tumours without a truncating APC mutation. A proportion of tumours not harbouring truncating APC mutations lack hMLH1 expression. We observed positive associations of smoking frequency and especially smoking duration in colorectal cancer without hMLH1 expression. This is in agreement with other previous case-control studies that have found cigarette smoking to be associated with microsatellite instability in colon²⁰ and colorectal^{21, 22} tumours, but not with another report.²³ Cigarette smoking may directly affect the methylation of the hMLH1 promotor region, resulting in absent hMLH1 expression and hence deficient DNA repair²¹. On the other hand, cigarette smoke contains numerous carcinogenic compounds that may cause mutations. When DNA repair mechanisms fail (e.g., by lack of hMLH1 expression) tumours may become more susceptible to mutations that eventually could lead to a carcinoma.

Polymorphisms in the genotype of the carcinogen metabolising glutathione S-transferases may alter colorectal cancer risk. One study found a borderline-significant twofold elevated risk associated with the *GSTM1* null genotype and smoking cigarettes in comparison with never smokers and presence of the *GSTM1* allele for rectal cancer in men,²⁸ but not for colon cancer.²⁶ No interaction was found for *GSTM1* in colon adenomas²⁴ or for *GSTM1* and *GSTT1* in colorectal cancer.^{25, 27}

A valid interpretation of the interaction parameter as estimated from a case-only study requires the assumption of independence between the gene polymorphism and exposure in the population.^{36, 37} In a large group of controls from the database of the International Collaborative Study on Genetic Susceptibility to Environmental Carcinogens, it was shown there is no association between smoking and the *GSTM1* or *GSTT1* genotypes.³⁸ We did not observe statistically significant interactions between *GSTM1* or *GSTT1* and any of the smoking variables. A borderline-significant interaction between smoking frequency and *GSTM1* genotype was observed. Although the OR for interaction between the *GSTM1* genotype and smoking frequency is difficult to interpret in the absence of information on the colorectal cancer risk conferred by the *GSTM1* null genotype per se, the effect of *GSTM1* genotype on cigarette smoking seems negligible. Because of the limited number of tumours displaying hMLH1 deficiency, no interaction of the *GSTM1* and *GSTT1* genotypes in this potentially interesting group could be estimated.

Although associations studied here were hypothesis-based, studying the different subsets of tumours resulted in multiple testing, which may have given rise to chance findings. The statistically significant results observed for overall colorectal cancer in this study are in accordance with results from other studies.³ Absence of hMLH1 is a rare occurrence in sporadic colorectal cancer, therefore just 53 patients were available in our reasonably large cohort of

patients to study associations between cigarette smoking and risk of colorectal tumours without hMLH1 expression. This limited number of patients may give rise to spurious findings, but the observed associations for hMLH1 deficient tumours are also in keeping with previous reports on colon cancer.²⁰⁻²²

This study indicates that smoking cigarettes may contribute to colorectal cancer. The association between frequency of cigarette smoking and colorectal cancer is most apparent in cancers that do not harbour a truncating mutation in the APC gene. Duration of smoking was associated with hMLH1 deficient tumours, though estimated risk ratios were moderate, and their corresponding confidence intervals wide. Finally, modification of the effect of cigarette smoking by the *GSTM1* and *GSTT1* null genotypes seems negligible.

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CHAPTER 7

GENERAL DISCUSSION

Diet is thought to be an important contributing factor to colorectal cancer risk. However, existing associations may be obscured by the heterogeneity of the cancer endpoint and the genetic make-up of the individual. In the studies described in this thesis, we investigated the occurrence of mutations in genes relevant to the early stages of colorectal carcinogenesis, i.e., the adenomatous polyposis coli (*APC*), *CTNNB1* and Kirsten ras (*K-ras*) genes, as well as expression of the human Mut-L homologue 1 (*hMLH1*) protein. Next, we investigated whether we could elucidate associations of meat, dietary fat, and smoking with colorectal cancer while accounting for these molecular aberrations in colorectal tumours or for genetic susceptibility determined by polymorphisms in the glutathione S-transferase M1 and T1 (*GSTM1*, *GSTT1*) genes. These studies were performed in a large group of colorectal cancer patients from the Netherlands Cohort Study on diet and cancer (NLCS). In this chapter, the main findings are summarised, methodological issues are discussed, and implications of the results and the perspectives for future research are presented.

MAIN FINDINGS

Mutations in the *APC* gene are considered a hallmark of colorectal cancer. Although our observation of 37% of tumours harbouring a truncating *APC* mutation seems low compared to the general assumption that up to 80% of colorectal tumours arise through a pathway involving loss of *APC* function,¹ it is in keeping with mutation frequencies of 30 to 70% observed in other studies.¹⁻⁹ Intriguingly, *APC* mutations, regardless of their putative effect, were a frequent finding in patients from our study. A large proportion of mutations would result in a single altered amino acid (52%) or no putative alteration (20%) of the *APC* protein, rather than to introduction of a stop codon (28%). Patients with rectosigmoid and rectal tumours had a higher frequency of truncating *APC* mutations as compared to patients with a proximal or distal colon tumour (53 and 45% versus 31 and 32%, respectively) (**chapter 2**).

In general, colorectal cancer is characterised by genetic instability. Whereas the majority of colorectal tumours display chromosomal instability, a small proportion (approximately 10 to 15%) is marked by microsatellite instability. In the widely accepted model for colorectal carcinogenesis, a carcinoma arises through the accumulation of mutations in a number of key genes (e.g. *APC*, *K-ras*, *TP53*).¹⁰ Results from our study, where we investigated the occurrence of *APC*, *CTNNB1* and *K-ras* gene mutations as well as *hMLH1* protein expression, indicated that colorectal cancer is more heterogeneous than predicted by the model, which is in accordance with a report from another cohort study.¹¹ Although *APC* and *K-ras* mutations were found to occur simultaneously more often than expected by chance alone, most tumours do not harbour mutations in both genes. Patients with *hMLH1* deficient tumours were older and more often women. Tumours lacking *hMLH1* expression were found almost exclusively in the proximal colon and showed less differentiation. In contrast to the report that approximately half of colorectal tumours lacking truncating *APC* mutations harbour a mutation in the *CTNNB1* gene,¹² the contribution of *CTNNB1* mutations to colorectal carcinomas seems negligible in our study (**chapter 3**).

In the study of associations between meat, meat products and fish consumption and the occurrence of different types of APC mutations as well as expression of hMLH1, the observed associations were weak. However, our data suggested that several types of fresh meat and meat products contribute differently to the aetiology of colon and rectal cancer, depending on APC mutation status of the tumour, but was not associated with absence of hMLH1 expression (**chapter 4**).

In the study on dietary fat intake and hMLH1 expression, APC and K-ras mutations, intake of total, saturated and mono-unsaturated fat was not associated with the risk of colon and rectal cancer or the different molecular subgroups. However, a striking positive association was observed for linoleic acid with colon tumours harbouring an activating K-ras mutation, but without an additional APC mutation or lacking hMLH1 expression (**chapter 5**).

Although estimated risk ratios were moderate, the association between cigarette smoking and colorectal cancer suggests that smoking of cigarettes contributes to colorectal cancer. The association with smoking frequency was most apparent in carcinomas that did not harbour a truncating mutation in the APC gene. Prolonged duration of smoking was associated with tumours lacking hMLH1 expression. Finally, modification of the effect of cigarette smoking by the *GSTM1* and *GSTT1* null genotypes seemed negligible (**chapter 6**).

METHODOLOGICAL CONSIDERATIONS

Before discussing the implications of the findings from the studies described in this thesis, it is important to highlight several methodological issues. In **chapters 2** through **6** strength and limitations of the studies have been described. In the following section these issues will be addressed in more detail.

SELECTION BIAS

Follow-up

Since the Netherlands Cancer Registry (NCR) and the Pathologisch Anatomisch Landelijk Geautomatiseerd Archief (PALGA) did not yet operate at a nationwide level at the start of the study in 1986, the choice of the municipalities included in the NLCS partially depended on the quality of coverage of the cohort by these databases, thus minimising loss to follow-up.¹³ Based on hospital discharge data, from 1988 onwards the estimated coverage of Dutch municipalities by the NCR and PALGA together was nearly 100% for the municipalities included in the cohort. The pathology laboratories in the municipalities not yet covered by PALGA in 1989 did not differ from the ones in municipalities that were completely covered by PALGA with respect to being located in an academic hospital or a cancer treatment centre. Therefore, it is not expected that differential selection bias took place with respect to dietary intake or patient and tumour characteristics including the *GSTM1* and *GSTT1* polymorphisms and APC, *CTNNB1* and K-ras mutation status or mismatch repair deficiency.

Additional analyses showed that there were no statistically significant differences in dietary intake levels nor in clinicopathological characteristics of the tumour (Dukes' stage and differentiation grade) or patient characteristics (age, family history of colorectal cancer) of patients lost to follow-up due to unavailable tumour tissue compared to the colorectal cancer patients whose tumour tissue blocks were used for subsequent analyses (**chapters 4 through 6**). Therefore, selection bias due to loss to follow-up of tumour tissue is unlikely.

Molecular analyses

A second source of selection bias may be related to the success rate of the analyses of the various endpoints, i.e. APC, CTNNB1, K-ras mutation analyses, hMLH1 immunohistochemistry, and GSTM1 and GSTT1 polymorphism detection. Tumour DNA was isolated from archival formalin-fixed, paraffin-embedded tumour tissue blocks that were macro-dissected using a haematoxylin and eosin stained section as a reference. Depending on the conditions of fixation and storage, the extracted archival DNA is more or less fragmented, which means that only DNA fragments from approximately 100 to 400 bp can be amplified. Mutations in the APC, K-ras, and CTNNB1 genes were detected by using a nested PCR protocol to amplify relevant parts of these genes, followed by direct sequencing.

In order to study the large mutation cluster region of the APC gene, amplification of overlapping segments with sizes ranging from 356 bp to 511 bp was performed. However, in 10% of patients for whom tumour DNA was available, the analysis could not be completed for all four APC fragments. In order to avoid a potential overrepresentation of false negative results, we have excluded those patients for whom one or more fragments could not be amplified, regardless of the data available on those fragments that were analysed.

With regard to K-ras mutation analysis, this constituted less of a problem, as the relevant codons 12 and 13 could be amplified and analysed for mutations in one single fragment of 114 bp, and in only 5% of cases this gene fragment could not be amplified.

Analysis of the CTNNB1 gene was restricted to tumours that did not harbour a truncating APC mutation and tumours that were hMLH1 deficient. CTNNB1 mutations were observed in too few patients to include this endpoint in the molecular epidemiological analyses.

Polymorphisms in the GSTM1 and GSTT1 genes were established by nested PCR followed by gel electrophoresis to assess presence or absence of the GST alleles. Analysis of the GSTM1 and GSTT1 polymorphisms could be completed in 85% of cases. This relatively low success rate is due to the fact that we only included patients for whom the analyses for both genes were complete and tumour DNA from some patients was unavailable as this was used earlier for APC and K-ras mutation analyses.

The success rate of the molecular analyses is partially dependent on the quality of the tumour DNA available, which relies on the methods of tissue fixation and storage of the pathology laboratories of the various hospitals that provided the tumour tissue blocks. These methods differ between laboratories and vary over

time. To what extent the success rate may be attributed to the tumour itself and its aetiology remains unclear, but it seems likely that any misclassification of the endpoints is random and not related to exposure.

VALIDITY AND REPRODUCIBILITY OF MOLECULAR ANALYSES

For the studies included in this thesis, a number of laboratory procedures have been carried out. Important to our data analyses was the validity and reproducibility of these assays.

Although the tumour areas of the tissue blocks were identified and isolated by macro-dissection, we may have included DNA from adjacent normal tissue that would potentially mask the detection of *APC*, *K-ras* and *CTNNB1* mutations in the tumour DNA. The sensitivity of our assays was estimated to be approximately 5% mutated DNA in a background of wild type DNA, which means that inclusion of a relatively small amount of normal DNA would not have hampered detection of mutations relevant to the tumour. However, this high sensitivity may have led to the detection of mutations that are present only in sub-clones of the tumour and thus represent tumour heterogeneity, rather than revealing a mutation that has been relevant for cancer development.

Expression of hMLH1 was determined by immunohistochemistry, and validated by comparison with BAT-26 analysis for a limited series of specimens, which revealed 99% correspondence between hMLH1 deficiency and microsatellite instability as determined by BAT-26 analysis. BAT-26 has previously been shown to identify microsatellite instability in sporadic colorectal cancer.¹⁴

In our study of genetic predisposition due to polymorphisms in the *GSTM1* and *GSTT1* genes, we could determine a homozygous deletion or the presence of at least one of the *GSTM1* and *GSTT1* alleles. No distinction could be made between heterozygous or homozygous presence of the *GSTM1* and *GSTT1* alleles. For both *GSTM1* and *GSTT1*, the hypothesized consequence of the null genotype is reduced or absent conjugation activity. Evidence is lacking whether heterozygosity in either *GSTM1* or *GSTT1* affects enzyme function.¹⁵

Reproducibility is important for interpretation of the results and was assessed by duplicate analyses of a number of gene fragments that were amplified from the isolated tumour DNA. The reproducibility for *APC* (85%), *K-ras* (88%), and *GSTM1/T1* (91%) gene analysis was good, taking into account the standardisation and validation procedures used for these molecular analyses. Since duplicate experiments performed on DNA obtained from colon cancer cell lines consistently yielded identical results, the lower level of reproducibility observed in the tissue samples may reflect heterogeneity of the tumour tissue.

Finally, all mutation and expression profiles were assessed by at least two observers to minimise errors arising from inter-observer variation. Considering the high sensitivity and good reproducibility of the assays, the quality of the molecular data used in the studies described in this thesis can be deemed satisfactory.

CHOICE OF STUDIED ENDPOINTS

When studying the associations between environmental exposures and mutations in genes involved in colorectal cancer, it is important to differentiate between mutations that have known oncogenic consequences versus those which tumourigenic effects are uncertain.

With regard to the *K-ras* mutations, we have focussed on the activating mutations in codons 12 and 13 and did not consider mutations of which effect on the activity of the *K-ras* protein is unknown. The latter were observed in 18 patients. Mutations in codon 61, located in exon 2, are also reported to affect *K-ras* activity, but these mutations were not determined since these represent approximately 5% of the activating alterations only.¹⁶ Therefore, and for efficiency reasons, mutation analysis of the *K-ras* oncogene was restricted to exon 1.

We have limited the mutation analysis of the *APC* gene to the mutation cluster region, in which 68 to 77% of somatic mutations are found. Therefore, we may have missed up to one third of all mutations that may occur in this large gene. However, most colorectal cancers that have *APC* mutations, exhibit at least one truncating mutation in the mutation cluster region¹⁷ and all truncating mutations in this region result in loss of the C-terminal part of the *APC* protein, which harbours functional domains that are critical to *APC*'s tumour suppressor function.¹⁸

An inherent problem of studying alterations in the *APC* gene is its character as a tumour suppressor gene, since it would require two hits to inactivate both alleles. However, there is evidence that in a tumour harbouring a mutation in the mutation cluster region of the *APC* gene the other allele is often deleted.¹⁷

Similar considerations as those with respect to the choice of *K-ras* mutations studied apply to the analyses with regard to mutations in the *APC* gene. We have focussed on patients harbouring a tumour with a point or frameshift mutation in the mutation cluster region that would lead to introduction of a stop codon and consequently to truncation of the *APC* protein and loss of function, as these are generally accepted to contribute to tumour formation. Following this line of reasoning with respect to specific point mutations hypothesized to be associated with environmental exposures, these analyses were also based on specific point mutations giving rise to introduction of a stop codon.

Since a large proportion of tumours harboured a missense mutation in the *APC* gene, this group was analysed separately with regard to the exposures under study. As the functional implication of these mutations is unknown, results are difficult to interpret. It is plausible that the occurrence of missense mutations partially represents increased mutation rates in the tumour rather than defective *APC* function.

Another impairment of *APC* function could be caused by gene silencing through promotor hypermethylation.^{19, 20} However, since this is a rare occurrence and probably reflects other mechanisms than the mechanisms hypothesized to induce specific *APC* mutations that were studied, this is likely not to have led to significant misclassification in our study.

Although *CTNNB1* mutations were suggested to occur in approximately 50% of tumours lacking a truncating mutation in the *APC* gene,¹² in our study only five of 464 patients without a truncating *APC* mutation and/or hMLH1 deficient tumour, harboured a mutation in the codons that encode phosphorylation sites. This very limited number of tumours harbouring a mutation in the *CTNNB1* gene did not allow for molecular epidemiological analyses to estimate associations between meat, fat and cigarette smoking and these genetic aberrations. In contrast to suggestions from literature,^{12, 21, 22} a major role for *CTNNB1* mutations in colorectal cancer seems unlikely.

Differences between microsatellite instable tumours and chromosomally instable tumours have been proposed to reflect environmental exposure²³ and therefore are an interesting group of tumours to study. Of the microsatellite instable tumours, approximately 90% show absence of hMLH1 expression.²⁴ In sporadic colorectal cancer, the absence of hMLH1 expression is generally due to hypermethylation of the promotor region²⁵⁻²⁷ and not to mutations in this gene as is the case in hereditary non-polyposis colorectal cancer.

VALIDITY AND REPRODUCIBILITY OF EXPOSURE ASSESSMENT

A crucial factor with regard to the studies described here, is the validity of the exposure data used. With regard to possible information bias, exclusion of the first 2.3 years of follow up is likely to have eliminated possible pre-clinical disease that may have affected exposure status in the case group. Under- or over-reporting of dietary and smoking factors at the start of a cohort study will most likely be non-differential, especially with regard to *APC*, *K-ras* mutation status, hMLH1 expression status or polymorphisms in *GSTM1* and *GSTT1*.

Data on consumption of various dietary factors were validated against a nine-day record method.²⁸ The observed Pearson correlation coefficients for meat (0.46), meat products (0.54) and fish (0.53) were modest.²⁸ The sex and energy-adjusted correlation coefficients for total fat (0.52), saturated fat (0.58) and polyunsaturated fat (0.75) were slightly better.²⁸ Although estimates of intake may not be as accurate as desirable, if misclassification occurs, it is more than likely to be non-differential. Therefore, it is possible that true associations are stronger but would still remain weak or undiscovered in this study.

Our data with regard to dietary factors were derived from the 150-item food frequency questionnaires that the study participants filled in at the start of the study. Diet was assessed only once, but the small decline in test-retest correlation coefficients over time (0.07 over five years) in the reproducibility study of the food frequency questionnaire suggests that the exposure is relatively stable over time.²⁹

One of our hypotheses with regard to the carcinogenic properties of meat consumption was based on the content of carcinogens, which are formed during meat preparation such as heterocyclic aromatic amines (HCA) and polycyclic aromatic hydrocarbons (PAH). Since we did not have data on habitual preparation of meat of all cases and subcohort members at baseline, the consumption of several types of meat was used as a proxy for intake of heat-induced carcinogens present in meat. Large variations in the intake of

HCA and PAH from meat can be expected as these are the result of varying preparation methods.³⁰ Data from meat preparation in subcohort members at a follow-up investigation, however, showed little variation in surface browning of meat, suggesting that the range in the content of heat-induced carcinogens would be limited.³¹ PAH levels in meat as prepared by the Dutch population under study are expected to be low, as PAH are induced by grilling or barbecuing of meat, which is not a common means of meat preparation in the Netherlands. The main source of PAH would be from tobacco smoke, and smoking status was included as a confounder in the used models and investigated separately in **chapter 6**. *N*-nitrosamines and their precursors are compounds that can be formed endogenously³² but are also found in red and processed meat.³³ Variations in intake of these meats could therefore reflect variations in exposure to *N*-nitrosamines.

CONFOUNDING

In **chapters 4** through **6**, we have examined the relationship between meat and fish consumption, dietary fat intake and smoking on the presence or absence of (specific) APC mutations and lack of hMLH1 expression. Key factors influencing risk of colon and rectal cancer were age, sex and family history of colorectal cancer. These factors were included in all analyses performed. Other possible confounding factors were chosen based on literature and their influence tested by introducing all of these factors as covariates to the multivariate models for overall colon and rectal cancer. The covariates that influenced the estimated risk ratios by 10% or more and/or contributed statistically significantly ($p < 0.05$) to the multivariate model in either the model for colon or rectal cancer or both were included in the model.

We did not investigate confounding factors specifically for the separate endpoints studied, because, as yet, there is insufficient information available on potential confounders for the exposures and these particular endpoints to take these into account. Possibly, factors that confound the relationship between the exposures of interest and colon and rectal cancer do not play an important role with regard to the molecular endpoints. Conversely, other factors may confound associations when considering these particular endpoints. However, examining large numbers of potential confounders in separate models for all the endpoints studied, would severely increase the chance of type I errors with regard to identifying confounders.

MULTIPLE COMPARISONS

Based on hypotheses prior to analysing our data, we have conducted several survival analyses with various endpoints (presence and absence of (specific) truncating and missense mutations in the APC gene and (specific) missense mutations in the *K-ras* gene, presence and absence of hMLH1 expression). Even though our analyses were hypothesis-based, the multiple comparisons for the exposures investigated in this thesis prompt us to consider the possibility of chance findings when interpreting the results from our studies.

INTERPRETATION OF FINDINGS

CURRENT MOLECULAR EPIDEMIOLOGICAL EVIDENCE

To the best of our knowledge only one other study has investigated dietary factors and the occurrence of truncating APC mutations in colorectal cancer.⁹ In this case-control study of dietary intake and risk of colon cancer with or without truncating APC mutations, moderate positive associations for colon tumours that harbour a truncating APC mutation were found with red meat and fish consumption,⁹ which is in contrast with results from our study (**chapter 4**). This may be due to the relatively small numbers of patients and controls (184 cases and 259 controls), but also to differences in definitions used with regard to types of meat as well as differences in exposure assessment between that study and our own study.

Just one other study included associations between diet and hMLH1 expression next to diet and microsatellite instability and found these associations to correspond.³⁴ Absence of hMLH1 expression appears to be good marker for microsatellite instability (**chapter 3** and references^{34, 35}). Therefore, the results obtained from the analyses based on absence of hMLH1 expression may be representative of microsatellite instable tumours and results from our study allow for comparison with results from studies that have estimated microsatellite instability.

Few other epidemiological studies have examined associations between diet and microsatellite instability.^{34, 36-38} Again, in contrast with our study in which no association between meat consumption and hMLH1 expression was observed, in a case-control study that addressed associations between dietary factors and microsatellite instability that was based on 40 microsatellite instable tumours, an inverse association of red meat with microsatellite instable tumours was observed.³⁴ Cigarette smoking was found to be associated with microsatellite instable colorectal tumours in three case-control studies,^{37, 39, 40} whereas another case-control study could not confirm this.⁴¹ Results from our study (**chapter 6**) indicated that smoking was associated with tumours that did not harbour an APC mutation, and this may partially be ascribed to tumours that lack hMLH1 expression.

TUMOUR INITIATION VERSUS PROGRESSION

Dietary and lifestyle habits were measured once at the start of our study, which may imply that dietary factors that have contributed to tumour progression rather than initiation are most likely to be observed. However, the group under study has been shown to have a relatively stable dietary pattern with a 0.07 decline in test-retest correlation coefficients over five years and can therefore probably also be extrapolated retrospectively over a period of at least five years.²⁹ However, whether this covers a period long enough to extend back to tumour initiation remains in question, as the period between tumour initiation and the clinical appearance of a colorectal tumour may be as long as 40 years.

We have investigated the associations between meat and fat intake as well as smoking and molecular endpoints of colorectal cancer, defined by (truncating) mutations in the tumour suppressor gene *APC*, activating mutations in the proto-oncogene *K-ras* and expression of the mismatch repair protein hMLH1. *APC* mutations are generally considered a gate-keeping event in colorectal carcinogenesis. *K-ras* mutations are also involved in the early stages of tumour development. The time window of mismatch repair deficiency, as represented by a.o. hMLH1 expression is still under debate, but probably also represents an early event. Studying these genetic alterations was based on the hypotheses that carcinogenic compounds derived from meat, fatty acid metabolism and cigarette smoke could induce mutations, and that mutations in genes affected during the early stages of colorectal cancer, that are relevant to its progression to a carcinoma, will still be present in carcinomas.

It has been suggested that different molecular pathways to colorectal cancer may reflect different environmental exposures.²³ In particular, Breivik and Gaudernack⁴² proposed that the type of instability in cancers may reflect the selection pressures exerted by specific carcinogens. They predicted that cells exposed to bulky-adduct forming agents will develop chromosomal instability, whereas cells exposed to methylating agents will develop mismatch repair defects and consequently microsatellite instability. Evidence to support this theory has been provided by a study in a colorectal cancer cell line, in which cells resistant to bulky-adduct forming agent 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) exhibited chromosomal instability, whereas cells resistant to the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) exhibited microsatellite instability and conversely, cells made chromosomally unstable are resistant to PhIP, whereas microsatellite unstable tumour cells are resistant to MNNG.⁴³

In our study of genetic alterations (**chapter 3**) the most pronounced differences with regard to age at diagnosis, sex, sub-localisation, and tumour differentiation were also found between tumours that did or did not express hMLH1. In addition, we observed differences between tumours with alterations in the *APC* and *K-ras* genes as well as expression of hMLH1 with regard to the studied exposures. However, they cannot all be related to the mechanism described by Breivik and Gaudernack.⁴² In our study of associations between meat and *APC* mutations (**chapter 4**), we found evidence for a positive association between beef (in colon) and other meat consumption (in colon and rectum) and truncating *APC* mutations, which may reflect the bulky-adduct pathway. No clear association between fat intake and truncating mutations in the *APC* gene was observed, but linoleic acid was strongly associated with tumours harbouring an activating *K-ras* mutation (**chapter 5**), again possibly reflecting a bulky-adduct pathway. On the other hand, cigarette smoking was associated with increased risk of colorectal cancer, and this association was most pronounced in tumours lacking a truncating *APC* mutation, and could partially be ascribed to hMLH1 deficiency (**chapter 6**). According to the proposed mechanism, this would reflect a methylating agents pathway, whereas cigarette smoke mainly contains bulky-adduct forming agents.⁴⁴ Additional studies may provide further insight on the role of possible carcinogens in different molecular pathways leading to colorectal cancer.

Although environmental factors may be involved in tumour initiation, they could also play a role in tumour progression. A mutation in the APC or K-ras gene or a mismatch repair defect may render the tumour more susceptible to environmental factors and thus the association studied would reveal more about the progression of the tumour rather than its initiation. From observational studies, like those described in this thesis, it is not possible to deduct clear clues as to whether diet and cigarette smoking play a part in tumour initiation or its progression. Studies of dietary factors and the occurrence of mutations in APC and K-ras and mismatch repair deficiency in adenomas may provide further insight in the actual initiation. In a case-control study on diet and sporadic colorectal polyps, red meat and high intake of fat were found to be positively associated with polyps lacking a truncating APC mutation.⁴⁵ As it is unknown what proportion of colorectal adenomas would develop into a carcinoma and which genetic alterations drive this progression, results from studies in adenomas cannot provide sufficient information on the relationship between dietary and lifestyle factors and colorectal cancer. However, these data could provide insight as to whether dietary factors play a role in the initiation or the progression of tumours.

Our findings support the idea that the exposures studied are involved in initiation of tumours through specific alterations although we cannot rule out that they may be associated with the progression of colorectal cancer.

TUMOUR LOCALISATION AND SEX

In the course of tumour development factors other than the ones under study in the framework of this thesis play a role. As reviewed by Iacopetta,⁴⁶ several differences exist between the right and left colon that could predispose the tumours originating at these sites to develop along different pathways, thus prompting consideration of separate analyses for various sites in the colon and rectum. Differences in genetic alterations or differences to various environmental exposures may reflect this. For example, the faecal bulk changes in composition during passage through the colorectal tract. Because these factors are intertwined, it is difficult to establish which factor determines the true underlying difference. By studying genetic alterations observed as several endpoints we may have differentiated enough. We have taken into account the potential differences between sub-localisations in the colorectal tract in the studies of associations between meat, fish and dietary fat consumption by stratifying the analysis for colon and rectal cancer. With regard to the associations studied between cigarette smoking and colorectal cancer, where exposure may be considered more systemic than through direct contact with the colorectal epithelium alone, we did not stratify on sub-localisation. No differences in risk estimates were observed, when analyses were performed for colon and rectal cancer and sub-groups with different molecular alterations separately.

We have not reported associations for men and women separately. When statistically assessing interaction by sex, it was found to be absent. Moreover, although the positive association between smoking and colorectal cancer was observed in men, but not women (**chapter 6**), the risk estimates for the various

exposures and endpoints did not differ much between men and women. However, it remains possible that sex influences risk for certain exposures or the formation of a colorectal cancer, but studying different groups, i.e. also stratifying for sex would inevitably lead to large reduction in power and enhance the chance of spurious findings.

GENERAL CONCLUSION

The studies described in this thesis, have contributed to the existing knowledge in several ways. In the first two studies described in this thesis, a distinct heterogeneity of the tumours with regard to mutations in *APC*, *K-ras* and *hMLH1* expression was observed. Moreover, in contrast to suggestions in literature, the contribution of *CTNNB1* mutations to the development of colorectal carcinomas seems very small. Mutation status appeared to be related to tumour sub-localisation. Although observed associations were not very strong and further research in this area is warranted, the studies of meat and fat consumption and cigarette smoking support the idea that taking into account the genetic heterogeneity of colorectal cancer endpoint by analysing groups of tumours according to genetic aberrations, may add to our understanding of the complex relationship between dietary and lifestyle factors and the development of a carcinoma. Therefore, it is recommendable to both expand this type of research to more exposure factors as well as investigating the colorectal cancer endpoint according to more than one molecular endpoint, as the combination of genetic aberrations seems to more accurately describe the heterogeneity in tumours than studying one single genetic feature.

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SUMMARY

Colorectal cancer affects a large number of people worldwide, in particular in Western countries. Like other cancers, cancer of the colon and rectum is a multi-factorial disease. Besides increasing age and a positive family history of colorectal cancer, dietary and lifestyle factors are likely candidates for affecting colorectal cancer risk. A high intake of fat, meat and alcohol as well as cigarette smoking are thought to increase the risk of developing a colorectal tumour, whereas vegetable, fibre and possibly fruit consumption may decrease the risk. However, observed associations are often weak and sometimes inconsistent.

The majority of colorectal carcinomas seem to arise through a series of well-defined histopathological changes, referred to as the adenoma-carcinoma sequence. A generally accepted paradigm for the mechanism underlying colorectal cancer development describes the sequential accumulation of genetic alterations in the adenomatous polyposis coli (APC), Kirsten ras (K-ras), and tumour protein 53 (TP53) genes. In addition to mutations in specific genes, most colorectal tumours display either chromosomal or microsatellite instability. In the majority of colorectal cancers chromosomal instability is observed. A smaller fraction (10 to 15%) is marked by microsatellite instability, which in approximately 90% of sporadic colorectal tumours is related to defective human Mut-L homologue 1 (hMLH1) function. These two types of genetic instability seem to represent distinct pathways, and possibly reflect differences in aetiology. Environmental factors may exert a direct effect on carcinogenesis through induction of DNA damage that results in mutations in genes relevant to tumour formation or may drive the selection of tumours harbouring specific mutations. Different molecular pathways in colorectal cancer may therefore reflect exposure to various environmental factors. The aims of the studies described in this thesis were to investigate the occurrence of genetic alterations thought to be important to the early stages of colorectal cancer development and to gain insight in the relation between environmental exposures and colorectal cancer as well as these genetic alterations.

The studies described in this thesis were carried out within the framework of the Netherlands Cohort Study on diet and cancer (NLCS) that was initiated in September 1986. At baseline, 120,852 men and women between 55 and 69 years of age, completed a 150-item food frequency and lifestyle questionnaire. A subcohort of 3,500 male and female subjects was randomly drawn from the cohort and followed-up biennially to determine accumulated person years. Through record linkage of the cohort with the Netherlands Cancer Registry and PALGA, 929 cases of colorectal cancer were identified after 7.3 years of follow-up of the cohort and excluding the first 2.3 years. From 54 pathology laboratories throughout the Netherlands, formalin-fixed, paraffin-embedded tumour material of 737 patients could be obtained, which was used for subsequent analyses of molecular alterations present in their tumours.

In the study described in **chapter 2**, the occurrence and type of mutations in the APC gene were studied in 665 patients for whom the mutation cluster region of the APC gene could be analysed completely. We observed a large number of mutations in total, which was in part due to the occurrence of multiple mutations in 40% of tumours. However, despite 72% of the carcinomas harbouring one or more mutations, only 37% of patients had a carcinoma with

at least one mutation that would result in a truncated and therefore inactivated APC protein, whereas 29% of patients harboured at least one missense mutation, but no truncating mutation and 6% of patients had a silent mutation, but no truncating or missense mutation. Rectosigmoid and rectal tumours relatively more frequently harboured C→T point mutations or frameshift mutations that would result in a truncated APC protein when compared to proximal and distal colon tumours, suggesting different aetiology of colon and rectal cancer.

Chapter 3 presents the results from our study on the occurrence of mutations in the APC, CTNNB1, K-ras genes and expression of hMLH1 in 656 carcinomas for which the analysis of these molecular features could be completed, and in which the characteristics of the patients and tumours were compared based on these genetic aberrations. Mutations at the phosphorylation sites of the CTNNB1 gene encoding β -catenin were a rare occurrence (5/464). Striking differences between hMLH1 deficient tumours and tumours harbouring a truncating APC and/or activating K-ras gene mutation were observed. Patients with a hMLH1 deficient tumour were older, more often women, their tumours were more often located in the proximal colon and showed poorer differentiation when compared to patients harbouring tumours with an APC and/or K-ras mutation.

Meat has been proposed as a risk factor for colorectal cancer based on its animal fat and haem iron content as well as its preparation. Due to varying means of preparation, different types of meat may contain heterocyclic aromatic amines, polycyclic aromatic hydrocarbons, and N-nitrosamines that may cause specific DNA damage. Thus, constituents of meat may influence the risk of developing tumours harbouring different genetic alterations, i.e. mutations in the APC gene or mismatch repair deficiency. The study on the associations between meat and fish consumption and genetic alterations in colon and rectal tumours, described in **chapter 4**, showed no association between total fresh meat, poultry or fish consumption and risk of colon or rectal cancer overall, nor tumours with or without specific genetic alterations. Meat product (processed meat) consumption, however, showed a positive association with colon tumours harbouring a truncating APC mutation (incidence risk ratio (RR) 1.61, 95% confidence interval (CI) 0.96-2.71 for highest versus lowest quartile of intake), whereas beef consumption was associated with an increased risk of colon tumours without a truncating APC mutation (RR 1.58, 95% CI 1.10-2.25 for highest versus lowest quartile of intake). None of the meat and fish variables were found to be associated with lack of hMLH1 expression, which led us to conclude that several types of meat may contribute differently to the aetiology of colon and rectal cancer, depending on APC mutation status, but not its hMLH1 expression status.

Dietary fats may both constitute a risk for developing colorectal cancer or exert a protective effect, depending on the type of fat. In **chapter 5**, the study on the relation between various dietary fats and molecular aspects of colorectal cancer as determined by the APC and K-ras mutation status and hMLH1 expression status is described. No clear associations were observed for intake of total, saturated and mono-unsaturated fat and risk of colon and rectal cancer or the different molecular subgroups. Linoleic acid, the main constituent of

polyunsaturated fatty acid intake, was associated with an increased risk of colon cancer with only a *K-ras* mutation and no additional truncating APC mutation or lack of hMLH1 expression (RR 6.74, 95% CI 2.36-19.51 for highest versus lowest quartile of intake). Linoleic acid intake was neither associated with risk of colon tumours without any of the gene defects, with tumours only lacking hMLH1 expressing, nor with tumours only harbouring APC mutations. Our data suggest a unique aetiology of tumours that harbour specific genetic aberrations.

Cigarette smoke contains numerous carcinogens that through direct or systemic contact with the colorectal epithelium may contribute to an increased risk of developing colorectal cancer. The aim of the study described in **chapter 6** was to study the contribution of cigarette smoking to sporadic colorectal cancer according to molecular alterations of the tumours and according to genetic susceptibility as determined by the glutathione S-transferase (GST) *GSTM1* and *GSTT1* genotypes. In comparison to never-smokers, a high smoking frequency was associated with an increased risk of colorectal cancer (RR 1.07, 95% CI 1.03-1.12 per five cigarettes/day increment) and this association was stronger in tumours without a truncating APC mutation (RR 1.11, 95% CI 1.05, 1.17). Long-term smoking was associated with tumours lacking hMLH1 expression (RR 1.17, 95% CI 1.00-1.37 per 10 years increment). No statistically significant interactions between smoking and *GSTM1* or *GSTT1* genotype were observed. We concluded that cigarette smoking is associated with risk of colorectal cancer, and that this association may depend on molecular characteristics of the tumour as defined by APC mutation and hMLH1 expression status.

In **chapter 7** the main findings from the studies described in this thesis are summarised and discussed in the light of methodological considerations, the limited number of other studies that have investigated the relation between environmental factors and the genetic aberrations. With regard to methodological considerations, we can conclude that the quality of the exposure data and the molecular analyses was satisfactory and that the choice of molecular endpoints was appropriate with regard to their proposed relevance to colorectal tumourigenesis, and did not result in important misclassification. It seems unlikely that the results were subject to extensive bias due to loss to follow-up of tumour tissue or the success rate of the molecular analyses. However, including different molecular endpoints in our analyses inevitably led to multiple testing and considering that our results are not always in line with the very limited number of publications of this kind, they have to be treated with some caution. It is impossible to draw firm conclusions on the role of dietary and lifestyle factors in the initiation or progression of the carcinogenic process, as the findings are derived from an observational study. Overall, our results support the hypothesis that various environmental exposures contribute differently to colorectal cancer risk depending on the genetic alterations that are considered to be important to its development. More research in this area is warranted and should ideally include more than one molecular endpoint as this seems to describe the heterogeneity in tumours more accurately than one single genetic feature.

SAMENVATTING

Colorectaal kanker treft wereldwijd een groot aantal mensen, met name in westerse landen. Zoals andere vormen van kanker, is kanker van het colon en het rectum een multifactoriële aandoening. Afgezien van een toenemende leeftijd en een positieve familiegeschiedenis van colorectaal kanker, zijn voedings- en leefstijlfactoren voor de hand liggende factoren die het risico op colorectaal kanker beïnvloeden. Een hoge inname van vet, vlees en alcohol alsmede het roken van sigaretten verhogen waarschijnlijk het risico op het ontwikkelen van een colorectaltumor, terwijl de consumptie van groente, vezel en mogelijk fruit het risico zou verkleinen. De gevonden associaties zijn echter vaak zwak en soms inconsistent.

De meerderheid van colorectaal carcinomen ontstaat via een serie goed gedefinieerde histopathologische veranderingen, welke bekend staat als de adenoom-carcinoom sequentie. Het mechanisme dat ten grondslag ligt aan de ontwikkeling van colorectaal kanker is het algemeen geaccepteerde paradigma van de stapsgewijze accumulatie van genetische veranderingen in de adenomateuze polyposis coli (APC), Kirsten ras (K-ras), en tumour protein 53 (TP53) genen. Buiten mutaties in specifieke genen, worden de meeste colorectaal tumoren gekenmerkt door chromosomale of microsatelliet instabiliteit. In de meerderheid van de colorectaal tumoren wordt chromosomale instabiliteit waargenomen. Een kleiner deel (10 tot 15%) wordt gekenmerkt door microsatelliet instabiliteit, die in ongeveer 90% van de sporadische colorectaal tumoren gerelateerd is aan een defecte human Mut-L homologue 1 (hMLH1) functie. Deze twee vormen van genetische instabiliteit lijken specifieke ontwikkelingsroutes te vertegenwoordigen en weerspiegelen mogelijk verschillen in etiologie.

Omgevingsfactoren hebben waarschijnlijk een direct effect op de carcinogenese via de inductie van DNA schade die resulteert in mutaties in genen die relevant zijn voor tumorvorming of sturen mogelijkwerijs de selectie van tumoren met specifieke mutaties. Verschillende moleculaire routes zouden daarom de blootstelling aan verschillende omgevingsfactoren kunnen weerspiegelen. Het doel van de studies die beschreven zijn in dit proefschrift, was om het voorkomen van genetische veranderingen die belangrijk zijn in de vroege ontwikkeling van colorectaal kanker te onderzoeken en inzicht te verwerven in de relatie tussen omgevingsfactoren en colorectaal kanker en deze genetische veranderingen.

De studies beschreven in dit proefschrift werden uitgevoerd binnen de Nederlandse Cohort Studie naar voeding en kanker (NLCS) die in september 1986 werd gestart. Bij aanvang van de studie vulden 120.852 mannen en vrouwen tussen de 55 en 69 jaar een voedselfrequentie- en leefstijlvragenlijst in. Een subcohort van 3.500 mannelijke en vrouwelijke deelnemers werd aselekt getrokken uit het cohort en werd tweejaarlijks gevolgd om de verzamelde persoonstijd vast te stellen. Via koppeling van het cohort met de Nederlandse Kankerregistratie en het Pathologisch Anatomisch Landelijk Geautomatiseerd Archief (PALGA) konden na 7,3 jaar follow-up van het cohort - en met uitsluiting van de eerste 2,3 jaar - 929 gevallen van colorectaal kanker worden geïdentificeerd. Van 737 patiënten kon tumorweefsel worden verzameld, dat gebruikt werd voor de analyse van genetische veranderingen in hun tumoren.

In de studie die beschreven wordt in **hoofdstuk 2**, werd het vóórkomen van en het type mutatie in het APC gen bestudeerd in 665 patiënten van wie de mutatie cluster regio van het APC gen volledig kon worden geanalyseerd. Er werd een groot aantal mutaties gevonden, wat gedeeltelijk toe te schrijven is aan het vóórkomen van multipale mutaties in 40% van de onderzochte tumoren. Hoewel in 72% van de carcinomen een of meerdere mutaties werd vastgesteld, had slechts 37% van de patiënten een tumor met tenminste één mutatie die zou leiden tot een verkort en daardoor inactief APC eiwit, terwijl 29% van de patiënten tenminste één missense, maar geen functionele mutatie bleek te hebben en 6% had een silent mutatie, maar geen functionele of missense mutatie. Tumoren in het rectosigmoid en rectum hadden relatief vaker een C→T puntmutatie of frameshift mutatie die zouden resulteren in een verkort eiwit in vergelijking met proximale en distale colontumoren, wat mogelijk duidt op een verschil in etiologie tussen het colon en het rectum.

Hoofdstuk 3 beschrijft de studie naar het voorkomen van mutaties in de APC, CTNNB1, K-ras genen en expressie van hMLH1 in 656 carcinomen van welke de analyse van deze moleculaire karakteristieken volledig geanalyseerd kon worden en naar de vergelijking van patiënt- en tumorkarakteristieken op basis van deze genetische veranderingen. Mutaties in de fosforyleringsplaatsen van het CTNNB1 gen dat codeert voor β -catenine waren zeldzaam (5/646). Opvallende verschillen werden waargenomen tussen hMLH1 deficiënte tumoren en tumoren die een functionele APC en/of een activerende K-ras mutatie hadden. Patiënten met een hMLH1-deficiënte tumor waren ouder, vaker vrouwen, hun tumoren waren vaker gelokaliseerd in het proximale colon en waren slechter gedifferentieerd dan patiënten met een tumor die een APC en/of K-ras mutatie bevatten.

Vlees zou een risicofactor voor colorectalkanker kunnen zijn op basis van het gehalte aan dierlijk vet en haemijzer alsook de bereidingswijze. Als gevolg van de verschillen in bereidingswijze kunnen de diverse vleessoorten heterocyclische aromatische amines, polycyclische aromatische koolwaterstoffen en N-nitrosamines bevatten, die specifieke DNA-schade kunnen veroorzaken. Zodoende zouden bestanddelen in vlees het risico op de ontwikkeling van tumoren met verschillende genetische veranderingen (mutaties in het APC gen of mismatch repair deficiëntie) kunnen beïnvloeden. De studie naar de associaties tussen vlees- en visconsumptie en genetische veranderingen in colon- en rectumtumoren, zoals beschreven in **hoofdstuk 4**, liet geen associatie zien tussen de consumptie van totaal vers vlees, gevogelte of vis en het risico op colon- en rectumkanker op zich, noch met tumoren met of zonder specifieke genetische veranderingen. Consumptie van vleeswaren liet echter een positief verband zien met colontumoren die een functionele APC mutatie hadden (relatief risico (RR) 1,61, 95% betrouwbaarheidsinterval (BI) 0,96-2,71 voor het hoogste t.o.v. het laagste kwartiel van inname), terwijl rundvleesconsumptie geassocieerd was met een verhoogd risico op colontumoren zonder een functionele APC mutatie (RR 1,58, 95% BI 1,10-2,25 voor het hoogste t.o.v. het laagste kwartiel van inname). Geen van de vlees- of visvariabelen bleek geassocieerd te zijn met de afwezigheid van hMLH1 expressie, wat leidde tot de conclusie dat verscheidene soorten vlees op verschillende wijze kunnen bijdragen aan de etiologie van colon- en

rectumkanker, afhankelijk van de APC mutatiestatus, maar niet de hMLH1 expressiestatus.

Voedingsvetten kunnen zowel een risicofactor vormen voor de ontwikkeling van colorectalkanker als een beschermend effect hebben, afhankelijk van het type vet. In **hoofdstuk 5** wordt de studie naar de relatie tussen diverse voedingsvetten en moleculaire aspecten van colorectalkanker op basis van de APC en K-ras mutatiestatus en hMLH1 expressiestatus beschreven. Voor totaal, verzadigd en mono-onverzadigd vet werden geen associaties gevonden met het risico op colon- en rectumkanker. Linolzuur, het belangrijkste vetzuur uit de groep meervoudig onverzadigde vetzuren, was geassocieerd met een verhoogd risico op colonkanker met alleen een K-ras mutatie en geen additionele functionele APC mutatie of hMLH1 deficiëntie (RR 6,74, 95% BI 2,36-19,51 voor het hoogste t.o.v. het laagste kwartiel van inname). De inname van linolzuur was niet geassocieerd met het risico op colon tumoren zonder enige genetische afwijking, met tumor die alleen hMLH1 deficiënt waren, noch met tumoren met alleen een APC mutatie. Onze resultaten suggereren dat tumoren met specifieke genetische afwijkingen een unieke etiologie hebben.

Sigarettenrook bevat veel carcinogene stoffen die via direct of systemisch contact met het colorectale epitheel een bijdrage zouden kunnen leveren aan de ontwikkeling van colorectalkanker. Het doel van de studie beschreven in **hoofdstuk 6** was de bijdrage van het roken van sigaretten aan sporadische colorectalkanker te onderzoeken, waarbij rekening werd gehouden met moleculaire veranderingen van de tumoren en de glutathion S-transferase (GST) GSTM1 en GSTT1 genotypen. In vergelijking met mensen die nooit gerookt hebben verhoogde een hoge rookfrequentie het risico op colorectalkanker (RR 1,07, 95% BI 1,03-1,12 per toename van vijf sigaretten per dag) en dit verband was sterker voor tumoren zonder een functionele APC mutatie (RR 1,11, 95% BI 1,05-1,17). Langdurig roken was geassocieerd met tumoren zonder hMLH1 expressie (RR 1,17, 95% BI 1,00-1,37 per toename van 10 jaar). Er werden geen statistisch significante interacties gevonden tussen roken en GSTM1 en GSTT1 genotype. Wij concludeerden dat het roken van sigaretten geassocieerd is met het risico op colorectalkanker en dat deze associatie afhankelijk zou kunnen zijn van de moleculaire karakteristieken van de tumor op basis van de APC mutatie en hMLH1 expressie status.

In **hoofdstuk 7** worden de belangrijkste resultaten van de studies die beschreven zijn in dit proefschrift samengevat en bediscussieerd in het licht van methodologische afwegingen en het beperkt aantal andere studies dat de relatie tussen omgevingsfactoren en genetische afwijkingen heeft onderzocht. Met betrekking tot de methodologische aspecten kunnen we concluderen dat de kwaliteit van de expositiedata en de moleculaire analyses goed was en dat de keuze voor de geanalyseerde eindpunten geschikt was met betrekking tot hun relevantie in de carcinogenese van het colon en rectum en niet geleid heeft tot belangrijke misclassificatie. Het lijkt onwaarschijnlijk dat onze resultaten sterk vertekend zijn door selectie door incomplete follow-up van de tumormateriaal of het succes van de moleculaire analyses. Het apart beschouwen van de verschillende moleculaire eindpunten heeft echter wel geleid tot het doen van multiple analyses. Daarnaast kwamen onze resultaten niet altijd overeen met de resultaten uit de beperkte vergelijkbare literatuur.

Daarom is het van belang onze resultaten met enige terughoudendheid te beschouwen. Over de rol van voedings- en leefstijlfactoren in de initiatie danwel progressie van het carcinogene proces kunnen geen definitieve conclusies worden getrokken, aangezien onze bevindingen afkomstig zijn uit observationeel onderzoek. In het algemeen ondersteunen onze resultaten de hypothese dat blootstelling aan verschillende omgevingsfactoren verschillend bijdraagt aan het risico op colorectalkanker, afhankelijk van de genetische veranderingen die belangrijk worden geacht voor de ontwikkeling ervan. Meer onderzoek op dit gebied is geboden en zou bij voorkeur meer dan één moleculair eindpunt beschouwen, aangezien dit de heterogeniteit van tumoren nauwkeuriger lijkt te beschrijven dan één enkele genetische component.

DANKWOORD

De afbeelding is een tekening van een menselijke figuur, die in een houding van dankbaarheid staat, met de handen gevouwen voor de borst. De figuur is eenvoudig getekend, met een hoofd, een torso en twee armen. De afbeelding is in zwart-wit en heeft een minimalistische stijl.

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De laatste pagina's van dit boekje wil ik graag gebruiken om de vele mensen die betrokken zijn geweest bij de totstandkoming ervan te bedanken.

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Mama en Jaap, *blood is thicker than water*. Altijd.

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