Dietary factors and K-ras oncogene mutations in sporadic colorectal cancer

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"Dietary factors and *K-ras* oncogene mutations in sporadic colorectal cancer"

M.Brink

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Dietary factors and *K-ras* oncogene mutations in sporadic colorectal cancer

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
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Introduction

BURDEN OF COLORECTAL CANCER

Colon and rectal cancer are the fourth most common types of cancer in the world (1). In both men and women, incidence of colon cancer occurs at a similar rate. However, cancer of the rectum has a 20-50% higher incidence in men than in women. In The Netherlands, colon and rectal cancer rank fourth (world standard annual incidence rate (WSR) 23 per 100,000 persons) and sixth (WSR 15 per 100,000 persons) on the list of most frequent cancers among men and second (WSR 19 per 100,000 persons) and eighth (WSR 9 per 100,000 persons) among women, respectively (Cancer Incidence in Five Continents, vol. VIII, 2002).

High-risk areas include North America, Western Europe, New Zealand and Australia. Central and South America, Asia and Africa are areas of low risk, but the incidence in these areas rises consistently with migration and westernisation of diet (2-4). Migrant and temporal trend studies suggest that colon and rectal cancer are determined largely by environmental exposures, such as diet. The cancer risk rates of migrants who move from low-to-high-risk countries tend to increase to the rates of the host countries within one or two generations, or even as early as within the migrating generation itself. Lichtenstein et al (5) suggested that environmental factors may contribute a proportion of approximately 65% to the susceptibility to colorectal cancer (CRC).

DIETARY FACTORS AND AETIOLOGY OF SPORADIC COLORECTAL CANCER

In a multitude of prospective cohort and case-control studies, the importance of dietary factors for CRC incidence has been investigated. The most common hypotheses from epidemiological studies suggest that an increased risk of CRC is the result of a typical western diet, rich in fat and low in fibre (6,7). However, most of the associations between dietary factors and CRC are still prone to debate.

Regarding dietary fat, it has been difficult to determine whether it is the fat content of diet or the associated consumption of meat, which is related to the increased risk of CRC. Epidemiological evidence, supported by identification of biological mechanisms through experimental evidence, suggests that a diet high in total fat and saturated fat possibly increases the risk of CRC (8-10). The evidence with respect to diets high in monounsaturated fat or polyunsaturated fat and the risk of CRC is inconsistent (11-13). Energy intake was suggested to be increased associated with colon cancer risk in some studies, after adjustment for physical activity (10,14). Regarding meat, associations have been found with red meat consumption (15), with the preparation of meat, like high temperature cooking, and with processed meat (4,9,16). Fish consumption is inversely associated with CRC (12,17).

A diet rich in dietary fibre, vegetables and fruit has been suggested to be protective of CRC (6,18,19). For a potential protective effect of dietary calcium on CRC, overall results from case-control and cohort studies indicate no effect (19-21). High intake of both folate and methionine are inversely associated with CRC risk (22,23), whereas deficient intakes of folate or methionine, in combination with high alcohol consumption, may be positively associated with CRC (24,25). For high intake of alcohol, a majority of epidemiological studies have reported either an increased risk of colon cancer (26,27) or of rectal cancer (28,29) or no association at all (30,31).

Although part of the inconsistent findings may be due to methodological issues related to the assessment of dietary intake, study design or chance, these are unlikely to be the only causes. These findings may also result from the oversimplified approach of CRC as a suddenly arising disease entity and which does not account for the different genes involved.

Therefore, a better aetiological understanding of CRC requires careful molecular analysis of the genetic changes involved in carcinogenesis in relation to exogenous factors.

DEVELOPMENT OF SPORADIC COLORECTAL CANCER

Approximately 5% of all CRCs are directly caused by inherited genetic abnormalities. The hereditary forms include familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC). FAP arises because of a mutation in the adenomatous polyposis coli (APC) gene, a tumour suppressor gene, whereas HNPCC results from mutations in DNA mismatch repair genes (32,33). Studies of these hereditary forms of CRC have given us many clues for understanding the molecular basis of sporadic CRC. In this thesis the focus will be on the sporadic, i.e. non-hereditary, form of CRC.

The adenoma-carcinoma sequence

The adenoma-carcinoma sequence (34), the model for pathogenesis of CRC proposed in 1989, is now widely accepted. According to this model, the initial colorectal lesion arises as a benign adenomatous polyp that later undergoes further disorganisation which leads to

Figure 1 The adenoma-carcinoma sequence and genetic alterations, adapted from Fearon and Vogelstein (35). The tumour suppressor genes APC and P53 are indicated in green, the oncogenes K-ras and β -catenin in yellow and other genes in pink.

changes in phenotype. Some earlier stages in the process have also been proposed: hyperproliferation of the upper crypt cells, aberrant crypt foci, and microadenomas. Both adenomatous polyps and microadenomas may progress to a malignant carcinoma, and finally metastasis may occur.

A genetic model for the adenoma-carcinoma sequence

A molecular basis for the adenoma-carcinoma progression has been presented (35) and this model describes the accumulation of alterations in various genes that control cell growth and differentiation, a complex multistep process which results in a neoplastic phenotype (figure 1). This process involves cumulative rather than sequential somatic genetic and epigenetic changes. Some tumour suppressor genes, in particular the *APC* gene, are known to prevent CRC through direct control of cell growth. Inactivation of these genes contributes directly to the neoplastic growth of the tumour; thus, the normal gene products function as "gatekeepers" and prevent uncontrolled growth (33,36,37). These traditional tumour suppressor genes may be joined by a number of genes responsible for repair of the genome, like human MutL homologue 1 (*hMLH1*) and human MutH homologue 2 (*hMSH2*), that indirectly suppress neoplasia. These genes encode DNA repair proteins that act as "caretakers" of the genome (33,36,37). Inactivation of these genes may result in greatly increased mutation rates and can be considered as equivalent to a constant exposure of mutagens (33,36). However, it is not clear whether such defects lead directly to cancer, as restoration of these functions in cancer cells has not been found to affect their growth (36).

One of the first events in the adenoma-carcinoma sequence is presumably an alteration in the *APC* gene that leads to a functional loss of the *APC* protein and occurs in 70-80% of sporadic colorectal adenomas and carcinomas (35,38). Evidence that *APC* mutations are involved in the development of an adenoma from normal colonic epithelium, was found in studies performed with FAP patients (32,33). A mutation in the *K-ras* oncogene is another early event in the adenoma-carcinoma sequence. *K-ras* mutations affect the progression from a small to a large adenoma. The gene has been found mutated in 10% of the screened adenomas smaller than 1 cm, and in 30-60% of adenomas larger than 1 cm and in adenocarcinomas (35,38). In addition to mutations in the *APC* and/or *K-ras* genes, mutations in other oncogenes and tumour suppressor genes may occur, such as in β -catenin and *p53*. Some events occur relatively early, including functional loss of the *APC* gene and mutation of the *K-ras* oncogene, whereas functional loss of other genes, such as *p53*, are usually late events (35,37).

The *K-ras* oncogene

In a significant proportion (30-60%) of the cancers in colon and rectum, the *K-ras* gene is found to be mutated. The *K-ras* gene product, a 21 kDa protein located at the inner plasma membrane, is activated transiently as a response to extracellular signals such as growth factors, cytokines and hormones that stimulate cell surface receptors. The Ras protein is involved in the transduction of mitogenic signals, and multiple downstream effectors of Ras have been revealed, including Raf-1, phosphatidylinositol-3 kinase, and RalGEF, which are involved in cell growth and proliferation. The hallmark of Ras protein function is the switch between its inactive state, in which the protein is bound to guanosine-diphosphate (GDP), and an active state in which conversion to guanosine-triphosphate (GTP) occurs. This transit is governed by two types of regulatory proteins: GDP-GTP exchange factors that catalyse the GDP-GTP exchange and GTPase-activating proteins that enhance the intrinsic capacity of Ras proteins to hydrolyse GTP into GDP, thereby returning the Ras protein into its inactive state.

Table 1 Overview of case-control and case-case studies of associations between dietary factors and *K-ras* mutation status in colorectal adenomas and carcinomas

Study population	Dietary factors investigated	General findings		
		Wild type <i>K-ras</i> vs. control	Mutated <i>K-ras</i> vs. control	Mutated <i>K-ras</i> vs. wild type <i>K-ras</i>
Cross-sectional study				
678 colorectal adenoma patients <i>Martinez et al, 1999 (42)</i>	<u>High energy intake</u>			N.S.*
	<u>Dietary fat</u>			
	high total fat, saturated fat			N.S.*
	<u>Dietary fibers</u>			N.S.*
	<u>Calcium</u>			
	high dietary calcium, total calcium			N.S.*
	<u>Folate</u>			
	high dietary folate			N.S.*
	high total folate (dietary + total)			Inverse association [#]
	<u>Meat</u>			
	red meat			N.S.*
	<u>Alcohol</u>			N.S.*
43 left-sided colorectal cancer patients <i>O'Brien et al, 2000 (43)</i>	high red meat			N.S.*
Case-control study				
108 colorectal cancer patients and 295 controls <i>Bautista et al, 1997 (46)</i>	<u>Energy intake</u>			
	high calories, proteins (total, animal or vegetable)			N.S.*
	<u>Dietary fat</u>			
	high total fats, polyunsaturated fat, saturated fat, cholesterol			N.S.*
	<u>High monounsaturated fat</u>			
	<u>Fibers</u>	Inverse association [#]	N.S.*	Increased association [#]
	high carbohydrates, fiber (total, vegetable, cereal, fruit or legume)			N.S.*
	<u>Minerals</u>			
	high phosphorous, sodium, potassium, iron, magnesium, zinc			N.S.*
	<u>Vitamins</u>			
	high vitamin B ₁ , vitamin B ₂ , vitamin B ₆ , vitamin B ₁₂ , niacin, folic acid, vitamin C, vitamin A, retinal, carotene, vitamin E, vitamin D			N.S.*
	<u>Calcium</u>	N.S.*	Inverse association [#]	Inverse association [#]
	185 colon cancer patients <i>Kampman et al, 2000 (45)</i>	<u>Meat</u>		
high total red meat, processed meat, poultry, fish				N.S.*
<u>Dairy products, calcium</u>				N.S.*
<u>Dietary fat</u>				
total fat, saturated fat, cholesterol				N.S.*

Table 1 continued

Study population	Dietary factors investigated	General findings		
		Wild type <i>K-ras</i> vs. control	Mutated <i>K-ras</i> vs. control	Mutated <i>K-ras</i> vs. wild type <i>K-ras</i>
185 colon cancer patients <i>Kampman et al, 2000 (45)</i>	Energy intake protein (total or animal)			N.S.*
1428 colon cancer patients and 2406 controls <i>Slattery et al, 2000 (44)</i>	Dietary fat high fat, saturated fat, monounsaturated fat, polyunsaturated fat, cholesterol	N.S.*	N.S.*	N.S.*
	Insulin-related factors high carbohydrate, glycemic index	N.S.*	N.S.*	N.S.*
	high refined grains	N.S.*	Increased association [#]	N.S.*
	DNA methylation factors low folate	Increased association [#]	N.S.*	N.S.*
	low vitamin B ₆	Increased association [#]	N.S.*	Inverse association [#]
	low vitamin B ₁₂ or methionine, high alcohol	N.S.*	N.S.*	N.S.*
	Carcinogen detoxification low cruciferous vegetables	Increased association [#]	N.S.*	Inverse association [#]

* N.S.: not significant, [#] statistically significant

Most mutations (90-95%) have been found in codons 12 (wild type GGT) and 13 (wild type GGC) of exon 1 and approximately 5% in codon 61 (wild type CAA) which is located in exon 2 (38-40). A mutation in one of these codons of *K-ras* causes the gene to remain longer in its active state, thereby stimulating cell growth and tissue proliferation (41). The most frequently observed types of mutation in codons 12 and 13 are G>A transitions and G>T transversions, whereas relatively few G>C transversions are found.

DIETARY RISK FACTORS, *K-RAS* GENE MUTATIONS AND COLORECTAL CANCER

Molecular epidemiological studies

To our knowledge, only two cross-sectional case-case studies (42,43) and three longitudinal case-control studies (44-46) on diet and *K-ras* mutations in sporadic colon and rectal cancer have been published and the findings were not consistent (see table 1). The most prominent results with respect to the *K-ras* mutation status will be discussed briefly, as of yet without distinguishing between the specific point mutations or affected codons.

Martinez *et al* (42) observed a significant increased association between low total folate intake (dietary and supplemental, but not dietary alone) and the risk of mutated *K-ras* colorectal adenomas as compared to wild type *K-ras* colorectal adenomas. No associations were observed for energy intake, total or saturated fat, dietary fibre, dietary and total calcium, dietary folate, red meat and alcohol. In a cross-sectional case-case study by O'Brien *et al* (43), no association was observed between current red meat consumption and rectal cancer risk with respect to the *K-ras* mutation status. Slattery *et al* (44) observed a significant increased association between prior intake of refined grains and the risk of mutated *K-ras* colon tumours versus controls. In addition, increased associations were observed between low intake of dietary folate and vitamin B₆ for associations with wild type

K-ras colon tumours versus controls. Low consumption of cruciferous vegetables was associated with an increased risk of wild type *K-ras* colon tumours as compared to controls. No associations were observed for saturated fat, monounsaturated fat (MUFA) and polyunsaturated fat (PUFA), cholesterol, carbohydrate, glycemic index, vitamin B₁₂, methionine and alcohol with both mutated and wild type *K-ras* tumours versus controls. In a case-control study by Kampman *et al* (45), no associations were observed in the case-case comparisons between the consumption of total red meat, beef, processed meat, poultry, fish, dairy products, total or saturated fat, cholesterol, total protein, animal protein and calcium, and the *K-ras* mutation status in colon cancer cases. Bautista *et al* (46) observed a significant inverse association between high intake of MUFA and wild type *K-ras* colorectal tumours as compared to controls. In addition, a significant inverse association was also observed for high intake of calcium with mutated *K-ras* colorectal tumours as compared to controls. No associations were observed for calories, proteins, animal proteins, vegetable protein, total fats, PUFA, saturated fat, cholesterol, carbohydrates, fibre, vegetable fibre, cereal fibre, fruit fibre, legume fibre, phosphorous, sodium, potassium, iron, magnesium, zinc, vitamin B₁, vitamin B₂, vitamin B₆, vitamin B₁₂, niacin, folic acid, vitamin C, vitamin A, retinal, carotene, vitamin E, vitamin D and colorectal cancer risk with respect to *K-ras* mutation status.

Experimental studies and proposed hypotheses on biological mechanism

Results from experimental studies suggest that specific compounds in the diet are relevant for colon and rectal cancer risk with respect to activating mutations in the *K-ras* oncogene. To date, animal and *in vitro* studies have yielded information on specific mutagenic factors present in our environment. The relevant results will be considered and several mutagenic factors will be discussed with respect to their role in DNA damage that may lead to oncogenic activity of the *K-ras* protein, i.e. heterocyclic aromatic amines (HAA), polycyclic aromatic hydrocarbons (PAH), and nitrosamines from meat proteins, ω -6 PUFA and folate.

Regarding meat intake, animal and *in vitro* studies have shown that HAA-DNA-adducts can cause transitions and transversions in DNA bases, as observed in the *K-ras* oncogene (47). On the other hand, studies in rodents have suggested that the HAA-adduct formation with DNA is associated with the wild type *K-ras* gene (48). Results from animal experiments are not entirely consistent. PAHs are suggested to be associated with a high prevalence of G>T transversions in the *K-ras* gene (49). High temperature cooking of meat is known to generate HAAs from meat proteins, whereas the process of grilling and smoking of meat forms PAHs as a result of fat dropping on open-flames. Another hypothesis linked to meat consumption involves the possible action of alkylating agents such as nitrosamines, formed from food preservatives in processed meat, on *K-ras* oncogene mutations (50). These agents are thought to induce G>A transitions at codon 12 in the *K-ras* gene, as was observed in rodents (51) and proposed for humans (52).

Experimental studies linking fat intake to DNA damage is confined to studies on MUFA and derivatives of PUFA (11,12). Several of these studies suggest that MUFAs and ω -6 PUFAs have opposite effects on the risk for colon cancer (12). Protective effects of a high MUFA intake, derived mainly from olive oil and a low intake of ω -6 PUFAs, were observed in experimental studies. Takeshita *et al* (53) found that the incidence of adenocarcinomas of the colon in 1,2-dimethylhydrazine-treated mice was significantly higher in animals fed a diet rich in linoleic acid (main source of ω -6 PUFA) than in animals fed a diet enriched with oleic acid (source of MUFA) or a low-fat diet. To date, no mechanism has been presented that can explain the effect of MUFAs. Peroxidation of ω -6 PUFAs, however, could lead to the accumulation of by-products like malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). These compounds can react with DNA to form adducts, mainly the pyrimidopurine adduct

of deoxyguanosine (M₁G) (54-56). The presence of the M₁G-adducts may result predominantly in G>A and G>T with very few G>C transversions in bacteria (54,57,58). In humans, colorectal biopsies from normal mucosa of participants in the United Kingdom Flexible Sigmoidoscopy Trial and the European Prospective Investigation on Cancer (EPIC) (n=162) were analysed for the presence of M₁G-adducts (56). Adduct levels were higher in individuals with adenomas than individuals without adenomas.

For folate, two plausible carcinogenic mechanisms to explain how sub-optimal folate status could pre-dispose to colon cancer have been proposed. Firstly, if the intracellular form of folate, 5, 10-methylenetetrahydrofolate (5, 10-methyleneTHF) is deficient, less methyl groups will be donated to uracil which may result in less available thymine (59). Eventually, the increased uracil incorporation in DNA may lead to DNA strand breaks and chromosome instability. Secondly, a sub-optimal folate status may result in global DNA hypomethylation and possibly in regional hypermethylation of gene promoters, which interferes with gene expression and can impair the expression of tumour suppressor genes, oncogenes and DNA repair genes (48,59,60). DNA strand breaks, global DNA hypomethylation and regional promoter hypermethylation are common events in colon cancer.

AIMS AND SCOPE OF THIS THESIS

The results from the reports that were described in the previous paragraph were inconsistent, which could be due to the study design, i.e. cross-sectional versus longitudinal studies, selection bias, recall bias or chance due to small sample sizes. In addition, three case-control studies used case-case comparisons to evaluate the aetiological heterogeneity of the risk factors for colon tumours with a wild type or mutated *K-ras* gene, but did not consistently report the case-control comparisons (44,45,46). It should be emphasized that case-case comparisons are useful to detect whether the associations between diet and end points used for cancer cases differ significantly between cases without and cases with the genetic alterations under study. A definite conclusion on how a dietary factor is related to the absence or presence of genetic alterations can be obtained with case-control comparisons, but never with case-case comparisons alone. Therefore, a prospective cohort study design, as used in this thesis, is appropriate to investigate associations between dietary factors and CRC risk with respect to *K-ras* mutations.

The studies described in this thesis (chapters 2 through 5) are embedded in the ongoing Netherlands Cohort Study (NLCS) on diet and cancer. The NLCS was initiated in 1986 and includes 58,279 men and 62,573 women, at baseline aged between 55 and 69 years old, who completed a self-administered questionnaire on diet, family history of cancer and other risk factors for cancer. The entire cohort is being monitored for cancer occurrence by annual record linkage to the Netherlands Cancer Registry (NCR) and to PALGA, a nationwide database of pathology reports. From 1989 until 1994, with exclusion of the first 2.3 years of follow-up, 819 incident cases of colon and rectal cancer have been identified, and all pathology laboratories in The Netherlands agreed to make these tissue blocks available for this study.

Previous studies on frequencies and specific types of point mutations in the *K-ras* oncogene in CRC in various countries have been reported (39,41,45,49,50,52,61-65). However, these results were often based on small numbers of selected patients, obtained from other populations that may differ from the genetic make-up and the environmental conditions in The Netherlands. Therefore, the first aim of this thesis was to evaluate the frequencies and specific types of mutations in the *K-ras* gene in a large number of unselected, incident colorectal cancer patients, identified in the NLCS. This study is described in chapter 2.

As described in the previous paragraph the link between consumption of meat and fish and the pattern of *K-ras* mutations in human colon and rectal cancer is not clear. It is hypothesized that high consumption of meat could contribute to the occurrence of point mutations in the *K-ras* gene, leading to oncogenic activation. In this respect it is expected that N-nitroso compounds, which may be endogenously formed or present in processed meat, induce G>A transitions in codons 12 and 13 of the *K-ras* gene. PACs originating from grilling and smoking of meat may cause G>T transversions, whereas HAAs, which are generated by high temperature cooking of meat proteins, may lead to transitions and transversions of DNA bases in the *K-ras* gene. These hypotheses form the basis of the associations between different types of fresh meat, meat products and fish, and the risk of CRC with or without specific point mutations in the *K-ras* gene, which were studied and described in chapter 3.

For the intake of fats and fatty acids and the risk for *K-ras* mutations, associations are inconsistent (unclear, not well-defined). Based on experimental studies it is hypothesized that peroxidation of ω -6 polyunsaturated fatty acids may lead to accumulation of malondialdehyde and 4-hydroxynonenal, that could form DNA adducts. The adducts could result in G>A transitions and G>T transversions. The associations of total, saturated, and MUFA and PUFA intake with the risk of CRC with or without specific point mutations in the *K-ras* gene were studied and described in chapter 4.

Folate plays an important role in one-carbon metabolism, in DNA synthesis and in methylation of DNA and proteins. Since a deficiency in intermediate folate metabolites leads to a reduced availability of thymine for DNA synthesis, and an increased incorporation of uracil results in more DNA strand breaks, genomic instability may be generated. In addition, folate deficiency may also result in global genome hypomethylation and regional hypermethylation in gene promoters. The latter mechanism inhibits gene expression, and has been demonstrated to evoke DNA repair deficiency. Based on these hypotheses, the association between dietary folate intake and the risk of CRC with or without *K-ras* mutations is studied and described in chapter 5.

Finally, the results and their implications are discussed in chapter 6.

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K-ras oncogene mutations in
sporadic colorectal cancer in
The Netherlands Cohort Study

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ABSTRACT

Activation of *K-ras* oncogene has been implicated in colorectal carcinogenesis, being mutated in 30-60% of the adenocarcinomas. In this study, 737 incident colorectal cancer (CRC) patients, originating from 120,852 men and women (55-69 years at baseline) participating in The Netherlands Cohort Study (NLCS), were studied in order to evaluate subgroups with respect to *K-ras* mutation status.

Mutation analysis of the exon 1 fragment of the *K-ras* oncogene, spanning codons 8 to 29, was performed on archival colorectal adenocarcinoma samples of all patients using macrodissection, nested PCR and direct sequencing of purified fragments. The method of mutation detection was validated by the confirmation of reported *K-ras* status in CRC cell lines, a good correlation between fresh-frozen and routinely-fixed, paraffin-embedded tissue, a detection limit of 5% mutated DNA and a good reproducibility. Various types of *K-ras* mutations were evaluated with respect to family history of CRC, tumour sublocalisation, Dukes' stage and tumour differentiation.

In 37% (271/737) of the patients, the exon 1 fragment of *K-ras* gene was found to be mutated. The predominant mutations are G>A transitions and G>T transversions, and codons 12 and 13 are the most frequently affected codons. Patients with a rectal tumour were found to have the highest frequency of G>T transversions as compared to patients with a colon or rectosigmoid tumour. This difference appeared to be confined to women with a rectal tumour harbouring G>T transversions. No significant differences were observed for Dukes' stage with respect to types of *K-ras* mutation, which does not support direct involvement of the *K-ras* oncogene in adenocarcinoma progression.

The equal distribution of *K-ras* mutations among cases with or without a family history of CRC argues against an important role for this mutation in familial colorectal cancer, and could imply that *K-ras* mutations are more likely involved in environmental mechanisms of colorectal carcinogenesis.

INTRODUCTION

The development of colorectal cancer (CRC) is a multistep process characterised by the accumulation of genetic alterations (1,2). The Fearon and Vogelstein model assumes the involvement of the APC (Adenomatous Polyposis Coli) gene in adenoma formation and the *K-ras* oncogene in the transition from intermediate adenomas to carcinomas in sporadic CRC (1).

The *K-ras* oncogene has been found mutated in 10-15% of the screened adenomas smaller than 1 cm and in 30-60% of adenomas larger than 1 cm. Also, 30-60% of the adenocarcinomas has a *K-ras* mutation (3,5). It was suggested, therefore, that a mutated *K-ras* gene contributes to the transition of an intermediate adenoma to a late adenoma or carcinoma (2). The *K-ras* gene product, a 21 kD protein located at the inner plasma membrane, is involved in the transduction of mitogenic signals. The Ras protein is activated transiently as a response to extracellular signals such as growth factors, cytokines and hormones that stimulate cell surface receptors (6). The hallmark of Ras function is a switch between an inactive state, in which the proteins are bound to guanosine-diphosphates (GDP) and an active state in which conversion to guanosine-triphosphates (GTP) has occurred. This transit is governed by two types of regulatory proteins: GDP-GTP exchange factors that catalyse the GDP/GTP exchange and GTPase-activating proteins that enhance the intrinsic capacity of Ras proteins to hydrolyse GTP into GDP, thereby returning Ras to the inactive state (7).

Mutant, activated forms of Ras proteins have an impaired intrinsic GTPase activity, which renders the protein resistant to inactivation by regulatory GTPase-activating proteins (4). Approximately 90% of the activating mutations is found in codons 12 (wild type GGT) and 13 (wild type GGC) of exon 1 and around 5% in codon 61 (wild type CAA) located in exon 2 (8,10). Previous studies from various countries have revealed specific point mutations in codons 12 and 13. The most frequently observed types of mutations are G>A transitions (11) and G>T transversions (12) and these alterations were found associated with sex and sublocalisation of the tumour (9).

To date, frequencies and specific types of point mutations in the *K-ras* oncogene in CRC have been investigated in several studies (4,5,10,13). These reports were generally based on small numbers of selected patients. In the current study, however, the frequency of *K-ras* mutations is studied in a large series of unselected, incident CRC patients from The Netherlands identified in a prospective cohort study. Potential differences in family history of CRC, tumour sublocalisation, Dukes' stage and tumour differentiation with respect to presence and type of the *K-ras* mutations are studied.

MATERIALS AND METHODS

Study Population

The participants in this study are incident CRC cases from The Netherlands Cohort Study on diet and cancer (NLCS). The NLCS has been described in detail elsewhere (14). Briefly, the prospective study was initiated in 1986 and includes 58,279 men and 62,573 women, aged between 55 and 69 years old, who completed a self-administered questionnaire on diet, family history of cancer and other risk factors for cancer at baseline. The entire cohort is being monitored for cancer occurrence by annual record linkage to The Netherlands Cancer Registry (NCR) and to PALGA, a nationwide database of pathology reports (15). From 1989 until 1994, with exclusion of the first 2.3 years of follow-up due to incomplete nationwide coverage of PALGA, 819 incident cases with histological confirmed CRC have been identified. The PALGA database was also used to identify the location of

tumour tissue storage in the Dutch pathology laboratories. CRC was classified according to site as follows: proximal colon, i.e. 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 CRC (at baseline) was retrieved from the NLCS database. Information about tumour sublocalisation, Dukes' stage and differentiation of the tumour was retrieved from the NCR database.

Table 1 Characteristics of CRC cases (N=737) subdivided into cases without a *K-ras* (N=466) mutation and cases with at least one *K-ras* mutation (N=271)

	Total (N=737)	Wild type <i>K-ras</i> (N=466)	<i>K-ras</i> mutation (N=271)	p-value ^a
Age at diagnosis (mean $\pm$ SD)	67.95 $\pm$ 4.26	67.75 $\pm$ 4.21	68.29 $\pm$ 4.32	0.10
Sex (% Men)	410 (56%)	257 (55%)	153 (57%)	0.73
Family history of CRC (% Yes)	76 (10%)	53 (11%)	23 (9%)	0.21

^a comparing cases with at least one *K-ras* mutation to cases without a *K-ras* mutation

Tissue Samples

Tumour material was collected after approval by the Medical Ethical Committees (MEC) of the Maastricht University, PALGA and the NCR. Subsequently, all pathology laboratories in The Netherlands agreed to make relevant tissue samples available for this study. Tumour tissue sample collection started in August, 1999 and was completed in December, 2001. The 819 tissue samples were distributed among 54 pathology laboratories throughout The Netherlands, and only 44 (5%) tumour tissue samples could not be traced. Finally, 775 (95%) of the eligible tissue samples have been retrieved and 737 (90%) of the tissue samples contained sufficient tumour material as confirmed by a pathologist (AdB) and hence were available for molecular analyses. Archival tissue sample blocks were registered and coded using a consecutive, unique identification number.

Five CRC cell lines, i.e. HT29, Colo205, CaCo2, SW480 and HCT116 (American Type Culture Collection (ATCC), Rockville, Md, USA) were used to check the specificity of *K-ras* mutation detection. In order to validate mutation analysis on paraffin-embedded tissue, 10

Table 2 Number and type of mutations, affected codons, and corresponding altered amino acids in exon 1 of the *K-ras* gene

Codon ^a	Type of point mutation ^a	Number of point mutations	Wild type codon ^a (amino acid ^b)	Mutated codon ^a (amino acid ^b)	Putative altered amino acid
8	A>G	3 (1%)	GTA (val)	GTG (val)	3
9	Insertion ^c	1 (0.4%)	GTT (val)	GTG (val)-GAG (glu)-CTT (leu)	1
10	G>A	1 (0.4%)	GGA (gly)	AGA (arg)	1
12	G>A	91 (33%)	GGT (gly)	GAT (asp)	75 (37%)
				AGT (ser)	16 (8%)
	G>T	87 (31%)		GTT (val)	70 (35%)
				TGT (cys)	17 (8%)
	G>C	23 (8%)		GCT (ala)	16 (8%)
				CGT (arg)	7 (3%)
13	G>A	58 (21%)	GGC (gly)	GAC (asp)	58 (94%)
	G>T	3 (1%)		TGC (arg)	3 (5%)
	G>C	1 (0.4%)		CGC (cys)	1 (2%)
15	G>A	1 (0.4%)	GGC (gly)	AGC (ser)	1
16	G>A	1 (0.4%)	AAG (lys)	AAA (lys)	1
19	G>A	1 (0.4%)	TTG (leu)	TTA (leu)	1
20	C>T	8 (3%)	ACG (thr)	ATG (met)	8
25	G>A	2 (0.8%)	CAG (glu)	CAA (glu)	2

^a For 10 patients, 2 mutations were found and these are included in this table

^b gly=glycine, asp=aspartic acid, ser=serine, val=valine, cys=cysteine, ala=alanine, arg=arginine, thr=threonine, met=methionine, glu=glutamine, leu=leucine, glz=glutamic acid

^c The insertion is 6 nucleotides (TGGAGC), located after the first position of codon 9, resulting in 3 altered codons

fresh CRC specimens were divided into two adjacent tissue blocks, one of which was fresh-frozen, and one routinely-fixed and embedded in paraffin. These specimens were obtained from patients who did not participate in the NLCS.

DNA Isolation

Sections (5 µm) were cut from paraffin-embedded tumour tissue blocks and stained with haematoxylin & eosin (H&E) for histopathological examination. For DNA isolation, five 20 µm sections of tumour tissue were used. Deparaffination of the sections was performed and, using the H&E section as a reference, tumour tissue was macrodissected from the normal colonic epithelium and scraped off.

Genomic DNA was extracted from macrodissected tumour tissue using the Puregene® DNA isolation kit (Gentra Systems). Briefly, 475 µl cell lysis solution and 25 µl proteinase K stock solution (20 mg/ml, obtained from Qiagen, St. Louis, MO, USA) were added to the tissue samples and incubated overnight at 55°C. Subsequently, DNA was extracted for 72 hours at 37°C, protein was removed and DNA was precipitated using 100% 2-propanol and dissolved in hydration buffer. The DNA concentration and purity was measured at 260 and 280 nm.

DNA from fresh, unfixed CRC cell lines and the fresh-frozen tissue samples was extracted as described for paraffin-embedded sections.

Mutation Analysis

An exon 1 fragment of the *K-ras* gene was amplified from isolated, genomic DNA using a nested PCR approach. In the first reaction, a fragment of 179 base pairs (bp) was generated, using the sense primer 5'-AGG CCT GCT GAA AAT GAC TGA ATA-3' and antisense primer 5'-CTG TAT CAA AGA ATG GTC CTG CAC-3'. The annealing temperature was 58°C. The resulting fragment was used as a template to amplify a 114 bp fragment spanning codons 8 to 29. This PCR is performed using the biotinylated, sense primer 5'-AAA ATG ACT GAA TAT AAA CTT GTG G-3' and the antisense primer 5'-CTC TAT TGT TGG ATC ATA TTC GTC-3' at an annealing temperature of 50°C. The inside products were checked for purity and size by electrophoresis on a 2% agarose gel and subsequently used for direct sequencing.

Mutations in the exon 1 fragment of the *K-ras* gene were detected by direct sequencing with a solid phase sequencing kit (Amersham Pharmacia) using the ALFexpress II DNA sequencer (Pharmacia Biotech). In brief, the biotinylated PCR product is captured on a sequencing comb coated with streptavidin. After removal of the non-biotinylated strands by alkaline denaturation, the remaining immobilized strand was used as a template for dideoxy sequencing reactions with a Cy5 labeled primer 5'-CTC TAT TGT TGG ATC ATA TTC GTC CAC-3' and T7 DNA polymerase. The sequence profile is analysed on the ALFexpress II DNA Analysis System. Evaluation was independently performed using ALFwin software (Amersham Pharmacia Biotech) by two observers, based on the criterium that at an increase of least a 5% is observed for the mutant peak as well as an a decrease of at least a 5% in the wild type peak, relative to the wild type pattern in the same sequence run. Data-entry was performed blindly by two independent observers.

The mutation detection limit was determined by mixing wild type DNA from the cell line CaCo2 and the homozygously mutated cell line SW480 in amounts varying from 0-100%.

Statistical Analysis

The overall frequency of *K-ras* mutations as well as the type of mutation and affected codon was computed for all 737 cases with respect to age at diagnosis, sex, family history of CRC, tumour sublocalisation, Dukes' stage and tumour differentiation. Differences in mean

Table 3 Characterisation of *K-ras* mutations at gene and codon level according to tumour sublocalisation, Dukes' stage and differentiation of the tumour

	Total CRC cases (N=737) ^a	Wild type <i>K-ras</i> (N=466)	<i>K-ras</i> mutation (N=271) ^a
Sublocalisation^a			
Proximal colon	240	149 (62%)	91 (38%)
Distal colon	224	156 (70%)	68 (30%)
Rectosigmoid	85	51 (60%)	34 (40%)
Rectum	176	102 (58%)	74 (42%)
<i>p</i> -value ^b			0.09
Dukes' stage^c			
A	179	114 (64%)	65 (36%)
B	238	154 (65%)	84 (35%)
C	183	120 (66%)	63 (34%)
D	85	47 (55%)	38 (45%)
<i>p</i> -value ^b			0.40

^a For 8 patients without a *K-ras* mutation and 4 patients with at least one *K-ras* mutation, the site of localization in the colon could not be determined (colon, NOS)

^b comparing patients without a *K-ras* mutation to patients with at least one *K-ras* mutation, patients with at least one G>A transition (N=153) or those with at least one G>T transversion (N=88) or G>C transversion (N=23)

^c For 21 patients without a *K-ras* mutation and 31 patients with at least one *K-ras* mutation, information on Dukes' stage was not available

^d For 10 patients, two mutations were found. These patients were included in the analyses and treated as patients with at least one *K-ras* mutation, etc.

^e The frequencies of G>A transition, G>T or G>C transversion per subsite were calculated by dividing the number of patients with G>A transitions (for example) through the total number of CRC cases

values of age at diagnosis as a continuous variable was evaluated using the Student t-test. Differences in the categorical variables sex, family history of CRC, tumour sublocalisation, Dukes' stage and tumour differentiation between patients without and with *K-ras* mutations were evaluated for significance with the χ^2 -test. In addition, differences in tumour sublocalisation, Dukes' stage and tumour differentiation between patients without a *K-ras* mutation and patients with at least one *K-ras* mutation, patients with at least one G>A transition (N=153) or those with at least one G>T transversion (N=88) or G>C transversion (N=23) were evaluated with the χ^2 -test. A p-value of 0.05 or less was considered statistically significant. Statistical analyses were performed using the SPSS software (version 9.0).

RESULTS

Validation of Techniques

In the colorectal cancer cell lines HT29, Colo205 and CaCo2, wild type *K-ras* was found, whereas homozygously mutated alleles in SW480 and heterozygously mutated alleles in HCT116 cells were revealed with direct sequence analysis. For the SW480, line, a GGT to GTT mutation in codon 12 was observed and for the HCT116 line, a GGC to GAC mutation in codon 13.

The effect of tissue processing was assessed by comparing an adjacent fresh-frozen and a paraffin-embedded tissue block in a series of 10 fresh colon tumour specimens. In nine specimens, the *K-ras* status in the paraffin-embedded tissue was identical to fresh, unfixed tissue, i.e. six specimens with wild type *K-ras*, one with a G>T transversion at the second position of codon 12, one with a G>C transversion at the second position of codon 12 and one with a G>C transversion at the second position of codon 13. In one specimen, a G>C transversion at the third position of codon 19 was observed in the routinely-fixed, paraffin-embedded tissue, but not in DNA extracted from the fresh tissue.

Table 3 continued

G>A transition (N=153) ^{a,e}	Point mutation		Affected codon	
	G>T transversion (N=88) ^{a,e}	G>C transversion (N=23) ^{a,e}	Codon 12 (N=195) ^{a,e}	Codon 13 (N=62) ^{a,e}
22 (23%)	27 (11%)	7 (3%)	61 (25%)	26 (11%)
38 (17%)	20 (9%)	6 (3%)	50 (22%)	12 (5%)
20 (24%)	12 (14%)	2 (2%)	21 (25%)	12 (14%)
37 (21%)	28 (16%)	9 (5%)	60 (34%)	11 (6%)
0.39	0.22	0.48	0.07	0.03
46 (26%)	15 (8%)	2 (1%)	46 (26%)	17 (9%)
39 (16%)	36 (15%)	7 (3%)	61 (26%)	18 (8%)
37 (20%)	17 (9%)	9 (5%)	43 (23%)	17 (9%)
16 (19%)	15 (18%)	4 (5%)	26 (31%)	8 (9%)
0.13	0.04	0.17	0.68	0.88

The detection limit of mutation detection was determined by mixing wild type DNA isolated from the CaCo2 cell line with decreasing concentrations of mutated DNA which was prepared from the homozygously mutated colorectal cell line SW480. The lowest level of detection was five percent mutant DNA in a background of wild type DNA as found in three independent experiments.

To establish the reproducibility of the mutation analysis, 32 NLCS adenocarcinoma specimens were subjected twice to the complete procedure, from tissue sectioning to DNA sequencing. In 88% (28/32) of the samples, the same *K-ras* status was observed in the duplicate experiments.

Types of Mutations

In the exon 1 fragment of the *K-ras* oncogene, a total of 281 mutations was found in 271 (37%) out of 737 patients. No significant differences were observed in mean age at diagnosis (67.75 ± 4.21 versus 68.29 ± 4.32), sex (55% versus 57%) and family history of CRC (11% versus 9%) between patients without and patients with one or more *K-ras* oncogene mutations in the tumour (table 1).

For ten patients, two different mutations were observed in exon 1. In six of these patients, both mutations were found in codon 12, and the other four patients showed one mutation in codon 12 or codon 13 and one mutation in another codon. In eighteen patients without aberrations in codon 12 or codon 13, a mutation was found in codons 8, 9, 10, 15, 16, 20 or 25.

Table 2 summarises the frequencies of genetic aberrations by type of mutation, affected codons and corresponding amino acids in the exon 1 fragment of the *K-ras* oncogene. The most frequently observed mutations in the gene are G>A transitions, G>T transversions and G>C transversions, i.e. 55% (155/281 mutations), 32% (90/281 mutations) and 9% (24/281 mutations), respectively. Of the total number of mutations in the exon 1 fragment, 72% (201/281) was observed in codon 12 (GGT) and 22% (62/281) in codon 13 (GGC). In codon 12, the GAT codon (37%) leading to aspartic acid and the GTT codon (35%) leading to valine were the most frequently observed (table 2). In codon 13, the G>A transition at the second base, which would lead to substitution of a glycine by aspartic acid, was by far the predominant mutation (94%). Furthermore, the point mutations (17/281) observed in codons 8, 10, 15, 16, 19, 20 and 25 were all transitions. In one case, an insertion of 6 nucleotides was observed in codon 9, leading to one altered codon and two inserted codons, however,

without a frameshift in the gene (table 2). The only alteration in the protein is the insertion of two extra amino acids.

Tumour Sublocalisation

Patients with rectal tumours have relatively the highest frequency of *K-ras* mutations as compared to patients with proximal or distal colon tumours or patients with a rectosigmoid tumour (42% versus 38%, 30% and 40%, respectively; $p=0.09$) (table 3). Moreover, different tumour sublocalisations showed different frequencies of G>T transversions as well as different frequencies of codon 12 or codon 13 mutations. Patients with a rectal tumour have the highest frequency of G>T transversions (16% versus 11%, 9% and 14% for proximal, distal and rectosigmoid tumours, respectively; $p=0.22$) and codon 12 mutations (34% versus 25%, 22% and 25%, respectively; $p=0.07$) (table 3). It should be noted that most G>T transversions are confined to codon 12 (table 2). Patients with a tumour in the rectosigmoid, however, have the highest frequency of codon 13 mutations (14% versus 11%, 5% and 6% for proximal, distal and rectal tumour, respectively; $p=0.03$) (table 3). Stratification by sex, in addition, reveals that women with a rectal tumour have the highest prevalence of *K-ras* mutations (53% versus 37%, 23% and 37% for proximal, distal and rectosigmoid tumour, respectively; $p=0.001$). This difference was confined, in particular, to the G>T transversions (27% for rectal tumour versus 8%, 11% and 7% for proximal and distal colon tumour and rectosigmoid tumour, respectively; $p=0.002$) (table 4).

Table 4 Stratification by sex with respect to tumour sublocalisation

		Wild type <i>K-ras</i>	<i>K-ras</i> mutation ^a	G>A transition ^c	G>T transversion ^c	G>C transversion ^c
Men	Sublocalisation^a	252	151	87	47	11
	Proximal colon	71 (61%)	45 (39%)	25 (22%)	17 (15%)	0 (0%)
	Distal colon	84 (64%)	47 (36%)	27 (21%)	10 (8%)	6 (5%)
	Rectosigmoid	25 (57%)	19 (43%)	9 (20%)	9 (21%)	1 (2%)
	Rectum	72 (64%)	40 (36%)	24 (21%)	11 (9%)	5 (4%)
	<i>p-value^b</i>		0.77	0.99	0.07	0.13
Women	Sublocalisation^a	206	116	66	40	12
	Proximal colon	78 (63%)	46 (37%)	30 (24%)	10 (8%)	7 (6%)
	Distal colon	72 (77%)	21 (23%)	11 (12%)	10 (11%)	0 (0%)
	Rectosigmoid	26 (63%)	15 (37%)	11 (27%)	3 (7%)	1 (2%)
	Rectum	30 (47%)	34 (53%)	13 (20%)	17 (27%)	4 (6%)
	<i>p-value^b</i>		0.001	0.09	0.002	0.10

^a For 5 male patients and 3 female patients without a *K-ras* mutation and 2 male patients and 2 female patients with at least one mutation, the site of localisation in the colon could not be determined (colon, NOS)

^b comparisons between patients without a *K-ras* mutation and patients with at least one *K-ras* mutation, patients with at least one G>A transition, G>T or G>C transversion

^c The frequencies of G>A transition, G>T or G>C transversion per sublocalisation and sex was calculated by dividing the number of patients with G>A transitions (for example) through the total number of CRC patients

We also evaluated the *K-ras* mutational status for tumours in the proximal and distal colon and for tumours in the colon versus the rectum (tumours in the rectosigmoid were excluded for this analysis). It was found that patients with a proximal colon tumour had a higher frequency of G>A transitions as compared to patients with a distal colon tumour ($p=0.11$) (table 3). This difference in frequencies of G>A transitions with respect to proximal and distal colon tumours was more pronounced for women ($p=0.02$) (table 4). Patients with a rectal tumour showed a relatively higher frequency of *K-ras* mutations ($p=0.08$), and in particular G>T transversions ($p=0.06$), as compared to patients with a colon tumour. Again, this difference was due to the high frequency of G>T transversions in rectal tumours observed in women ($p=0.0003$) (table 4).

Dukes' Stage

Most tumours were staged as Dukes' B (35%, 238/685), and tumours with Dukes' D stage (12%, 85/685) constitute the smallest group (table 3). Patients with a Dukes' A staged tumour more often have G>A transitions (26% versus 16%, 20% and 19% for Dukes' B, C and D, respectively; $p=0.13$), whereas patients with a Dukes' D staged tumour have relatively a higher frequency of G>T transversions (18% versus 8%, 15% and 9% for Dukes' A, B or C, respectively; $p=0.04$). No clear differences were observed in the frequencies of codon 12 or codon 13 mutations with respect to the Dukes' stage of the tumour (table 3).

Tumour Differentiation

No differences were observed in frequencies of *K-ras* mutations, G>A transitions, G>T or G>C transversions or mutations in codon 12 or codon 13 ($p>0.05$) with respect to tumour differentiation.

DISCUSSION

Overall, we observed that the frequency of *K-ras* oncogene mutations in a large representative sample of CRC patients from The Netherlands (age at diagnosis between 57 and 76 years) was 37%. The frequency of *K-ras* gene mutations in CRC patients reported in the literature, ranges from 30-60% (3,9,11,12,16-27). This broad range of reported frequencies of *K-ras* mutations may be due to various factors, such as the sensitivity and specificity of mutation detection methods, small series of, selected, patients (3,12,17,20,21,23) and/or variability in analysed gene region, i.e. only codons 12 (21), 13 (12,20,22,23,28) and/or 61 (11). Also, environmental factors may be involved. Techniques used for mutation screening such as temperature gradient gel electrophoresis (TGGE) (3) or SSCP (17,29) and methods for mutation detection like PCR-restriction fragment length polymorphism (RFLP) (20,28) or PCR-based mutant allele-specific amplification (MASA) (20) may show differences in sensitivity and/or specificity of mutation detection in the *K-ras* gene. In this study, the analysis of *K-ras* mutations is based on a highly sensitive and specific detection method, i.e. direct sequencing of purified PCR fragments. This method identifies mutations in DNA samples which contain at least five percent or more mutated DNA. The reported frequency of *K-ras* mutations could, therefore, be an underestimation. Direct sequencing was validated by mutation analysis of five CRC cell lines, and of adjacent blocks with paraffin-embedded and fresh tissue from a series of 10 CRC specimens. The mutation analysis of five CRC cell lines confirmed the reported sequences of exon 1 fragments of the *K-ras* oncogene (30). In addition, the *K-ras* mutation status as determined in paraffin-embedded tumour tissue was also found in fresh tumour tissue in 9 out of 10 specimens. In one specimen, the mutation detected in paraffin-embedded tissue was not found in fresh tissue which may be related to heterogeneity in the tumour tissue or a lack in the reproducibility of this sample. These results indicate that tissue processing does not significantly affect the reliability of mutation analysis in archival specimens. The reproducibility of the identification technique used in this study was found to be good, since duplication of the complete analytical procedure yielded the same results for 28 out of 32 (88%) adenocarcinomas. The *K-ras* gene mutation analysis is based on a relatively large series of non-selected, incident CRC patients, which indicates that the found frequency of *K-ras* mutations is representative for CRC patients in the Dutch population.

Slattery *et al* (31) have evaluated the association between several genetic alterations and the presence or absence of family history of colorectal cancer using incident colon cancer cases. These authors did not find an association with overall *K-ras* mutations, although patients with a G>T transversion of the second base of codon 12 were more likely

to have a family history of colorectal cancer compared to those without this specific type of point mutation. In our study, no significant differences were observed in family history of CRC between patients without and with a *K-ras* mutation. Our findings suggest that these genetic alterations in sporadic colorectal tumours are not associated with family history of CRC and that therefore diet, environment and/or lifestyle factors may contribute to the acquirement of *K-ras* gene mutations involved in the early phases of carcinogenesis. However, more studies evaluating other genetic markers and/or a different kind of population study, i.e. twin studies, may be necessary to address this issue.

Studies from various countries have analysed the frequency of the type of *K-ras* point mutation in colorectal cancer. These studies were conducted in the UK (24,25), former Yugoslavia (12), Czech Republic (21), Norway (9), Switzerland (18), Mexico (11), USA (26) and The Netherlands (17,19,20). All studies, except for the study performed in former Yugoslavia (12), have identified the G>A transition as the most frequently found type of *K-ras* mutation. In the current study, the G>A transition appeared also to be the predominant mutation. The pattern of specific alterations observed, i.e. G>A transitions and G>T transversions, could be due to differences in diet and/or other lifestyle factors. N-nitroso compounds, for example, in red and processed meat could induce G>A transitions (25) and this is supported by previous experimental studies (32,33). Two mechanisms, which could explain the G>A transition are the formation of guanine-adducts in the DNA and the silencing of the O⁶-methylguanine DNA methyltransferase (MGMT). MGMT is a DNA repair protein that removes adducts from the O⁶-position of guanine (34) in DNA. Promotor hypermethylation of the *MGMT* gene, a phenomenon often seen in colon cancer cells (28,35) and which leads to silencing of the gene, results in conversions of guanine-cytosine pairs to adenine-thymine pairs. Guanine-to-thymine transversions, however, are more likely to be induced by carcinogenic agents like the polycyclic aromatic hydrocarbons (PAHs) found in smoked and barbecued meat (36), dietary fats (37) and cigarette smoke (38).

In the present study, codons 12 and 13 were affected in 94% of the tumours with a *K-ras* mutation in the exon 1 fragment, and the majority of mutations would result in an amino acid substitution of glycine by aspartic acid (57%) or by valine (33%). Mutations in either of these codons could lead to an activated Ras protein. Al-Mulla *et al* (39) compared detailed crystal structures of the Ras protein with these two most frequently observed mutations in codon 12, i.e. substitutions of glycine by aspartic-acid or valine. The tightly bound complex of GTP in codon 12 glycine-to-valine mutant Ras protein may generate a more stable signal as compared to the codon 12 glycine-to-aspartic acid mutant Ras protein or the codon 12 wild type Ras protein. Moreover, Andreyev *et al* (27) have shown that the presence of the glycine to valine substitution in codon 12 leads to a decreased survival of CRC patients and suggest this alteration is important for cancer progression and also that it may predispose to a more aggressive biological behaviour in patients with advanced CRC. Span *et al* (19) also observed an association between specific *K-ras* point mutations and cancer progression. Recently, Bazan *et al* (40) reported biological relevance for codon 13 mutations in terms of colorectal cancer clinical outcome, such as Dukes' stage ($p < 0.05$). They also reported a possible role for codon 12 mutations in the mucinous differentiation pathway. Associations between specific *K-ras* point mutations and cancer progression were, however, not supported by our and other studies (3,26,41).

Eighteen patients were observed with an affected codon other than codons 12 or 13. It is unknown whether mutations in these codons lead to a constituent activation of the Ras protein. Our results show that mutations in codons other than codons 12 or 13 of exon 1 are rare. The observed pattern of mutated codons, i.e. 94% of the mutations were found in codons 12 and 13, is probably due to a selective growth advantage (42).

Previous studies have presented frequencies of *K-ras* mutations based on tissue samples of selected series of patients with various distributions of Dukes' staged tumours.

Some suggested an increase in the frequencies of the *K-ras* oncogene mutations with more advanced stages of Dukes' classification (12,17), whereas others (3,9,42) did not find any association between frequencies of point mutations and Dukes' stage. In the current study, all incident CRC cases were included regardless of the Dukes' stage. No significant differences in the distribution of Dukes' stage were observed between patients without a *K-ras* mutation and those with a specific *K-ras* gene mutation. It suggests that *K-ras* mutations are not involved in the progression of adenocarcinomas and that genetic aberrations occur in pathways, which do not depend on *K-ras* mutations during the more advanced stages of colorectal cancer.

Various topographical subdivisions of the large intestine have been proposed, according to different criteria (22,43,44,45). The so-called rectosigmoid can be considered as a rather more clinically applied term than an anatomically defined transitional zone between the colon and rectum. Although the rectum is considered to comprise the last 12 cm of the intestine proximal of the anal verge, clinicopathological data are inconsistent as to whether the tumours in this region can be assigned to the colon or rectum (46). In our study, the NCR database contains the clinicopathological data, including the sublocalisation of the tumour, as supplied by the clinicians. Therefore, in the current multicenter study, for so-called rectosigmoid tumours it was not possible to definitively assign a tumour to either the colon or rectum. Consequently, the rectosigmoid was excluded from analysis when comparing colonic versus rectal adenocarcinomas. Among similar lines, another topographical issue which we addressed was the comparison of proximal versus distal colon tumours. Differences could be expected between these two with respect to faecal content and composition, microbial flora and activity, and the local variations in distribution of intestinal epithelial cell types. Owing to the mentioned unclear definition of the term rectosigmoid tumour, analyses were performed with both the ex- and inclusion of rectosigmoid tumours within the group of distal colon tumours. The resulting asymmetry between proximal and distal colon tumours was maintained, regardless of these differences in type of analysis.

Patients with a rectal tumour have a relatively higher frequency of G>T transversions as compared to patients with a distal colon tumour and this difference appeared to be most pronounced for female patients. This was not found in other studies (3,21,26,44). However, Breivik *et al* (9) reported that *K-ras* mutations were not found in tumours located proximal to the descending colon of men under the age of 70, whereas rare mutations such as G>C transversions were almost exclusively observed in tumours of the rectum of women. Again, results based on a smaller number of samples (123 men and 125 women, stratified by age) could lead to biased, inconsistent associations. In the current study, G>C transversions also constitute a small group of specific point mutations, but were mainly observed in men with tumours in the distal colon and in women with tumours in the proximal colon. Plausible explanations for differences in tumour site could be the role of diet with respect to bowel transit time and bacterial fermentation of carbohydrates (47), production of volatile fatty acids (9) or exposure of colonic epithelium to potential dietary carcinogens (48). The relatively high frequency of G>T transversions in the rectum of women might be related to sex differences in faecal concentration and transit time. Both bowel transit time and frequency of constipation have been reported to be substantially higher in women than in men under similar conditions (49). The G>T transversions and also the generally higher frequency of *K-ras* mutations in women might be related to the time of contact with, and the concentration of, particular carcinogens. More aetiological insight in the underlying mechanisms is required to clarify this issue.

The *K-ras* mutational status was evaluated for differences in frequencies with respect to tumour differentiation. Most tumours were classified as 'moderately' differentiated. Generally, the distinction between good or moderately differentiated tumours is often

ambiguous. Therefore, the classification of tumour differentiation could be biased. Our findings do not support a role of the *K-ras* oncogene in tumour differentiation.

In conclusion, we observed a frequency of 37% for mutations in exon 1 fragment of the *K-ras* oncogene, predominantly in codons 12 and 13. The G>A transition and the G>T transversion are the most frequently observed mutations, with the G>T transversion primarily confined to codon 12. Patients with a rectal tumour have a relatively higher frequency of G>T transversion as compared to patients with distal colon tumour and this is confined to female patients. The pattern of *K-ras* point mutations observed in the several sublocalisations of the colorectal tract is suggestive for the involvement of dietary factors.

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3

Meat consumption and *K-ras* mutations in sporadic colon and rectal cancer in The Netherlands Cohort Study

Meat consumption and *K-ras* mutations in sporadic colon and rectal cancer in The Netherlands Cohort Study

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ABSTRACT

Associations between meat and fish consumption and *K-ras* mutations in colon and rectal cancer were investigated in The Netherlands Cohort Study on diet and cancer.

After 7.3 years of follow-up, excluding the first 2.3 years, 448 colon and 160 rectal cancer patients, and 2948 subcohort members were available for data-analyses. *K-ras* gene mutation analysis was performed on all archival adenocarcinomas. Case-cohort analyses were used to compute adjusted incidence rate ratios (RR) and 95% confidence intervals (CI) for colon and rectal cancer and for *K-ras* mutation status subgroups.

Total fresh meat, most types of fresh meat and fish were not associated with colon or rectal cancer, neither overall nor with *K-ras* mutation status. However, several weak associations were observed for wild type *K-ras*, i.e. an increased association for beef and colon tumours, and an inverse association for pork and colon and rectal tumours. For meat products, an increased association was observed with wild type *K-ras* tumours in the colon.

Although neither total fresh meat nor fish appeared to be associated with colon or rectal cancer risk, beef, pork and meat products are differentially associated with colon and rectal cancer risk, depending on *K-ras* mutation status.

INTRODUCTION

The current epidemiological evidence for associations of different types of meat, meat products and fish with colorectal cancer (CRC) risk is controversial (1-3). These observed inconsistencies could, in part, be due to the heterogeneity of the colon and rectal cancer endpoint that is studied. Associations may become more apparent when the molecular events involved in colorectal carcinogenesis are taken into account.

The majority of colon and rectal tumours develops from small adenomatous polyps through a well-defined sequence of morphological changes (4), a process that is associated with the acquisition of somatic mutations (5,6). A genetic alteration that occurs in adenomas (10%) as well as in carcinomas (40%) of the colon and rectum, is the oncogenic activation of the *K-ras* gene by mutations. Activating mutations are mainly found in codons 12 and 13 (5,7-9). The most frequently observed types of point mutations are the G>A transitions, G>T and G>C transversions (9-11).

The link between several types of fresh meat, meat products and fish, and the pattern of *K-ras* mutations in human colon and rectal cancer is not clear. To date, only a few case-control and case-case studies have been conducted on the association between the consumption of dietary factors and *K-ras* mutation status (12-17). Four studies reported on several types of meat consumption (12,14,15,17).

It has been suggested that N-nitroso compounds, present in processed meat or endogenously formed (12), may induce G>A transitions at the second base of codon 12 or 13 of the *K-ras* gene in the human colon (13). High temperature cooking of meat proteins generates heterocyclic aromatic amines (HAA), whereas the process of grilling and smoking of meat generates polycyclic aromatic amines (PAH) as a result of fat dropping on open flames. HAA-DNA-adducts can cause transitions and transversions, whereas PAH's could induce G>T transversions in the *K-ras* gene. Results from animal experiments, however, are not consistent (14).

Consequently, high consumption of meat and fish could contribute to the acquired genetic alterations in the *K-ras* oncogene observed in colon and rectal tumours. Therefore, associations between dietary consumption of different types of meat, meat products and fish, and the risk of specific point mutations in the *K-ras* oncogene in patients with colon and rectal cancer were studied within the framework of The Netherlands Cohort Study on diet and cancer (NLCS).

MATERIALS AND METHODS

Study Population

The study subjects are incident colon and rectal cancer cases and subcohort members from the NLCS, which has been described in detail elsewhere (15). Briefly, the cohort study was initiated in 1986 and includes 58,279 men and 62,573 women, aged 55-69 years old at baseline, who originated from 204 Dutch municipalities with computerized population registries. A self-administered questionnaire on diet and other risk factors for cancer was completed at baseline. The entire cohort is being monitored for cancer occurrence by annual record linkage to The Netherlands Cancer Registry (NCR, nine cancer registries in The Netherlands) and to PALGA, a nationwide network and registry of histo- and cytopathology (www.palga.nl) (16). In the municipalities included in the NLCS, the NCR and PALGA, together, have nearly 100% coverage since the start of the study (17-19). PALGA also provides necessary information on the identification of the pathology laboratory location of the storage of paraffin-embedded blocks of the eligible CRC patients. Accumulation of

person-time in the cohort has been estimated through biennial vital status follow-up of a subcohort of 3,500 men and women who were randomly selected after baseline exposure measurement. Cases with prevalent cancer other than non-melanoma skin cancer were excluded from the subcohort, which left 3,346 men and women for analyses.

The first 2.3 years of follow-up were excluded due to incomplete coverage of PALGA alone in some of the municipalities included in the NLCS. Within this period, 83 subcohort members were either deceased or diagnosed with cancer other than non-melanoma skin cancer, leaving 3,263 men and women for analysis. From 1989 till 1994, 929 incident cases with histologically confirmed CRC were observed of whom 819 could also be linked to a PALGA report of the lesion. The PALGA database was used to identify and locate tumour tissue in Dutch pathology laboratories. CRC was classified according to site as follows: colon i.e. cecum through sigmoid colon (ICD-O-1 codes: 153.0, 153.1, 153.2, 153.3, 153.4, 153.5, 153.6, 153.7, 153.8, 153.9), rectosigmoid (ICD-O-1 code 154.0) and rectum (ICD-O-1 code 154.1). Information about age at baseline, sex and family history of CRC (at baseline) was retrieved from the NLCS database.

Tissue Samples

This study is based on data of gene mutation analysis from CRC patients, described in detail elsewhere (9). Briefly, tumour material of all CRC patients was collected after approval by the Medical Ethics Committees of Maastricht University, the NCR and PALGA. Subsequently, all pathology laboratories in The Netherlands agreed to make relevant tissue samples available upon request from PALGA. Tissue samples of the 819 cases were distributed among 54 pathology laboratories throughout The Netherlands. Tumour tissue specimen collection started in August 1999 and was completed in December 2001. The loss to follow-up of tissue samples of cases amounted to 5%. Tissue samples from nine patients registered in one pathology laboratory could not be retrieved due to administrative inconsistencies, leaving 810 tissue samples for collection. For 34 cases, paraffin-embedded material was not available in the archives of pathology laboratories, leaving 776 cases for the determination of the *K-ras* mutation status. For 39 cases (5%), the *K-ras* mutation status could not be determined, i.e. for 20 cases only normal colonic mucosa was available, 10 cases were revised with an benign adenoma (AdB) instead of an adenocarcinoma, for six cases the yield of DNA was not sufficient to determine *K-ras* mutation status and for three cases the available tissue did not include malignant CRC tissue. Finally, tumour material from 737 incident colorectal adenocarcinoma cases was available of whom 476 were colon cancer cases, 85 were rectosigmoid cancer cases and 176 were rectal cancer cases. Statistical analyses were performed separately for colon and rectal cancer as differences in the aetiology of colon and rectal cancer have been reported (1). 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-analyses. Moreover, the number of patients with a rectosigmoid tumour was too small for adequate stratified analyses (9).

Detection of *K-ras* Mutations

Mutation analysis of the exon 1 fragment of the *K-ras* oncogene, spanning codons 8-29, was performed on archival colorectal adenocarcinoma specimens of all 737 CRC patients using macrodissection, nested Polymerase Chain Reaction (PCR) and direct sequencing of purified fragments, which has been described in detail elsewhere (9). The method of mutation detection was validated by the confirmation of reported *K-ras* status in CRC cell lines and a good correlation between fresh-frozen and routinely-fixed, paraffin-embedded tissue. The detection limit was 5% mutated DNA. Duplicate analyses revealed a good

reproducibility (88%) (9). Two observers (GR and ML) independently performed evaluation of mutation analysis and data entry.

The Food Frequency Questionnaire

The dietary section of the questionnaire was a 150-item semi-quantitative food frequency questionnaire, which concentrated on habitual consumption of food and beverages during the year preceding the start of the study. Daily mean nutrient intakes were calculated using the computerized Dutch food composition table (20), by multiplying frequencies and portion sizes of all food items with their tabulated nutrient contents. The questionnaire was validated against a 9-day diet record (21). The Spearman correlation coefficients for total fresh meat, meat products and fish were 0.46, 0.54 and 0.53, respectively (21). 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 257 subjects (28 incident colon adenocarcinoma cases, 16 incident rectal adenocarcinoma cases and 215 subcohort members; two subcohort members were also colon or rectal cancer cases), dietary data were incomplete or inconsistent, and they were excluded from the analyses. These subjects either 1) left 60 or more (out of 150) questionnaire items blank and ate fewer than 35 items at least once per month and/or 2) left one or more item blocks (groups of items, i.e., beverages) blank. Additional details are given elsewhere (21). Hence, 448 colon and 160 rectal cases and 3048 subcohort members were available for data-analyses.

The food frequency questionnaire contained 14 items on the consumption of meat with the main (hot) meal (mainly fresh meat, including chicken), 5 items on the consumption of meat products, which are used as sandwich filling, and 3 items on fish consumption. For the serving size of total fresh meat, a question was included on the quantity of meat usually purchased (per person, per meal). Fresh meat is defined as meat that has not undergone some form of preservation, i.e. smoking, fermentation, and/or treatment with nitrate and/or nitrite salt ("curing") and which includes beef, pork, minced meat, chicken, liver and other meat (i.e. sausages). Coding of fresh meat items was based on raw weight to take into account the amount of fat originally present in the meat but ultimately ending up into the gravy, which is usually consumed as well. Meat products are defined as meat items that have undergone some form of preservation (mostly cured, sometimes also smoked or fermented). For chicken and fish, standard serving sizes were used. Meat items included in the questionnaire were converted into mean daily consumption in grams.

Quartiles of the consumption of total fresh meat, beef, pork, liver, minced meat, other meat, and meat products were computed for men and women separately, based on the distribution of subcohort members as described in detail later. For chicken and fish, groups were classified into a non-user and three user categories (5.3-13.2, 13.2-22.8 and ≥ 22.8 g/day for chicken and 0-10, 10-20 and ≥ 20 g/day for fish) and this classification was used for both men and women.

Daily intake of dietary fibre (g/day), alcohol (g/day), fruit (g/day), vegetables (g/day) and total energy (kcal/day) and age at baseline (years), sex (men/women), Quetelet Index (QI; kg/m^2), physical activity (<30 min/d, 30-60 min/d, 60-90 min/d, >90 min/d), family history of CRC (yes/no) and smoking status (never/ex/current) were regarded as potential confounders.

Table 1 Meat variables and other characteristics (mean $\pm$ SD) of the study population at baseline

	Subcohort	Colon cancer	
		Wild type <i>K-ras</i>	Mutated <i>K-ras</i>
N	3048	297	151
Sex (% Men)	48.4	51.5	58.9
Age (y)	61.4 $\pm$ 4.2	62.7 $\pm$ 4.0	63.8 $\pm$ 4.1
<i>Meat variables</i>			
Total fresh meat (g/day)	99.7 $\pm$ 42.2	98.4 $\pm$ 37.0	99.3 $\pm$ 39.1
Beef (g/day)	25.7 $\pm$ 22.5	27.9 $\pm$ 21.8	27.6 $\pm$ 23.6
Pork (g/day)	38.4 $\pm$ 30.3	36.3 $\pm$ 30.1	37.6 $\pm$ 29.6
Minced meat (g/day)	18.3 $\pm$ 17.3	17.2 $\pm$ 13.4	18.0 $\pm$ 16.0
Liver (g/day)	2.0 $\pm$ 4.4	1.9 $\pm$ 4.2	1.8 $\pm$ 4.1
Chicken (g/day)	13.7 $\pm$ 15.6	13.2 $\pm$ 15.5	12.4 $\pm$ 15.2
Other meat (g/day)	2.5 $\pm$ 6.4	2.9 $\pm$ 8.0	2.7 $\pm$ 5.9
Meat products (g/day)	13.2 $\pm$ 15.0	15.0 $\pm$ 16.6	11.7 $\pm$ 13.2
Fish and shellfish (g/day)	12.7 $\pm$ 15.0	11.4 $\pm$ 13.5	13.2 $\pm$ 15.7
<i>Other dietary factors</i>			
Fiber (g/day)	27.0 $\pm$ 8.2	26.7 $\pm$ 7.6	27.7 $\pm$ 8.8
Alcohol (g/day)	10.1 $\pm$ 14.1	11.0 $\pm$ 15.4	10.8 $\pm$ 14.2
Fruit (g/day)	176.3 $\pm$ 117.7	172.7 $\pm$ 123.7	176.9 $\pm$ 122.2
Vegetable (g/day)	193.6 $\pm$ 82.3	183.3 $\pm$ 78.2	198.0 $\pm$ 87.0
Energy (kcal/day)	1919.4 $\pm$ 518.1	1916.5 $\pm$ 494.4	1902.7 $\pm$ 472.9
<i>Other characteristics</i>			
QI (kg/m ²)	25.1 $\pm$ 3.1	25.5 $\pm$ 3.2	25.8 $\pm$ 3.3
Family history of CRC (% Yes)	5.6	13.5	9.3
Smoker (%)			
Never	37.0	36.7	37.1
Ex smoker	35.2	43.1	46.4
Current smoker	27.8	20.2	16.6
Physical activity (%)^a			
<30 minutes/day	20.9	19.7	20.3
30-60 minutes/day	32.7	33.7	32.4
60-90 minutes/day	30.9	29.6	29.1
>90 minutes/day	15.5	17.0	18.2

^a comparing cases with at least one *K-ras* mutation to cases without a *K-ras* mutation

¹ 6 out of 151 colon cancer cases had more than one *K-ras* mutation

² 3 out of 67 rectal cancer cases had more than one *K-ras* mutation

* For 41 subcohort members and 9 colorectal cancer cases, information on physical activity was not available

Statistical Analysis

The overall frequency of *K-ras* mutations as well as the type of mutation were computed for all colon and rectal cancer cases as described elsewhere (9). Mean values of the continuous variables age at baseline (years), consumption of total fresh meat, beef, pork, minced meat, chicken, liver, other meat, meat products, fish, dietary fiber, alcohol, fruit, vegetables, total energy and QI were evaluated for subcohort members and colon and rectal cancer cases with wild type and mutated *K-ras* gene. Distributions of the categorical variables sex, family history of CRC, smoking status and physical activity were evaluated for subcohort members and colon and rectal cancer patients with wild type and mutated *K-ras* gene and tested for differences between patient groups with the χ^2 -test. Differences in mean values of the continuous variables between patients with wild type and mutated *K-ras* gene were tested with the Student T-test or the Mann-Whitney-U-test if the variables were not normally distributed. The statistical software package SPSS (version 9) was used for these analyses.

Incidence rate ratios (RR) and corresponding 95% confidence intervals (CI) were estimated for colon and rectal cancer cases with wild type or mutated *K-ras* gene tumours. RRs were computed using Cox regression models with the STATA statistical software package (intercooled STATA, version 7) according to consumption of quartiles or

Table 1 continued

<i>p</i> -value ^a	Rectal cancer		<i>p</i> -value ^a
	Wild type <i>K</i> -ras	Mutated <i>K</i> -ras	
	93	67	
0.14	72.0	55.2	0.03
0.006	62.6 ± 4.1	62.2 ± 4.0	0.50
0.82	100.6 ± 40.3	95.7 ± 36.6	0.43
0.90	27.6 ± 24.6	22.5 ± 23.3	0.19
0.67	32.2 ± 27.0	40.8 ± 29.3	0.06
0.57	23.2 ± 25.2	16.7 ± 12.4	0.03
0.29	2.1 ± 4.1	1.9 ± 4.4	0.90
0.55	14.5 ± 15.6	12.3 ± 12.8	0.62
0.95	2.2 ± 4.1	2.7 ± 4.8	0.84
0.02	13.5 ± 14.7	15.0 ± 12.9	0.51
0.24	12.4 ± 13.1	11.0 ± 10.3	0.71
0.02	27.8 ± 8.0	27.6 ± 7.8	0.87
0.59	13.8 ± 17.6	11.2 ± 12.2	0.30
0.73	184.3 ± 145.8	180.7 ± 129.9	0.87
0.07	192.9 ± 72.4	188.0 ± 110.6	0.74
0.78	2027.3 ± 517.4	1997.0 ± 449.1	0.70
0.42	24.9 ± 2.8	25.5 ± 2.9	0.20
0.20	9.7	11.9	0.66
	25.8	34.3	
	41.9	43.3	
0.62	32.3	22.4	0.31
	18.5	22.7	
	27.2	27.3	
	32.6	34.8	
0.98	21.7	15.2	0.73

categories and one standard deviation (SD) of increase in fresh meat, meat products and fish, all based on the distribution in the subcohort. The lowest quartile or category of consumption was regarded as the reference category. The person-years at risk, estimated from the subcohort, were used in the denominator of the incidence rates (15). Standard errors were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling from the cohort. This method is equivalent to the variance-covariance estimator as presented by Barlow (22). The proportional hazards assumption was tested using the scaled Schoenfeld residuals (23). Those variables that were found to contribute substantially ($p < 0.10$) to the multivariate model for colon and/or rectal cancer (age, sex, QI, smoking, energy intake and family history of CRC) were included as covariates in all multivariate analyses. Interactions between total fresh meat, meat products and fish consumption on one hand and sex on the other hand were tested for colon and rectal cancer separately and not found to be statistically significant. Therefore, results for men and women are presented together. Finally, age at baseline, sex, family history of CRC, smoking status, QI and the intake of energy were confounders for either one or both of the models, i.e. with colon or rectal cancer cases, and were therefore included as covariates for all models to be tested. For each analysis, linear trends were evaluated with the Wald test of the regression coefficient estimate by fitting ordinal exposure variables (quartiles/categories of consumption) as continuous terms.

Table 2 Incidence rate ratios (RR) and 95% confidence intervals (CI) for colon (N=448) and rectal (N=160) cancer patients overall according to the intake of total fresh meat, meat products and fish

		Quartile/Category of intake				P for trend	RR (95% CI) for one SD increase in intake ^a
		1 ¹	2	3	4		
Total fresh meat							
Median intake (g/d)							
men		61.0	91.0	110.7	150.8		
women		50.7	80.3	103.4	139.2		
colon cancer cases		109	112	123	104		
rectum cancer cases		40	48	40	32		
personyears		3661	3701	3660	3715		
RR (95% CI) ²	colon	1.00	1.03 (0.77-1.37)	1.16 (0.88-1.54)	0.99 (0.74-1.33)	0.80	0.97 (0.89-1.07)
	rectum	1.00	1.21 (0.78-1.86)	1.03 (0.65-1.61)	0.82 (0.51-1.33)	0.33	0.94 (0.80-1.09)
RR (95% CI) ³	colon	1.00	1.05 (0.78-1.41)	1.12 (0.83-1.49)	0.96 (0.70-1.31)	0.92	0.96 (0.87-1.06)
	rectum	1.00	1.11 (0.71-1.73)	0.93 (0.59-1.47)	0.72 (0.44-1.19)	0.14	0.89 (0.76-1.05)
Beef							
Median intake (g/d)							
men		4.1	16.1	30.0	51.4		
women		3.0	14.0	25.5	46.9		
colon cancer cases		100	98	108	142		
rectum cancer cases		39	49	32	40		
personyears		3648	3742	3507	3840		
RR (95% CI) ²	colon	1.00	0.94 (0.70-1.27)	1.08 (0.80-1.44)	1.23 (0.93-1.63)	0.09	1.05 (0.96-1.15)
	rectum	1.00	1.22 (0.79-1.88)	0.84 (0.52-1.37)	0.90 (0.57-1.43)	0.35	0.95 (0.79-1.13)
RR (95% CI) ³	colon	1.00	0.98 (0.72-1.33)	1.09 (0.81-1.48)	1.28 (0.96-1.72)	0.06	1.06 (0.97-1.17)
	rectum	1.00	1.25 (0.80-1.96)	0.82 (0.49-1.36)	0.92 (0.57-1.49)	0.38	0.94 (0.78-1.14)
Pork							
Median intake (g/d)							
men		7.9	28.0	44.3	76.0		
Women		5.0	22.5	40.1	66.2		
colon cancer cases		121	120	109	98		
rectum cancer cases		44	46	36	34		
personyears		3659	3658	3688	3733		
RR (95% CI) ²	colon	1.00	1.03 (0.78-1.35)	0.94 (0.71-1.24)	0.86 (0.76-1.37)	0.24	0.96 (0.87-1.07)
	rectum	1.00	1.07 (0.70-1.64)	0.84 (0.53-1.33)	0.80 (0.50-1.28)	0.22	0.90 (0.76-1.07)
RR (95% CI) ³	colon	1.00	0.98 (0.74-1.30)	0.90 (0.67-1.20)	0.77 (0.57-1.04)	0.07	0.93 (0.83-1.04)
	rectum	1.00	1.01 (0.66-1.56)	0.81 (0.51-1.28)	0.70 (0.43-1.13)	0.09	0.87 (0.72-1.03)
Minced Meat							
Median intake (g/d)							
men		3.2	11.5	21.0	37.8		
Women		0.0	9.6	18.1	32.9		
colon cancer cases		104	119	128	97		
rectum cancer cases		33	38	54	35		
personyears		3648	3718	3692	3679		
RR (95% CI) ²	colon	1.00	1.12 (0.84-1.49)	1.28 (0.97-1.70)	0.95 (0.70-1.28)	0.99	0.95 (0.86-1.04)
	rectum	1.00	1.13 (0.70-1.82)	1.67 (1.07-2.61)	1.07 (0.66-1.74)	0.38	1.09 (0.94-1.26)
RR (95% CI) ³	colon	1.00	1.11 (0.83-1.50)	1.26 (0.94-1.69)	0.93 (0.68-1.27)	0.88	0.95 (0.85-1.05)
	rectum	1.00	1.06 (0.66-1.73)	1.60 (1.01-2.52)	1.01 (0.62-1.67)	0.50	1.08 (0.92-1.27)
Liver							
Median intake (g/d)							
men		0.0	4.1				
Women		0.0	3.7				
colon cancer cases		295	153				
rectum cancer cases		99	61				
personyears		9589	5148				
RR (95% CI) ²	colon	1.00	1.02 (0.83-1.26)			0.84	1.00 (0.91-1.11)
	rectum	1.00	1.16 (0.83-1.62)			0.38	1.02 (0.88-1.18)
RR (95% CI) ³	colon	1.00	1.04 (0.84-1.29)			0.71	1.02 (0.92-1.12)
	rectum	1.00	1.11 (0.79-1.57)			0.54	1.02 (0.88-1.18)

Table 2 continued

		Quartile/Category of intake				P for trend	RR (95% CI) for one SD increase in intake ^a
		1 ¹	2	3	4		
Chicken							
Median intake (g/d)							
men/women		0.0	5.3	13.2	22.8		
colon cancer cases		125	101	98	124		
rectum cancer cases		35	44	33	48		
personyears		3457	3564	3616	4100		
RR (95% CI) ²	colon	1.00	0.81 (0.61-1.07)	0.78 (0.58-1.04)	0.86 (0.66-1.13)	0.30	0.96 (0.86-1.07)
	rectum	1.00	1.26 (0.80-2.00)	0.92 (0.56-1.50)	1.18 (0.76-1.86)	0.77	1.00 (0.86-1.16)
RR (95% CI) ³	colon	1.00	0.85 (0.63-1.14)	0.81 (0.60-1.08)	0.87 (0.66-1.15)	0.34	0.95 (0.85-1.07)
	rectum	1.00	1.26 (0.79-2.03)	0.94 (0.57-1.55)	1.12 (0.70-1.79)	0.96	0.98 (0.83-1.14)
Other meat							
Median intake (g/d)							
men		0.0	6.0				
Women		0.0	7.0				
colon cancer cases		313	135				
rectum cancer cases		105	55				
personyears		10421	4316				
RR (95% CI) ²	colon	1.00	1.13 (0.91-1.41)			0.25	1.05 (0.96-1.15)
	rectum	1.00	1.29 (0.91-1.81)			0.15	0.98 (0.86-1.11)
RR (95% CI) ³	colon	1.00	1.16 (0.92-1.46)			0.20	1.08 (0.98-1.18)
	rectum	1.00	1.34 (0.95-1.91)			0.10	1.00 (0.88-1.15)
Meat products*							
Median intake (g/d)							
men		1.0	7.4	15.8	33.3		
women		0.0	4.3	10.5	22.4		
colon cancer cases		113	94	118	123		
rectum cancer cases		44	30	39	47		
personyears		3588	3624	3794	3732		
RR (95% CI) ²	colon	1.00	0.87 (0.65-1.17)	1.06 (0.80-1.40)	1.13 (0.86-1.49)	0.22	1.04 (0.95-1.14)
	rectum	1.00	0.70 (0.43-1.12)	0.88 (0.57-1.38)	1.09 (0.71-1.66)	0.54	1.01 (0.89-1.16)
RR (95% CI) ³	colon	1.00	0.89 (0.65-1.21)	1.06 (0.78-1.42)	1.17 (0.86-1.59)	0.19	1.05 (0.94-1.16)
	rectum	1.00	0.68 (0.41-1.11)	0.84 (0.53-1.35)	1.04 (0.64-1.68)	0.70	0.97 (0.84-1.13)
Fish							
Median intake (g/d)							
men		0.0	4.6	14.8	30.5		
women		0.0	4.6	15.5	28.2		
colon cancer cases		123	122	116	87		
rectum cancer cases		48	33	48	31		
personyears		4235	3238	4444	2820		
RR (95% CI) ²	colon	1.00	1.31 (1.00-1.73)	0.92 (0.70-1.21)	1.05 (0.78-1.41)	0.64	0.94 (0.85-1.04)
	rectum	1.00	0.86 (0.54-1.36)	0.93 (0.62-1.41)	0.89 (0.56-1.42)	0.70	0.91 (0.79-1.06)
RR (95% CI) ³	colon	1.00	1.30 (0.97-1.73)	0.82 (0.62-1.09)	1.03 (0.76-1.40)	0.40	0.93 (0.83-1.04)
	rectum	1.00	0.88 (0.55-1.43)	0.97 (0.64-1.48)	0.94 (0.59-1.52)	0.89	0.93 (0.80-1.08)

¹ reference category/quartile of intake² RR adjusted for age and sex³ RR adjusted for age, sex, QI, smoking, energy intake and family history of CRC^a see for one standard deviation of increase based on the intake of the subcohort (table 1)^{*} RR per increment of 15 g/day (SD in subcohort), equivalent to one standard sandwich filling

RESULTS

The overall frequency and spectrum of mutations in the *K-ras* gene have been presented in detail elsewhere (9). In brief, a total of 227 mutations was found in 218 (36%) out of 608 colon and rectal cancer patients. The most frequently observed mutations are the

Table 3 Adjusted RR¹ for colon and rectal cancer patients with a K-ras mutation status² according to the intake of fresh meat, meat products and fish

		Quartile/Category of intake				P for trend	RR (95% CI) for one SD increase in intake ^a
		1 ³	2	3	4		
Total fresh meat							
<i>Cases (K-ras-/K-ras+)</i>							
	colon	68/35	80/29	76/45	66/35		
	rectum	25/15	21/24	25/14	18/12		
RR _{K-ras} (95% CI)	colon	1.00	1.16 (0.82-1.64)	1.05 (0.74-1.49)	0.94 (0.65-1.36)	0.61	0.95 (0.84-1.07)
	rectum	1.00	0.85 (0.47-1.55)	1.02 (0.58-1.80)	0.76 (0.41-1.44)	0.56	0.93 (0.75-1.15)
RR _{K-ras+} (95% CI)	colon	1.00	0.83 (0.50-1.37)	1.25 (0.79-2.00)	1.01 (0.61-1.66)	0.57	0.99 (0.85-1.16)
	rectum	1.00	1.53 (0.80-2.94)	0.82 (0.39-1.72)	0.68 (0.31-1.47)	0.10	0.85 (0.67-1.07)
Beef							
<i>Cases (K-ras-/K-ras+)</i>							
	colon	62/32	66/29	66/39	96/44		
	rectum	21/16	25/23	18/12	25/14		
RR _{K-ras} (95% CI)	colon	1.00	1.04 (0.72-1.49)	1.05 (0.72-1.51)	1.36 (0.96-1.93)	0.08	1.08 (0.97-1.20)
	rectum	1.00	1.16 (0.64-2.12)	0.89 (0.46-1.71)	1.06 (0.57-1.94)	0.93	1.02 (0.81-1.27)
RR _{K-ras+} (95% CI)	colon	1.00	0.87 (0.52-1.47)	1.18 (0.73-1.92)	1.14 (0.71-1.83)	0.38	1.04 (0.89-1.22)
	rectum	1.00	1.36 (0.70-2.63)	0.74 (0.34-1.61)	0.75 (0.35-1.61)	0.20	0.84 (0.61-1.16)
Pork							
<i>Cases (K-ras-/K-ras+)</i>							
	colon	85/33	74/42	68/40	63/29		
	rectum	31/12	25/19	18/18	15/16		
RR _{K-ras} (95% CI)	colon	1.00	0.86 (0.61-1.20)	0.77 (0.55-1.08)	0.72 (0.51-1.02)	0.05	0.91 (0.80-1.05)
	rectum	1.00	0.81 (0.48-1.39)	0.58 (0.32-1.04)	0.50 (0.26-0.93)	0.01	0.74 (0.57-0.96)
RR _{K-ras+} (95% CI)	colon	1.00	1.30 (0.80-2.09)	1.24 (0.76-2.02)	0.90 (0.53-1.53)	0.68	0.97 (0.82-1.15)
	rectum	1.00	1.52 (0.73-3.17)	1.38 (0.65-2.94)	1.21 (0.56-2.60)	0.75	1.03 (0.82-1.29)
Minced Meat							
<i>Cases (K-ras-/K-ras+)</i>							
	colon	66/34	79/37	89/36	56/37		
	rectum	19/14	17/19	32/20	21/12		
RR _{K-ras} (95% CI)	colon	1.00	1.16 (0.82-1.64)	1.37 (0.97-1.92)	0.85 (0.58-1.25)	0.68	0.93 (0.82-1.04)
	rectum	1.00	0.89 (0.46-1.74)	1.77 (0.99-3.19)	1.17 (0.62-2.19)	0.21	1.18 (0.99-1.40)
RR _{K-ras+} (95% CI)	colon	1.00	1.04 (0.64-1.68)	1.06 (0.64-1.74)	1.07 (0.65-1.75)	0.78	0.98 (0.83-1.16)
	rectum	1.00	1.28 (0.64-2.56)	1.36 (0.68-2.72)	0.81 (0.37-1.79)	0.66	0.87 (0.68-1.12)
Liver							
<i>Cases (K-ras-/K-ras+)</i>							
	colon	184/98	106/46				
	rectum	55/41	34/24				
RR _{K-ras} (95% CI)	colon	1.00	1.10 (0.86-1.42)			0.44	1.02 (0.91-1.14)
	rectum	1.00	1.15 (0.74-1.79)			0.54	1.05 (0.89-1.24)
RR _{K-ras+} (95% CI)	colon	1.00	0.92 (0.63-1.33)			0.65	1.01 (0.85-1.19)
	rectum	1.00	1.07 (0.63-1.80)			0.80	0.97 (0.74-1.28)
Chicken							
<i>Cases (K-ras-/K-ras+)</i>							
	colon	73/42	73/27	62/35	82/40		
	rectum	20/13	24/19	16/17	29/16		
RR _{K-ras} (95% CI)	colon	1.00	0.96 (0.68-1.36)	0.81 (0.57-1.16)	0.91 (0.65-1.28)	0.44	0.96 (0.84-1.09)
	rectum	1.00	1.19 (0.64-2.18)	0.76 (0.39-1.49)	1.23 (0.69-2.21)	0.75	1.04 (0.85-1.26)
RR _{K-ras+} (95% CI)	colon	1.00	0.64 (0.39-1.06)	0.80 (0.51-1.28)	0.80 (0.51-1.26)	0.52	0.95 (0.79-1.14)
	rectum	1.00	1.39 (0.68-2.85)	1.20 (0.57-2.53)	0.97 (0.46-2.03)	0.75	0.88 (0.68-1.15)
Other meat							
<i>Cases (K-ras-/K-ras+)</i>							
	colon	201/110	89/43				
	rectum	57/43	32/22				
RR _{K-ras} (95% CI)	colon	1.00	1.16 (0.89-1.52)			0.28	1.08 (0.96-1.21)
	rectum	1.00	0.90 (0.63-2.23)			0.14	0.97 (0.81-1.17)

Table 3 continued

		Quartile/Category of intake				P for trend	RR (95% CI) for one SD increase in intake ^a
		1 ¹	2	3	4		
Other meat							
RR _{K-ras+} (95% CI)	colon	1.00	1.16 (0.79-1.70)			0.44	1.07 (0.93-1.23)
	rectum	1.00	1.24 (0.73-2.10)			0.43	1.04 (0.87-1.24)
Meat products							
Cases (<i>K-ras</i> +/ <i>K-ras</i> +) <i>K-ras</i>							
	colon	65/40	59/33	76/41	90/30		
	rectum	28/14	20/9	18/20	23/22		
RR _{K-ras+} (95% CI)	colon	1.00	0.91 (0.63-1.33)	1.10 (0.76-1.57)	1.42 (1.00-2.03)	0.03	1.115 (0.99-1.25)
	rectum	1.00	0.71 (0.39-1.30)	0.62 (0.34-1.16)	0.84 (0.45-1.58)	0.51	0.91 (0.73-1.14)
RR _{K-ras+} (95% CI)	colon	1.00	0.84 (0.52-1.37)	1.00 (0.63-1.59)	0.77 (0.45-1.32)	0.51	0.90 (0.73-1.11)
	rectum	1.00	0.62 (0.26-1.46)	1.26 (0.61-2.61)	1.41 (0.67-2.99)	0.17	1.05 (0.86-1.27)
Fish							
Cases (<i>K-ras</i> +/ <i>K-ras</i> +) <i>K-ras</i>							
	colon	84/37	81/36	74/35	51/36		
	rectum	25/19	20/11	24/24	20/11		
RR _{K-ras+} (95% CI)	colon	1.00	1.30 (0.93-1.81)	0.80 (0.57-1.12)	0.87 (0.60-1.26)	0.13	0.88 (0.77-1.02)
	rectum	1.00	0.97 (0.53-1.79)	0.86 (0.48-1.52)	1.06 (0.58-1.94)	0.99	0.97 (0.81-1.17)
RR _{K-ras+} (95% CI)	colon	1.00	1.29 (0.79-2.10)	0.87 (0.54-1.42)	1.38 (0.85-2.25)	0.52	1.00 (0.85-1.19)
	rectum	1.00	0.76 (0.35-1.61)	1.13 (0.61-2.07)	0.78 (0.37-1.65)	0.84	0.86 (0.69-1.07)

¹ Multivariate adjusted RR for age, sex, QI, smoking, energy intake and family history of CRC and their 95% confidence intervals

² Wild type *K-ras* (*K-ras*-): no mutation in the exon 1 fragment of the gene, mutated *K-ras* (*K-ras*+: at least one mutation in the exon 1 fragment of the gene

³ Reference category/quartile of intake

⁴ See for one standard deviation of increase based on the intake of the subcohort (table 1)

⁵ RR per increment of 15 g/day (SD in subcohort), equivalent to one standard sandwich filling

G>A transitions (54%), G>T transversions (33%) and G>C transversions (7%). The observed frequencies of the mutations in this series of patients are similar to the frequencies of the 737 CRC cases, including the rectosigmoid cancer cases, for whom *K-ras* mutation status was determined (9).

Table 1 shows the baseline characteristics of the study population. Colon and rectal cancer cases were more often men, were older, more frequently reported a family history of CRC, had a higher daily alcohol intake and were less frequently never smokers as compared to the subcohort. Colon cancer cases with a *K-ras* mutation in their tumour had a lower daily consumption of meat products, a higher intake of dietary fibers and were significantly older than colon cancer cases with a wild type *K-ras* tumour (p-values 0.02, 0.02 and 0.006, respectively). There were no statistically significant differences between colon cancer cases with and without a *K-ras* mutation in their tumour in dietary consumption of total fresh meat, beef, pork, minced meat, liver, chicken, other meat, fish and other factors presented in table 1. Rectal cancer cases with a *K-ras* mutation in their tumour had a lower daily consumption of minced meat, a higher daily consumption of pork and were less frequently men than rectal cancer cases with a wild type *K-ras* gene in their tumour (p-values 0.03, 0.06 and 0.03, respectively). No statistically significant differences were observed between rectal cancer cases with and without a *K-ras* mutation in their tumours for other factors presented in table 1.

Associations between the consumption of total fresh meat, different types of fresh meat, i.e. beef, pork, minced meat, liver, chicken and other meat, meat products and fish, and the risk of colon or rectal cancer are presented in table 2. RR and 95% CI for colon and rectal cancer were presented after adjustment for age and sex and after adjustment for age,

Table 4 Adjusted RR¹ for colon and rectal cancer patients with specific point mutations² in the *K-ras* oncogene according to the intake of fresh meat, meat products and fish

		Quartile/Category of intake				P for trend	RR (95% CI) for one SD increase in intake ^a
		1 ^b	2	3	4		
Total fresh meat							
<i>Cases (G>A/ G>T or C)</i>							
	colon	16/16	16/12	31/13	19/15		
	rectum	11/5	12/13	7/6	3/8		
RR _{G>A} (95% CI)	colon	1.00	0.98 (0.48-1.98)	1.81 (0.97-3.40)	1.11 (0.55-2.22)	0.34	1.02 (0.85-1.22)
	rectum	1.00	1.11 (0.49-2.51)	0.62 (0.24-1.64)	0.28 (0.08-1.03)	0.02	0.71 (0.55-0.93)
RR _{G>T,C} (95% CI)	colon	1.00	0.75 (0.35-1.61)	0.83 (0.40-1.73)	1.00 (0.48-2.08)	0.97	1.01 (0.78-1.32)
	rectum	1.00	2.44 (0.86-6.92)	1.01 (0.31-3.27)	1.19 (0.38-3.68)	0.62	0.94 (0.65-1.36)
Beef							
<i>Cases (G>A/ G>T or C)</i>							
	colon	16/14	19/7	19/18	28/17		
	rectum	7/9	14/8	7/6	5/9		
RR _{G>A} (95% CI)	colon	1.00	1.13 (0.57-2.26)	1.13 (0.57-2.21)	1.41 (0.74-2.66)	0.30	1.11 (0.91-1.36)
	rectum	1.00	2.00 (0.79-5.06)	1.08 (0.37-3.11)	0.68 (0.21-2.26)	0.25	0.67 (0.47-0.95)
RR _{G>T,C} (95% CI)	colon	1.00	0.48 (0.19-1.19)	1.26 (0.62-2.58)	1.02 (0.50-2.08)	0.47	0.99 (0.78-1.26)
	rectum	1.00	0.81 (0.31-2.17)	0.63 (0.21-1.87)	0.82 (0.30-2.21)	0.64	1.06 (0.69-1.61)
Pork							
<i>Cases (G>A/ G>T or C)</i>							
	colon	19/13	23/18	25/12	15/13		
	rectum	6/6	10/11	10/7	7/8		
RR _{G>A} (95% CI)	colon	1.00	1.23 (0.65-2.30)	1.28 (0.69-2.40)	0.77 (0.38-1.56)	0.53	0.86 (0.70-1.06)
	rectum	1.00	1.67 (0.60-4.66)	1.71 (0.60-4.90)	1.23 (0.40-3.77)	0.72	0.92 (0.68-1.25)
RR _{G>T,C} (95% CI)	colon	1.00	1.43 (0.68-2.98)	0.98 (0.43-2.22)	1.07 (0.48-2.41)	0.88	1.10 (0.83-1.46)
	rectum	1.00	1.72 (0.63-4.71)	0.99 (0.33-2.97)	1.07 (0.37-3.13)	0.74	1.06 (0.74-1.51)
Minced meat							
<i>Cases (G>A/ G>T or C)</i>							
	colon	20/12	15/18	19/16	28/10		
	rectum	7/7	11/8	9/11	6/6		
RR _{G>A} (95% CI)	colon	1.00	0.71 (0.36-1.40)	0.94 (0.49-1.81)	1.34 (0.73-2.46)	0.24	1.09 (0.91-1.31)
	rectum	1.00	1.56 (0.61-4.01)	1.27 (0.47-3.47)	0.90 (0.30-2.72)	0.75	0.86 (0.61-1.22)
RR _{G>T,C} (95% CI)	colon	1.00	1.44 (0.69-3.04)	1.36 (0.62-2.95)	0.83 (0.36-1.95)	0.64	0.89 (0.67-1.18)
	rectum	1.00	1.06 (0.39-2.89)	1.51 (0.59-3.88)	0.77 (0.25-2.38)	0.85	0.88 (0.61-1.27)
Liver							
<i>Cases (G>A/ G>T or C)</i>							
	colon	56/36	26/20				
	rectum	23/18	10/14				
RR _{G>A} (95% CI)	colon	1.00	0.91 (0.55-1.49)			0.70	1.01 (0.82-1.26)
	rectum	1.00	0.79 (0.38-1.65)			0.53	0.53 (0.31-0.91)
RR _{G>T,C} (95% CI)	colon	1.00	1.12 (0.64-1.94)			0.70	1.10 (0.90-1.36)
	rectum	1.00	1.45 (0.69-3.05)			0.32	1.18 (0.92-1.50)
Chicken							
<i>Cases (G>A/ G>T or C)</i>							
	colon	24/17	14/10	21/13	23/16		
	rectum	7/6	11/10	8/9	7/7		
RR _{G>A} (95% CI)	colon	1.00	0.57 (0.29-1.13)	0.84 (0.46-1.53)	0.78 (0.43-1.42)	0.66	0.94 (0.74-1.18)
	rectum	1.00	1.59 (0.61-4.19)	1.08 (0.38-3.07)	0.85 (0.29-2.46)	0.52	0.88 (0.59-1.32)
RR _{G>T,C} (95% CI)	colon	1.00	0.59 (0.27-1.29)	0.74 (0.36-1.53)	0.81 (0.41-1.61)	0.70	0.95 (0.69-1.30)
	rectum	1.00	1.52 (0.55-4.19)	1.36 (0.47-3.91)	0.86 (0.29-2.59)	0.65	0.80 (0.54-1.18)
Other meat							
<i>Cases (G>A/ G>T or C)</i>							
	colon	55/42	27/14				
	rectum	20/22	13/10				
RR _{G>A} (95% CI)	colon	1.00	1.33 (0.82-2.17)			0.25	1.12 (0.97-1.31)
	rectum	1.00	1.57 (0.79-3.13)			0.20	1.15 (0.95-1.39)

Table 4 continued

		Quartile/Category of intake				P for trend	RR (95% CI) for one SD increase in intake*
		1 ³	2	3	4		
Other meat							
RR _{G>T,C} (95% CI)	colon	1.00	0.94 (0.50-1.77)			0.85	0.99 (0.76-1.29)
	rectum	1.00	1.10 (0.50-2.41)			0.81	0.90 (0.63-1.27)
Meat products*							
Cases (G>A/G>T or C)							
	colon	20/16	16/16	24/16	22/8		
	rectum	6/7	5/5	11/10	11/10		
RR _{G>A} (95% CI)	colon	1.00	0.79 (0.40-1.55)	1.11 (0.60-2.08)	1.08 (0.54-2.16)	0.61	0.98 (0.78-1.23)*
	rectum	1.00	0.89 (0.27-2.99)	1.98 (0.69-5.62)	2.37 (0.75-7.51)	0.07	1.14 (0.90-1.45)*
RR _{G>T,C} (95% CI)	colon	1.00	1.06 (0.52-2.17)	1.03 (0.50-2.11)	0.53 (0.22-1.28)	0.20	0.82 (0.55-1.23)*
	rectum	1.00	0.65 (0.20-2.16)	1.16 (0.41-3.24)	1.08 (0.38-3.05)	0.63	1.02 (0.77-1.35)*
Fish							
Cases (G>A/G>T or C)							
	colon	20/16	19/14	18/16	25/10		
	rectum	10/9	5/5	16/10	2/8		
RR _{G>A} (95% CI)	colon	1.00	1.27 (0.65-2.48)	0.81 (0.42-1.57)	1.73 (0.92-3.24)	0.27	1.12 (0.91-1.36)
	rectum	1.00	0.61 (0.21-1.81)	1.42 (0.65-3.12)	0.28 (0.06-1.26)	0.48	0.78 (0.58-1.06)
RR _{G>T,C} (95% CI)	colon	1.00	1.19 (0.57-2.50)	0.98 (0.48-1.98)	0.95 (0.43-2.10)	0.80	0.83 (0.65-1.07)
	rectum	1.00	0.78 (0.25-2.40)	1.01 (0.40-2.51)	1.20 (0.45-3.22)	0.68	0.96 (0.72-1.28)

¹ Multivariate adjusted RR for age, sex, QI, smoking, energy intake and family history of CRC and their 95% confidence intervals

² the presence of G>A transitions or G>T or G>C transversions in the exon 1 fragment of the K-ras gene

³ reference category/quartile of intake

* see for one standard deviation of increase based on the intake of the subcohort (table 1)

* RR per increment of 15 g/day (SD in subcohort), equivalent to one standard sandwich filling

sex, smoking, QI, energy intake and family history of CRC. The age-and-sex adjusted RR and the multivariate RR were similar. Frequent consumption of total fresh meat, minced meat, liver, chicken, other meat, meat products and fish was not significantly associated with the risk of colon or rectal cancer (table 2). A high intake of beef was borderline positively associated with the risk of colon cancer (RR for highest versus lowest quartile of consumption 1.28, 95% CI 0.96-1.72; p_{trend} 0.06). There was no association between beef and rectal cancer risk. A high consumption of pork was, however, borderline inversely associated with the risk of colon cancer (RR for highest versus lowest quartile of consumption 0.77, 95% CI 0.57-1.04; p_{trend} 0.07) and rectal cancer (RR for highest versus lowest quartile of consumption 0.70, 95% CI 0.43-1.13; p_{trend} 0.09).

Associations of total fresh meat, beef, pork, minced meat, liver, chicken, other meat, and meat products and fish with wild type or mutated K-ras gene in colon or rectal tumours are presented in table 3. Results will first be presented for tumours with a wild type K-ras gene. No clear associations were observed for the highest versus the lowest consumption of total fresh meat, minced meat, liver, chicken, other meat and fish and colon and rectal tumours with a wild type K-ras gene. A nonsignificant, increased association was observed between beef and wild type K-ras colon tumours (RR for highest versus lowest quartile of consumption 1.36, 95% CI 0.96-1.93; p_{trend} 0.08). No association was observed for beef and rectal tumours with a wild type K-ras gene. On the other hand, a high consumption of pork was inversely associated with wild type K-ras colon and rectal tumours (RR for highest versus lowest quartile of consumption 0.72, 95% CI 0.51-1.02 and 0.50, 95% CI 0.26-0.93, respectively). A high consumption of meat products showed a significant increased association with colon tumours with a wild type K-ras gene (RR for highest versus lowest quartile of intake 1.42, 95% CI 1.00-2.03; p_{trend} 0.03). No clear association was found for

high consumption of meat products and rectal tumours with a wild type *K-ras* gene. Regarding colon and rectal tumours that harbour a *K-ras* mutation, no clear associations were observed between highest versus lowest quartile of consumption of different types of fresh meat, meat products and fish.

Subgroup analyses were performed to evaluate the associations between total fresh meat, different types of fresh meat, meat products and fish, and specific types of *K-ras* point mutations (G>A transitions and G>T or G>C transversions) in colon and rectal tumours (table 4). Results will first be presented for tumours with a G>A transition in the *K-ras* gene. Total fresh meat consumption was not associated with G>A transitions in the colon tumours, but an inverse association with G>A transitions in the rectal tumours was observed. However, none of the specific types of fresh meat, i.e. beef, pork, minced meat, liver, chicken, other meat, neither fish were associated with the risk of G>A transitions in both colon and rectal tumours. For meat products, a borderline significant trend with increased risk of rectal tumours harbouring G>A transitions was observed (RR for highest versus lowest quartile of intake 2.37, 95% CI 0.75-7.51; p_{trend} 0.07). Regarding G>T or G>C transversions in colon and rectal tumours, no clear associations were observed for total fresh meat, the different types of fresh meat, meat products and fish.

DISCUSSION

In this large cohort study with 448 incident colon and 160 incident rectal cancer patients, no associations were observed between total fresh meat and fish, and the risk of colon or rectal cancer, either overall or after *K-ras* mutation status was taken into account. This was also observed for specific types of fresh meat. However, several weak associations were observed regarding tumours harbouring a wild type *K-ras* gene. An increased association for high consumption of beef and an inverse association for high consumption of pork, and the risk of colon tumours were observed. In addition, consumption of pork was also inversely associated with rectal tumours with a wild type *K-ras* gene. For meat products, an increased association was observed with wild type *K-ras* tumours in the colon and with G>A transitions in the *K-ras* gene in rectal tumours.

Earlier results on meat and CRC in the NLCS, based on 3.3 years of follow-up, showed no association for high consumption of total fresh meat and fish and colon cancer risk in men and women. A positive association for both men and women was observed for meat products which consists mainly of cured meat (24). Rectal cancer was not included in these analyses. These findings were in line with the findings of a meta-analysis conducted by Norat *et al* (3). In total, 29 studies were evaluated, including 22 case-control and 7 cohort studies for investigating the association between processed meat and CRC. A high consumption of processed meat was found to be associated with a moderate, but significant, increase in CRC risk. In the current study, after 7.3 years of follow-up and with the exclusion of the first 2.3 years, these findings were similar to the earlier results on meat and CRC in the NLCS, i.e. neither an association was observed for total fresh meat, different types of fresh meat, nor for fish. However, in the current study, no significant association was observed between meat products and colon or rectal cancer risk overall.

With regard to *K-ras* mutations, two case-control studies with colon cancer patients, one with 2418 patients and 2410 controls (25), the other with 185 patients (26,27), and one cross-sectional case-case study with rectal cancer patients (28) have previously been conducted and have reported on the association between meat consumption and *K-ras* mutations. In none of these studies an association between meat consumption and colon or rectal cancer was observed after the *K-ras* gene mutation status was taken into account.

The current cohort study on total fresh meat, different types of fresh meat, meat products and fish and specific *K-ras* mutations is, to our knowledge, the only prospective study performed to date. The prospective design of this study and the high completeness of follow-up of cancer incidence and of the subcohort, make information and selection bias unlikely. In addition, as a result of the exclusion of the first 2.3 years of follow-up, the chance of information bias due to potential preclinical colorectal cancer is minimal.

Epidemiological studies have indicated that consumption of broiled, fried, barbequed or smoked meats may increase the risk of CRC (29-31), although these findings were not consistent. Augustsson *et al* (31) observed an inverse association between high intake of heterocyclic aromatic amines (HAA) and the risk of colon and rectal cancer. However, Gerhardsson de Verdier *et al* (29) observed an increased association for total meat intake as well as frequent consumption of brown gravy and a preference for a heavily browned meat surface and the risk of CRC. The relative risks (RR) were higher for rectal than for colon cancer. High temperature cooking of meat proteins generates HAA, whereas the process of grilling and smoking of meat forms polycyclic aromatic amines (PAH) as a result of fat dropping on open flames. Animal and *in vitro* studies have shown that HAA-DNA-adducts can cause transitions and transversions, as observed in the *K-ras* oncogene. However, results from animal experiments are not entirely consistent (14). In humans, a higher prevalence of G>T transversions in the *K-ras* gene was observed in 37 colorectal tumours from Yugoslavia which the authors explain as possibly being due to the extensive use of barbequed and smoked meat throughout the year in Yugoslavia (11). Unfortunately, information on meat preparation was not collected at baseline in the current study. An increased association was found for high consumption of beef and overall colon cancer risk. When taking into account the presence or absence of a *K-ras* mutation, this association appeared to be confined to colon tumours with a wild type *K-ras* gene. A high consumption of pork, however, appeared to be inversely associated with both colon and rectal cancer with a wild type *K-ras* gene. In addition, consumption of beef and pork was not associated with the risk of colon and rectal tumours harbouring *K-ras* point mutations. Consequently, consumption of beef and pork seems to be of importance for colon and rectal tumours with a *K-ras* wild type genotype and may, exert its action through another pathway than the *K-ras* signalling route. The inverse associations for high consumption of pork can not readily be explained and need replication in other studies. In general, multiple comparisons were performed and, therefore, it could be that some of the observed associations are chance findings. Therefore, caution is warranted in interpreting the results.

It is hypothesized that fat content of meat could influence CRC risk by increasing the excretion of bile acids (3). The products of the bile acid excretion may act as tumour promoters by a non-specific effect that increases cell proliferation in the mucosa layer (3). However, total fat intake (32) as well as total fresh meat consumption were not associated with overall colon and rectal cancer risk nor with *K-ras* mutation status. In the previous study (32), only ω -6 polyunsaturated fat (PUFA) was observed to be associated with specific *K-ras* mutations, however, this type of fat is not predominantly present in meat.

In the current study, no clear association was observed for daily consumption of meat products and overall colon and rectal cancer risk. When the absence or presence of *K-ras* mutations was taken into account in the current study, a high intake of meat products was found to be positively associated with colon tumours with a wild type *K-ras* gene. As for high consumption of beef, the high consumption of meat products may be positively associated with colon cancer risk, although through another pathway than the *K-ras* signalling route. In addition, subgroup analysis of specific point mutations in the *K-ras* gene showed that high consumption of meat products is positively associated with rectal tumours harbouring a G>A transition. This is in line with the biological evidence obtained in animal studies (33) and

humans (13). A plausible explanation is the action of alkylating agents, such as nitrosamines. Nitrosamines and their precursors are compounds observed in red and processed meat (34) or can be endogenously formed (35). Alkylating DNA agents like nitrosamines could generate O⁶-methylguanines and these have been detected in human colonic tissue (36). N-nitroso compounds could also induce G>A transitions in codons 12 or 13 of the *K-ras* gene of rat colon carcinomas (33,37,38) or in human colonic tissue (35). Why the association in our study is confined to the rectum and is not observed for the colon remains unclear. A plausible explanation for differences in tumour site could be due to the duration of contact with, and the concentration of, the potential dietary carcinogens like nitrosamines. Possibly, the lower transit time of stool in the rectum as compared to the colon leads to an increased exposure time for the rectum. However, more aetiological insight in the underlying mechanisms is required to clarify this issue.

Our results suggest that total fresh meat and fish are not associated with colon or rectal cancer risk or with the *K-ras* mutation status of these cancer types. A high consumption of beef, pork and meat products is of importance, in particular for the aetiology of colon tumours without a *K-ras* mutation. Therefore, beef, pork and meat products may exert their actions in colon cancer through another pathway, which does not depend on the *K-ras* mutation status. Regarding rectal cancer, a high consumption of meat products appears to be a risk factor for G>A transitions in the *K-ras* gene.

In conclusion, although neither total fresh meat nor fish appeared to contribute to the risk of colon and rectal cancer, the consumption of beef, pork and meat products is differentially associated with colon and rectal cancer risk depending on *K-ras* mutation status.

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Fat and *K-ras* mutations in sporadic colon and rectal cancer in The Netherlands Cohort Study

Fat and *K-ras* mutations in sporadic colon and rectal cancer in The Netherlands Cohort Study

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ABSTRACT

Associations between dietary intake of various fats and specific *K-ras* mutations in colon and rectal cancer were investigated within the framework of The Netherlands Cohort Study on diet and cancer.

After 7.3 years of follow-up and with exclusion of the first 2.3 years, 448 colon and 160 rectal cancer patients and 2948 subcohort members (55-69 years at baseline) were available for data-analyses. Mutation analysis of the *K-ras* gene was performed on all archival colon and rectal adenocarcinoma specimens. Case-cohort analyses were used to compute adjusted incidence rate ratios (RR) and 95% confidence intervals (CI) for colon and rectal cancer cases and for *K-ras* mutation status subgroups.

Intake of total, saturated and monounsaturated fat was not significantly associated with colon or rectal cancer. High intake of dietary polyunsaturated fat (PUFA) and, specifically, linoleic acid is associated with an increased risk of mutated *K-ras* colon tumours. The RRs for one standard deviation of increase of PUFA and linoleic acid were 1.21 (95% CI 1.05-1.41) and 1.22 (95% CI 1.05-1.42), respectively and similar associations were observed for both G>A transitions and G>T or G>C transversions in the colon. In contrast, no significant associations were observed with rectal cancer risk, overall or with specific *K-ras* mutation status.

A high intake of PUFA, in particular linoleic acid, may be an important dietary risk factor for *K-ras* mutated colon tumours, possibly by generating G>A transitions or G>T or G>C transversions in the *K-ras* oncogene.

INTRODUCTION

The current epidemiological evidence for the association of total fat and specific fatty acids with colorectal cancer (CRC) risk is controversial (1,2). The observed inconsistencies could, in part, be due to the heterogeneity of the colon and rectal cancer endpoint that is studied. Associations may become more apparent when the molecular events involved in colorectal carcinogenesis are taken into account.

The majority of colon and rectal tumours develops from small adenomatous polyps through a well-defined sequence of cytological and morphological changes (3), a process that is associated with the acquisition of somatic mutations (4,5). A genetic alteration that occurs in adenomas (10%) as well as carcinomas (40%) in colon and rectal cancer, is the oncogenic activation of the *K-ras* gene by mutations. Activating mutations are mainly found in codons 12 and 13 (4,6-8). The most frequently observed types of point mutations are G>A transitions (8,9), and G>T and G>C transversions (10).

The link between fat intake and the pattern of mutations in human colon and rectal cancer is not clear. Only a few epidemiological studies have been conducted up to date on the association between the intake of fat and *K-ras* mutation status (11-14) and results are inconsistent. Experimental studies suggest that peroxidation of ω -6 poly-unsaturated fats (PUFA) could lead to the accumulation of by-products like malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). These compounds could react with DNA to form adducts, mainly the pyrimidopurine adduct of deoxyguanosine (M₁G) (15-17). The presence of the M₁G-adducts resulted predominantly in G>A and G>T, with very few G>C transversions in bacteria (16,18,19).

Consequently, exposures to specific dietary fat and fatty acids could contribute to the heterogeneity of acquired genetic alterations in the *K-ras* oncogene observed in colon and rectal tumours. Associations between dietary intakes of fat and specific fatty acids and the risk of specific point mutations in the *K-ras* oncogene in patients with colon and rectal cancer were studied within the framework of the Netherlands Cohort Study on diet and cancer (NLCS).

MATERIALS AND METHODS

Study Population

The participants in this study are incident, colon and rectal cancer cases and subcohort members from the NLCS, which has been described in detail elsewhere (20). Briefly, the study was initiated in 1986 and includes 58,279 men and 62,573 women, aged 55-69 years old at baseline, who originated from 204 Dutch municipalities with computerized population registries. A self-administered questionnaire on diet and other risk factors for cancer was completed at baseline. The entire cohort is being monitored for cancer occurrence by annual record linkage to the Netherlands Cancer Registry (NCR, nine cancer registries in the Netherlands) and to PALGA, a nationwide network and registry of histo- and cytopathology (www.palga.nl) (21). In the municipalities included in the NLCS, the NCR and PALGA, together, have nearly 100% coverage since the start of the study (22-25). PALGA also provides necessary information on the identification of the pathology laboratory location of the storage of paraffin-embedded blocks of the eligible CRC patients. Accumulation of person-time in the cohort has been estimated through biennial vital status follow-up of a subcohort of 3,500 men and women who were randomly selected after baseline exposure measurement (21). Cases with prevalent cancer other than non-melanoma skin cancer were excluded from the subcohort, which left 3,346 men and women for analysis.

Table 1 Nutrient intake (mean $\pm$ SD) and other characteristics of the study population at baseline

	Subcohort	Colon cancer	
		Wild type <i>K-ras</i>	Mutated <i>K-ras</i>
N	2948	297	151
Sex (% Men)	48.2	51.5	58.9
Age (y)	61.3 $\pm$ 4.2	62.7 $\pm$ 4.0	63.8 $\pm$ 4.1
<i>Fat variables*</i>			
Total fat (g/d)	83.8 $\pm$ 15.8	84.3 $\pm$ 15.4	86.7 $\pm$ 14.8
Saturated fat (g/d)	33.2 $\pm$ 7.5	33.5 $\pm$ 6.9	33.6 $\pm$ 6.7
MUFA (g/d)**	31.4 $\pm$ 7.0	31.7 $\pm$ 6.5	32.1 $\pm$ 6.9
PUFA (g/d)***	17.3 $\pm$ 7.5	17.2 $\pm$ 7.3	19.3 $\pm$ 7.6
Linolenic acid (g/day)	1.3 $\pm$ 0.6	1.6 $\pm$ 0.7	1.3 $\pm$ 0.6
Linoleic acid (g/day)	16.0 $\pm$ 7.5	1.2 $\pm$ 0.5	18.0 $\pm$ 7.7
<i>Other dietary factors</i>			
Fiber (g/d)	27.0 $\pm$ 8.2	26.7 $\pm$ 7.6	27.7 $\pm$ 8.8
Alcohol (g/d)	10.1 $\pm$ 14.1	11.0 $\pm$ 15.4	10.8 $\pm$ 14.2
Fruit (g/d)	177.0 $\pm$ 118.0	172.7 $\pm$ 123.7	176.9 $\pm$ 122.2
Vegetable (g/d)	193.8 $\pm$ 82.2	183.3 $\pm$ 78.2	198.0 $\pm$ 87.0
Energy (kcal/d)	1919.1 $\pm$ 517.1	1916.5 $\pm$ 494.4	1902.7 $\pm$ 472.9
<i>Other characteristics</i>			
QI (kg/m ²)	25.1 $\pm$ 3.1	25.5 $\pm$ 3.2	25.8 $\pm$ 3.3
Family history [§] (% Yes)	5.7	13.5	9.3
Smoker (%)			
Never	36.9	36.7	37.1
Ex smoker	35.2	43.1	46.4
Current smoker	27.8	20.2	16.6
Physical activity (%)			
<30 minutes/day	20.1	19.7	20.3
30-60 minutes/day	32.2	33.7	32.4
60-90 minutes/day	31.1	29.6	29.1
>90 minutes/day	15.4	17.0	18.2

* comparing cases with at least one *K-ras* mutation to cases without a *K-ras* mutation

* adjusted for energy by the residual method (45)

** monounsaturated fat

*** polyunsaturated fat

§ family history of CRC

The first 2.3 years of follow up were excluded due to incomplete coverage of PALGA alone in some of the municipalities included in the NLCS. Within this period, 83 subcohort members were either deceased or diagnosed with cancer other than non-melanoma skin cancer, leaving 3,263 men and women for analysis. From 1989 till 1994, 929 incident cases with histologically confirmed CRC were observed of whom 819 could also be linked to a PALGA report of the lesion. The PALGA database was used to identify and locate tumour tissue in Dutch pathology laboratories. CRC was classified according to site as follows: colon i.e. cecum through sigmoid colon (ICD-O-1 codes: 153.0, 153.1, 153.2, 153.3, 153.4, 153.5, 153.6, 153.7, 153.8, 153.9), rectosigmoid (ICD-O-1 code 154.0) and rectum (ICD-O-1 code 154.1). Information about age at baseline, sex and family history of CRC (at baseline) was retrieved from the NLCS database.

Tissue Samples

This study is based on data of gene mutation analysis from CRC patients, described in detail elsewhere (8). Briefly, tumour material of all CRC patients was collected after approval by the Medical Ethics Committees of Maastricht University, the NCR and PALGA. Subsequently, all pathology laboratories in The Netherlands agreed to make relevant tissue samples available upon request from PALGA. Tissue samples of the 819 cases were distributed among 54 pathology laboratories throughout The Netherlands. Tumour tissue

Table 1 Continued

<i>p</i> -value ^a	Rectal cancer		<i>p</i> -value ^a
	Wild type <i>K-ras</i>	Mutated <i>K-ras</i>	
	93	67	
0.14	72.0	55.2	0.03
0.006	62.6 ± 4.1	62.2 ± 4.0	0.50
0.12	86.7 ± 15.3	86.0 ± 14.2	0.76
0.89	34.1 ± 6.8	34.3 ± 6.9	0.87
0.54	32.2 ± 6.6	32.5 ± 5.6	0.80
0.006	18.5 ± 8.9	17.5 ± 8.2	0.46
0.18	1.2 ± 0.6	1.3 ± 0.5	0.41
0.009	17.4 ± 8.9	16.2 ± 8.3	0.41
0.02	27.8 ± 8.0	27.6 ± 7.8	0.87
0.59	13.8 ± 17.6	11.2 ± 12.2	0.30
0.73	184.3 ± 145.8	180.7 ± 129.9	0.87
0.07	192.9 ± 72.4	188.0 ± 110.6	0.74
0.78	2027.3 ± 517.4	1997.0 ± 449.1	0.70
0.42	24.9 ± 2.8	25.5 ± 2.9	0.20
0.20	9.7	11.9	0.66
	25.8	34.3	
	41.9	43.3	
0.62	32.3	22.4	0.31
	18.5	22.7	
	27.2	27.3	
	32.6	34.8	
0.98	21.7	15.2	0.73

specimens collection started in August 1999 and was completed in December 2001. The loss to follow-up of tissue samples of cases amounted to 5%. Tissue samples from nine patients registered in one pathology laboratory could not be retrieved due to administrative inconsistencies, leaving 810 tissue samples for collection. For 34 cases, paraffin-embedded material was not available in the archives of pathology laboratories, leaving 776 cases for the determination of the *K-ras* mutation status. For 39 cases (5%), the *K-ras* mutation status could not be determined, i.e. for 20 cases only normal colonic mucosa was available, 10 cases were revised with an benign adenoma (AdB) instead of an adenocarcinoma, for six cases the yield of DNA was not sufficient enough to determine *K-ras* mutation status and for three cases the available tissue did not include malignant CRC tissue. Finally, tumour material from 737 incident colorectal adenocarcinoma cases was available of whom 476 were colon cancer cases, 85 were rectosigmoid cancer cases and 176 were rectal cancer cases. Statistical analyses were performed separately for colon and rectal cancer as differences in the aetiology of colon and rectal cancer have been reported (1). 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 analyses. Moreover, the number of patients with a rectosigmoid tumour was too small for adequate stratified analyses (8).

Detection of *K-ras* Mutations

Mutation analysis of the exon 1 fragment of the *K-ras* oncogene, spanning codons 8-29, was performed on archival colorectal adenocarcinoma specimens of all 737 CRC patients using macrodissection, nested Polymerase Chain Reaction (PCR) and direct sequencing of

purified fragments, which has been described in detail elsewhere (8). The method of mutation detection was validated by the confirmation of reported *K-ras* status in CRC cell lines and a good correlation between fresh-frozen and routinely-fixed, paraffin-embedded tissue. The detection limit was 5% mutated DNA. Duplo analyses revealed a good reproducibility (88%) (8). Evaluation of mutation analysis and data entry was independently performed by two observers (GR and ML).

The Food Frequency Questionnaire

The dietary section of the questionnaire was a 150-item semi-quantitative food frequency questionnaire, which concentrated on habitual consumption of food and beverages during the year preceding the start of the study. Daily mean nutrient intakes were calculated using the computerized Dutch food composition table (26), by cumulating the multiplied frequencies and portion sizes of all food items with their tabulated nutrient contents. The questionnaire was validated against a 9-day diet record (27). Crude and energy-gender-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 PUFA (27). For energy intake the correlation coefficient was 0.74. On average, the questionnaire covered 91% of the record intake explaining part of the underestimation of energy intake from the questionnaire data (as presented in table 1). 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 257 subjects (28 incident colon adenocarcinoma cases, 16 incident rectal adenocarcinoma cases and 215 subcohort members, two subcohort members were also colon or rectal cancer cases), dietary data were incomplete or inconsistent, and they were excluded from the analyses. These subjects either 1) left 60 or more (out of 150) questionnaire items blank and ate fewer than 35 items at least once per month and/or 2) left one or more item blocks (groups of items, e.g., beverages) blank. Additional details are given elsewhere (27). Hence, 608 colon and rectal cancer cases and 3048 subcohort members were available for data analyses.

Intake of specific fatty acids was based on a food composition database with specific fatty acids derived from the TRANSFAIR study (28). 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 on average 93 percent, but varies across food sources. Daily intakes of total fat (g/day), saturated fat (g/day), monounsaturated fat (MUFA) (g/day), PUFA (g/day), and linolenic acid (g/day) and linoleic acid (g/day) as main constituents of PUFA, were used as exposure variables. Linolenic and linoleic acid were used as the most abundant sources of ω -3 PUFA and ω -6 PUFA. For data-analyses, quartiles of the intake of fat and fatty acids were computed based on the distribution of subcohort members. Daily intake of dietary fiber (g/day), alcohol (g/day), fruit (g/day), vegetables (g/day) and total energy (kcal/day) and age at baseline (years), sex (men/women), Body Mass Index (BMI; kg/m²), physical activity (<30 min/d, 30-60 min/d, 60-90 min/d, >90 min/d), family history of CRC (yes/no) and smoking status (never/ex/current) were regarded as potential confounders.

Statistical Analysis

The overall frequency of *K-ras* mutations as well as the type of mutation was computed for all colon and rectal cancer cases as described elsewhere (8). Fat intake was adjusted for energy by the residual method (29). Mean values of the continuous variables age at

baseline (years), intake of total fat, saturated fat, MUFA, PUFA, linolenic acid, linoleic acid, dietary fiber, alcohol, fruit, vegetables and total energy and QI were evaluated for subcohort members and colon and rectal cancer cases with wild type and mutated *K-ras* gene. Distributions in the categorical variables sex, family history of CRC, smoking status and physical activity were evaluated for subcohort members and colon and rectal cancer patients with wild type and mutated *K-ras* gene. Differences in mean values of the continuous variables and the distributions in categorical variables between patients with wild type and mutated *K-ras* tumours were tested with the Student T-test and χ^2 -test, respectively, using the statistical software package SPSS (version 9).

Incidence rate ratios (RR) and corresponding 95% confidence intervals (CI) for colon and rectal cancer cases with wild type or mutated *K-ras* gene tumours were estimated according to intakes of quartiles (with the lower quartile of intake regarded as the reference group) and of one standard deviation (SD) of increase of intake of total fat, saturated fat, MUFA, PUFA, linolenic acid and linoleic acid using Cox proportional hazards models with the STATA statistical software package (intercooled STATA, version 7). The total person-years at risk, estimated from the subcohort, were used in the analyses (27). Standard errors were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling from the cohort. This method is equivalent to the variance-covariance estimator as presented by Barlow (30). The proportional hazards assumption was tested using the scaled Schoenfeld residuals (31). Using the backwards stepwise procedure, all confounders were tested in the overall colon and rectal cancer models separately. Interactions between fat and specific fatty acid intake and sex were tested for colon and rectal cancer separately and never found to be statistically significant. Therefore, results for men and women are presented together. Finally, age at baseline, sex, family history of CRC, smoking status, QI and the intake of energy were confounders for either one or both of the models and were therefore included as covariates for all models to be tested. Since 100 subcohort members had missing values for QI, results in the tables will be presented for 2948 subcohort members. For each analysis, trends were evaluated with the Wald test by fitting ordinal exposure variables (quartiles of intake) as continuous terms.

RESULTS

The overall frequency and spectrum of mutations in the *K-ras* gene have been presented in detail elsewhere (8). In brief, a total of 227 mutations was found in 218 (36%) out of 608 colon and rectal cancer patients. The most frequently observed mutations are the G>A transitions (54%), G>T transversions (33%) and G>C transversions (7%). The observed frequencies of the point mutation are similar to the frequencies of the 737 CRC cases, including rectosigmoid cancer cases, for whom *K-ras* status was determined (8).

Table 1 shows various types of fat intake and other baseline characteristics of the study population. Colon and rectal cancer cases were more often men, were older, more frequently reported a family history of CRC and had a higher daily alcohol intake as compared to the subcohort. Colon cancer cases with a mutated *K-ras* tumour were significantly older, had higher daily intakes of PUFA, linoleic acid, fibers and vegetables than colon cancer cases with a wild type *K-ras* tumour. There were no statistically significant differences between colon cancer cases with a wild type *K-ras* tumour and a mutated *K-ras* tumour in dietary intakes of total fat, saturated fat, MUFA, linolenic acid and other factors presented in table 1. Rectal cancer cases with a mutated *K-ras* tumour were less frequently men as compared to rectal cancer cases with a wild type *K-ras* tumour. No statistically significant differences between rectal cancer cases with mutated *K-ras* tumours and wild

Table 2 Incidence rate ratios (RR) and 95% confidence intervals (CI) for colon (N=448) and rectal cancer patients (N=160) according to the intake of total fat and specific fatty acids

		Quartile of intake				P for trend	RR (95% CI) for one SD increase in intake ^a
		1 ¹	2	3	4		
Total fat							
Median intake (g/d)							
men		78.1	90.3	98.5	109.1		
Women		63.0	71.5	77.6	85.3		
colon cancer cases		113	126	95	114		
rectum cancer cases		43	42	34	40		
personyears		3657	3675	3741	3664		
RR (95% CI) ²	colon	1.00	1.09 (0.82-1.43)	0.80 (0.60-1.08)	0.99 (0.74-1.32)	0.49	1.02 (0.90-1.15)
	rectum	1.00	0.96 (0.62-1.49)	0.75 (0.47-1.20)	0.92 (0.59-1.44)	0.52	0.94 (0.78-1.13)
RR (95% CI) ³	colon	1.00	1.09 (0.82-1.44)	0.74 (0.55-1.01)	0.95 (0.71-1.27)	0.28	1.00 (0.88-1.13)
	rectum	1.00	0.89 (0.56-1.39)	0.74 (0.46-1.19)	0.86 (0.55-1.36)	0.41	0.91 (0.76-1.09)
Saturated fat							
Median intake (g/d)							
men		28.9	33.7	38.3	45.8		
Women		23.9	27.8	31.0	36.6		
colon cancer cases		101	123	119	105		
rectum cancer cases		43	35	46	36		
personyears		3645	3703	3709	3679		
RR (95% CI) ²	colon	1.00	1.20 (0.90-1.59)	1.14 (0.85-1.51)	0.97 (0.72-1.30)	0.72	0.98 (0.89-1.09)
	rectum	1.00	0.80 (0.50-1.26)	1.04 (0.67-1.59)	0.80 (0.51-1.26)	0.58	0.97 (0.83-1.15)
RR (95% CI) ³	colon	1.00	1.19 (0.88-1.60)	1.10 (0.82-1.49)	0.93 (0.69-1.26)	0.51	0.97 (0.87-1.08)
	rectum	1.00	0.79 (0.49-1.28)	1.02 (0.65-1.59)	0.73 (0.46-1.17)	0.37	0.95 (0.80-1.12)
MUFA**							
Median intake (g/d)							
men		28.2	33.2	36.9	42.5		
Women		22.4	26.1	28.9	33.0		
colon cancer cases		101	124	119	104		
rectum cancer cases		45	34	41	40		
personyears		3660	3650	3714	3713		
RR (95% CI) ²	colon	1.00	1.23 (0.92-1.63)	1.17 (0.88-1.56)	1.03 (0.76-1.37)	0.97	1.00 (0.89-1.11)
	rectum	1.00	0.76 (0.48-1.21)	0.91 (0.59-1.41)	0.89 (0.57-1.38)	0.77	0.93 (0.79-1.10)
RR (95% CI) ³	colon	1.00	1.19 (0.89-1.60)	1.12 (0.82-1.51)	0.98 (0.73-1.33)	0.77	0.99 (0.88-1.12)
	rectum	1.00	0.70 (0.44-1.13)	0.91 (0.57-1.44)	0.87 (0.56-1.37)	0.80	0.93 (0.78-1.11)
PUFA***							
Median intake (g/d)							
men		11.6	16.0	20.9	29.1		
Women		8.8	12.4	16.2	22.5		
colon cancer cases		95	124	113	116		
rectum cancer cases		47	33	39	41		
personyears		3656	3683	3726	3667		
RR (95% CI) ²	colon	1.00	1.35 (1.01-1.81)	1.23 (0.92-1.66)	1.24 (0.92-1.66)	0.26	1.05 (0.96-1.16)
	rectum	1.00	0.71 (0.45-1.13)	0.84 (0.54-1.30)	0.87 (0.56-1.34)	0.68	1.00 (0.84-1.19)
RR (95% CI) ³	colon	1.00	1.38 (1.02-1.86)	1.24 (0.91-1.68)	1.21 (0.89-1.64)	0.37	1.03 (0.94-1.14)
	rectum	1.00	0.74 (0.46-1.19)	0.86 (0.55-1.34)	0.83 (0.53-1.29)	0.54	0.98 (0.83-1.16)
Linolenic acid							
Median intake (g/d)							
men		0.8	1.2	1.5	2.0		
Women		0.6	0.9	1.2	1.6		
colon cancer cases		108	108	124	108		
rectum cancer cases		44	40	36	40		
personyears		3642	3684	3657	3750		
RR (95% CI) ²	colon	1.00	0.97 (0.73-1.30)	1.15 (0.87-1.52)	1.01 (0.76-1.35)	0.65	0.99 (0.90-1.09)
	rectum	1.00	0.89 (0.57-1.38)	0.81 (0.51-1.28)	0.90 (0.58-1.41)	0.59	0.94 (0.79-1.11)
RR (95% CI) ³	colon	1.00	0.95 (0.70-1.30)	1.10 (0.82-1.47)	1.01 (0.75-1.36)	0.70	0.98 (0.89-1.09)
	rectum	1.00	0.91 (0.57-1.47)	0.87 (0.55-1.38)	0.91 (0.58-1.44)	0.67	0.95 (0.80-1.12)

Table 2 continued

		Quartile of intake				P for trend	RR (95% CI) for one SD increase in intake ^a
		1 ¹	2	3	4		
RR (95% CI) ³	colon	1.00	1.38 (1.02-1.86)	1.24 (0.91-1.68)	1.21 (0.89-1.64)	0.37	1.03 (0.94-1.14)
	rectum	1.00	0.74 (0.46-1.19)	0.86 (0.55-1.34)	0.83 (0.53-1.29)	0.54	0.98 (0.83-1.16)
Linoleic acid							
Median intake (g/d)							
men		10.0	14.8	19.5	27.9		
Women		7.4	11.1	14.9	21.2		
colon cancer cases		91	126	113	118		
rectum cancer cases		41	40	35	44		
person-years		3658	3700	3699	3676		
RR (95% CI) ²	colon	1.00	1.44 (1.07-1.92)	1.29 (0.96-1.73)	1.30 (0.97-1.75)	0.17	1.07 (0.97-1.18)
	rectum	1.00	0.98 (0.63-1.54)	0.86 (0.54-1.37)	1.06 (0.68-1.64)	0.96	1.02 (0.86-1.21)
RR (95% CI) ³	colon	1.00	1.49 (1.10-2.02)	1.32 (0.97-1.80)	1.31 (0.97-1.77)	0.19	1.06 (0.96-1.17)
	rectum	1.00	1.03 (0.65-1.64)	0.90 (0.56-1.45)	1.03 (0.66-1.62)	0.96	1.00 (0.84-1.18)

^a adjusted for energy by the residual method (45)

¹ reference category of quartiles of intake

² see for one standard deviation of increase table 1

³ relative risk adjusted for age and sex

⁴ relative risk adjusted for age, sex, QI, smoking, energy intake and family history of CRC

⁵ monounsaturated fat

⁶ polyunsaturated fat

type *K-ras* tumours were observed for total fat, specific fatty acids and other (dietary) factors.

Associations between the intake of total fat, saturated fat, MUFA, PUFA, linolenic acid and linoleic acid with the risk of colon or rectal cancer are presented in table 2. Incidence rate ratios (RR) and 95% CI for colon and rectal cancer are adjusted for age and sex and for age, sex, smoking, QI, energy intake and family history of CRC. The age-sex adjusted RR and the multivariate RR were similar. Frequent consumption of total fat, saturated fat, MUFA, PUFA, linolenic acid and linoleic acid were all not associated with the risk of colon or rectal cancer (table 2).

Associations of total fat, saturated fat, MUFA, PUFA, linolenic and linoleic acid with wild type *K-ras* tumours and mutated *K-ras* tumours in the colon or rectum are presented in table 3. Results will first be discussed for wild type *K-ras* tumours. No clear associations were observed for high intakes of total fat, saturated fat, MUFA, PUFA, linolenic acid and linoleic acid and the risk of colon and rectal cancer with a wild type *K-ras* gene. With regard to the colon mutated *K-ras* tumours, positive associations were observed for high intakes of PUFA (RR for highest versus lowest quartile of intake 2.09, 95% CI 1.25-3.49, p_{trend} 0.002) and specifically linoleic acid (RR for highest versus lowest quartile of intake 2.19, 95% CI 1.30-3.67, p_{trend} 0.002), but not linolenic acid. These positive associations were also observed for one SD of increase of intake (table 3). No significant associations were observed for rectal mutated *K-ras* tumours and for highest versus lowest intakes of total fat, saturated fat, MUFA and linolenic acid and colon and rectal cancer with mutated *K-ras* tumours.

Subgroup analyses were performed to evaluate the associations between total fat, saturated fat, MUFA, PUFA, linolenic acid and linoleic acid intakes and specific types of *K-ras* point mutations (G>A transitions and G>T or G>C transversions) in colon and rectal tumours (table 4). High intake of PUFA and specifically linoleic acid showed positive associations with colon tumours harbouring G>A transitions (RR for highest versus lowest quartile of intake 1.95, 95% CI 1.01-3.77 and RR 1.80, 95% CI 0.92-3.51, respectively) or G>T or G>C transversions (RR 2.17, 95% CI 0.98-4.80 and RR 2.69, 95% CI 1.19-6.08, respectively). These positive associations were also observed for one SD of increase of intake (table 4). There were no associations found between PUFA and linoleic acid intake

Table 3 Adjusted RR¹ for colon and rectal cancer patients with wild type *K-ras* gene (*K-ras*⁻) and with mutated *K-ras* gene (*K-ras*⁺) according to the intake of total fat and specific fatty acids

		Quartile of intake				P for trend	RR (95% CI) for one SD increase in intake ^a
		1 ²	2	3	4		
Total fat							
<i>Cases (K-ras-/K-ras+)</i>							
	colon	75/35	81/44	57/32	77/33		
	rectum	25/18	26/13	21/12	16/22		
RR _{K-ras-} (95% CI)	colon	1.00	1.04 (0.74-1.45)	0.71 (0.49-1.02)	0.99 (0.70-1.38)	0.48	0.98 (0.84-1.13)
	rectum	1.00	1.00 (0.57-1.76)	0.80 (0.44-1.47)	0.63 (0.33-1.20)	0.11	0.81 (0.64-1.02)
RR _{K-ras+} (95% CI)	colon	1.00	1.19 (0.75-1.90)	0.82 (0.49-1.36)	0.88 (0.54-1.44)	0.32	1.03 (0.84-1.26)
	rectum	1.00	0.72 (0.35-1.51)	0.65 (0.31-1.37)	1.18 (0.63-2.23)	0.65	1.07 (0.81-1.41)
Saturated fat							
<i>Cases (K-ras-/K-ras+)</i>							
	colon	60/39	86/34	76/39	68/32		
	rectum	25/18	21/13	26/18	17/16		
RR _{K-ras-} (95% CI)	colon	1.00	1.42 (0.99-2.02)	1.22 (0.84-1.75)	1.06 (0.74-1.54)	0.96	1.01 (0.89-1.15)
	rectum	1.00	0.82 (0.45-1.52)	1.02 (0.57-1.80)	0.64 (0.34-1.21)	0.28	0.86 (0.70-1.06)
RR _{K-ras+} (95% CI)	colon	1.00	0.85 (0.52-1.38)	0.92 (0.58-1.48)	0.73 (0.45-1.18)	0.26	0.90 (0.75-1.07)
	rectum	1.00	0.75 (0.35-1.59)	1.02 (0.52-2.01)	0.86 (0.43-1.72)	0.89	1.08 (0.85-1.39)
MUFA							
<i>Cases (K-ras-/K-ras+)</i>							
	colon	61/37	81/41	73/40	75/26		
	rectum	29/15	19/12	21/19	20/19		
RR _{K-ras-} (95% CI)	colon	1.00	1.27 (0.89-1.82)	1.16 (0.80-1.68)	1.18 (0.82-1.68)	0.53	1.01 (0.88-1.17)
	rectum	1.00	0.64 (0.35-1.17)	0.71 (0.39-1.28)	0.69 (0.38-1.24)	0.27	0.82 (0.64-1.06)
RR _{K-ras+} (95% CI)	colon	1.00	1.06 (0.66-1.70)	1.04 (0.64-1.69)	0.67 (0.40-1.13)	0.14	0.94 (0.77-1.16)
	rectum	1.00	0.81 (0.38-1.76)	1.31 (0.64-2.68)	1.22 (0.61-2.44)	0.34	1.10 (0.89-1.37)
PUFA							
<i>Cases (K-ras-/K-ras+)</i>							
	colon	68/23	87/31	72/41	63/49		
	rectum	24/21	18/14	25/14	22/16		
RR _{K-ras-} (95% CI)	colon	1.00	1.36 (1.00-1.91)	1.05 (0.73-1.49)	0.91 (0.63-1.31)	0.32	0.94 (0.83-1.07)
	rectum	1.00	0.76 (0.41-1.43)	1.03 (0.58-1.82)	0.89 (0.49-1.62)	0.96	0.97 (0.78-1.21)
RR _{K-ras+} (95% CI)	colon	1.00	1.44 (0.82-2.52)	1.83 (1.08-3.11)	2.09 (1.25-3.49)	0.002	1.21 (1.05-1.41)
	rectum	1.00	0.72 (0.36-1.45)	0.67 (0.33-1.34)	0.75 (0.39-1.44)	0.38	0.99 (0.77-1.27)
Linolenic acid							
<i>Cases (K-ras-/K-ras+)</i>							
	colon	69/38	71/33	79/38	71/35		
	rectum	30/12	21/17	18/18	20/18		
RR _{K-ras-} (95% CI)	colon	1.00	1.02 (0.70-1.46)	1.16 (0.82-1.64)	1.05 (0.74-1.49)	0.63	0.95 (0.85-1.08)
	rectum	1.00	0.65 (0.36-1.18)	0.57 (0.32-1.04)	0.66 (0.37-1.17)	0.14	0.86 (0.70-1.11)
RR _{K-ras+} (95% CI)	colon	1.00	0.84 (0.50-1.41)	1.00 (0.62-1.60)	0.95 (0.59-1.55)	0.97	1.04 (0.88-1.23)
	rectum	1.00	1.57 (0.71-3.50)	1.62 (0.76-3.45)	1.54 (0.73-3.25)	0.27	1.07 (0.88-1.29)
Linoleic acid							
<i>Cases (K-ras-/K-ras+)</i>							
	colon	64/22	89/33	72/40	65/49		
	rectum	20/19	26/13	20/15	23/18		
RR _{K-ras-} (95% CI)	colon	1.00	1.46 (1.03-2.06)	1.14 (0.79-1.63)	1.01 (0.70-1.45)	0.63	0.97 (0.86-1.10)
	rectum	1.00	1.32 (0.73-2.39)	0.99 (0.53-1.86)	1.12 (0.61-2.07)	0.97	0.99 (0.80-1.23)
RR _{K-ras+} (95% CI)	colon	1.00	1.60 (0.92-2.80)	1.87 (1.09-3.22)	2.19 (1.30-3.67)	0.002	1.22 (1.05-1.42)
	rectum	1.00	0.72 (0.34-1.51)	0.81 (0.40-1.63)	0.93 (0.48-1.79)	0.91	1.00 (0.77-1.29)

¹ Multivariate adjusted RR for age, sex, QI, smoking, energy intake and family history of CRC and their 95% confidence intervals and adjusted for energy by the residual method (45)² reference category of quartiles of intake^a see for one standard deviation of increase table 1

** monounsaturated fat

***polyunsaturated fat

and rectal tumours with G>A transitions or G>T or G>C transversions. In addition, no significant associations were observed between highest versus lowest quartile of intake of total fat, saturated fat, MUFA and linolenic acid and the risk of G>A transitions or G>T or G>C transversions in colon and rectal tumours.

DISCUSSION

In this study, no associations were observed between intakes of specific fats or fatty acids and overall colon and rectal cancer risk. However, associations were observed when taking into account the presence or absence of specific point mutations in the *K-ras* gene of colon and rectal cancer patients. No significant associations were observed for total fat, saturated fat, and MUFA intakes and the risk of colon and rectal tumours without or with a *K-ras* mutation. In contrast, positive associations were observed for high intake of PUFA, specifically linoleic acid but not linolenic acid, and the risk of colon tumours harbouring *K-ras* oncogene mutations. Subgroup analyses of specific point mutations in the *K-ras* gene revealed that these positive associations were observed for G>A transitions and G>T and G>C transversions.

This cohort study on fat and specific fatty acids intake in relation to specific *K-ras* mutations in colon and rectal cancer is, to our knowledge, the only prospective study performed to date. One large case-control study with colon cancer patients (11), two case-control studies of limited size among colon (13), and colon and rectal cancer patients (12) and one cross-sectional case-case study with colorectal adenoma patients (14) have previously been conducted. The prospective design of the current study and high completeness of follow-up of cancer incidence and subcohort, make information and selection bias unlikely. In addition, as a result of the exclusion of the first 2.3 years of follow-up, the chance of information bias due to potential preclinical colorectal cancer is minimal. Finally, tumour tissue was available for 84% of the eligible cases (776 out of 929) and there were no significant differences in fat intake levels nor in clinicopathological characteristics of the tumour (Dukes' stage and differentiation grade) of these patients compared to the CRC patients with a known *K-ras* mutation status of their tumour (data not shown). Therefore, selection bias due to loss to follow-up is unlikely.

In general, the validation of the fat intake variables was satisfactory (see methods section and (27)). Although total fat intake and energy intake may have been underestimated as compared to the nine-day record method in the validation study (27) and, in addition, there is inevitably a certain degree of misclassification of fat intake variables, it is not expected that this is differential with respect to the *K-ras* mutation status of the tumour. Therefore, the associations observed in this study are probably attenuated. In other epidemiological studies similar inaccuracies occur in the measurement of fat intake since similar methods of intake assessment were employed (11,12,14).

One of the hypotheses for the association between fat intake and CRC is that meat consumption instead of fat intake determines the increased risk. We also investigated meat consumption as a potential risk factor for *K-ras* mutations in colon and rectal cancer, and total fresh meat was not associated with risk. Therefore, we did not additionally adjust for this potential confounder in these analyses.

In the current study, a high intake of total and saturated fats was not associated with overall colon or rectal cancer risk and with *K-ras* mutation status and these findings are supported by others (11,13,14). Bautista *et al* (12) observed an inverse association between a high intake of total fat and colorectal tumours with a *K-ras* wild type gene (odds ratio for highest versus lowest tertile of intake 0.47, 95% CI 0.22-0.99). However, the results were

Table 4 Adjusted RR¹ for colon and rectal cancer patients with G>A transitions or with G>T or G>C transversions in the *K-ras* oncogene according to the intake of intake total fat and specific fatty acids

		Quartile of intake				P for trend	RR (95% CI) for one SD increase in intake ^a
		1 ²	2	3	4		
Total fat							
Cases (G>A/ G>T or C)							
RR _{G>A} (95% CI)	colon	20/15	23/20	17/12	22/9		
	rectum	12/7	6/7	6/7	9/11		
	colon	1.00	1.11 (0.60-2.07)	0.78 (0.40-1.52)	1.02 (0.54-1.90)	0.79	1.09 (0.82-1.44)
	rectum	1.00	0.45 (0.17-1.22)	0.43 (0.16-1.19)	0.74 (0.31-1.79)	0.50	0.87 (0.57-1.32)
RR _{G>T,C} (95% CI)	colon	1.00	1.26 (0.64-2.48)	0.72 (0.33-1.56)	0.57 (0.25-1.30)	0.07	0.90 (0.69-1.17)
	rectum	1.00	1.09 (0.37-3.20)	1.07 (0.37-3.11)	1.50 (0.58-3.89)	0.42	1.17 (0.84-1.63)
Saturated fat							
Cases (G>A/ G>T or C)							
RR _{G>A} (95% CI)	colon	23/14	16/17	23/15	20/10		
	rectum	9/10	5/8	13/3	6/11		
	colon	1.00	0.69 (0.36-1.34)	0.95 (0.52-1.74)	0.76 (0.41-1.42)	0.60	0.95 (0.75-1.19)
	rectum	1.00	0.51 (0.17-1.55)	1.31 (0.55-3.10)	0.64 (0.23-1.84)	0.88	1.01 (0.70-1.45)
RR _{G>T,C} (95% CI)	colon	1.00	1.19 (0.57-2.47)	1.00 (0.47-2.10)	0.63 (0.28-1.43)	0.21	0.79 (0.62-1.01)
	rectum	1.00	0.91 (0.34-2.44)	0.33 (0.08-1.25)	1.07 (0.45-2.55)	0.84	1.15 (0.80-1.64)
MUFA^{**}							
Cases (G>A/ G>T or C)							
RR _{G>A} (95% CI)	colon	19/17	21/18	25/13	17/8		
	rectum	10/4	6/7	7/13	10/8		
	colon	1.00	1.09 (0.57-2.09)	1.34 (0.70-2.56)	0.84 (0.43-1.66)	0.78	1.05 (0.80-1.39)
	rectum	1.00	0.53 (0.19-1.46)	0.59 (0.22-1.66)	0.96 (0.40-2.33)	0.99	1.06 (0.71-1.60)
RR _{G>T,C} (95% CI)	colon	1.00	1.01 (0.51-1.98)	0.72 (0.34-1.51)	0.46 (0.20-1.07)	0.04	0.78 (0.60-1.02)
	rectum	1.00	2.00 (0.57-7.07)	4.00 (1.25-12.8)	1.93 (0.57-6.56)	0.12	1.12 (0.91-1.37)
PUFA^{***}							
Cases (G>A/ G>T or C)							
RR _{G>A} (95% CI)	colon	14/9	18/13	23/14	27/20		
	rectum	11/10	6/9	11/4	5/9		
	colon	1.00	1.44 (0.71-2.94)	1.76 (0.89-3.49)	1.95 (1.01-3.77)	0.04	1.18 (0.97-1.42)
	rectum	1.00	0.51 (0.18-1.43)	0.86 (0.35-2.11)	0.42 (0.14-1.20)	0.21	0.80 (0.57-1.12)
RR _{G>T,C} (95% CI)	colon	1.00	1.51 (0.64-3.60)	1.57 (0.67-3.70)	2.17 (0.98-4.80)	0.06	1.24 (0.98-1.57)
	rectum	1.00	1.10 (0.44-2.74)	0.45 (0.14-1.46)	0.94 (0.38-2.30)	0.59	1.02 (0.72-1.45)
Linolenic acid							
Cases (G>A/ G>T or C)							
RR _{G>A} (95% CI)	colon	24/14	13/18	24/14	21/10		
	rectum	6/6	8/9	9/11	11/6		
	colon	1.00	0.54 (0.26-1.11)	1.02 (0.57-1.85)	0.91 (0.49-1.70)	0.79	1.04 (0.83-1.29)
	rectum	1.00	1.04 (0.33-3.28)	1.39 (0.47-4.04)	1.75 (0.62-4.95)	0.22	1.21 (0.92-1.59)
RR _{G>T,C} (95% CI)	colon	1.00	1.26 (0.57-2.80)	1.00 (0.47-2.14)	0.75 (0.32-1.72)	0.38	0.95 (0.73-1.25)
	rectum	1.00	1.91 (0.64-5.66)	2.21 (0.81-6.03)	1.09 (0.35-3.38)	0.75	0.92 (0.71-1.20)
Linoleic acid							
Cases (G>A/ G>T or C)							
RR _{G>A} (95% CI)	colon	14/8	19/14	24/12	25/22		
	rectum	11/10	6/7	10/6	7/9		
	colon	1.00	1.51 (0.75-3.07)	1.85 (0.93-3.66)	1.80 (0.92-3.51)	0.06	1.19 (0.98-1.44)
	rectum	1.00	0.56 (0.19-1.60)	0.89 (0.35-2.26)	0.65 (0.24-1.74)	0.59	0.81 (0.57-1.15)
RR _{G>T,C} (95% CI)	colon	1.00	1.83 (0.76-4.40)	1.52 (0.61-3.77)	2.69 (1.19-6.08)	0.03	1.27 (1.00-1.61)
	rectum	1.00	0.83 (0.31-2.27)	0.70 (0.25-1.94)	0.94 (0.38-2.31)	0.83	1.06 (0.74-1.53)

¹ Multivariate adjusted RR for age, sex, QI, smoking, energy intake and family history of CRC and their 95% confidence intervals and adjusted for energy by the residual method (45)

² reference category of quartiles of intake

^a see for one standard deviation of increase table 1

^{**} monounsaturated fat

^{***} polyunsaturated fat

based on only 108 CRC cases in a population-based case-control setting, and therefore estimates of associations must be interpreted with caution.

Animal studies indicated an inverse association of high MUFA intake, derived mainly from oleic acid with CRC risk (32,33). However, in epidemiological studies, no clear associations were observed with CRC risk (34-36). Regarding the *K-ras* mutation status, no associations were observed for high intake of MUFA and the risk of colon and rectal cancer in the current study. This is in line with Slattery's observation (11). However, Bautista *et al* (12) observed that a high intake of MUFA was inversely associated with the risk of having a wild type *K-ras* gene. This study was conducted in Spain where the main source of oleic acid is olive oil. This is not the case for the current cohort and could be an explanation for the different findings. The association was not reported in the other studies (13,14).

To our best knowledge, this is the first study reporting on specific polyunsaturated fatty acids in relation to *K-ras* mutation status. Overall PUFA intake was studied by Slattery *et al* (11) and Bautista *et al* (12) and no associations were observed with *K-ras* mutation status.

In the current study, no clear associations were observed between the high intake of linolenic acid as the main source of ω -3 PUFA, and the risk of colon or rectal cancer with or without specific point mutations in the *K-ras* gene. Epidemiological, clinical, and experimental data indicate a protective effect of fish oil-derived ω -3 PUFA on overall colon cancer (35,37-44). Collett *et al* (37) demonstrated that the major ω -3 PUFA constituent of fish oil, docosahexaenoic acid (DHA) reduces the Ras protein localization to the plasma membrane without affecting posttranslational lipidation and lowers the GTP-binding of the Ras protein in mouse colonocytes treated with azoxymethane (colon carcinogen). These findings were corroborated by *in vivo* studies (45). Another hypothesis is that the tumour-promoting activity of ω -6 PUFA was abrogated by competitive inhibition of ω -3 PUFA from the metabolism of arachidonic acid and therefore reducing malondialdehyde (MDA) levels and subsequently pyrimidopurine-deoxyguanosine (M_1G) adducts $M_1G>T$ and $M_1G>C$ transversions (38). Summarized, ω -3 PUFA may exert their effect through inhibition of the Ras protein activity and not by generating functional aberrations in the exon 1 fragment of the *K-ras* oncogene. This could explain the lack of association between linolenic acid and *K-ras* mutation status of colon or rectal tumours in this study.

In the current study, no significant association was observed for linoleic acid, as the main source of ω -6 PUFA intake, and the overall risk of colon or rectal cancer. This is in line with most epidemiological evidence up to date (46). However, a positive effect was found between high intake of linoleic acid and colon tumours harbouring *K-ras* gene mutations. Subgroup analyses of specific point mutations in the *K-ras* gene revealed that colon tumours harbouring G>A transitions or G>T or G>C transversions are positively associated with high linoleic acid intake. These associations are in line with the biological evidence (16,18) and are possibly the result of an increased formation of G-adducts resulting in G>A transitions or G>T or G>C transversions. Increased intake of ω -6 PUFA enhances tumorigenesis in experimental animals and *in vitro* systems by several mechanisms (47). The conjugated double bonds in poly-unsaturated fatty acids are highly sensitive to lipid peroxidation, and may generate fatty acid hydroperoxides. These hydroperoxides will be reduced by glutathione peroxidase to non-reactive fatty acid alcohols or may react with metal ions to yield alkoxyl radicals. The fatty acid alcohols do not lead to DNA damage and will not be discussed further. However, alkoxyl radicals are reduced to aldehydes, with 2,3-epoxy-4-hydroxynonanal (4-HNE) and MDA as the most prominent forms. The bifunctional alkylating agent 4-HNE can react with DNA to yield etheno and other base adducts which are thought to promote the carcinogenic process. Etheno-dG induces mainly transitions to A and transversions to T in bacteria (16,33,48,49) and etheno-dC induces transitions to T in the kidneys of monkeys (50). MDA is a major genotoxic carbonyl compound, which is also a by-product of the arachidonic acid metabolism in the synthesis of prostaglandines. MDA is

mutagenic in bacterial and mammalian cells, and carcinogenic in rats (18). MDA reacts with DNA to form adducts, predominantly with deoxyguanosine to generate M₁G (15). The most common mutations in progeny molecules are G>T transversions and G>A transitions, with a minor contribution by G>C transversions (16,18,19). In the current study, the associations with G>A transitions, G>T and G>C transversions in the *K-ras* oncogene were only observed for colon cancer patients and not for rectal cancer patients. The reason for this apparent difference in the aetiology remains unclear. In addition, the observed significant association found for colon cancer could be a result of a type I error due to the numerous associations analysed in this study. Although the studied associations were mainly hypotheses-driven, caution is warranted in interpreting these results and further investigations are needed.

Our results suggest that PUFA, and in particular linoleic acid, is an important dietary risk factor for colon tumours with *K-ras* mutations possibly by generating G>A transitions or G>T or G>C transversions in the exon 1 fragment of the *K-ras* oncogene. This implies that, for some dietary exposures like polyunsaturated fat intake, it is meaningful to account for somatic mutations in the *K-ras* oncogene in the aetiology of colon and rectal cancer.

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5 Dietary folate intake and *K-ras* mutations in sporadic colon and rectal cancer in The Netherlands Cohort Study

Dietary folate intake and *K-ras* mutations in sporadic colon and rectal cancer in The Netherlands Cohort Study

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ABSTRACT

The association between dietary folate and specific *K-ras* mutations in colon and rectal cancer was investigated in The Netherlands Cohort Study on diet and cancer.

After 7.3 years of follow-up, 448 colon and 160 rectal cancer patients and 3048 subcohort members (55-69 years at baseline) were available for data-analyses. Mutation analysis of the *K-ras* gene was performed on all archival adenocarcinoma specimens. Case-cohort analyses were used to compute adjusted incidence rate ratios (RR) and 95% confidence intervals (CI) for colon and rectal cancer overall and for *K-ras* mutation status subgroups according to 100 µg/d increased intake in dietary folate.

Dietary folate intake was not significantly associated with colon cancer risk for men or women, either overall or with *K-ras* mutation status. For rectal cancer, high folate intake was inversely associated with the disease risk in men and was most pronounced for *K-ras* mutated tumours, whereas an increased association was observed for women. Regarding the *K-ras* mutation status in women, an increased association was observed for both wild type and mutated *K-ras* tumours. Specifically, folate intake was positively associated with G>T and G>C transversions in rectal tumours (RR 2.69, 95% CI 1.43-5.09), but inversely associated with G>A transitions (RR 0.08, 95% CI 0.01-0.53).

Our data suggest that the effect of folate intake on rectal cancer risk is different for men and women, and depends on the *K-ras* mutation status of this cancer type.

INTRODUCTION

To date, results from epidemiological studies (1-4) generally show an inverse association between dietary folate and the risk of colorectal cancer (CRC). However, associations may be different for men versus women and for colon versus rectum (4,5). In addition, the results from most animal studies support the epidemiological data, but results were not always consistent (1,2).

Folate plays an important role in one-carbon metabolism, a series of interrelated biochemical reactions involved in DNA synthesis and methylation of DNA, RNA, and proteins (6) in animals and humans. Two pathways have been described linking a low folate status to colon and rectal carcinogenesis (6,7). First, a deficient intracellular form of folate, 5, 10-methylenetetra-hydrofolate (5, 10-methyleneTHF) leads to a decreased availability of methyl groups for uracil which may result in less available thymine. Eventually, the increased uracil incorporation in DNA may lead to DNA strand breaks and chromosome instability. Secondly, sub-optimal folate status may result in global DNA hypomethylation and regional hypermethylation in gene promoters which interferes with gene expression and can impair DNA repair (8,9). DNA strand breaks, global DNA hypomethylation and regional promoter hypermethylation are common events in colon cancer.

The association between folate and CRC was evaluated earlier in The Netherlands Cohort Study on diet and cancer (NLCS), based on 7.3 years of follow-up (3,10). In this study (3), the association between folate intake and colon cancer tended to be inverse for men and women after the exclusion of the first year of follow-up, but did not reach statistical significance. For rectal cancer, folate was inversely associated with the disease risk in men, although borderline significant, but not in women. Evaluating these findings, it would be interesting to study the association between dietary folate and CRC when specific mutations in genes involved in colorectal carcinogenesis are taken into account.

The oncogenic activation of *K-ras* by mutations is a genetic alteration that occurs in adenomas (10%) as well as in carcinomas (40%) in colon and rectal cancer. Activating mutations are mainly found in codons 12 and 13 (11-14). The most frequently observed types of point mutations are G>A transitions, and G>T and G>C transversions (14-16). Possibly, the G>A transitions frequently observed in the *K-ras* gene are a result of impaired promoter methylation of the DNA-repair gene O⁶-methylguanine DNA methyltransferase (MGMT) (9) which could, in part, be due to a low folate status (10).

To date, only three epidemiological studies report on the association between folate intake and *K-ras* mutation status in colorectal adenomas (7) and in colon(rectal) tumours (17,18) with varying results. However, it is difficult to compare the results from different studies due to differences in case groups and reference groups. In the current study, the association between dietary folate intake and the risk of specific point mutations in the *K-ras* oncogene in patients with colon and rectal cancer were studied for men and women within the framework of the NLCS.

MATERIALS AND METHODS

Study Population

The participants in this study are incident colon and rectal cancer cases and subcohort members from the NLCS, which has been described in detail elsewhere (19). Briefly, the study was initiated in 1986 and includes 58,279 men and 62,573 women, aged 55-69 years old at baseline, who originated from 204 Dutch municipalities with computerized population registries. A self-administered questionnaire on diet and other risk factors for cancer was completed at baseline. The entire cohort is being monitored for cancer occurrence by annual

Table 1 Nutrient intake (mean $\pm$ SD) and other characteristics among colon cancer cases and subcohort members

	Subcohort	Men		
		Wild type <i>K-ras</i>	<i>K-ras</i> mutation	<i>p-value</i> ^a
N	1475	153	89	
Age (y)	61.3 $\pm$ 4.2	62.5 $\pm$ 4.2	63.7 $\pm$ 4.2	0.04
Folate (μ g/day)	224.2 $\pm$ 72.0	225.4 $\pm$ 62.9	225.1 $\pm$ 83.6	0.97
<i>Other dietary factors</i>				
Alcohol (g/day)	14.5 $\pm$ 16.4	15.7 $\pm$ 16.4	15.6 $\pm$ 15.9	0.95
Fiber (g/day)	28.7 $\pm$ 8.8	28.8 $\pm$ 7.2	29.7 $\pm$ 8.9	0.40
Energy (kcal/day)	2165.6 $\pm$ 512.1	2157.1 $\pm$ 452.6	2069.4 $\pm$ 438.6	0.14
Fresh meat (g/day)	105.9 $\pm$ 43.3	101.5 $\pm$ 35.9	103.6 $\pm$ 36.9	0.66
Vitamin B6 (mg/day)	1.5 $\pm$ 0.4	1.6 $\pm$ 0.3	1.6 $\pm$ 0.4	0.90
Vitamin C (mg/day)	98.1 $\pm$ 41.5	100.9 $\pm$ 41.5	108.5 $\pm$ 42.9	0.18
Iron (mg/day)	13.2 $\pm$ 3.2	13.5 $\pm$ 2.9	13.7 $\pm$ 3.3	0.68
Methionine (g/day)	1.7 $\pm$ 0.4	1.7 $\pm$ 0.4	1.7 $\pm$ 0.4	0.16
<i>Other characteristics</i>				
QI (kg/m ²)	25.0 $\pm$ 2.6	25.3 $\pm$ 2.6	25.9 $\pm$ 3.1	0.11
Family history (% Yes)	5.7	13.7	11.2	0.58
Smoker (%)				
Never	13.4	11.8	11.2	
Ex smoker	51.3	62.1	67.4	
Current smoker	35.3	26.1	21.3	0.67
Physical activity (%)				
<30 minutes/day	15.0	10.6	11.4	
30-60 minutes/day	29.7	31.8	27.3	
60-90 minutes/day	34.8	32.5	38.6	
>90 minutes/day	20.4	25.2	22.7	0.76

^a comparing cases with at least one *K-ras* mutation to cases without a *K-ras* mutation¹ 2 out of 89 male colon cancer cases had more than one *K-ras* gene mutation² 4 out of 62 female colon cancer cases had more than one *K-ras* gene mutation^{*} For 41 subcohort members and 6 colon cancer cases, information on physical activity was not available

record linkage to The Netherlands Cancer Registry (NCR, nine cancer registries in The Netherlands) and to PALGA, a nationwide network and registry of histo- and cytopathology (www.palga.nl) (20). In the municipalities included in the NLCS, the NCR and PALGA, together, have nearly 100% coverage since the start of the study (21-23). PALGA also provides necessary information on the identification of the pathology laboratory location of the storage of paraffin-embedded blocks of the eligible CRC patients. Accumulation of persontime in the cohort has been estimated through biennial vital status follow-up of a subcohort of 3,500 men and women who were randomly selected after baseline exposure measurement. Cases with prevalent cancer other than non-melanoma skin cancer were excluded from the subcohort, which left 3,346 men and women for analysis.

The first 2.3 years of follow-up were excluded due to incomplete coverage of PALGA alone in some of the municipalities included in the NLCS. Within this period, 83 subcohort members were either deceased or diagnosed with cancer other than non-melanoma skin cancer, leaving 3,263 men and women for analysis. From 1989 till 1994, 929 incident cases with histologically confirmed CRC were observed of whom 819 could also be linked to a PALGA report of the lesion. The PALGA database was used to identify and locate tumour tissue in Dutch pathology laboratories. CRC was classified according to site as follows: colon i.e. cecum through sigmoid colon (ICD-O-1 codes: 153.0, 153.1, 153.2, 153.3, 153.4, 153.5, 153.6, 153.7, 153.8, 153.9), rectosigmoid (ICD-O-1 code 154.0) and rectum (ICD-O-1 code 154.1). Information about age at baseline, sex and family history of CRC (at baseline) was retrieved from the NLCS database.

Table 1 Continued

Subcohort	Women		
	Wild type <i>K-ras</i>	<i>K-ras</i> mutation	<i>p</i> -value ^a
1573	144	62	
61.4 ± 4.3	62.8 ± 4.1	63.9 ± 3.6	0.06
199.8 ± 64.7	189.4 ± 67.3	192.9 ± 61.6	0.72
5.7 ± 9.4	6.0 ± 12.4	3.9 ± 7.0	0.22
25.5 ± 7.2	24.4 ± 7.4	24.9 ± 7.9	0.68
1691.4 ± 408.5	1660.9 ± 400.7	1663.5 ± 416.7	0.97
93.8 ± 40.4	95.2 ± 38.0	93.1 ± 41.6	0.73
1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	0.58
108.2 ± 43.8	102.0 ± 47.1	105.7 ± 47.4	0.61
11.7 ± 2.7	11.4 ± 2.4	11.2 ± 2.9	0.69
1.5 ± 0.4	1.5 ± 0.3	1.5 ± 0.4	0.46
25.1 ± 3.5	25.7 ± 3.7	25.5 ± 3.6	0.78
5.5	13.2	6.5	0.16
59.1	63.2	74.2	
20.2	22.9	16.1	
20.7	13.9	9.7	0.31
26.3	29.4	33.3	
35.4	35.7	40.0	
27.3	26.6	15.0	
11.0	8.4	11.7	0.34

Tissue Samples

Tumour material of all CRC patients was collected after approval by the Medical Ethics Committees (MEC) of Maastricht University, the NCR and PALGA (14). Subsequently, all pathology laboratories in The Netherlands agreed to make relevant tissue samples available upon request from PALGA. Tissue samples of the 819 cases present in 54 pathology laboratories throughout The Netherlands were collected between August 1999 and December 2001. For 43 (5%) untraceable tumour tissue specimens and 39 specimens (5%) with insufficient CRC tumour material, *K-ras* mutation status could not be determined. Finally, tumour material from 737 incident colorectal adenocarcinoma cases was available of whom 476 were colon cancer cases, 85 were rectosigmoid cancer cases and 176 were rectal cancer cases. Statistical analyses were performed separately for colon and rectal cancer as differences in the aetiology of colon and rectal cancer have been reported (24). 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-analyses. Moreover, the number of patients with a rectosigmoid tumour was too small for adequate stratified analyses (14).

Detection of *K-ras* mutations

Mutation analysis of the exon 1 fragment of the *K-ras* oncogene, spanning codons 8-29, was performed on archival colorectal adenocarcinoma specimens of all 737 CRC patients using macrodissection, nested PCR and direct sequencing of purified fragments, which has been described in detail elsewhere (14).

Table 2 Nutrient intake (mean $\pm$ SD) and other characteristics among rectal cancer cases and subcohort members

	Subcohort	Men		
		Wild type <i>K-ras</i>	Rectal cancer <i>K-ras</i> mutation	<i>p</i> -value
N	1475	67	37	
Age (y)	61.3 $\pm$ 4.2	62.3 $\pm$ 4.2	62.4 $\pm$ 4.1	0.89
Folate (μ g/day)	224.2 $\pm$ 72.0	222.4 $\pm$ 52.5	212.3 $\pm$ 67.6	0.40
<i>Other dietary factors</i>				
Alcohol (g/day)	14.5 $\pm$ 16.4	16.9 $\pm$ 19.3	14.7 $\pm$ 12.5	0.53
Fiber (g/day)	28.7 $\pm$ 8.8	29.2 $\pm$ 8.3	29.9 $\pm$ 9.1	0.67
Energy (kcal/day)	2165.6 $\pm$ 512.1	2182.6 $\pm$ 483.2	2165.0 $\pm$ 467.0	0.86
Fresh meat (g/day)	105.9 $\pm$ 43.3	101.3 $\pm$ 38.6	92.8 $\pm$ 40.2	0.30
Vitamine B6 (mg/day)	1.5 $\pm$ 0.4	1.5 $\pm$ 0.4	1.5 $\pm$ 0.4	0.91
Vitamine C (mg/day)	98.1 $\pm$ 41.5	108.5 $\pm$ 46.8	105.0 $\pm$ 56.9	0.74
Iron (mg/day)	13.2 $\pm$ 3.2	13.6 $\pm$ 2.9	13.3 $\pm$ 3.1	0.83
Methionine (g/day)	1.7 $\pm$ 0.4	1.7 $\pm$ 0.4	1.6 $\pm$ 0.4	0.20
<i>Other characteristics</i>				
QI (kg/m ²)	25.0 $\pm$ 2.6	24.9 $\pm$ 2.5	25.1 $\pm$ 2.7	0.66
Family history (% Yes)	5.7	7.5	16.2	0.17
Smoker (%)				
Never	13.4	9.0	13.5	
Ex smoker	51.3	55.2	54.1	
Current smoker	35.3	35.8	32.4	0.76
Physical activity (%)^a				
<30 minutes/day	15.0	9.1	16.7	
30-60 minutes/day	29.7	27.3	30.6	
60-90 minutes/day	34.8	37.9	30.6	
>90 minutes/day	20.4	25.8	22.2	0.64

^a comparing cases with at least one *K-ras* mutation to cases without a *K-ras* mutation

¹ 2 out of 37 male rectal cancer cases had more than one *K-ras* gene mutation

² 1 out of 30 female rectal cancer cases had more than one *K-ras* gene mutation

^a For 41 subcohort members and 2 rectal cancer cases, information on physical activity was not available

The Food Frequency Questionnaire

The dietary section of the questionnaire was a 150-item semi-quantitative food frequency questionnaire, which concentrated on habitual consumption of food and beverages during the year preceding the start of the study. Daily mean nutrient intakes were calculated by cumulating the multiplied frequencies and portion sizes of all food items with their tabulated nutrient contents from the Dutch food composition table (25). The questionnaire was validated against a 9-day diet record (26). 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.

Folate data were derived from a validated liquid chromatography trienzyme method (27) used to analyse the 125 most important dutch foods contributing to folate intake (28). The mean daily intakes of all other relevant nutrients were calculated using the computerized Dutch Food Composition Table (25). For data-analyses, an increment of 100 μ g/day increase in folate intake for men and women was computed based on the current recommendations of the Dutch Health Council.

For 257 subjects (28 incident colon adenocarcinoma cases, 16 incident rectal adenocarcinoma cases and 215 subcohort members; two subcohort members were also colon or rectal cancer cases), dietary data were incomplete or inconsistent, and they were excluded from the analyses. These subjects either 1) left 60 or more (out of 150) questionnaire items blank and ate fewer than 35 items at least once per month and/or 2) left one or more item blocks (groups of items, i.e., beverages) blank. Additional details are given elsewhere (26). Hence, 608 colon (242 men and 206 women) and rectal cases (104 men

Table 2 Continued

Subcohort	Women		
	Wild type <i>K-ras</i>	Rectal cancer <i>K-ras</i> mutation	<i>p</i> -value
1573	26	30	
61.4 ± 4.3	63.5 ± 4.0	62.0 ± 3.9	0.17
199.8 ± 64.7	212.2 ± 70.7	216.2 ± 94.8	0.86
5.7 ± 9.4	5.3 ± 6.5	6.6 ± 10.3	0.60
25.5 ± 7.2	24.2 ± 5.9	24.6 ± 4.5	0.74
1691.4 ± 408.5	1627.0 ± 370.6	1789.7 ± 328.4	0.09
93.8 ± 40.4	98.8 ± 45.2	99.2 ± 32.0	0.97
1.3 ± 0.3	1.4 ± 0.3	1.4 ± 0.3	0.88
108.2 ± 43.8	109.5 ± 43.1	111.3 ± 45.8	0.88
11.7 ± 2.7	11.6 ± 2.2	11.6 ± 2.2	0.95
1.5 ± 0.4	1.6 ± 0.4	1.5 ± 0.3	0.63
25.1 ± 3.5	25.1 ± 3.5	26.0 ± 3.1	0.27
5.5	15.4	6.7	0.29
59.1	69.2	60.0	
20.2	7.7	30.0	
20.7	23.1	10.0	0.07
26.3	42.3	30.0	
35.4	26.9	23.3	
27.3	19.2	40.0	
11.0	11.5	6.7	0.39

and 56 women) and 3048 subcohort members (1475 men and 1573 women) were available for data-analyses.

Daily intake of dietary fibre (g/day), vitamin B₂ (mg/day), vitamin B₆ (mg/day), vitamin C (µg/day), iron (mg/day), alcohol (g/day), fresh meat (g/day), and total energy (kcal/day) and age at baseline (years), Quetelet Index (QI; kg/m²), physical activity (<30 min/d, 30-60 min/d, 60-90 min/d, >90 min/d), family history of CRC (yes/no) and smoking status (never/ex/current) were regarded as potential confounders.

Statistical Analysis

Analyses were conducted for men and women separately. The overall frequency of *K-ras* mutations as well as the type of mutation were computed for all colon and rectal cancer cases as described elsewhere (14). Mean values of the continuous variables age at baseline (years), intake of total folate, dietary fiber, alcohol, fresh meat, vegetables and total energy and QI were evaluated for subcohort members and colon and rectal cancer cases with wild-type and mutated *K-ras* gene. Differences in mean values of the continuous variables between patients with wild type and mutated *K-ras* gene were tested with the Student T-test or the Mann-Whitney-U-test if the variables were not normally distributed. Distributions of the categorical variables family history of CRC, smoking status and physical activity were evaluated for subcohort members and colon and rectal cancer patients with wild-type and mutated *K-ras* gene and tested for differences between patient groups with the X²-test. The statistical software package SPSS (version 9) was used for these analyses.

Age-adjusted and multivariate-adjusted incidence rate ratios (RR) and their 95% confidence intervals (CI) for colon and rectal cancer risk were calculated for men and women separately, using Cox regression models with the STATA statistical software package (intercooled STATA, version 7). Standard errors were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling

from the cohort. This method is equivalent to the variance-covariance estimator as presented by Barlow (29). The proportional hazards assumption was tested using the scaled Schoenfeld residuals (30). Those variables that were found to contribute substantially ($p < 0.10$) to the multivariate model for colon and/or rectal cancer (age, smoking, QI, total fresh meat consumption, and intake of iron and vitamin C) were included as covariates in all multivariate analyses. Additional adjustment for dietary fiber and energy intake was performed, in line with the previous report on folate and overall colon and rectal cancer risk in the Netherlands Cohort Study (3). The person-years at risk, estimated from the subcohort, were used in the analyses.

To date, foods are not fortified with folic acid in The Netherlands. For the time being, an intake of 200 g of vegetables and two pieces of fruit daily is recommended. This is approximately twice the amount of fruit and vegetables found to be consumed in the Dutch National Food Consumption Survey (DNFCS) carried out in 1992. The fruit and vegetable intake accounts for approximately 50 μg folate (28). We calculated RRs according to an increase in intake of 100 $\mu\text{g}/\text{d}$ of folate. Approximately twice this additional amount would be necessary to reach the US recommended daily allowance of 400 $\mu\text{g}/\text{d}$ in the majority of the men and women of this cohort.

RESULTS

The overall frequency and spectrum of mutations in the *K-ras* gene have been presented in detail elsewhere (14). In brief, a total of 227 mutations was found in 218 out of 608 (36%) colon and rectal cancer patients. The most frequently observed mutations are the G>A transitions (54%), G>T transversions (33%) and G>C transversions (7%). The observed frequencies of the mutations in this series of patients are similar to the frequencies of the 737 colorectal cancer cases (including the rectosigmoid) for whom *K-ras* status was determined (14).

Table 1 shows the folate intake and other baseline characteristics for colon cancer cases and subcohort members, separately for men and women. The mean age of the subcohort members was 61.3 years for men and 61.4 years for women. Overall, colon cancer cases were older than the subcohort members. Colon cancer patients with a mutated *K-ras* gene were significantly older than the patients with a wild type *K-ras* gene (p -values 0.04 and 0.06 for men and women, respectively). Dietary folate intake was higher among subcohort members as compared to the different colon cancer subgroups. No statistically significant differences for other (dietary) factors were observed for colon cancer cases without and with a *K-ras* mutation in men and women as presented in table 1.

For rectal cancer cases and subcohort members, the mean folate intake and other baseline characteristics are presented in table 2. Rectal cancer patients were older than the subcohort members. Dietary folate intake among subcohort members and the different rectal cancer subgroups was similar. Women with a mutated *K-ras* rectal tumour were more often ex-smoker as compared to women with a wild type *K-ras* rectal tumour and subcohort members. No statistically significant differences for other (dietary) factors were observed for rectal cancer cases without and with a *K-ras* mutation in men and women as presented in table 2.

For colon cancer, associations between folate intake and risk of disease, as well as *K-ras* mutation status are presented for men and women in table 3. No significant association was observed between dietary folate and overall colon cancer risk or with the *K-ras* mutation status after adjustment for age, QI, smoking, alcohol, fresh meat, energy intake and family history of CRC, iron intake, vitamin C intake and dietary fibre.

Table 3 Incidence rate ratios (RR) and 95% confidence intervals (CI) for colon cancer patients overall and with a (specific) *K-ras* mutation status (N=448) according to dietary folate intake

	RR (95% CI) per increment of 100 µg/day increase of dietary folate intake			
	N	Men	N	Women
colon cancer				
RR ¹	231	1.08 (0.91-1.30)	199	0.87 (0.67-1.15)
RR ²	231	0.87 (0.66-1.14)	199	0.98 (0.62-1.56)
<i>K-ras</i>				
RR ²	148	0.94 (0.69-1.27)	141	0.89 (0.52-1.52)
<i>K-ras</i> *				
RR ²	83	0.76 (0.47-1.22)	58	1.22 (0.52-2.82)
G>A*				
RR ²	47	0.78 (0.40-1.53)	34	1.29 (0.48-3.49)
G>T*, G>C*				
RR ²	29	0.77 (0.41-1.45)	25	1.56 (0.49-4.95)

¹RR adjusted for age²RR adjusted for age, QI, smoking, alcohol, fresh meat, energy intake and family history of CRC, vitamin C, iron, fiber

For rectal cancer, associations between folate intake and risk of disease overall, as well as *K-ras* mutation status are presented for men and women in table 4. A significant, inverse association was observed between folate and overall rectal cancer risk in men (RR 0.58, 95% CI 0.36-0.93), as well as with a *K-ras* mutation in the tumour (RR 0.40, 95% CI 0.17-0.89). The association was strongest for G>T or G>C transversions in the *K-ras* gene (RR 0.19, 95% CI 0.03-1.07). For women, a significant positive association was observed between folate and overall rectal cancer risk (RR 1.85, 95% CI 1.13-3.02) and this association was observed for rectal tumours without and with a *K-ras* oncogene mutation (RR 1.94, 95% CI 1.03-3.66 and 1.83, 95% CI 0.91-3.68, respectively). After taking into account the specific point mutations in the *K-ras* gene, an increased risk was observed for rectal tumours harbouring G>T or G>C transversions (RR 2.69, 95% CI 1.43-5.09). However, a significant inverse association was observed between folate and G>A transitions (RR 0.08, 95% CI 0.01-0.53).

Table 4 Incidence rate ratios (RR) and 95% confidence intervals (CI) for rectal cancer patients overall and with a (specific) *K-ras* mutation status (N=160) according to dietary folate intake

	RR (95% CI) per increment of 100 µg/day increase of dietary folate intake			
	N	Men	N	Women
rectal cancer				
RR ¹	99	0.89 (0.68-1.16)	51	1.43 (0.98-2.08)
RR ²	99	0.58 (0.36-0.93)	51	1.85 (1.13-3.02)
<i>K-ras</i>				
RR ²	63	0.69 (0.40-1.19)	24	1.94 (1.03-3.66)
<i>K-ras</i> *				
RR ²	36	0.40 (0.17-0.89)	27	1.83 (0.91-3.68)
G>A*				
RR ²	22	0.68 (0.36-1.28)	11	0.08 (0.01-0.53)
G>T*, G>C*				
RR ²	13	0.19 (0.03-1.07)	17	2.69 (1.43-5.09)

¹RR adjusted for age²RR adjusted for age, QI, smoking, alcohol, fresh meat, energy intake and family history of CRC, vitamin C, iron, fiber

DISCUSSION

In a large cohort with 448 incident colon cancer patients and 160 incident rectal cancer patients, no significant associations were observed between folate intake and the risk of colon cancer in men and women, either overall or after the *K-ras* mutation status was taken into account. For men, a protective effect was observed for folate and rectal cancer risk, overall and most pronounced, with *K-ras* mutated tumours. For women, however, an increased association was observed between folate and rectal cancer risk, overall and also when the *K-ras* mutation status was taken into account. Regarding the specific point mutations in the *K-ras* gene of rectal tumours in women, dietary folate was positively associated with G>T or G>C transversions, however, inversely associated with G>A transitions.

The current cohort study on folate intake and specific *K-ras* mutations is, to our knowledge, the only prospective study performed to date taking into account the *K-ras* mutation status. The prospective design of this study and high completeness of follow-up of cancer incidence and subcohort make information and selection bias unlikely. In addition, as a result of the exclusion of the first 2.3 years of follow-up, the chance of information bias due to potential preclinical CRC is minimal.

Although several epidemiological studies have been conducted on dietary factors and *K-ras* mutations in colon and rectal cancer, only three have reported on folate intake, i.e. one large case-control study with colon cancer patients (17), one case-control study with colon and rectal cancer patients (20) and one cross-sectional case-case study with colorectal adenoma patients (7). Slattery *et al* (17) observed a statistically significant inverse association between low dietary folate intake and the risk of colon tumours with a wild type *K-ras* gene (odds ratio (OR) for the lowest versus the highest tertile of intake 1.4, 95% CI 1.1-1.8; $p_{\text{trend}} < 0.01$). In another smaller case-control study with colon and rectal cancer patients, no statistical differences in dietary folate intake as observed between cases with a *K-ras* mutation compared to cases with a wild type *K-ras* (18). Therefore, case-control analyses regarding dietary folate intake was not further explored in the study by Bautista *et al* (18). In a large case-case comparison of colorectal adenoma patients (7), individuals in the upper tertile of total folate intake (dietary and supplemental folate), compared with individuals in the lowest tertile, had a nearly 50% lower risk of having *K-ras* mutation-positive adenomas (OR 0.52, 95% CI 0.30-0.88; $p_{\text{trend}} 0.02$). This reduction in risk of *K-ras* mutation-positive adenomas was not observed for dietary folate intake alone (OR 0.91, 95% CI 0.50-1.64; $p_{\text{trend}} 0.75$). Caution is warranted in interpreting these results, because case-case comparisons, including both male and female colon and rectal adenomas, were performed. Case-case comparisons are valuable for the estimation of etiologic heterogeneity with regard to *K-ras* mutation status. However, they do not give information on the direction of the association between folate and *K-ras* wild type or mutated tumours, respectively. This can only be obtained with case-control comparisons. In addition, we observed distinct differences in associations between men and women and colon and rectal cancer. Our results do not support the protective effect of folate for mutated *K-ras* colon tumours observed in the other studies. One explanation could be that dietary folate intake in The Netherlands is too low to evoke its potentially protective effect in colon and rectal cancer, although a protective effect was observed for male rectal cancer. In the NLCS, 22% of the study population used vitamin B supplements in 1986 (31). However, due to legislative restrictions in The Netherlands, it was not allowed until 1994 to use folic acid in vitamin supplements. Therefore, the overall effect of folic acid supplements in this cohort is presumably negligible.

In the current study, an inverse association was observed for both men and women between high folate intake and rectal tumours harbouring a G>A transition in the *K-ras*

oncogene. This was also observed by Slattery *et al* (17), although not statistically significant, for G>A transitions at the second position of codon 12 (OR for lowest tertile versus highest tertile of intake 0.7, 95% CI 0.4-1.3; P_{trend} 0.11). This could be an indirect consequence of promoter hypermethylation of mismatch repair genes, like the O⁶-methylguanine DNA methyltransferase (MGMT) gene. Van Engeland *et al* (10) evaluated the association between promoter hypermethylation of genes involved in colorectal carcinogenesis, like MGMT, and folate and alcohol intake within the framework of the NLCS. MGMT is a DNA repair protein that removes adducts from the O⁶ position of guanine (32) in DNA, thereby preventing G>A or C>T mutations. Despite the limited size of the study performed by van Engeland *et al* (10), the data suggest that low folate and high alcohol intakes may be associated with promoter hypermethylation in genes involved in CRC, i.e. MGMT. No clear associations were observed for folate and G>A transitions in men, either in the colon or the rectum. More aetiological insight in the underlying mechanisms relating the differences in tumour site, gender and K-ras mutation status in CRC is required to clarify this issue. Moreover, due to the overall small number of cases when studying subgroups of K-ras mutated tumours, the results of this study should be interpreted with caution, and need replication in future larger studies.

We observed an increased risk of rectal cancer with increased folate intake in women. This was observed for both wild type and mutated K-ras gene, i.e. G>T and G>C transversions, but not for G>A transitions as described above. For men, however, an inverse association with G>T and G>C transversions in rectal tumours was observed. Another recent study also showed elevated risks for rectal cancer in women with increased dietary folate intake, though not statistically significant (4). With the folate food fortifications and extensive use of (multi)vitamin supplements including folate in different countries, it is important to further investigate this issue in prospective studies.

In conclusion, in this study dietary folate is not significantly associated with colon cancer risk, either overall or with the K-ras mutation status. Moreover, our data suggest that the effect of high folate intake on rectal cancer risk is different for men and women, and depends on the K-ras mutation status of the tumour.

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INTRODUCTION

In this chapter, we present a general discussion of the results of the study. We first discuss the theoretical background of the study, and then we discuss the results of the study. We then discuss the implications of the study for future research and for practice.

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6

General Discussion

CONCLUSIONS

The results of the study are summarized in the following conclusions:

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CHAPTER 6

INTRODUCTION

In this molecular epidemiological investigation, alterations in the *K-ras* oncogene were studied with respect to their frequency and relation to dietary risk factors in sporadic colorectal adenocarcinomas. In the current chapter, the findings and methodology of other relevant molecular epidemiological studies and our study on *K-ras*, dietary factors and CRC are compared and its merits are discussed. Current epidemiological evidence, including the results of this thesis, on meat, fat and folate in the diet in relation to *K-ras* mutations in colorectal tumours is summarized in tables 1, 2 and 3. Next, the strengths and limitations of epidemiological studies incorporating molecular endpoints will be discussed. Finally, the implications for the interpretation of our findings and for future research will be addressed.

EPIDEMIOLOGICAL EVIDENCE FOR ASSOCIATIONS BETWEEN DIETARY FACTORS AND *K-RAS* MUTATIONS IN COLORECTAL CANCER

The current evidence

The molecular epidemiological investigations conducted to date include two cross sectional studies (1, 2), three case-control studies (3-5), and our prospective cohort study. The main findings of these studies are presented in tables 1, 2 and 3.

Red meat was not related to the *K-ras* status, of either colorectal adenomas (1) or colorectal carcinomas (2) (table 1) in the cross-sectional studies. In addition, Slattery *et al* (4) did not observe a significant association between red meat or processed meat and the risk of colon cancer with or without *K-ras* mutations (table 6.1) within a case-control study. In case-case comparisons within the case-control study by Kampman *et al* (3), the consumption of poultry was associated with a 15-fold increased risk of *K-ras* mutations in codon 12 compared with mutations in codon 13. To further evaluate the observed aetiological heterogeneity between codons 12 and 13 for poultry consumption, these authors compared both case groups with a population-based control group. A non-significant, positive association was observed with codon 12 mutations (OR 1.2, 95% CI 0.8-1.6), whereas a non-significant, inverse association was found with codon 13 mutations (OR 0.4, 95% CI 0.1-1.2). No substantial differences in *K-ras* mutation status were observed with regard to the consumption of total red meat, beef, processed meat and fish. Our results (table 6.1 and chapter 3), on the other hand, suggest that beef, pork and meat products exert their effect via another route than the *K-ras* signalling pathway, since associations were mainly observed with wild type *K-ras*.

The associations between total fat and specific fatty acids and the risk of *K-ras* mutations in CRC risk were studied by Martinez *et al* (1), Bautista *et al* (5), Kampman *et al* (3), Slattery *et al* (4) and Brink *et al* (6) (table 2). Except for Bautista *et al* (5), none of the cross-sectional or case-control studies (1,3) reported significant associations between total fat and the *K-ras* mutation status in CRC. A strong association for total fat and mono-unsaturated fat (MUFA) with an increased risk of mutated *K-ras* tumours was observed for *K-ras* mutated CRC patients compared to wild type *K-ras* CRC patients (5). Results from case-control analyses showed that the strong associations with total fat and MUFA were restricted to CRC patients bearing a wild type *K-ras* gene, i.e. there was a significant protective effect of MUFA on wild type *K-ras* tumours. These associations were not reported in the other studies (1,3). In addition, no associations were observed between total fat and MUFA and *K-ras* mutation status in the current study (table 2). The study by Bautista *et al* (5) was conducted in Spain where olive oil is the main source of oleic acid and thus of MUFA. This is not the case for the current cohort and this could be an explanation for the different findings. Results from our study indicate an increased association between the risk

Table 1 Main findings of the molecular epidemiological studies on the associations between the consumption of meat, meat products and fish, and *K-ras* mutations in sporadic CRC

Study population	Dietary factor	Contrast	Comparison	OR or RR* (95% CI)
Cross-sectional study				
678 colorectal adenoma cases <i>Marinez et al, 1999 (1)</i>	red meat	highest (>71 g/day) vs. lowest (<33.3 g/day) tertile	mutated <i>K-ras</i> /wild type <i>K-ras</i>	1.13 (0.63-2.05)
43 left-sided colorectal cancer cases <i>O'Brien et al, 2000 (2)</i>	red meat	mean intake mutated <i>K-ras</i> (92.4 ± 9.7 g/day) vs. wild type <i>K-ras</i> (82.3 ± 7.7 g/day)	mutated <i>K-ras</i> /wild type <i>K-ras</i>	difference in mean intake is not statistically significant
Case-control study				
185 colon cancer cases <i>Kampman et al, 2000 (3)</i>	total red meat	increment of 43 g increase/day	mutated <i>K-ras</i> /wild type <i>K-ras</i> codon12/codon 13 transition/transversion	1.1 (0.8-1.6) 1.3 (0.5-3.4) 1.4 (0.7-2.9)
	beef	increment of 26 g increase/day	mutated <i>K-ras</i> /wild type <i>K-ras</i> codon12/codon 13 transition/transversion	1.0 (0.7-1.4) 1.7 (0.7-4.2) 0.9 (0.5-1.8)
	processed meat	increment of 23 g increase/day	mutated <i>K-ras</i> /wild type <i>K-ras</i> codon12/codon 13 transition/transversion	1.0 (0.7-1.5) 1.0 (0.5-2.2) 1.1 (0.6-1.8)
	poultry	increment of 17 g increase/day	mutated <i>K-ras</i> /wild type <i>K-ras</i> codon12/codon 13 transition/transversion	1.1 (0.8-1.5) 15.0 (1.4-160.0) 0.9 (0.5-1.8)
	fish	increment of 20 g increase/day	mutated <i>K-ras</i> /wild type <i>K-ras</i> codon12/codon 13 transition/transversion	1.2 (1.0-1.5) 1.8 (0.8-4.4) 0.9 (0.7-1.1)
1428 colon cancer cases and 2406 controls <i>Slattery et al, 2000 (4)</i>	red meat	highest (>1.0 g/1000 kcal) vs. lowest (<0.3 g/1000 kcal) tertile	mutated <i>K-ras</i> /controls wild type <i>K-ras</i> /controls mutated <i>K-ras</i> /wild type <i>K-ras</i>	0.9 (0.6-1.3) 1.1 (0.9-1.4) 0.8 (0.6-1.2)
	processed meat	highest (>0.23 g/1000 kcal) vs. lowest (≤0.08 g/1000 kcal) tertile	mutated <i>K-ras</i> /controls wild type <i>K-ras</i> /controls mutated <i>K-ras</i> /wild type <i>K-ras</i>	0.9 (0.7-1.2) 1.0 (0.8-1.3) 0.9 (0.6-1.2)
Cohort study				
608 incident colon and rectal cancer cases and 2948 subcohort members <i>Brink et al, 2004 (6)*</i>	total fresh meat (beef, pork, minced meat, liver, chicken, other meat) ¹	highest (150.8 g/day) vs. lowest (61.0 g/day) quartile for men, highest (139.2 g/day) vs. lowest (50.7 g/day) quartile for women	<i>Colon</i>	
			wild type <i>K-ras</i> /subcohort	0.94 (0.65-1.36)
			mutated <i>K-ras</i> /subcohort	1.01 (0.61-1.66)
	meat products	highest (33.3 g/day) vs. lowest (1.0 g/day) quartile for men, highest (22.4 g/day) vs. lowest (0.0 g/day) quartile for women	<i>Rectum</i>	
			wild type <i>K-ras</i> /subcohort	0.76 (0.41-1.44)
			mutated <i>K-ras</i> /subcohort	0.68 (0.31-1.47)
			<i>Colon</i>	
			wild type <i>K-ras</i> /subcohort	1.42 (1.00-2.03)
			mutated <i>K-ras</i> /subcohort	0.77 (0.45-1.32)
			<i>Rectum</i>	
			wild type <i>K-ras</i> /subcohort	0.84 (0.45-1.58)
			mutated <i>K-ras</i> /subcohort	1.41 (0.67-2.99)

Table 1 continued

Study population	Dietary factor	Contrast	Comparison	OR or RR* (95% CI)
Cohort study				
608 incident colon and rectal cancer cases and 2948 subcohort members <i>Brink et al, 2004 (6)*</i>	fish	highest (30.5 g/day) vs. lowest (0.0 g/day) quartile for men,	Colon	0.87 (0.60-1.26)
		highest (28.2 g/day) vs. lowest (0.0 g/day) quartile for women	mutated <i>K-ras</i> /subcohort	1.38 (0.85-2.25)
			Rectum	1.06 (0.58-1.94)
			mutated <i>K-ras</i> /subcohort	0.78 (0.37-1.65)

*RR only in study by Brink *et al*, in all other studies the OR is used

[†] RRs for the specific types of meat, see chapter 3 of the thesis

of colon tumours with a mutated *K-ras* gene and ω -6 PUFA intake, in particular linoleic acid (table 2). Moreover, these associations were also observed for G>A transitions and G>T and G>C transversions in the *K-ras* gene (chapter 4). To our knowledge, the association between ω -6 PUFA, in particular linoleic acid, and *K-ras* mutation status and the specific *K-ras* point mutations has not been investigated previously in CRC.

For total (dietary+supplemental) folate intake (table 3), Martinez *et al* (1) observed a substantial inverse association for the risk of colorectal adenomas with a mutated *K-ras* gene as compared to adenomas with a wild type *K-ras* gene, but these authors did not observe an association for dietary folate alone (table 3). Slattery *et al* (4) found that a low intake of dietary folate was associated with an increased risk of wild type *K-ras* colon tumours versus controls. No association was observed for mutated *K-ras* colon tumours versus controls. However, low intake of dietary folate was associated with an inverse risk of G>A transitions in codon 12 of the *K-ras* gene in colon tumours versus wild type *K-ras* colon tumours. In the current study, an inverse association was seen for dietary folate and mutated *K-ras* rectal tumours in men, whereas an increased association was found for women (table 3 and chapter 5). For men, this inverse association was observed for G>A transitions as well as G>T or G>C transversions (chapter 5). For women, the increased association was confined to G>T or G>C transversions, whereas an inverse association was observed for G>A transitions.

Case-case comparisons versus case-control comparisons

In reviewing the molecular epidemiological evidence, as summarised in tables 1 to 3, it is important to distinguish between results based on case-case comparisons and case-control/subcohort comparisons. Although case-case comparisons may give information on the degree of heterogeneity of risk factors between cases with a mutated *K-ras* tumour and cases with a wild type *K-ras* tumour, these comparisons can not give any information on the risk of mutated *K-ras* or wild type *K-ras* tumours versus a control group, i.e. a non-case group. Case-case comparisons may give clues as to whether risk factors differ for subgroups, but additional case-control/subcohort comparisons are necessary to elucidate the direction and strength of associations. For example, Bautista *et al* (5) observed a strong association for MUFA intake with mutated *K-ras* CRC cases versus wild type *K-ras* cases. However, additional case-control analyses were conducted and showed an inverse association between MUFA and the risk of wild type *K-ras* colorectal tumours, whereas no significant association with the risk of mutated *K-ras* tumours was found. Thus, MUFA appears to be a protective factor for wild type *K-ras* CRC cases, whereas it has no effect on *K-ras* mutated CRC cases. Thus, the case-case comparisons could be misleading if case-control comparisons are not presented.

The choice of dietary factors evaluated in the current thesis was based on biological mechanisms enumerating from previous experimental and molecular epidemiological studies (see chapters 3 through 5). Biological mechanisms for meat, fat and dietary folate in relation to *K-ras* mutations as described in the literature support the associations that we found between these dietary factors and the risk of colorectal tumours with a *K-ras* mutation (chapters 3 through 5). With respect to wild type *K-ras* tumours, inverse associations were observed in two prior studies for folate (4) and MUFA (5). None of the other molecular epidemiological studies presented associations between the dietary factors under study and the risk of wild type *K-ras* tumours (1-3). Therefore, in order to evaluate the differential effects of dietary factors on the risk of tumours with and without a *K-ras* mutation, case-cohort analyses were performed for these different categories of cases.

STRENGTHS AND LIMITATIONS OF EPIDEMIOLOGICAL STUDIES INCORPORATING MOLECULAR ENDPOINTS

The degree of coverage and completeness of the follow-up will be discussed in order to

Table 2 Main findings of the molecular epidemiological studies on the associations between fat intake and *K-ras* mutations in sporadic CRC

Study population	Dietary factor	Contrast	Comparison	OR or RR* (95% CI)
Cross-sectional study				
678 colorectal adenoma cases <i>Marinez et al, 1999 (1)</i>	total fat	highest (>82.3 g/day) vs. lowest (<55.5 g/day) tertile	mutated <i>K-ras</i> /wild type <i>K-ras</i>	1.55 (0.76-3.16)
	saturated fat	highest (>27.2 g/day) vs. lowest (≤18.0 g/day) tertile	mutated <i>K-ras</i> /wild type <i>K-ras</i>	1.06 (0.54-2.07)
Case-control study				
108 colorectal cancer cases and 295 controls <i>Bautista et al, 1997 (5)</i>	total fat	highest vs. lowest tertile	mutated <i>K-ras</i> /wild type <i>K-ras</i>	3.04 (0.95-9.76)
			mutated <i>K-ras</i> /controls	1.4 (0.55-3.7)
			wild type <i>K-ras</i> /controls	0.47 (0.22-0.99)
	MUFA	highest vs. lowest tertile	mutated <i>K-ras</i> /wild type <i>K-ras</i>	10.71 (2.84-40.4)
			mutated <i>K-ras</i> /controls	1.6 (0.63-4.2)
			wild type <i>K-ras</i> /controls	0.25 (0.10-0.60)
	PUFA	highest vs. lowest tertile	mutated <i>K-ras</i> /wild type <i>K-ras</i>	0.73 (0.25-2.18)
			mutated <i>K-ras</i> /controls	0.46 (0.18-1.2)
			wild type <i>K-ras</i> /controls	0.86 (0.43-1.7)
	saturated fat	highest vs. lowest tertile	mutated <i>K-ras</i> /wild type <i>K-ras</i>	0.53 (0.16-1.71)
			mutated <i>K-ras</i> /controls	0.90 (0.34-2.4)
			wild type <i>K-ras</i> /controls	0.79 (0.38-1.7)
185 colon cancer cases <i>Kampman et al, 2000 (3)</i>	total fat	increment of 20 g increase/day	mutated <i>K-ras</i> /wild type <i>K-ras</i>	1.2 (0.9-1.6)
			codon12/codon 13	1.2 (0.6-2.3)
			transition/transversion	0.9 (0.6-1.4)
	saturated fat	increment of 16 g increase/day	mutated <i>K-ras</i> /wild type <i>K-ras</i>	1.1 (0.8-1.4)
			codon12/codon 13	1.4 (0.7-2.6)
			transition/transversion	1.1 (0.7-1.6)

Table 2 continued

Study population	Dietary factor	Contrast	Comparison	OR or RR* (95% CI)
Case-control study				
1428 colon cancer cases and 2406 controls Slattery et al, 2000 (4)	total fat	highest (>40.0 g/1000 kcal) vs. lowest (≤30.0 g/1000 kcal) tertile	mutated <i>K-ras</i> /controls	1.1 (0.8-1.5)
			wild type <i>K-ras</i> /controls	1.0 (0.8-1.2)
			mutated <i>K-ras</i> /wild type <i>K-ras</i>	1.2 (0.8-1.7)
	saturated fat	highest (>15.0 g/1000 kcal) vs. lowest (≤10.0 g/1000 kcal) tertile	mutated <i>K-ras</i> /controls	1.1 (0.8-1.5)
			wild type <i>K-ras</i> /controls	0.8 (0.7-1.1)
			mutated <i>K-ras</i> /wild type <i>K-ras</i>	1.3 (0.9-1.9)
	MUFA	highest (>14.2 g/1000 kcal) vs. lowest (≤11.0 g/1000 kcal) tertile	mutated <i>K-ras</i> /controls	1.1 (0.8-1.4)
			wild type <i>K-ras</i> /controls	1.0 (0.8-1.2)
PUFA	highest (>8.0 g/1000 kcal) vs. lowest (≤5.5 g/1000 kcal) tertile	mutated <i>K-ras</i> /controls	1.1 (0.8-1.4)	
		wild type <i>K-ras</i> /controls	1.1 (0.9-1.4)	
Cohort study				
608 incident colon and rectal cancer cases and 2948 subcohort members Brink et al, 2004 (6)*	total fat	highest (109.1 g/day) vs. lowest (78.1 g/day) quartile for men, highest (85.3 g/day) vs. lowest (63.0 g/day) quartile for women	Colon	
			wild type <i>K-ras</i> /subcohort	0.99 (0.70-1.38)
			mutated <i>K-ras</i> /subcohort	0.88 (0.54-1.44)
			Rectum	
			wild type <i>K-ras</i> /subcohort	0.63 (0.33-1.20)
			mutated <i>K-ras</i> /subcohort	1.18 (0.63-2.23)
	saturated fat	highest (45.8 g/day) vs. lowest (28.9 g/day) quartile for men, highest (36.6 g/day) vs. lowest (23.9 g/day) quartile for women	Colon	
			wild type <i>K-ras</i> /subcohort	1.06 (0.74-1.54)
			mutated <i>K-ras</i> /subcohort	0.73 (0.45-1.18)
			Rectum	
			wild type <i>K-ras</i> /subcohort	0.64 (0.34-1.21)
			mutated <i>K-ras</i> /subcohort	0.86 (0.43-1.72)
	MUFA	highest (42.5 g/day) vs. lowest (28.2 g/day) quartile for men, highest (33.0 g/day) vs. lowest (22.4 g/day) quartile for women	Colon	
			wild type <i>K-ras</i> /subcohort	1.18 (0.60-1.26)
			mutated <i>K-ras</i> /subcohort	0.67 (0.40-1.13)
			Rectum	
			wild type <i>K-ras</i> /subcohort	0.69 (0.38-1.24)
			mutated <i>K-ras</i> /subcohort	1.22 (0.61-2.44)
	PUFA	highest (29.1 g/day) vs. lowest (11.6 g/day) quartile for men, highest (22.5 g/day) vs. lowest (8.8 g/day) quartile for women	Colon	
			wild type <i>K-ras</i> /subcohort	0.91 (0.63-1.31)
			mutated <i>K-ras</i> /subcohort	2.09 (1.25-3.49)
			Rectum	
			wild type <i>K-ras</i> /subcohort	0.89 (0.49-1.62)
			mutated <i>K-ras</i> /subcohort	0.75 (0.39-1.44)
	linolenic acid	highest (2.0 g/day) vs. lowest (0.8 g/day) quartile for men, highest (1.6 g/day) vs. lowest (0.6 g/day) quartile for women	Colon	
			wild type <i>K-ras</i> /subcohort	1.05 (0.74-1.49)
			mutated <i>K-ras</i> /subcohort	0.95 (0.59-1.55)
			Rectum	
			wild type <i>K-ras</i> /subcohort	0.66 (0.37-1.17)
			mutated <i>K-ras</i> /subcohort	1.54 (0.73-3.25)
	linoleic acid	highest (27.9 g/day) vs. lowest (10.0 g/day) quartile for men, highest (21.2 g/day) vs. lowest (7.4 g/day) quartile for women	Colon	
			wild type <i>K-ras</i> /subcohort	1.01 (0.70-1.45)
			mutated <i>K-ras</i> /subcohort	2.19 (1.30-3.67)
			Rectum	
			wild type <i>K-ras</i> /subcohort	1.12 (0.61-2.07)
			mutated <i>K-ras</i> /subcohort	0.93 (0.48-1.79)

*RR only in study by Brink et al, in all other studies the OR is used

evaluate the influence of selection and information bias on misclassification. In addition, implications of possible measurement errors in the assessments of dietary factors and *K-ras* mutations, followed by confounding and multiple testing will be discussed.

Selection bias

Since the person-year follow-up was nearly 100% complete (98% for 7.3 years of follow-up), selection bias in general is unlikely to be important in The Netherlands Cohort Study on diet and cancer (NLCS). The cohort was recruited from samples drawn from a large number of municipal population registries. Municipalities were selected according to the degree of coverage of their inhabitants by the cancer registries (CR) and PALGA, minimising loss to follow-up (7). The mean coverage appeared to increase from 98.5% at the start of the study to 100% in 1988. Therefore, tumour tissue of CRC patients was

Table 3 Main findings of the molecular epidemiological studies on the associations between dietary folate intake and *K-ras* mutations in sporadic CRC

Study population	Dietary factor	Contrast	Comparison	OR or RR* (95% CI)
Cross-sectional study				
678 colorectal adenoma cases <i>Marinez et al, 1999 (1)</i>	dietary folate	highest (>318 µg/day) vs. lowest (<226 µg/day) tertile	mutated <i>K-ras</i> /wild type <i>K-ras</i>	0.91 (0.50-1.64)
	dietary + supplemental folate	highest (>571 µg/day) vs. lowest (<279 µg/day) tertile	mutated <i>K-ras</i> /wild type <i>K-ras</i>	0.52 (0.30-0.88)
Case-control study				
108 colorectal cancer cases and 295 controls <i>Bautista et al, 1997 (5)</i>	folic acid	highest vs. lowest tertile	mutated <i>K-ras</i> /wild type <i>K-ras</i>	2.03 (0.68-6.11)
1428 colon cancer cases and 2406 controls <i>Slattery et al, 2000 (4)</i>	folate	lowest (≤125 mcg /1000 kcal) vs. highest (>220 mcg /1000 kcal) tertile**	mutated <i>K-ras</i> /controls	1.2 (0.8-1.7)**
			wild type <i>K-ras</i> /controls	1.4 (1.1-1.8)**
			mutated <i>K-ras</i> /wild type <i>K-ras</i>	0.8 (0.6-1.2)**
			2 G>A/wild type <i>K-ras</i>	0.7 (0.4-1.3)**
Cohort study				
608 incident colon and rectal cancer cases and 2948 subcohort members <i>Brink et al, 2004 (6)*</i>	dietary folate	increment of 100 µg increase/day	wild type <i>K-ras</i> /subcohort	
			<i>Colon</i>	
			men	0.94 (0.69-1.27)
			women	0.89 (0.52-1.52)
			<i>Rectum</i>	
			men	0.69 (0.40-1.19)
			women	1.94 (1.03-3.66)
			mutated <i>K-ras</i> /subcohort	
			<i>Colon</i>	
			men	0.76 (0.47-1.22)
			women	1.22 (0.52-2.82)
			<i>Rectum</i>	
			men	0.40 (0.17-0.89)
			women	1.83 (0.91-3.68)

*RR only in study by Brink et al, in all other studies the OR is used

**the reference category is the highest level of intake of folate (instead of the lowest level of intake, as in the other studies in the table)

collected for the patients identified between 2.3 and 7.3 years after the start of the NLCS (in 1986), i.e. patients diagnosed with CRC between 1989 and 1994.

Within this 5-year period, 929 incident CRC cases with histologically confirmed tumours were identified of whom 819 cases could also be linked to a PALGA report of the lesion. The linkage to the PALGA database, and thus to the availability of the individual pathological reports, was necessary in order to locate the tumour material at a specific laboratory. Reasons for not finding PALGA reports for 12% of the eligible patients include: 1) PALGA alone reached national coverage from 1991 onwards, although the NCR and PALGA combined reached a coverage of near 100% in the municipalities included in our study by 1988, 2) approximately 6% of histologically confirmed cases in the NCR are not reported by PALGA due to administrative reasons (data Comprehensive Cancer Center Limburg 1990-1996, personal communication L. Schouten) and 3) mistakes in identifying information, leading to incorrect linkage, i.e. name, date of birth and gender. The pathology laboratories that did, or did not deliver their reports to PALGA after 1991 were not different with respect to location in an academic hospital or a cancer specialty centre. Therefore, it is not expected that differential selection bias occurred with respect to dietary intake or tumour characteristics.

In this study, tissue blocks were available for 83% of the cases (776 out of 929) since the PALGA report of the lesion was unavailable for 12% of cases (110 out of 929 resulting in 819) and an additional 5% (43 out of 819, i.e. 776) of the tumour blocks could not be traced. Reasons for this loss to follow-up of tumour blocks ($N=43$) were 1) for 9 patients registered in one pathology laboratory, tissue could not be retrieved due to administrative inconsistencies and 2) for 34 patients the material could not be found (8). In addition, for 5% of the patients (i.e. 39 out of 776) the *K-ras* mutation status could not be determined due to either the presence of only normal colon mucosa (20 out of 39), a revision with a benign adenoma instead of an adenocarcinoma (10 out of 39), an insufficient yield of DNA (6 out of 39) or the absence of malignant CRC tissue (3 out of 39) (6).

Nearly all tumour material of CRC patients was collected from the various pathology laboratories by one person, usually by retrieval directly from the archives on location instead of requesting the material by mail. This approach could have increased the invaluable willingness of the pathologists to cooperate in the current study, since this way of collecting the tumour material was both time and cost efficient for the pathology departments. Both the degree of participation and the yield of the samples are not expected to be related to either characteristics of the tumour or dietary intake.

Taken together, the recovery of the tumour tissue is considered as more than satisfactory, and it is not anticipated that selection bias would lead to differential misclassification of the *K-ras* mutation status of the tumour. The series of samples that were analysed consist of non-selected, incident CRC patients, representative for the NLCS.

Information bias

It is unlikely that systematic misclassification of exposure is of significant importance in the current study, since all prevalent cases (i.e. clinically diagnosed) were excluded at baseline. All other participants completed a validated, semi-quantitative food frequency questionnaire at baseline. In addition, due to the exclusion of the first 2.3 years of follow-up, influence of pre-clinical CRC on dietary intake was minimised.

Assessment of dietary factors

The intake data were validated against a nine-day record method (9). The observed correlations were reasonable and were not different from the correlations observed in other studies (chapters 3 through 5). Even though estimations of intake may not be as accurate as we would wish them to be, possible misclassification is expected to be non-differential with

respect to *K-ras* mutation status. Therefore, the associations observed are probably an underestimation of the true association.

Assessment of *K-ras* mutations

Mutation analysis of tumour samples, in particular when routinely-fixed, paraffin-embedded tissue is used obtained within a multi-centred setting, is prone to false positive as well as false negative results. The sensitivity and specificity of the methods used for *K-ras* mutations in the current study are discussed in detail in chapter 2. Findings regarding the reproducibility and validity of these techniques will be discussed briefly in this paragraph.

Reproducibility

To establish the reproducibility of the technique in our study, a subset of DNA specimens was subjected twice to the complete procedure, from tissue sectioning to DNA sequencing (chapter 2). The reproducibility of the mutation analysis was found to be good (88%), in view of the poor DNA template quality of a significant proportion of the archival tissue samples. Surprisingly little has been published on reproducibility of *K-ras* mutation identification in the other reported molecular epidemiological studies (1-5). In our study, the reproducibility appeared to be strongly dependent on the quality of the DNA after tissue processing. The degree of fragmentation of the DNA templates, mainly due to effects of tissue lysis and formalin fixation, is critical for amplification of the *K-ras* fragments, in particular in the first cycles of the PCR. The reproducibility is also dependent on the heterogeneity of the tumour tissue, i.e. on the presence of a variety of clones with different genetic make-up in the tumour. However, the effect of tissue heterogeneity has not been tested experimentally.

In the current study, routinely-fixed, paraffin-embedded colorectal tumour specimens from 54 pathology departments were used for the determination of the *K-ras* mutation status. Variation in procedures for post-operative storage, fixation, dehydration and paraffin-embedding of the tissue between tumour samples as well as between pathology laboratories affect the degree of DNA fragmentation and hence the quality of the DNA to be analysed. Therefore, we took several precautions to warrant a reliable mutation analysis. The exon 1 fragment of the *K-ras* gene, including codons 12 and 13, is a small fragment (114 bp) that can be amplified easily and reliably. Only purified, complete fragments were used for sequencing, the accepted standard for determination of the *K-ras* mutation status. In the end, the *K-ras* mutation status could be determined for all 737 colorectal tumour specimens included in this study, the reproducibility is 88%, and the frequency of the *K-ras* mutations is in line with the frequencies observed in the literature.

Validity

Several experiments were conducted to validate the assessment of the *K-ras* mutation status of the archival samples (chapter 2). The mutation analysis was validated by amplification and sequencing DNA fragments from CRC cell lines, and from adjacent blocks with paraffin-embedded and fresh tissue from a series of CRC specimens. The validation of the used techniques indicated that the observed point mutations of the CRC cell lines are the same as those reported in the literature. Tissue processing under controlled conditions was found not to affect the reliability of our *K-ras* mutation analysis of archival specimens. Therefore, errors in the assessment of *K-ras* mutations may occur at low frequency, mainly due to an intrinsic, poor quality of DNA from samples.

Most importantly, differential misclassification of the *K-ras* mutation status of tumours is not very plausible, since molecular analysis and evaluation of data was done blinded with respect to dietary intake, potential confounders or tumour characteristics such as clinicopathological data of tumours. In addition, evaluation of sequence patterns and data

entry were done by two different laboratory technicians, results were compared, and in case of dissimilarities, data were reviewed together until consensus was obtained.

In conclusion, potential misclassification of cases regarding the *K-ras* mutation status of their tumour, due to minor faults in the reproducibility and validity of the used techniques, is expected to be non-differential with respect to other aspects of the tumour as well as dietary intake.

Confounding

Meat consumption, fat intake and dietary folate were evaluated for their relation with overall colon and rectal cancer and with the *K-ras* mutation status in chapters 3, 4 and 5, respectively. The influence of possible confounders, based on the literature, was tested in the overall colon and rectal cancer models, i.e. without stratification on presence or absence of a *K-ras* mutation. Hence, the variables, significant in either one or both of the models, were included as covariates for all models to be tested, i.e. models for specific molecular endpoints as determined by the *K-ras* mutation status. Possibly, confounders included in the overall models are not important confounders for the associations studied with the *K-ras* mutation status in colon or rectal cancer. In addition, unknown confounders specific to the diet-*K-ras* mutation status associations studied may have played a role. However, we would rather not explore all potential confounders for the associations studied in our dataset because of the increased chance of type-I errors. Since little is known about potential confounders, we restricted our evaluations to the known confounders for the specific diet-CRC associations.

Multiple testing

In total, sixteen food groups or nutrients were considered in this thesis. As differences in the aetiology of colon and rectal cancer have been reported (10), statistical data-analyses were performed separately for colon and rectal cancer as described in chapters 3, 4 and 5. In addition, results were presented separately for men and women in chapter 5. Including the *K-ras* mutation status of the tumours, 170 Cox proportional Hazard tests were performed. A p -value ≤ 0.05 was considered statistically significant. In general, 5% of these tests (9 out of 170 tests) may be found statistically significant, based on chance alone. We observed 18 statistically significant associations. Therefore, caution is warranted in interpreting our results. However, the choice of associations we investigated was primarily based on prior hypotheses, generally generated from experimental studies.

IMPLICATIONS OF THE FINDINGS OF THIS STUDY AND FUTURE PERSPECTIVES

The relevance of the diet-gene associations with respect to the different aetiology of colon and rectal cancer, the importance to the specific molecular alterations in the *K-ras* gene versus the wild type gene, and other relevant mechanisms for the risk of CRC, will be discussed.

Differences in aetiology between colon and rectal cancer

Many of the associations observed in this study were either confined to cancer in the colon or cancer in the rectum, rarely both. Considering our hypotheses set out in chapter 1, these differences were unexpected. However, in view of the finding that the *K-ras* mutation status of tumours differed along the colorectal tract (chapter 2), and suggestions in literature for differences in the aetiology of tumours in the colon versus the rectum (10,11), differences according to localization in the colorectal tract are not surprising, after all. Nevertheless, the differences in associations of dietary factors with colon and rectal cancer with or without

mutations in the *K-ras* gene cannot readily be explained with underlying biological mechanisms that are known at present. Therefore, stratified analyses at different sites in the colorectal tract are preferable if allowed by the power of the study. In our study, patients with a rectosigmoid tumour ($n=85$) were excluded, since the tumours in this rather more clinically than anatomically defined region could not be assigned to either the colon or the rectum. Moreover, the number of patients with rectosigmoid tumours is too small for adequate stratified analyses regarding the *K-ras* mutations status. Finally, replication of our findings with regard to the *K-ras* gene for both colon and rectal cancer would be reassuring.

The importance of point mutations in the *K-ras* gene versus wild type *K-ras* gene

Overall, some hypotheses suggested in chapter 1 were confirmed, some were not and, surprisingly, some associations were observed for the wild type *K-ras* gene for which there were no prior hypotheses.

In chapter 3, it was suggested that meat preparation is relevant for development of CRC, i.e. broiled, fried, barbequed or smoked meats may increase the risk through the generation of high concentrations of carcinogens, like PAA's and PAH's. Subsequently, these carcinogens may induce specific point mutations. Information on meat preparation was not collected at baseline in the current study. Since fresh meat generally needs further preparation, this type of meat may be an important source of these carcinogens. Hence, it is plausible that associations between fresh meat and colon or rectal cancer with specific point mutations in the *K-ras* gene exist. However, in this study no association was found between any of the fresh meat variables and risk of colon or rectal cancer with specific point mutations (chapter 3). This could be due to the expected low content of carcinogens in prepared fresh meat consumed in this cohort, and also to the lack of correlation between meat preparation and the amount of fresh meat consumed (12). Surprisingly, associations were observed between certain fresh meat variables and cancer with a wild type *K-ras* gene (chapter 3), i.e. a positive association between colon cancer risk and beef consumption and inverse associations between both colon and rectal cancer risk and pork consumption. Associations with the risk of colon tumours harbouring a wild type *K-ras* gene were also observed with meat products consumption. Although associations were weak and of opposite direction for different meat variables, and may be the result of type I errors, these associations are intriguing and require replication and further study. More specifically, it is of interest to focus on the *K-ras* independent pathway in relation to meat consumption in future studies. For rectal tumours, our observations regarding the increased risk of tumours harbouring G>A transitions support our initial hypothesis regarding the consumption of meat products (chapter 3). However, the lack of this association for colon cancer remains puzzling.

Regarding fat intake, an increased association was observed between PUFA, in particular linoleic acid, and G>A transitions and G>T or G>C transversions in the *K-ras* gene of colon cancer patients (chapter 4). These findings are in line with the observations in previous experimental studies, but need replication in other observational epidemiological studies. On the other hand, the hypotheses regarding MUFA and ω -3 PUFA and *K-ras* mutation status in CRC were not confirmed in the current study. One possible explanation is that the intake of MUFA and ω -3 PUFA in The Netherlands is too low to evoke its potentially protective effect. Another explanation could be that MUFA and ω -3 PUFA are not involved in the development of CRC via a *K-ras*-dependent pathway. Indeed, for MUFA intake, Bautista observed a significant inverse association with the risk of CRC with a wild type *K-ras* gene (5) in a case-control study in Spain where the intake of MUFA is higher than in The Netherlands. This underlines, once again, the potential importance the *K-ras* independent route to colorectal carcinogenesis in relation to dietary intake. The uncertain role of MUFA

and ω -3 PUFA in colorectal carcinogenesis with respect to the *K-ras* gene needs further study.

An inverse association was observed for dietary folate and the risk of rectal tumours with a mutated *K-ras* gene in men, irrespective of the type of point mutation involved (chapter 5). For women, however, a positive association was observed with the risk of rectal tumours harbouring a wild type *K-ras* gene or a *K-ras* mutation. Moreover, the increased association with *K-ras* mutations was confined to G>T or G>C transversions. An inverse association was also seen with rectal tumours harbouring a G>A transition and this finding is in line with the hypothesis. Possibly, the effect of dietary folate is differential for men and women. However, these findings also need replication in other prospective cohort studies.

Overall, our results suggest that meat, fat and folate appear to be of less importance for the risk of colon and rectal tumours with specific point mutations in the *K-ras* gene than was attributed to these factors at the start of the study. However, these factors may be of aetiological importance for the risk of colon or rectal tumours with a wild type *K-ras* gene. Several literature reports never focussed on the findings observed with wild type *K-ras* tumours, partly because a definite conclusion on the risk of wild type *K-ras* tumours alone was hampered by the case-case approach only (1,2). On the other hand, both Bautista and Slattery observed significant associations between dietary factors, i.e. MUFA (5), folate (4) and cruciferous vegetables (4) and the risk of tumours with the wild type *K-ras* gene. It would therefore be useful to study associations between dietary factors and the risk of colon and rectal tumours with molecular characteristics compatible with wild type *K-ras*, such as microsatellite instability of the tumour.

Importance of mechanisms other than the mutation status of the *K-ras* gene

Although we focused on hypotheses related to specific point mutations in the *K-ras* gene, it is interesting to consider other mechanisms relevant for the risk of CRC.

Other (epi)genetic mechanisms

As indicated above, other genes relevant for colorectal carcinogenesis (13,14) should also be studied in this respect. The most important genes include the tumour suppressor gene *APC*, the oncogene *β -catenin* and the DNA repair enzymes (see fig 1 in chapter 1). The focus should not only be on specific point mutations in these genes but could also be on epigenetic aspects such as promotor methylation of the *APC* gene. Methylation of the promotor region of genes is important for the expression of genes, and therefore for normal functioning of gene products (15,16). A number of dietary factors are thought to influence methylation (16). Therefore, (epi)genetic factors involved in colorectal carcinogenesis may be of importance, to be investigated in relation to dietary factors, in addition to the *K-ras* gene alone.

Another aspect to be explored in the future may be the association between dietary factors and types of mutations, not with respect to a specific gene, but genome-wide. When regarding the underlying biological mechanisms for associations between dietary factors and specific point mutations, an association between for example the consumption of PUFA and the increased risk of G>A transitions occurring throughout the genome in tumour cells from CRC patients may be plausible. However, caution is warranted, as the assessment of e.g. G>A transitions will be performed in colorectal tumour tissue from patients already diagnosed with the cancer. Therefore, the assessment of G>A transitions will reflect the genetic make-up of the tumour at the time of diagnosis and is the result of the effects of many different factors responsible for the selective growth advantage of the tumour, and not only dietary factors. This also applies to the findings in this thesis, since the mutation status of the *K-ras* gene is also the status of the tumour cells at the time of diagnosis.

Dietary factors as indirect cancer promoting effectors

Dietary factors may act as promotor-stimulating compounds in the development of CRC instead of as initiating factors by causing specific point mutations. Cells may become more or less susceptible to cell division, depending on dietary intake as well as on the molecular make-up of the cells, for example, the *K-ras* mutation status of the cell. As a result, associations between dietary factors and colon or rectal tumours with a mutated *K-ras* gene may be observed. Whether this may be an explanation for our findings is not clear and needs further exploration in laboratory experiments.

Finally, it would be interesting to consider the role of polymorphisms in metabolic enzymes as effect-modifiers in the relationship between dietary factors and *K-ras* mutation status in CRC patients. Examples of polymorphic enzymes proposed to be involved in colorectal carcinogenesis are the *NAT* genes (17), involved in meat metabolism, the *COX-2* gene (18), involved in fat metabolism, and the polymorphic genes involved in methyl metabolism (19), and thus potentially important to consider when studying the effects of folate.

CONCLUSION

Overall, our results suggest that meat, fat and folate appear to be of less importance for the risk of colon and rectal tumours with specific point mutations in the *K-ras* gene than was attributed to the factors at the start of the study, on the basis of biological mechanisms. However, these factors may be of aetiological importance for the risk of colon or rectal tumours with a wild type *K-ras* gene, strongly suggesting the effects of dietary factors on other pathways leading to CRC. This study shows that incorporating molecular endpoints in classical epidemiological studies of diet and cancer is a relevant approach to unravel the aetiology of colon and rectal cancer.

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Current knowledge of the
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Summary

улытка

Current epidemiological evidence for associations between dietary factors with overall colorectal cancer (CRC) risk is weak or inconsistent. This could be due to methodological factors such as study design, selection bias and information bias. It could also be due to the heterogeneity of the cancer endpoint that is studied. Subtypes of colorectal cancer patients could be distinguished by the absence or presence of alterations in genes involved in the development of colorectal cancer. Therefore, more insight in the aetiology of colorectal cancer can be obtained by studying the associations between dietary factors and alterations in genes involved in the early development of cancer. The majority (95%) of colorectal cancer is non-hereditary, i.e. sporadic, and develops from small adenomatous polyps through a well-defined sequence of cytological changes and this development is associated with the accumulation of alterations in tumour suppressor genes and oncogenes regulating cell division and growth. Among the early alterations that occur in 40% of the colorectal carcinomas are somatic mutations in the *K-ras* oncogene. Approximately 90% of the activating mutations in this gene is found in codons 12 and 13 of exon 1 and around 5% in codon 61 located in exon 2. The most prevalent point mutations in exon 1 are the G>A transitions, the G>T transversions and the G>C transversions. The aim of this molecular epidemiological study was to obtain more insight in the associations between dietary factors and the risk of colorectal tumours with or without specific point mutations in exon 1 of the *K-ras* oncogene.

The studies described in this thesis are embedded in The Netherlands Cohort Study on diet and cancer (NLCS). The NLCS was initiated in 1986 and includes 58,279 men and 62,573 women (55-69 years of age) who originated from 204 Dutch municipalities with computerized population registries and who completed a self-administered questionnaire on diet, family history of cancer and other risk factors for cancer. The entire cohort is monitored for cancer occurrence by annual linkage to The Netherlands Cancer Registry (NCR) and to PALGA, a nationwide network and registry of histo- and cytopathology. Accumulation of person-time in the cohort has been estimated through biennial vital status follow-up of a subcohort of 3,500 men and women who were randomly selected after baseline exposure measurement. After 7.3 years of follow-up and with the exclusion of the first 2.3 years, 929 incident cases with histologically confirmed CRC were observed of whom 819 could also be linked to a PALGA report of the lesion. The tumour tissue samples of these 819 cases were distributed over 54 pathology laboratories throughout The Netherlands. After approval of the Medical Ethical committees, PALGA, the pathologists and the NCR the tissue blocks were made available for this study. Of the 819 tumour tissue samples that were identified, 775 (95%) could be traced and for 737 of these (95% of the available blocks), the *K-ras* mutation status of exon 1 (including exons 12 and 13) could be determined.

In **chapter 1** an overview is presented of the associations between dietary factors and colorectal cancer, and of the (genetic) development of colorectal cancer with special attention for the *K-ras* oncogene. Although much research is performed on diet and colorectal cancer so far, there are still only few studies available on the associations between dietary factors and *K-ras* oncogene mutations in colorectal cancer.

The frequency of *K-ras* oncogene mutations in tumours of incident colorectal cancer patients from the NLCS is described in **chapter 2**. Moreover, the method of mutation detection used in the current study, i.e. direct sequencing of amplified and purified *K-ras* gene fragments, was validated by the confirmation of reported *K-ras* mutation status in CRC cell lines and by a good correlation between fresh-frozen and routinely-fixed, paraffin-embedded tissue. The detection limit of mutated DNA was 5%. The reported frequency of *K-ras* mutations was observed to be 37% among 737 incident colorectal cancer patients, with codons 12 and 13 as the most frequently affected codons (94%). The G>A transition and the G>T and G>C transversions were the most frequently observed point mutations, with the G>T transversion primarily confined to codon 12. Rectal tumours were found to have the

highest frequency of G>T transversions, as compared to colon or rectosigmoid tumours. Stratification by gender revealed that this difference was confined to the female patients. No significant differences between tumours with or without specific *K-ras* mutations were observed for Dukes' stage or tumour differentiation grade.

Subsequently, associations between several dietary factors and the risk of colon or rectal tumours with or without *K-ras* gene mutations were studied. The choice of dietary factors evaluated in the current thesis was based on biological mechanisms underlying the development of specific *K-ras* mutations in colorectal cancer. Differences in the *K-ras* mutation pattern at various subsites in the colorectal tract and in the aetiology of colon and rectal cancer have been reported, and therefore, summarised results from the NLCS are presented separately for colon and rectal cancer (chapters 3 through 5). Patients with a rectosigmoid tumour (n=85) were excluded due to the criterion for classifying rectosigmoid tumours and due to the small number of patients for adequate stratified analyses regarding the *K-ras* mutation status.

It was hypothesized that high levels of heterocyclic aromatic amines (HAA), generated by high temperature cooking of meat proteins, and polycyclic aromatic amines (PAH) originating from grilling and smoking of meat contribute to the occurrence of *K-ras* point mutations. In **chapter 3**, associations between the intake of (several types of) meat, meat products and fish, and the risk of colon or rectal tumours with or without a *K-ras* mutation. Significant associations were mainly observed between meat and meat products and tumours with a wild type *K-ras* gene. A high consumption of beef and meat products was associated with an increased risk of wild type *K-ras* tumours in the colon, but not in the rectum. A high consumption of pork was inversely related with wild type *K-ras* tumours in both colon and rectum. Based on these results, it was concluded that high consumption of (several types of) meat and meat products may be relevant for the aetiology of colon and rectal cancer.

Based on experimental results, it was hypothesized that ω -6 polyunsaturated fat (PUFA) peroxidation leads to the formation and accumulation of malondialdehyde and 4-hydroxynonenal. These by-products could form DNA-adducts with guanine bases which finally may result in G>A transitions or G>T transversions. Is a high ω -6 PUFA intake associated with an increased risk of G>A transitions or G>T transversions in the *K-ras* gene of colon and rectal cancer patients? This was the aim of **chapter 4**. Null findings were observed for the intakes of total fat, saturated fat, monounsaturated fat and ω -3 PUFA, and colon and rectal cancer, overall and also after the *K-ras* mutation status was taken into account. Intake of dietary PUFA (total intake of ω -3 and ω -6 fatty acids) and specifically linoleic acid (as the main source of ω -6 fatty acids) was associated with an increased risk of mutated *K-ras* colon tumours. Similar associations were observed for the specific *K-ras* point mutations, i.e. the G>A transitions, G>T and G>C transversions, in colon tumours. For rectal cancer, however, no associations were observed with overall risk or with *K-ras* mutation status. Possibly, intake of PUFA, especially the ω -6 PUFA, is associated with an increased risk of colon tumours with a *K-ras* gene mutation.

Folate plays an important role in one-carbon metabolism, in DNA synthesis and in methylation of DNA and proteins. Deficient folate intake may generate genomic instability through reduced availability of thymine for DNA synthesis and through increased incorporation of uracil, which may lead to more DNA strand breaks. In addition, global genome hypomethylation and regional hypermethylation in gene promoters could occur as another result of deficient folate intake. In **chapter 5** the relation between folate and *K-ras* mutation status in colon or rectal cancer patients was studied. For men, folate appears to be inversely associated with the overall risk of rectal cancer, and this association was confined to mutated *K-ras* tumours. For women, however, an increased association was observed between high folate intake and the risk of overall rectal cancer. With respect to the *K-ras*

mutation status, the increased association remained for both wild type and mutated *K-ras* tumours. Moreover, this increased association was confined to rectal tumours harbouring G>T and G>C transversions, whereas a significant inverse association was observed with rectal tumours harbouring a G>A transition. Folate seems to play a role in the aetiology of rectal cancer through the involvement of the *K-ras* oncogenes. Results from this study indicate that future molecular epidemiological research on folate intake need to account for sex differences.

In **chapter 6**, the most important findings of our study are summarized and compared with the findings of other relevant molecular epidemiological studies. Overall, our results suggest that meat, fat and folate appear to be of less importance for the risk of colon and rectal tumours with specific point mutations in the *K-ras* gene than was attributed to these factors at the start of the study. However, these factors may be of aetiological importance for the risk of colon or rectal tumours with a wild type *K-ras* gene. In addition, the possibilities and pitfalls of molecular epidemiological studies are discussed in this chapter. Moreover, other genetic factors are pointed out which can be studied in future molecular epidemiological research. This study shows that incorporating molecular endpoints in classical epidemiological studies of diet and cancer is a relevant approach the aetiology of colon and rectal cancer.

Samenvatting

opdrachten

Epidemiologische studies hebben tot nu toe zwakke of tegenstrijdige relaties laten zien tussen voedingsfactoren en het risico op kanker van de dikke darm (colorectum: colon, rectosigmoïd en rectum). Dit kan te maken hebben met methodologische factoren zoals de onderzoeksopzet, selectiebias en informatiebias. Een andere verklaring kan zijn dat dikke darmkanker te heterogeen is om beschouwd te kunnen worden als één ziektebeeld. Mogelijk zijn er meerdere subgroepen van patiënten te onderscheiden door ze in te delen op basis van de af- of aanwezigheid van afwijkingen in genen die betrokken zijn bij de ontwikkeling van dikke darmkanker. Meer inzicht in het ontstaan van dikke darmkanker kan worden verkregen door de relaties te onderzoeken tussen voedingsfactoren en afwijkingen in genen die betrokken zijn bij de vroege ontwikkeling van kanker. Het merendeel (ongeveer 95%) van dikke darmkanker behoort tot de sporadische vorm, dat wil zeggen dat ze niet zijn ontstaan uit erfelijke genetische afwijkingen. Volgens het gangbare model voor de ontwikkeling van dikke darmkanker ontstaan de meeste tumoren uit adenomateuze poliepen in een stapsgewijs proces. Tijdens dit proces stapelen afwijkingen zich op in tumor-suppressor-genen en oncogenen die de groei en differentiatie van dikke darmcellen reguleren. Somatische mutaties in het *K-ras* gen behoren tot de vroege veranderingen die plaatsvinden in een aanzienlijk deel (40%) van tumoren in de dikke darm. De meeste mutaties in dit gen (90%) worden gevonden in codons 12 en 13 van exon 1 en een klein deel (5%) wordt gevonden in codon 61 in exon 2. De puntmutaties in exon 1 zijn vooral G>A transitities, G>T transversies en G>C transversies. Het doel van dit moleculair-epidemiologisch onderzoek was om meer inzicht te krijgen in de relatie tussen verschillende voedingsfactoren en het risico op dikke darmkanker waarbij onderscheid is gemaakt tussen tumoren met en tumoren zonder specifieke mutaties in exon 1 van het *K-ras* gen.

De studies die in dit proefschrift beschreven staan, zijn uitgevoerd binnen het kader van de Nederlandse Cohort Studie naar voeding en kanker (NLCS). De NLCS is gestart in 1986 met ruim 120.000 mannen en vrouwen (55-69 jaar) in 204 Nederlandse gemeenten met een geautomatiseerd bevolkingsregister, verspreid over het land. Bij de aanvang hebben de deelnemers een lijst met vragen over voedingsgewoonten, kanker in de familie en andere risicofactoren voor kanker ingevuld. Vervolgens is het optreden van kanker bij de deelnemers nagegaan door middel van jaarlijkse koppelingen met de Nederlandse Kanker Registratie (NKR) en met het Pathologisch Anatomisch Landelijk Geautomatiseerd Archief (PALGA). Tevens is bij aanvang van de NLCS een aselect subcohort gekozen, bestaande uit 3500 mannen en vrouwen, waarmee de persoonstijd voor het totale cohort wordt geschat. Na 7,3 jaar follow-up, met uitzondering van de eerste 2,3 jaar, werden 929 patiënten met dikke darmkanker geïdentificeerd met behulp van koppeling met de NKR. Voor 819 van de 929 patiënten was ook een PALGA rapport beschikbaar. De paraffine-blokjes met het tumorweefsel van deze 819 patiënten waren opgeslagen in 54 pathologie laboratoria verspreid door heel Nederland. Na toestemming door de betrokken medisch-ethische commissies, PALGA, de pathologen en de NKR was het weefsel voor deze studie beschikbaar. In totaal kon voor 775 van de 819 patiënten (95%) het tumormateriaal worden teruggevonden. Voor 737 van hen (95% van de beschikbare weefselblokjes) kon de *K-ras* mutatie status van exon 1 (waarin codons 12 en 13 zijn gelocaliseerd) worden bepaald.

In **hoofdstuk 1** wordt een literatuuroverzicht gegeven van de relaties tussen voeding en dikke darmkanker, en van de (genetische) ontwikkeling van dikke darmkanker met daarbij speciale aandacht voor het *K-ras* gen. Alhoewel veel onderzoek is verricht naar voeding en dikke darmkanker, zijn er maar weinig studies geweest naar de relatie tussen voeding en *K-ras* mutaties.

In **hoofdstuk 2** is de frequentie van *K-ras* mutaties in tumoren van de incidentie dikke darmkanker patiënten uit de NLCS beschreven. Tevens werd de mutatiedetectie-techniek, namelijk het op directe wijze bepalen van de basenvolgorde van geamplificeerde en gezuiverde *K-ras* genfragmenten, op verschillende manieren gevalideerd. De detectielimiet

werd bepaald op 5% gemuteerd DNA. Er werd een goede correlatie gevonden tussen de bepaling van *K-ras* mutaties in bevroren tumorweefsel en in gefixeerd, paraffine-ingebeld tumorweefsel. Tevens konden de *K-ras* gen mutaties die beschreven zijn in cellijnen afkomstig van dikke darmkanker met onze techniek worden bevestigd. Uiteindelijk werd bij 37% van de 737 patiënten een mutatie in het *K-ras* gen gevonden. De meest voorkomende types puntmutaties zijn G>A transities, en G>T en G>C transversies, waarbij G>T transversies voornamelijk in codon 12 werden gedetecteerd. Tevens werden G>T transversies vooral in rectum tumoren bij vrouwen gevonden. Er werden geen significante verschillen aangetoond voor Dukes' stadiëring of tumordifferentiatie tussen tumoren met of zonder specifieke *K-ras* mutaties.

Vervolgens werd onderzocht of bepaalde voedingsfactoren geassocieerd zijn met het risico op colon of rectum tumoren met of zonder een *K-ras* mutatie. De keuze van de bestudeerde voedingsfactoren in dit proefschrift is gebaseerd op de kennis van de biologische mechanismen die ten grondslag liggen aan deze *K-ras* mutaties. De resultaten in hoofdstukken 3 tot en met 5 zijn apart gepresenteerd voor colon en rectum kanker aangezien gerapporteerd is dat er verschillen bestaan tussen *K-ras* mutatiepatronen in de diverse sublokalisaties van de dikke darm, en dat colon- en rectumkanker mogelijk een verschillende etiologie hebben. Patiënten met een rectosigmoïd tumor werden uitgesloten voor verdere analyses, omdat 1) het classificeren van een tumor in het rectosigmoïd niet gebeurt volgens een duidelijk criterium en 2) het aantal patiënten met een rectosigmoïd tumor te klein was om adequate analyses te kunnen uitvoeren.

Er zijn vele studies die erop wijzen dat hoge concentraties van heterocyclische aromatische amines, die ontstaan als vlees op zeer hoge temperatuur wordt verhit, en van polycyclische aromatische amines, die gevormd worden door vlees te grillen of te roken, *K-ras* puntmutaties kunnen veroorzaken. In **hoofdstuk 3** worden associaties tussen de inname van (verschillende soorten) vlees, vleeswaren en vis en het risico op colon- of rectumtumoren met of zonder een *K-ras* mutatie beschreven. Een hoge consumptie van rundvlees en vleeswaren bleek significant geassocieerd te zijn met een verhoogd risico op tumoren met een wild type *K-ras* gen in het colon, maar niet in het rectum. Een hoge consumptie van varkensvlees was geassocieerd met een verlaagd risico op tumoren met een wild type *K-ras* gen in zowel het colon als het rectum. Op basis van de resultaten van deze studie werd geconcludeerd dat een hoge inname van (verschillende soorten) vlees en vleeswaren relevant kan zijn voor de etiologie van colon- en rectumkanker.

Een hoge inname van vetten lijkt ook een rol te spelen bij het ontstaan van colon- en rectumkanker. Uit experimentele studies blijkt dat peroxidatie van ω -6 poly-onverzadigd vet, dat via de voeding wordt opgenomen, leidt tot vorming van malonaldehyde en 4-hydroxynonenal. In dierexperimenteel onderzoek is aangetoond dat deze producten leiden tot DNA-adducten aan guanine basen, welke uiteindelijk worden omgezet in G>A transities en G>T transversies. Is een hoge inname van ω -6 poly-onverzadigd vet geassocieerd met een verhoogd risico op G>A transities dan wel G>T transversies in het *K-ras* gen in patiënten met colon of rectum kanker? Deze vraag komt aan de orde in **hoofdstuk 4**. Er zijn geen significante associaties gevonden tussen de inname van totaal vet, verzadigd vet en mono-onverzadigd vet enerzijds en het risico op colon en rectum kanker anderzijds, ook niet nadat rekening is gehouden met de *K-ras* mutatiestatus. Een hoge inname van poly-onverzadigd vet (de totale inname van ω -3 en ω -6 poly-onverzadigde vetzuren) en meer specifiek van linolzuur (een hoofdbron van ω -6 poly-onverzadigd vet) zijn wel geassocieerd met een verhoogd risico op colontumoren met een *K-ras* mutatie en deze associatie blijkt onafhankelijk van het type mutatie in de tumor. Voor rectumkanker werden deze relaties echter niet gevonden. Dus, een hoge inname van poly-onverzadigd vet blijkt relevant voor de ontstaanswijze van colon tumoren met een *K-ras* mutatie.

Foliumzuur speelt een belangrijke rol in het koolstofmetabolisme, in de synthese van DNA en in de methylering van DNA en eiwitten. Te weinig inname van foliumzuur kan leiden tot instabiliteit van het genoom; breuken in DNA-ketens kunnen ontstaan doordat thymine onvoldoende voor de DNA-synthese beschikbaar is en doordat er een verhoogde inbouw van uracil plaatsvindt. Tevens kan globale hypomethylering van het hele genoom en regionale hypermethylering in de promotorregio's van genen optreden door een deficiënte foliumzuurinname. In **hoofdstuk 5** wordt de relatie tussen foliumzuurinname en *K-ras* mutaties in patiënten met colon- of rectum kanker beschreven. In deze studie werden verschillen gevonden voor mannen en vrouwen. Een hoge foliumzuurinname blijkt geassocieerd te zijn met een verlaagd risico op rectumtumoren bij mannen, ongeacht de *K-ras* mutatiestatus. Echter, bij vrouwen werd een verhoogd risico op rectumtumoren gevonden. Dit verhoogd risico werd gevonden voor tumoren met *K-ras* mutaties, maar ook voor tumoren met een wild type *K-ras* gen. Interessant is dat een hoge inname van foliumzuur bij vrouwelijke patiënten met een *K-ras* gemuteerde tumor een sterk beschermend effect blijkt te hebben voor G>A transitities. Foliumzuur lijkt een duidelijke rol te spelen in de ontwikkeling van rectumkanker, en het *K-ras* gen lijkt hierbij betrokken te zijn. Deze studie toont ook aan dat in toekomstig moleculair epidemiologisch onderzoek naar foliumzuur de man-vrouw verschillen moeten worden meegenomen.

In **hoofdstuk 6** wordt een slotbeschouwing gegeven waarin de belangrijkste bevindingen van onze studie worden samengevat en vergeleken met andere moleculair epidemiologische studies. De resultaten suggereren dat een hoge inname van vlees en vet en een lage inname van foliumzuur van minder groot belang zijn op het risico van die colon of rectum tumoren met puntmutaties in het *K-ras* gen dan was verondersteld. Echter, deze factoren kunnen van etiologisch belang zijn voor het risico op colon of rectum tumoren met een wild type *K-ras* gen. Tevens worden de mogelijkheden en beperkingen van moleculair epidemiologische studies besproken. Ook wordt in dit hoofdstuk aangegeven welke andere genetische factoren in toekomstig moleculair epidemiologisch onderzoek kunnen worden bestudeerd. Deze studie laat zien dat het inbouwen van moleculaire eindpunten in de klassieke epidemiologische onderzoeken naar voeding en kanker een relevante aanpak is om de etiologie van colon- en rectumkanker gedeeltelijk op te helderen.

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Dankwoord en Gebeden

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MIJURODIJA...mijn thuishaven! Lieve pap en mam, de basis voor het boekje dat hier ligt, hebben jullie mij gegeven. Jullie zijn onmisbaar! Judith (mijn echte zus), Tony, Roland en Ingrid, jullie maken MIJURODIJA compleet!

Joris, jou zing ik toe... ♪ ♪ Moppie, moppie, moppie, ik vind jou helemaal toppie! ♪ ♪

Bailamos deze vrijdagavond, 19 november 2004!

Mirian Brink werd op 3 oktober 1973 geboren te Gieten (Drenthe). In 1990 behaalde ze het M.A.V.O diploma aan 'de Esakker' te Gieten. Vervolgens behaalde ze in 1992 het H.A.V.O. diploma aan het D.A.G. Hammar skjöld college/Dr. Nassau college te Assen. In datzelfde jaar begon ze met de studie 'Voeding & Diëtetiek' aan de Hanze Hogeschool te Groningen waarvoor ze het propedeusecertificaat verkreeg. Hiermee werd ze in 1993 toegelaten tot de studie Biologie aan de Rijksuniversiteit Groningen, waar ze in 1998 afstudeerde in de medisch-biologische richting. Tijdens deze studie heeft ze onderzoekservaring opgedaan bij de vakgroepen Biologische Psychiatrie en Pathologie van het Groningen Universitair Medisch Centrum. Na het afstuderen trad zij als assistent in opleiding in dienst bij de afdelingen Epidemiologie en Pathologie van de Universiteit Maastricht. Gedurende deze periode werd het door het Koningin Wilhelmina Fonds (KWF) Kankerbestrijding gesubsidieerde en in dit proefschrift beschreven onderzoek uitgevoerd onder begeleiding van Prof. dr. ir. Piet A. van den Brandt (Epidemiologie), Dr. ir. Matty P. Weijnenberg (Epidemiologie) en Dr. Anton F.P.M. de Goeij (Pathologie). Naast werkzaamheden als het analyseren, schrijven en publiceren van gegevens, heeft ze veel energie en tijd gestoken in het coördineren en uitvoeren van het verzamelen van het tumormateriaal uit verschillende laboratoria in Nederland en (deels) het coördineren van de *K-ras* mutatiestatus-bepaling van het patiëntenmateriaal (het opzetten van de gebruikte technieken en het verrichten van deze bepalingen is gedaan door twee analisten). Ook heeft ze een coördinerende bijdrage geleverd in de opzet van twee databases waarin gegevens over de voorraad van het tumormateriaal en de *K-ras* mutatiestatus van dikke darmkanker patiënten werden opgeslagen. In deze periode behaalde zij haar registratie voor Epidemiologie-A. Sinds januari 2004 is ze werkzaam bij de Koninklijke Nederlandse Akademie van Wetenschappen (KNAW) in Amsterdam als secretaris van de wetenschapsverkenning 'Multifactoriële aandoeningen in het Genomics-tijdperk' onder begeleiding van Dr. Marij Stukart en uitgevoerd door een verkenningcommissie onder voorzitterschap van Prof. dr. Dirkje S. Postma.