DIET-GENE INTERACTIONS IN SPORADIC AND HEREDITARY COLORECTAL CARCINOGENESIS

Epidemiological perspectives

Dorien Voskuil

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STELLINGEN

1. De frequentie van zowel K-ras mutaties als p53 overexpressie is niet duidelijk verschillend in de vroege adenoom-fase van Hereditair Non-Polyposis Colorectale Carcinogenese (HNPCC) en sporadische colorectale carcinogenese. *(dit proefschrift)*

2. De positieve associatie tussen vleesconsumptie en colorectale adenomen en carcinomen is duidelijker wanneer de vleesbereiding hierbij wordt betrokken. *(dit proefschrift)*

3. Associaties tussen genmutaties en voedingsfactoren worden niet gevonden als alle mutaties samen worden bestudeerd, daar carcinogenen in de voeding mogelijk zeer specifieke mutaties (fingerprints) in een gen veroorzaken. *(dit proefschrift)*

4. Mutatie-analyse van het p53-gen is nodig ter bevestiging van de hypothese dat voedingsfactoren mutaties in p53 kunnen veroorzaken. Daarentegen is vaststelling van overexpressie van het p53 eiwit toereikend om aan te tonen dat p53 status het effect van voedingsfactoren kan modificeren. *(dit proefschrift)*

5. Men kan het ook te bruin bakken.

6. Het gebruikelijke onderscheid tussen endogene processen en exogene (voedings-) factoren houdt onvoldoende rekening met het concept "gen-omgeving-interacties".

7. Afhankelijk van de fase waarin wetenschappelijk onderzoek zich bevindt, dient het belang van een studie niet alleen afgewogen te worden aan de statistische "power" van de studie.

8. "Collectebusfondsen" zijn onontbeerlijk voor het onderzoek naar de rol van voeding bij de preventie van kanker; en moeten ook wetenschappelijk onderzoek mogelijk maken naar de rol van voeding bij de behandeling van kankerpatiënten.

9. Het is onterecht als mensen met een erfelijke aandoening daar extra onder moeten lijden doordat zij geen levensverzekering kunnen afsluiten. Bij HNPCC patiënten geldt dit in het bijzonder, omdat door screening het risico aan de ziekte te overlijden sterk afneemt.

10. De wetenschapper die de beste kansen heeft voor een hogere wetenschappelijke functie is degene die al zijn tijd en inzet daartoe aangewend heeft en niet afgeleid wordt door welke wereldse zaak dan ook. (Barbara van Balen, 1998, in *De universiteit als modern mannenklooster*.)

11. De begrippen "poliep" en "kanker" krijgen een andere lading als ze te dichtbij komen.

12. De overeenkomst tussen dikke darmpoliepen en koraalpoliepen is niet alleen hun uitstulpende vorm, maar ook de mate waarin ze de aandacht vast kunnen houden van respectievelijk een duikende onderzoeker en een onderzoekende duiker.

Stellingen behorend bij het proefschrift *'Diet-gene interactions in sporadic and hereditary colorectal carcinogenesis. Epidemiological perspectives'*.
Dorien Voskuil, 12 mei 1999

ABSTRACT

Thesis Diet-gene interactions in sporadic and hereditary colorectal carcinogenesis
Epidemiological perspectives

Dorien Voskuil

Colorectal cancer is known to develop by accumulation of alterations in regulatory genes. Both familial and environmental factors play a role in the etiology of colorectal cancer and its adenomatous precursor lesions. This thesis examines diet-gene interactions in sporadic and hereditary colorectal carcinogenesis, from two different epidemiological perspectives, as outlined in AIMS 1 and 2.

AIM 1: *to examine evidence for a role of dietary risk factors in the etiology of Hereditary Non-Polyposis Colorectal Cancer (HNPCC); are some aspects of the etiology similar to sporadic colorectal carcinogenesis?*

In a descriptive epidemiological study of HNPCC first degree relatives, the cumulative incidence of colorectal cancer at age 75 increased from 19% in ancestors, to 32% in the first generation and 55% in the second generation, which is largely attributable to a birth cohort effect. The earlier age of onset in successive HNPCC generations thus appears not to be a biological feature of HNPCC but reflects a time trend in cancer occurrence which is similar to trends in the general population.

Subsequently, we assessed frequencies of mutations in the K-ras gene and overexpression of the p53 protein, in 48 HNPCC and 59 sporadic adenomas. Frequencies of these abnormalities were indeed similar in HNPCC (31% and 25% for K-ras and p53 respectively) and sporadic (32% and 25% for K-ras and p53 respectively) adenomas. This points towards similar molecular pathways to early stages of colorectal carcinogenesis with regard to K-ras and p53, and therefore suggests that environmental factors may play a similar role.

To study the association between risk of HNPCC colorectal adenomas and dietary factors related to meat consumption and preparation directly, we conducted a case control study of HNPCC and sporadic adenomas (n=62 and 57 respectively), and endoscopy control groups for both (n=83 and 65 for HNPCC and sporadic respectively). We observed red meat consumption and darkly browned meat surface to be positively associated with risk of adenomas in the sporadic group (Odds Ratio (OR) 4.1 and 3.0 respectively, 95% confidence interval (95% CI) 0.7-23.0 and 1.2-7.5 respectively), but not in the HNPCC group (OR 0.4 and 0.7, 95% CI 0.1-2.2 and 0.3-1.4 respectively). Although this may suggest that meat consumption and preparation are only relevant in the etiology of

sporadic adenomas, it does not exclude that (other) dietary factors play a role in some phases of HNPCC colorectal carcinogenesis.

AIM 2: *to examine evidence for a differential effect of dietary risk factors on K-ras/p53 dependent and independent pathways to colorectal carcinogenesis.*
In a case control study on dietary factors and the risk of sporadic colon cancer, we examined carcinoma tissue (n=185) for mutations in the K-ras and p53 genes.
We found high intakes of animal protein and calcium to be positively associated with carcinomas harboring K-ras codon 12 mutations (OR 1.5 and 1.2, 95% CI 1.0-2.1 and 0.9-1.6 respectively), but inversely with carcinomas harboring codon 13 mutations (OR 0.4 and 0.6, 95% CI 0.2-1.0 and 0.3-1.2 respectively). Transition and transversion mutations were not differently associated with diet.
We examined both p53 mutations and p53 overexpression in relation to diet, and observed a positive association for (saturated) fat, only in the p53-independent pathway (for saturated fat, no overexpression OR 1.5, 95% CI 1.1-2.0) and not in the p53-dependent pathway (for saturated fat, with overexpression OR 1.1, 95% CI 0.8-1.5). This difference was more pronounced with p53 overexpression than with p53 mutation as endpoint. Interestingly, when examining specific mutations we found similar dietary factors only to be associated with transversion mutations (for saturated fat, OR 2.0, 95% CI 1.0-4.1), and not with mutations at CpG islands (for saturated fat, OR 0.9, 95% CI 0.6-1.6).
Both studies suggest that if diet is associated with K-ras and p53 mutations in colon cancer, very specific types of mutations may be involved.

Our studies have been among the first to examine diet-gene interactions in colorectal carcinogenesis, using an epidemiological approach. Such "early" studies into a new area, tend to be of small size, and they are therefore not sufficient to provide "proof" of diet-gene interactions. However, these studies have served well to to suggest directions for future research, and to develop and expand the relevant research infrastructures.

Future studies on diet-gene interactions in colorectal carcinogenesis should continue to aim at disentangling the effects of diet in all molecular phases of the adenoma-carcinoma sequence, and additionally, clarifying the possible role of environmental factors in hereditary colorectal cancer.

CONTENTS

INTRODUCTION

CHAPTER 1 INTRODUCTION

BACKGROUND

Cancer of the colon and rectum is the fourth most common type of cancer throughout the world [1]. Large differences in incidence have been observed between countries (e.g. 45, 36 and 4 per 100.000 person-years in North-America, The Netherlands and India, respectively). Colorectal cancer incidence in The Netherlands has increased gradually since the 1970s, especially with increasing age [2]. Mean age at diagnosis increased from 61 in the 1960s to about 67 more recently.

Migrant studies have shown that the rates in groups moving from low-risk to high-risk areas adapt to the rates of the high-risk areas within one or two generations [3], suggesting this cancer is largely determined by environmental factors. Doll and Peto (1981) have estimated 90% of colorectal cancer risk to be attributable to environmental factors, especially dietary factors [4]. A hereditary component is likely as well, as a positive family history of colorectal cancer increases the relative risk about two-fold [5]. Epidemiological studies suggest that 5-10% of colorectal cancer incidence can be attributed to dominantly inherited susceptibility genes [6].

Interaction between genetic and dietary factors in the etiology of colorectal carcinogenesis is the subject of this thesis. This is studied from two epidemiological perspectives:

- possible role of dietary factors in the etiology of Hereditary Non-Polyposis Colorectal Carcinogenesis (HNPCC) (as environmental factors may also play a role in HNPCC, and if so, HNPCC may function as a model for sporadic colorectal carcinogenesis)
- differential effect of diet in sporadic colorectal cancer, depending on mutational status of K-ras and p53 genes (to provide further clues to specific effects of diet in the etiology of colorectal carcinogenesis)

In the following paragraphs of this introductory chapter, the main concepts of the etiology of colorectal carcinogenesis will be described. The last paragraph provides an outline of the thesis and the aims of the studies described.

COLORECTAL CARCINOGENESIS

The adenoma-carcinoma sequence

Colorectal cancer can be seen as a genetic disease, i.e. requiring alteration, mutation or loss of at least 4 to 5 oncogenes and tumor-suppressor genes for a malignant tumor to

develop [7]. The products of these regulatory genes normally mediate signal transduction, control the cell cycle, maintain genomic stability, and mediate programmed cell death (apoptosis). Through clonal evolution a malignant tumor develops out of one single cell which acquires a mutation in one regulatory gene, thereby gaining growth advantage. One cell in this clone may then acquire a mutation in another regulatory gene, and so on. Malignant cells fail to respond to regulatory mechanisms, leading to uncontrolled and excessive tissue growth and spread to distant sites.
During this process of accumulation of alterations, mutations or loss of key genes (Figure 1.1), the normal colonic epithelium goes through several phases: hyperproliferation, a benign tumor or adenoma will form; growth, the adenoma can transform into a malignant carcinoma; metastasis, the carcinoma spreads to distant sites in the body [8]. Although some (epi)genetic abnormalities occur mostly in specific stages of colorectal carcinogenesis, the order in which they may occur is basically random.

The Adenomatous Polyposis Coli gene. Mutations or loss of the APC gene, also known as a "gatekeeper gene", is frequently one of the first steps in colorectal carcinogenesis, and occurs in 70-80% of sporadic colorectal cancers [9]. Subsequent to a mutation in this "gatekeeper", mutations in other oncogenes and tumor-suppressor genes may occur, such as K-ras and p53. If mutated in germline cells, APC is responsible for the autosomal dominantly inherited form of cancer called Familial Adenomatous Polyposis, which is characterized by multiple adenomas (>100) at a very young age.

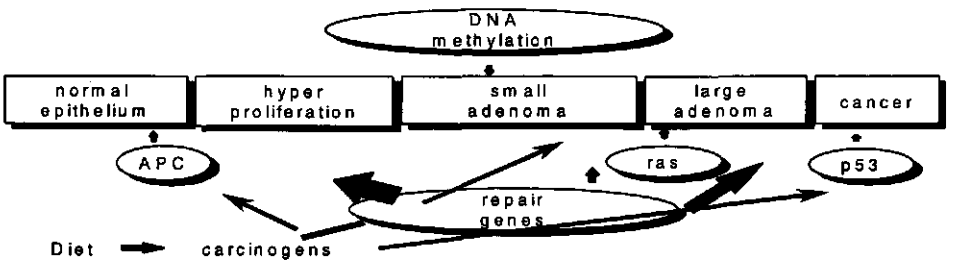


Figure 1.1. *The adenoma-carcinoma sequence, as derived from Vogelstein et al.* [8]

The K-ras oncogene. The K-ras gene, also called Ki-ras or Kirsten-ras, is mutated in approximately 40-50% of colorectal carcinomas and large adenomas and in a lower

percentage of small adenomas [8]. K-ras mutations are therefore thought to occur early in the colorectal carcinogenic process, during the progression from small to large adenoma. The gene product functions as a molecular switch in signal transduction pathways of growth factors. Normal functioning K-ras can switch between its active and inactive form. However, a mutation in at least one allele of K-ras causes the gene to remain in its active form, thereby stimulating cell growth and tissue proliferation [10].
Most mutations (90-95%) occur in codon 12 and 13 of the K-ras gene, and few in codon 61. Some mutations can be found in the N-ras and H-ras genes, which have the same function as K-ras [10]. Frequency and type of K-ras mutations vary substantially between different types of cancer. Environmental factors may play a role in inducing mutations. Interestingly, the type of mutation characteristically caused by mutagenic alkylating agents (G-A transitions at the second base), occurs most frequently [11]. Tumors with this type of mutation are thought to be more invasive [12]. As there are some suggestions that tumor characteristics and prognosis may differ for tumors with or without K-ras mutations and for tumors with different types of K-ras mutations, K-ras may also play a role in the promotion and progression phases of colorectal carcinogenesis.

The p53 tumor suppressor gene. Mutation or loss of the p53 tumor-suppressor gene is observed in about 50% of colorectal carcinomas but in a lower percentage of adenomas [8]. Therefore, p53 mutations are thought to occur during transformation from adenoma to carcinoma. P53 plays a major role in many types of cancer and is involved in gene transcription, DNA synthesis and repair, and programmed cell death (apoptosis) [13]. When functioning normally the p53 protein puts a halt to the cell cycle if DNA is damaged, thereby providing the cell with time to repair the DNA damage or to go into apoptosis if the damage is too severe. When one allele of the p53 gene is mutated, the other one is usually lost by allelic deletion, leaving the cell without p53 tumor suppressor function. The frequency and type of mutations vary strongly between different types of cancers (Figure 1.2) [14].
These different mutation spectra presumably reflect differences in exogenous carcinogens and endogenous biological processes known to cause mutations. In lung cancer G:C-T:A transversion mutations occur in high frequencies and are thought to be caused by smoking [15]. Although G:C-T:A transversions also occur in colorectal cancer, the most frequent type of mutation in colorectal cancer is the G:C-A:T transition at CpG dinucleotides, which is known to be the result of spontaneous deamination of methylated CpG sites [14]. The environmental or nutritional determinants of this genetic instability are not known yet.

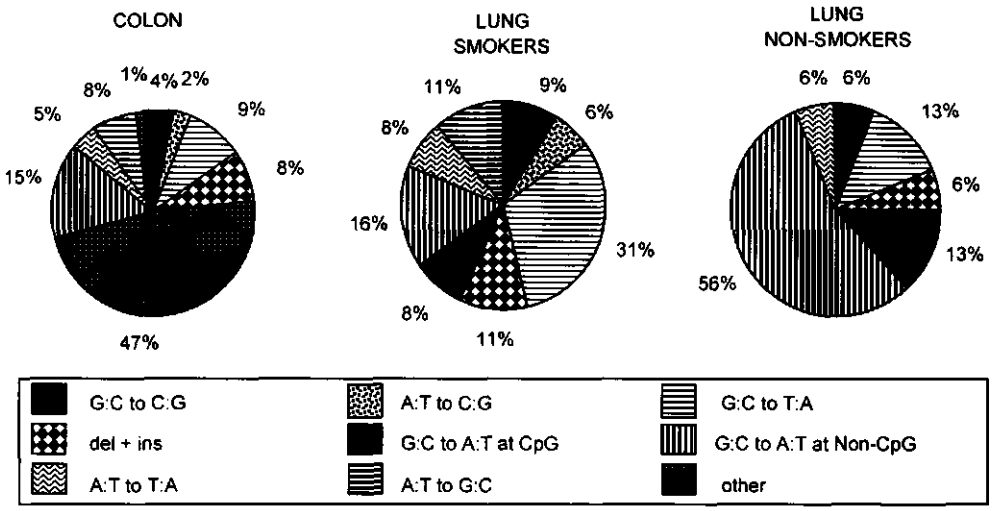
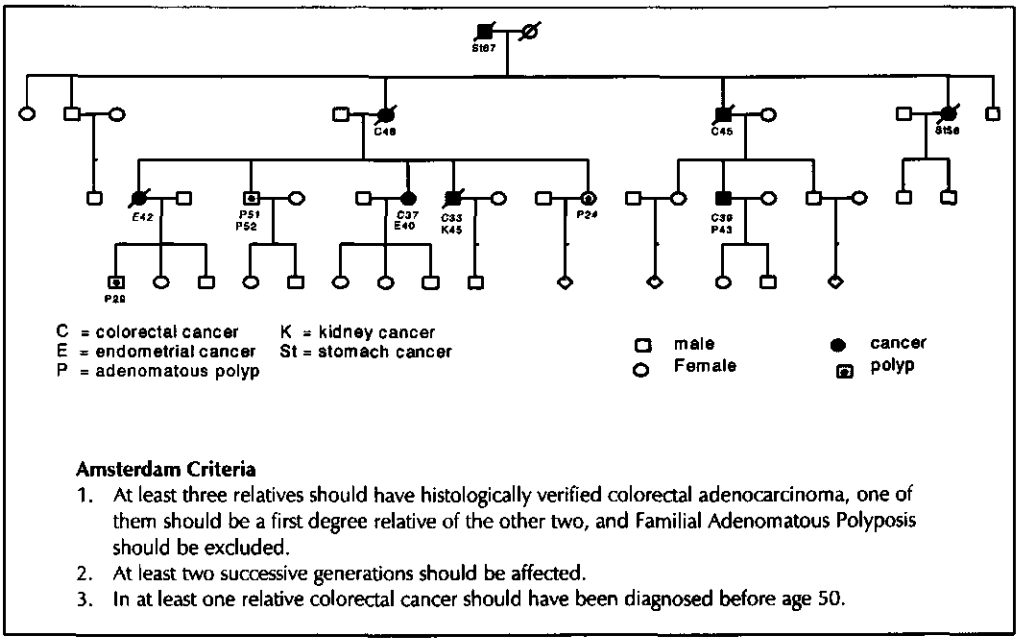


Figure 1.2. *Mutation spectra of the p53 gene in several types of cancer (derived from Greenblatt et al.)* [14].

Mismatch repair genes. Mutation or inactivation due to methylation, of one of the mismatch repair genes, results in microsatellite instability. About 10 to 15% of sporadic colorectal cancer show this microsatellite instability [16;17]. However, this frequency is much higher (80-90%) in Hereditary Non-Polyposis Colorectal Cancer (HNPCC) [17;18], where a germline mutation in one of the mismatch repair genes is responsible for this type of genetic instability.

Hereditary Non-Polyposis Colorectal Cancer

Approximately 1 to 5% of colorectal cancer is due to a genetic disorder called Hereditary Non-Polyposis Colorectal Cancer (HNPCC) [19]. HNPCC was first described by Warthin in 1913 [20], and was named 'cancer family syndrome' in the 1960s [21]. This disease is characterized by a susceptibility to colorectal cancer, frequently localized in the proximal colon and is characterized by an early age of onset [22;23]. Furthermore, members of HNPCC families appear to develop extracolonic tumors (e.g. of the endometrium, stomach, urinary tract) more often than the general population [22;24]. Until recently the genetic background of this disease was largely unknown, therefore only clinical characteristics could be used for diagnosis. In 1990 the International Collaborative Group on HNPCC proposed a set of clinical diagnostic criteria, later named the Amsterdam criteria (Figure 1.3) [25].



Amsterdam Criteria

1. At least three relatives should have histologically verified colorectal adenocarcinoma, one of them should be a first degree relative of the other two, and Familial Adenomatous Polyposis should be excluded.
2. At least two successive generations should be affected.
3. In at least one relative colorectal cancer should have been diagnosed before age 50.

Figure 1.3. **Genealogical tree of a HNPCC family fulfulling the Amsterdam criteria.**

The disease follows an autosomal dominant inheritance pattern, caused by a germline mutation in one of at least five mismatch repair genes (MSH2, MLH1, PMS1, PMS2, and MSH6/GTBP) [18]. Errors in microsatellites, which are short repeated sequences of DNA, occur often, but are usually repaired during normal DNA replication. Due to the mutational inactivation of one of the mismatch repair genes these replication errors (RER) are not repaired in HNPCC, leading to widespread genetic instability, also called microsatellite instability (MSI). This "mutator phenotype" expresses itself by the rapid accumulation of somatic mutations in oncogenes and tumor-suppressor genes, thereby rapidly progressing through the adenoma-carcinoma sequence.

The tumors in HNPCC not only develop in the colon and rectum, but also at many other sites. Interestingly, the tumor spectrum has changed over several generations [26], which suggests a role of environmental factors in the etiology of this hereditary disease. Additionally, incomplete penetrance of the disease (80-90%) [22], earlier onset of disease in successive generations (anticipation) [23;27], and occurrence of somatic gene mutations generally thought to be exogenous of origin (e.g. APC, K-ras, p53) [12;28;29], are characteristics which support a role for environmental factors in the etiology of HNPCC.

ENVIRONMENTAL RISK FACTORS

Lifestyle and dietary risk factors

Many studies have been conducted on the association between diet and the risk of colorectal cancer. As mentioned, already in the early 1980s Doll & Peto (1981) estimated that 90% of the risk of colorectal cancer could be attributed to environmental factors, most likely dietary factors [4]. Recently two reports have been published on diet and cancer: one by the Committee on Medical Aspects of Food and Nutrition Policy in the UK [30], and the other by the World Cancer Research Fund in the USA [1]. Both have drawn conclusions on the evidence from epidemiological studies regarding dietary risk factors for colorectal cancer (summarized in Table 1.1).

High vegetable consumption is supported by the strongest evidence, and may possibly prevent half of the colorectal cancer cases. Vegetables are also considered protective in colorectal adenoma etiology, although evidence is somewhat weaker. So far it is unknown which components in vegetables account for the decrease in risk. Possible candidates could be bioactive compounds such as antioxidants and folate. However, evidence is inconsistent and insufficient to draw conclusions about a protective role for these specific dietary factors. Fiber may decrease the risk of colorectal cancer by dilution of carcinogens in the bowel and/or by decreasing bowel transit time and thus decreasing duration of exposure of bowel epithelium to carcinogens. The expected decrease in risk due to fiber intake is similar to that of vegetables, but the evidence is somewhat weaker. Evidence for a protective role of fruit is inconsistent and insufficient. For lifestyle related factors such as physical activity and aspirin or other NSAID intake, moderate to strong evidence points towards a protective role in the etiology of both colorectal adenomas and carcinomas.

With respect to positive risk factors, a diet high in fat and meat is thought to increase risk, although, evidence is only moderate. The classic hypothesis was that a diet high in meat elevates the risk of colorectal cancer through a high intake of fat, which increases bile acid production and the luminal concentration of free fatty acids [3]. More recently it has been suggested that the increased risk is related to other components of meat, e.g. heterocyclic amines [31], N-nitroso compounds [32], and iron [33]. Some experimental studies have shown that these components possibly increase risk of colorectal cancer by causing mutations in genes such as APC and K-ras [11;34;35].

Table 1.1. *Overview of dietary factors and other lifestyle factors possibly related to the risk of colorectal adenomas and carcinomas.*

risk factor	association		epidemiological evidence		
	direction	strength	carcinomas	adenomas	proposed mechanisms
Diet, risk factors					
red meat	increase	double	moderate	weak	heterocyclic amines, N-nitroso compounds, iron/haem
total fat	increase	double at most	weak	weak	increased secondary bile acids & free fatty acids, increased cell proliferation
alcohol	increase	variable	moderate	moderate	possible antagonist in DNA methylation, cytotoxic effect
Diet, protective factors					
vegetables	decrease	half	moderate/strong	moderate	fiber, micro-nutrients, bioactive compounds
fruit	decrease	half at most	inconsistent/insufficient		fiber, micro-nutrients, bioactive compounds
fiber	decrease	half	moderate	moderate/weak	dilution + binding carcinogens in bowel, decreased bowel transit time
antioxidants	decrease	variable	inconsistent		free radical scavengers
folate	decrease	?	insufficient	insufficient	DNA methylation and synthesis
calcium	decrease	low or no	moderate/weak		reduced cell proliferation by binding free fatty acids & secundary bile acids, direct effect on cell proliferation
other lifestyle factors					
physical activity	decrease	at least half	moderate/strong	insufficient	colonic motility
smoking	increase	double	weak	moderate	source of carcinogens
aspirin, NSAIDs	decrease	half	moderate/strong	moderate/strong	prostaglandin metabolism
Body Mass Index	increase	?	weak	insufficient	energy balance, sedentary lifestyle, related to physical activity.

Meat consumption in relation to colorectal adenomas and carcinomas

The epidemiological evidence regarding meat consumption and the risk of colorectal adenomas and carcinomas is summarized in Table 1.2 (see also Appendixes 1.1 and 1.2)

Table 1.2. *Summary of results from cohort and case-control studies on the association between meat consumption and the risk of colorectal adenomas and carcinomas**.

type of study	endpoint	number of studies	mean RR†	range RRs†	% RRs† positive	% significant RR¶†
cohort	carcinomas	8	1.2	0.8-1.9	54%	25%
case-control	carcinomas	25	1.5	0.4-3.3	64%	47%
cohort	adenomas	1	1.2	-	-	-
case-control	adenomas	6	1.6	0.8-2.4	57%	33%

* Overview of studies in Appendix 1.1 (cohort studies) and Appendix 1.2 (case-control studies)
† RR=relative risk, or OR (odds ratio) for case-control studies), using 95% Confidence Interval
¶ % studies reporting significantly positive RR (based on 8, 17 and 3 studies respectively which reported CI)

In eight cohort studies total meat or red meat intake were examined in relation to risk of colon or colorectal cancer (see Appendix 1.1). In two large United States cohort studies high intake of red meat was found to be associated with increased risk in both women (RR, 95% CI: 1.8, 1.1-2.9) [36] and men (RR, 95% CI: 1.7, 1.2-2.6) [37], while in two other US prospective studies no increased risk was found (RR, 95% CI: 1.0, 0.6-1.8) [38] and (RR, 95% CI: 1.2, 0.7-2.0) [39]. Gaard *et al.* found in a Norwegian cohort study that total meat intake was associated with colon cancer risk, but only in women (RR, 95% CI: 1.9, 0.8-4.9) and not in men [40]. Three other cohort studies also reported no increased risk with intake of total meat [41], red meat [42], and fresh meat [43]. In the latter, a Dutch cohort study, an increased risk with consumption of processed meat was found [43]. Giovannucci *et al.* reported on the only cohort study on colorectal adenomas so far, and failed to show an increased risk (RR, 95% CI: 1.2, 0.7-2.1) [44].

Since 1980, in 25 case control studies the association of total meat intake or several other indices of red meat intake and the risk of colon or colorectal cancer has been examined. The risk of colorectal adenomas was examined in 6 case control studies (Table 1.2, see also Appendix 1.2) [45-75]. The mean Odds Ratio in these studies was about 1.5, both for colorectal adenomas (range 0.8-2.4) and carcinomas (range 0.4-3.3). Overall, the findings are somewhat more consistent for the risk of colorectal carcinomas, with almost half of the studies reporting a significantly increased risk.

Risk associated with meat preparation and heterocyclic amines

As mentioned, the risk associated with consumption of meat may also be related to meat preparation. In the past ten years twelve studies reported on varying factors related to the preparation of meat and their association with colorectal adenomas or carcinomas (Table

1.3). In four studies (including two cohort studies in Finland and Norway) the risk of colorectal cancer was found not to increase with increasing frequency of eating fried meat or well-done meat [40;76-78]. The remaining eight studies all reported risk to be increased with one or more factors relating to meat preparation. Young & Wolf found the risk of colorectal cancer to be increased (RR=1.3, 95% CI=1.0-1.8) with high vs low consumption of pan-fried foods [79]. Peters et al. observed an increased risk with frequent consumption (≥5x/week) of fried ham or bacon and barbecued or smoked meat, and Welfare et al. with frequent consumption of gravy (>2x/week) and roast meat (≥1x/week), only for proximal colon cancer [53;80]. Relative risks of similar magnitude (around RR=3) were reported for increased consumption of barbequed meat and bacon [54], well done meat [81], and fried meat [72]. Gerhardsson de Verdier et al. explored the association with several indicators of meat preparation in a Swedish case control study [58]. They found increased risks of both colon and rectum cancer with frequent use of brown gravy, heavily browned meat surface and high frying temperature. Frequent consumption of fried or roasted meat did not increase the risk of colorectal cancer in this study. Probst-Hensch et al. reported an increased risk of colorectal adenomas with a combination of several factors related to meat consumption and preparation [82]. Consumption of red meat more than once a week, of which at least 10% is fried, with a preference for a dark surface, is associated with a two-fold increased risk (OR, 95% CI: 2.2, 1.1-4.3) as compared to consuming red meat less than once a week, of which less than 10% is fried, with a preference for a light surface.
Summarizing, in 8 of 12 studies significantly increased risks relating to meat preparation were found. For the two cohort studies no increase in risk was reported. On average a two-fold increase in risk was reported, with relative risk estimates between 0.6 and 5.0.

Recently, attention has been focused on the interaction between genetic polymorphisms of metabolic enzymes (N-acetyltransferase, Cytochrome P450, Glutathion-S-Transpherase), and the consumption of meat in relation to the risk of colorectal adenomas and carcinomas. Of the five studies conducted, four found significantly increased risk of colorectal adenomas and/or carcinomas with rapid NAT2 polymorphism (in combination with NAT1 or CYP1A2) and frequent consumption of total meat, red meat, well done meat or fried meat [39;80;83;84]. In one study only a marginally increased risk with processed meat intake in the intermediate or rapid NAT2 group was found [75]

Concluding, an increase in risk of colorectal adenomas and carcinomas with red meat consumption seems to be more consistent when factors relating to preparation are taken into account. Possibly, the increase in risk is higher for those who are genetically susceptible to (pre)carcinogens from meat.

Table 1.3. *Meat preparation related factors and the risk of colorectal adenomas and carcinomas: case control and cohort studies.*

Author year, country	cases/ contr source contr	type of exposure	contrast in exposure	relative risk estimates	
Lyon & Mahoney [76] 1988	246/484 population	fried meat	≥11.5 vs ≤6x/wk	1.2 men	(0.8-1.9)
			≥8 vs <4x/wk	1.3 women	(0.8-2.1)
		broiled meat	>1 vs ≤/wk	0.7 men	(0.5-1.0)
			>1.5 vs ≤0.25x	1.1 women	(0.7-1.7)
Young & Wolf [79] 1988, USA	353/618 population	pan-fried foods	high vs low	1.3	(1.0-1.8)
Peters et al. [53] 1989, USA	147/147men population	fried ham/ bacon	≥5 vs ≤1 x/wk	1.0 all	(0.4-2.4)
				2.6 prox.colon	(0.9-7.9)
		meat bbq or smoked	≥5 vs ≤1 x/wk	1.3 all	(0.6-2.7)
				2.9 prox.colon	(1.2-7.3)
Wohlleb et al. [54] 1990, USA	43/41 men hospital	bbq meat	≥1 vs <1 x/wk	3.3	(1.2-9.2)
		bacon	≥1 vs <1 x/wk	5.0	(1.0-25.0)
Schiffman & Felton [81] 1990, USA	50	doneness	well done vs rare to med rare	3.5	(1.3-9.6)
Gerhardsson et al. [58] 1991, Sweden	559/505 population	brown gravy	>1x/wk vs <3/month	1.6 colon	(1.1-2.3)
				1.9 rectum	(1.2-3.0)
		meat surface	heavily vs lightly browned	2.0 colon	(1.2-3.6)
				3.4 rectum	(1.7-6.7)
		frying temp	high vs med/low	1.9 colon	(1.4-2.5)
				1.5 rectum	(1.1-2.1)
		meat fried or roasted	highest vs lowest quintile	0.9 colon	NG
				1.4 rectum	NG
Muscat & Wynder [77] 1994, USA	511/500 hospital	doneness	well done vs rare	1.2 men	(0.6-2.4)
				1.0 women	(0.6-1.5)
Knekt et al. [78] 1994, Finland	853 men 9,990, 24 yr	fried meat	highest vs lowest tertile	1.0	(0.6-1.9)
Gaard et al. [40] 1996, Norway	143 50,535, 11y	meat fried or roasted	≥5 vs <1x/wk	0.6 men	(0.2-1.7)
				0.9 women	(0.3-3.3)
Welfare et al. [80] 1997, England	174/174 population	gravy	>2 vs <1 x/wk	3.2 prox col	(1.1-11.2)
		roast meat	≥1 vs <1 x/wk	3.0 prox col	(1.1-9.2)
De Stefani et al. [72] 1997, Uruguay	250/500 hospital	fried meat	highest vs lowest quartile	2.6	(1.6-4.1)
Probst-H. et al. [82] 1997, USA	488/488 adenomas*	red meat incl. preparation	high risk vs low risk category*	2.2	(1.1-4.3)

NG= Confidence Interval not given

* 488 adenoma cases vs 488 sigmoidoscopy controls; red meat >1x/wk, >10% fried, and dark surface preference vs red meat <1x/wk, <10% fried, light surface preference

AIMS AND OUTLINE OF THE THESIS

The aims of the studies described in this thesis were:
AIM 1:to examine evidence for a role of dietary risk factors in the etiology of HNPCC, are some aspects of the etiology similar to sporadic colorectal carcinogenesis?
AIM 2 to examine evidence for a differential effect of dietary risk factors on K-ras-/p53-dependent and -independent pathways to colorectal carcinogenesis.

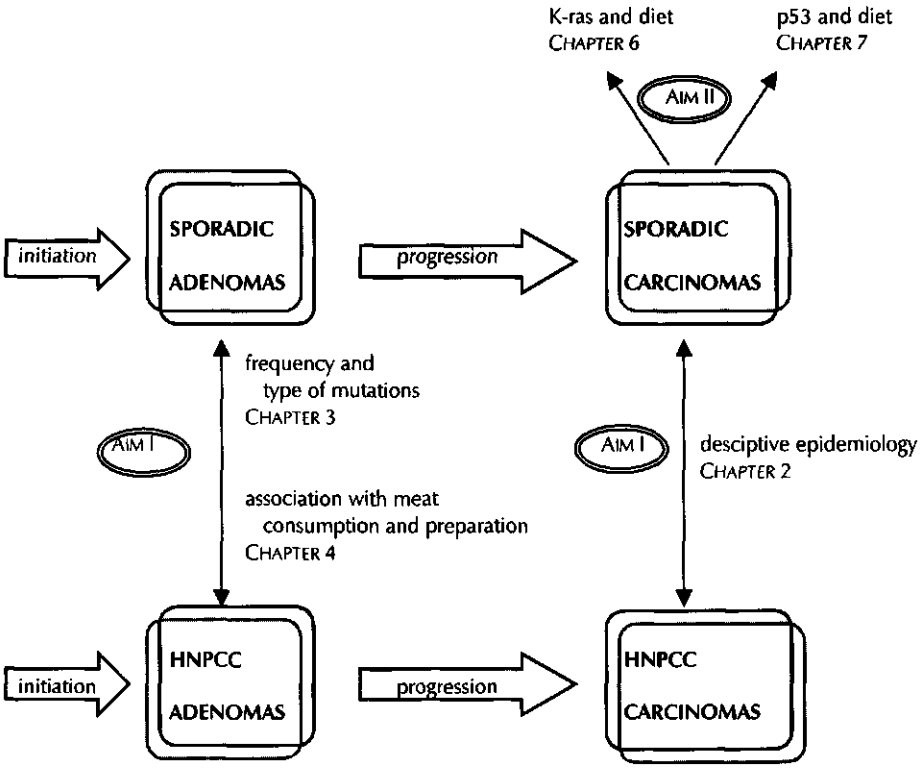


Figure 1.4. *Schematic overview of the studies described in chapters 2, 3, 5-7 of this thesis. Chapter 4 describes a methodological study and is therefore not included in this scheme.*

CHAPTER 2 *How much higher is the risk of colorectal cancer in HNPCC families, as compared to the general population, and does the risk increase over successive generations?* To answer these questions descriptive analyses have been conducted on data from the Netherlands Foundation for the Detection of Hereditary Tumors, which

keeps a registry of HNPCC families in the Netherlands, and the population-based Netherlands Cancer Registry, which collects data on all malignant neoplasms occurring in The Netherlands since 1989.

CHAPTER 3 *Do frequencies of K-ras and p53 abnormalities differ between sporadic and HNPCC adenomas?* In a case control study on dietary risk factors, genetic susceptibility, and DNA-damage in colorectal adenomas, we collected adenomatous tissue from 59 sporadic and 48 HNPCC cases in order to detect K-ras mutations and p53 protein overexpression.

CHAPTER 4 *Would a substantial amount of information be lost when using a reduced questionnaire on meat consumption and preparation?* Analyses for this methodological study were performed on the data from a Swedish case control study on the relation between the intake of heterocyclic amines and the risk of cancer in a population of Stockholm elderly, using a very extensive questionnaire to assess the intake of heterocyclic amines.

CHAPTER 5 *Are meat consumption and preparation associated with the risk of sporadic and HNPCC colorectal adenomas in a similar fashion?* In the case-control study of sporadic and HNPCC colorectal adenomas we studied the association with factors relating to meat consumption and preparation, as assessed by a questionnaire especially developed for this purpose.

CHAPTER 6 *Is diet differently associated with colon cancer with or without (specific) mutations in K-ras?* In a previously conducted case-control study on dietary risk factors in colon cancer, archival tumor tissue was retrieved to detect mutations in codon 12 and 13 of the K-ras gene. We studied whether differences in intake of nutrients and foods of animal origin are related to (specific) mutations in K-ras.

CHAPTER 7 *Is diet differently associated with colon cancer with or without (specific) p53 abnormalities?* In the same case-control study of colon cancer, p53 protein overexpression and p53 gene mutations were detected in archival tumor tissue. Nutrients and food groups of both animal and plant origin were studied in relation to p53 abnormalities in colon cancer. Both p53 protein overexpression and p53 gene mutations were assessed to examine whether the use of either of these endpoints would lead to different results in an epidemiological study.

CHAPTER 8 The integrated results of CHAPTERS 2-7 are discussed in the light of the strengths and limitations of the studies. The biological plausibility of the examined diet-gene interactions is outlined, and results from previously published observational and experimental studies are discussed. Finally, some general remarks are made about our studies and possible lines of future research into diet-gene interactions are presented.

Appendix 1.1. *Meat intake and the risk of colorectal adenomas and carcinomas: cohort studies*

Author, year, country	no. of cases, endpoint[†]/ size cohort - yrs follow-up	type of meat	contrast in meat intake	relative risk estimates	
Philips et al., 1985, USA [41]	182, colorectal 25,493 - 21 years	total meat	≥4x/wk vs <1x/wk	0.9	(0.6-1.5)
Willett et al., 1990, USA [36]	150, colon 88,751 women - 6 years	red meat	≥134g/day vs <59g/day	1.8	(1.1-2.9)
Thun et al., 1992, USA [42]	1,150, colon 764,343 - 6 years	red meat	highest vs lowest quintile	1.2 men 1.0 women	NG* NG*
Giovannucci et al., 1992, USA [44]	170, colorectal adenoma 7,284 men - 6 years	red meat	>110 vs <24 g/day	1.2 men	(0.7-2.1)
Bostick et al., 1994, USA [38]	212, colon 35,215 women - 4 years	red meat	>11x/wk vs <4x/wk	1.0	(0.6-1.8)
Giovannucci et al., 1994, USA [37]	205, colon 47,949 men - 6 years	red meat	highest (median 129.5 g/day) vs lowest (18.5 g/d) quintile	1.7	(1.2-2.6)
Goldbohm et al., 1994, The Netherlands [43]	215, colon 120,852 - 3.3 years	'fresh' meat	highest vs lowest quintile medians ca. 150 vs 50 g/day	0.8	(0.5-1.4)
Gaard et al., 1996, Norway [40]	143, colon 50,535 - 11.4 years	total meat total meat	≥5x/wk vs ≤2x/wk	0.8 men 1.9 women	(0.4-1.9) (0.8-4.9)
Chen et al., 1998, USA [39]	212 colorectal 22,071 men - 13 years	red meat	>1x/day vs ≤0.5x/day	1.2	(0.7-2.0)

* NG= confidence interval not given

† Carcinoma if not mentioned otherwise

Appendix 1.2. *Meat intake and the risk of colorectal adenomas and carcinomas: case control studies*

Author year, country	cases[1]/contr source contr	type of meat	contrast in meat intake	relative risk estimates	
Manousos et al. [45] 1983, Greece	100/100 hospital	beef	from 1x to 2x /wk	1.8	NG
Miller et al. [46] 1983, Canada	542/ 542+535 population+ hospital	beef	>110 vs <64.2 g/day (m)	1.2 colon, men	NG
			>42.4 vs 0 g/day (f)	1.7 rectum, men	
				1.0 colon, women	
			>23.1 vs <6.9 g/day (m)	1.7 rectum, women	
		pork		1.1 colon, men	
			>14.0 vs <4.3 g/day (f)	1.3 rectum, men	
				1.4 colon, women	
				2.7 rectum, women	
Macquart-M. et al. [47] 1986, France	399/399 hospital	total meat	highest vs lowest quartile	0.9	NG
Kune et al. [48] 1987, Australia	715/727 population	beef	highest vs lowest quintile	1.8	(1.2-2.4)
		pork		0.6	(0.4-0.7)
Macquart-M. et al. [49] 1987, France	252/238 adenomas hospital	total meat	highest vs lowest quartile	0.8	NG
La Vecchia et al. [50] 1988, Italy	575/778 hospital	beef/veal	highest vs lowest tertile	2.1 colon	NG
				2.3 rectum	NG
Tuyns et al. [51] 1988, Belgium	818/2,851 population	beef	>538g/wk vs <226g/wk	2.1 colon	NG
			>509g/wk vs <200g/wk	0.7 rectum	NG
		pork		0.4 colon	NG
				0.7 rectum	NG
Lee et al. [52] 1989, Singapore	203/425 hospital	red meat	highest vs lowest tertile	1.3	(0.8-2.0)
Peters et al. [53] 1989, USA	147/147men population	beef	≥5x/wk vs ≤1x/wk	1.0	(0.6-1.6)
Wohlleb et al. [54] 1990, USA	43/41 men hospital	pork	≥1x/wk vs <1x/wk	3.3	(1.3-8.1)
Benito et al. [55] 1990, Spain	286/295 population	total meat	highest vs lowest quartile	2.9 colon	NG
				2.4 rectum	NG
Kato et al. [56] 1990, Japan	adenoma+ carcinoma			inverse association*	
Kune et al. [57] 1991, Australia	49/727 adenomas population	beef	>360 g/wk vs <360 g/wk	2.4 men	(1.0-5.8)
Gerhardsson et al. [58] 1991, Sweden	559/505 population	total meat	highest vs lowest quintile	1.9 colon	NG
				3.2 rectum	NG
Bidoli et al. [59] 1992, Italy	248/699 hospital	red meat	highest vs lowest tertile	1.6 colon	NG
				2.0 rectum	NG

Iscovich et al. [60] 1992, Argentina	110/220 population	red meat	highest vs lowest terile	0.8	(0.4-1.7)
Peters et al. [61] 1992, USA	746/746 population	red meat	per 10x/month	1.2	(1.1-1.3)
Zaridze et al. [62] 1992, Russia	217/217 population	total meat	highest vs lowest quartile	1.0	(0.5-2.0)
Steinmetz& Potter [63] 1993, Australia	220/438 population	red meat	≥8.3 vs ≤3.9 x/wk ≥7.2 vs ≤3.4 x/wk	1.6 men 1.5 women	(0.8-3.1) (0.7-3.0)
Sandler et al. [64] 1993, USA	236/409 adenomas colonoscopy	beef	≥2.6 vs ≤0.6 x/wk ≥2.3 vs ≤0.5 x/wk	2.1 men 1.6 women	(0.8-5.2) (0.7-3.5)
Kono et al. [65] 1993, Japan	187/1,557 adenomas colonoscopy	total meat	≥4 vs <2 x/wk	1.5	(0.9-2.4)
Benito et al. [66] 1993, Spain	adenomas			no association*	
Centonze et al. [67] 1994, Italy	119/119 population	'fresh' meat	≥132 vs <88 g/day	0.7	(0.4-1.4)
Kampman et al. [68] 1995, Netherland	232/259 population	red meat	>94 vs <52 g/day	0.9 men 2.4 women	(0.4-1.8) (1.0-5.7)
Kotake et al. [69] 1995	363/363	beef pork		1.7 colon 0.8 rectum 0.8 colon 1.6 rectum	ns * ns ns s
La Vecchia et al. [70] 1996, Italy	1,326/2,024 hospital	red meat	≥4x/wk vs <4x/wk	1.6	(1.4-1.9)
Shannon et al. [71] 1996, USA	424/414 population	red meat	highest vs lowest quartile	1.5 men 0.7 women	(0.8-2.7) (0.4-1.4)
De Stefani et al. [72] 1997, Uruguay	250/500 hospital	red meat	highest vs lowest quartile	2.6 colon 2.9 rectum	(1.5-4.5) (1.5-5.5)
Franceschi et al. [73] 1997, Italy	1,953/4,154 hospital	red meat	≥6.3 vs <3.5 x/wk	1.1	(0.9-1.4)
LeMarchand et al. [74] 1997,Hawaii(US)	1,192/1,192 population	red meat	≥82 vs <23 g/day	1.8 men 1.0 women	(1.2-2.7) (0.6-1.5)
Kampman et al. [75] 1999, USA	1,542/1,860 population	red meat	>8.8x/wk vs vs ≤2.2 x/wk >6.2x/wk vs vs ≤1.5 x/wk	0.9 men 1.0	(0.7-1.3) (0.7-1.5)

NG= Confidence Interval not given

* paper not available

† carcinoma if not mentioned otherwise

COLORECTAL CANCER RISK IN HNPCC FAMILIES: DEVELOPMENT DURING LIFETIME AND IN SUCCESSIVE GENERATIONS

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CHAPTER 2 COLORECTAL CANCER RISK IN HNPCC FAMILIES: DEVELOPMENT DURING LIFETIME AND IN SUCCESSIVE GENERATIONS

Members of hereditary non-polyposis colorectal cancer (HNPCC) families develop colorectal cancer at a much higher rate, and at a much younger age, than the general population. To quantify lifetime colorectal cancer risk in HNPCC family members, we calculated the cumulative incidence (CI) in different age categories, and compared this to the general population. Furthermore, we investigated whether successive generations of HNPCC families had earlier onset of disease.

In 51 HNPCC families, selected according to the "Amsterdam criteria", the CI of colorectal cancer at age 75 was 40%, compared to only 4% in the general population. The CI ratio (CIR) of HNPCC family members relative to the general population was 148 at age 40, 79 at age 50 and 11 at age 75. Comparing successive generations of HNPCC families, the CI at age 75 increases from 19% in the ancestors to 32% in the first generation and 55% in the second generation. However, Cox proportional hazard analysis showed that this generation effect (RR per generation: 1.8, 95% CL = 1.4-2.2) largely disappears after adjustment for year of birth.

In summary, at young ages, HNPCC family members experience an up-to-150 times higher risk for colorectal cancer than the general population. This risk difference declines from age 60 onwards. The earlier age of onset in successive HNPCC generations does not appear to be a biological feature of HNPCC, but reflects a secular time trend in cancer occurrence in these families, similar to that in the general population.

INTRODUCTION

Colorectal cancer is one of the most common types of cancer in the Western world. Besides environmental and dietary factors, genetic or hereditary factors are considered relevant, both with respect to adenomatous precursors and the malignancy itself. Fuchs *et al.* found, in the Nurses' Health Study and the Health Professionals Follow-up Study, a 1.7-fold increased risk of colorectal cancer in subjects with a family history of colorectal cancer in first-degree relatives, as compared with those without a family history of the disease [5]. Besides this increased risk in the general population, some cancer-prone families experience an extremely high risk of colorectal carcinomas. There are several hereditary diseases with an excess risk of colorectal cancer, e.g., familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). HNPCC, which

was first described by Warthin (1913) [20] and named cancer family syndrome in the 1960s [21], is a disease with a definite hereditary component. This autosomal-dominant disease is caused by a germline mutation in one of at least 4 known mismatch repair genes (h*MSH2*, h*MLH1*, h*PMS1*, h*PMS2*) [85]. It is characterised by susceptibility to colorectal cancer, frequently localised in the proximal colon, and with an early age of onset [22;23]. HNPCC probably accounts for about 1-5% of cases of colorectal cancer [86;87]. Age at diagnosis of colorectal cancer in individuals from HNPCC families is approximately 45 years [22-24;88-92], whereas only 5% of the colorectal cancer patients in the general population is diagnosed before the age of 45 (Netherlands Cancer Registry, 1989-1992). Furthermore, members of HNPCC families appear to develop extracolonic tumours more often than the general population. In particular, tumours of the stomach, urinary tract and endometrium belong to the HNPCC tumour spectrum [22;24]. In HNPCC families, an earlier age at diagnosis in successive generations has been described [23;27], which might reflect an earlier onset of disease, *i.e.* anticipation.

The presence of a registry of hereditary tumours, as well as a complete nationwide cancer registry in The Netherlands, provides the opportunity to compare colorectal cancer risk in HNPCC families and in the general population. The purpose of our study was 2-fold: first, to quantitatively assess the risk of colorectal carcinomas in HNPCC families, and to compare this risk with the risk of colorectal carcinomas in the general population; and second, to investigate the development of colorectal cancer risk over a lifetime, and to determine whether this risk differs in successive generations of HNPCC families.

POPULATION AND METHODS

HNPCC families

In the Netherlands, registration and screening of HNPCC families is coordinated by the Foundation for the Detection of Hereditary Tumours. If HNPCC is suspected in a family, a genealogical tree of the family is constructed. For inclusion of a family in this study, the "Amsterdam criteria" proposed by the International Collaborative Group on HNPCC (ICG-HNPCC) in 1991 were used. These are as follows: 1) at least three relatives with histologically verified colorectal cancer, one of them being a first-degree relative of the other two; 2) at least two successive generations should be affected; 3) in one of the relatives colorectal cancer should be diagnosed before age 50; and 4) Familial Adenomatous Polyposis should be excluded [93].

In September 1994, 51 registered families, consisting of 313 cases and their 1,108 non-affected first-degree relatives, were included on the basis of the "Amsterdam criteria". All families met these criteria except that in 14 of them the diagnosis was confirmed by medical reports instead of pathological reports. Small families with few cases and families

with the majority of colorectal cancer patients in the older generations could not be included exclusively on the basis of histological verifications. Excluding these families from the analyses did not notably change the results. Therefore, in all analyses presented, these 14 families are included. Mutation analysis of the h*MSH2* and h*MLH1* mismatch repair genes was conducted in a clinical setting in 23 families, in 12 of which a mutated gene could be detected. This number of families is too small to allow separate analyses of families with and without a mutation.

Colorectal cancer in the general population

The Netherlands Cancer Registry (NCR) is a population-based cancer registry which systematically collects data on all malignant neoplasms occurring in The Netherlands since 1989 [94]. After notification, mainly by pathology and haematology departments, the medical records of newly diagnosed patients (and tumours) are collected. Relevant information for the cancer registry is abstracted by trained registration clerks and submitted to a regional cancer registry. After extensive checks for duplicate records, the data are entered into the national database. The systematic completeness of the registration is estimated to be 98.5%. We used incidence data from the NCR in 1989-1992, as well as information on the age distribution of the population of The Netherlands in 1992 [94].

Data analysis

From the 51 genealogical trees, only those cases of colorectal cancer for which a pathological-anatomical or other medical report was available, were included in the analyses. Thus, subjects whose colorectal cancer could only be based on family history information were excluded (n=41). Because the data used in this study are partly derived from genealogical trees that go back to the beginning of this century, not all information on age at diagnosis and death is available. As these dates are required to determine the person years of observation, people for whom this information was missing could not be included (60 cases, 134 relatives) in the analyses. This resulted in inclusion of a total of 212 patients with verified colorectal cancer and 974 non-affected first-degree relatives. Whenever date of birth was included in the survival analysis, another 24 (of the 974) non-affected relatives, of whom age at diagnosis or death was known but not their date of birth, were excluded.

To quantify lifetime risk, we calculated the cumulative incidence (CI_t), being the probability for an individual to develop the disease before age t [95]. The estimated CI at a certain age (CI_t) is based on the age-specific incidence rates, taking into account the length of the age categories as well as the proportion of people at risk at the beginning of each subsequent age category [96]. The 95% confidence limits (95% CL) of CI_t are derived from the Poisson distribution of the observed number of cases [97]. In the data from The Netherlands Cancer

Registry, the age-specific incidence rate is the number of new cases, in 1989-1992, in a 5-year age group per 100,000 individuals of that 5-year age group. To study anticipation, we calculated CI_t for successive generations in HNPCC families. Each family was subdivided into generations, starting with one (or a pair of) common ancestor(s), and each following generation being the next horizontal line in the genealogical tree. The first generation consists of the children of the ancestors, the second generation consists of all the children of the cases in the first generation, and so on.

Time from date of birth until diagnosis was used as survival time. Subjects who were not affected by colorectal cancer or had died from other causes before the end of the study period were treated as "censored" observations. To account for differences in gender, year of birth and year of diagnosis, we conducted survival analysis using Cox proportional hazards regression modelling [98]. Generation, birth cohort or year of birth, and gender, were entered in the model separately and in combination. The proportional hazards assumptions were found to be valid for each of the variables.

Results

Description of the HNPCC population

Table 2.1. *Descriptive data of histologically verified colorectal cancer patients in 51 HNPCC families.*

Characteristics		Total (n=212)	Men (n=115)	Women (n=97)	General Population*
Age at diagnosis mean ± sd		43.6 ± 12.4	44.3 ± 12.5	42.9 ± 12.1	69 ± 13
Localization tumour	n (%)				
Proximal		112 (52.8)	62 (53.9)	50 (51.5)	817 (31.6)
Distal		87 (41.0)	45 (39.1)	42 (43.3)	1767 (68.4)
Unknown		13 (5.6)	8 (7.0)	5 (5.2)	n.a.†
Stage at diagnosis	n (%)				
A		10 (4.7)	7 (6.1)	3 (3.1)	434 (16.2)
B		90 (42.4)	49 (42.6)	41 (42.3)	822 (30.8)
C		58 (27.4)	33 (28.7)	25 (25.8)	538 (20.1)
D		17 (8.0)	7 (6.1)	10 (10.3)	481 (18.0)
Unknown		37 (17.4)	19 (16.5)	18 (18.6)	397 (14.8)

* General population data, from "Comprehensive Cancer Centre Middle Netherlands"

† n.a., not applicable

Fifty-one HNPCC families consisted on average of 3 generations, with 28 first-degree at-risk members per family, of which an average of 6 had a colorectal carcinoma. Table 2.1 describes the characteristics of the histologically verified colorectal cancer patients in the 51 HNPCC families. Mean age at diagnosis of patients with colorectal cancer was 43.6 years in the overall group, whereas it was 65 (n=3), 49 (n=73), 41 (n=116) and 33 (n=20) years in the ancestors, and first, second and third generation, respectively. No cases have occurred in the fourth generation so far. More than 50% of the tumours were localised in the proximal colon. The majority of the tumours (70%) were in Dukes stage B and C at diagnosis. Of the patients with colorectal cancer, 24% (n=51, data not shown) developed more than one colorectal carcinoma. Sixty-two other primary, extra-colonic, tumours were diagnosed before or after the colorectal tumour in these patients. Twenty-one were tumours located in the endometrium, 5 in the urinary tract including the bladder, 6 in the stomach, 3 in the small intestine, 3 in the prostate, 4 in the ovaries, 4 in the breast, 1 in the lung, 9 at other sites and 6 at unknown sites.

Table 2.2. *Cumulative incidence data (95% confidence limits) for colorectal cancer in 51 HNPCC families in comparison with the general population (NCR, 1995).*

	HNPCC families		CI at age t (Cit) (95% confidence limits)		
Age (years)	Cumulative # of cases	Initially at risk	HNPCC (per 1000)	NCR (per 1000)	Cumulative Incidence Ratio (CIR)
0-19	2	1186	1.8 (0-4.2)	0.1 (0.06-0.16)	16.4
20-24	6	1071	5.6 (1.8-9.4)	0.2 (0.15-0.21)	31.2
25-29	24	1007	24.3 (15.7-32.8)	0.3 (0.23-0.30)	92.5
30-34	55	905	59.6 (47.4-71.7)	0.4 (0.36-0.46)	144.3
35-39	90	799	104 (90-118)	0.7 (0.63-0.77)	148.4
40-44	122	662	153 (136-169)	1.4 (1.3-1.5)	110.7
45-49	153	517	210 (190-229)	2.7 (2.5-2.8)	78.9
50-54	167	395	242 (226-259)	5.0 (4.7-5.2)	48.7
55-59	184	301	291 (268-313)	8.8 (8.5-9.4)	33.0
60-64	195	233	331 (308-353)	14.9 (14.5-15.3)	22.2
65-69	207	171	386 (355-415)	24.2 (23.7-24.8)	15.9
70-74	210	123	405 (383-426)	36.7 (36.0-37.3)	11.0
75-79	212	81	425 (397-451)	53.2 (52.3-54.1)	8.0
80-84	212	43	-*	72.6 (71.4-73.7)	-*
85+	212	17	-*	108 (105-110)	-*

* Not calculated because of the small number of subjects initially at risk in HNPCC families.

Comparison of lifetime risk to the general population

In HNPCC families the CI of colorectal cancer at age 75 (CI_{75}) is 40.5% (Table 2.2), whereas in the general population it is only 3.7% at that age. In the general population the $CI_{lifetime}$ is about 10% and increases most clearly after age 65 (Figure 2.1). In HNPCC families CI_{75} exceeds 40% and increases mainly in the age categories from 30-65 years. The CI is somewhat higher among men than among women, both in HNPCC families and the general population (Figure 2.1).

Due to the above pattern the CIR of HNPCC families relative to the general population is highest in the younger categories (CIR = 148.4). Because of the increase in colorectal cancer incidence in the general population after age 60, the CIR decreases over lifetime to about 10.

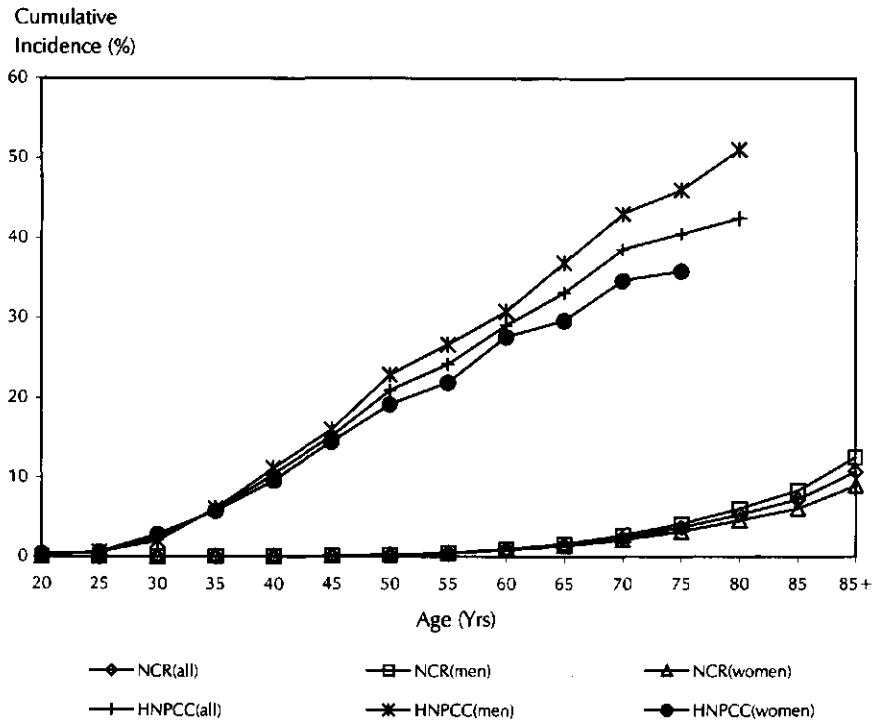

Figure 2.1. *CI of colorectal cancer by gender in HNPCC families and the general population.*

Anticipation within successive HNPCC generations

Figure 2.2 shows the CI according to age in successive generations of HNPCC families. The CI increases in each successive generation, which indicates that the age-specific incidence rates tend to increase more rapidly. In the ancestors there are no histologically verified cases before age 55; the CI_{75} is 18.9%. In the first generation the CI is 14.6% at age 50 and increases further to 32.4% at age 75. CI_{50} in the second generation is 26.8% and CI_{75} is 55.0%. In the third generation CI_{45} is 27.8%. No estimate can be given for the CI_{50} and CI_{75} since there has been no observation time in the older age categories so far. To account for the differences in observation time within the different generations, survival analysis was conducted. The survival curves of time from birth until diagnosis of colorectal cancer differ significantly between the successive generations in HNPCC families. Hazard rate ratios (RR) increase significantly in the second and third generation, as compared to the first generation in a univariate model (Table 2.3). There is an increasing trend over successive generations (RR per generation: 1.78; 95% CL 1.43-2.20).

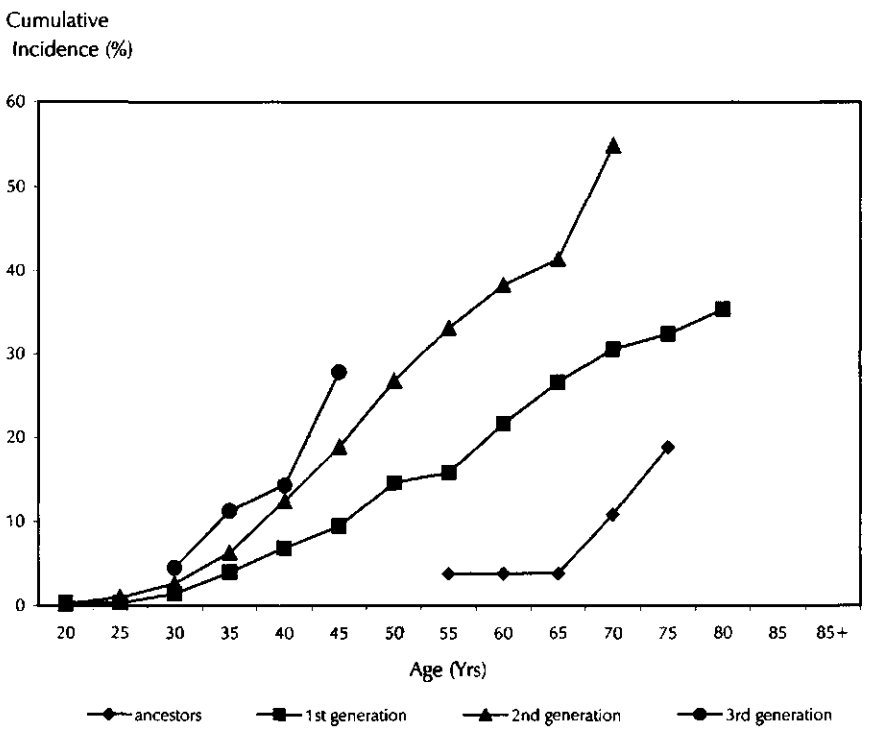

Figure 2.2. *CI of colorectal cancer in successive generations of HNPCC families.*

To study whether the generation effect can be explained by a secular time trend, we repeated the analysis using birth cohorts, categorised in five 25-year intervals for year of birth, as the explanatory variable. We observed an increase in RRs over successive birth cohorts (Table 2.3), with a significant trend (RR per birth cohort: 1.72; 95% CL 1.40-2.10). Moreover, when adding both generation and year of birth to the model, the RRs for generation effect were no longer significant (Table 2.3, RR third generation: 1.21), whereas year of birth remained significant. Since the colorectal cancer risk in the general population is slightly lower for women (Figure 2.1), and to compensate for potentially different sex distributions in successive HNPCC generations, the multivariate model was gender-adjusted. Estimates were not notably affected, although we indeed observed a 20% lower risk in women (95% CL = 0.61-1.06).

Table 2.3. *Cox proportional hazards regression model comparing HNPCC generations.*

	number of subjects		RR (95% confidence limits)			
	total no. of cases	initially at risk	univariate Model*		multivariate model†	
generation¶						
ancestors	3	37	0.31	(0.10-0.97)	0.71	(0.21-2.42)
first	37	311	1.00‡		1.00‡	
second	116	529	1.93	(1.42-2.64)	1.25	(0.85-1.84)
third	20	291	2.47	(1.44-4.22)	1.21	(0.63-2.32)
birthcohort						
< 1899	5	55	0.34	(0.14-0.85)		
1900-1924	64	235	1.00‡			
1925-1949	108	395	1.86	(1.32-2.62)		
1950-1974	35	418	2.34	(1.44-3.81)		

* RR=1.78 (95% CL = 1.43-2.20) when generations scored 1-5; for birth cohort RR=1.72 (95% CL = 1.40-2.10)

† adjustment of generation for birth cohort was done using year of birth as a continuous variable

¶ the fourth generation contributed 'survival time' but no cases

‡ reference category

Discussion

In this population of Dutch HNPCC families, cancers of the colon and rectum occur up to 150 times more frequently than in the general Dutch population, especially at younger ages. In the general population, the majority of colorectal cancer cases develop after age 65, whereas in HNPCC family members, most tumours develop before this age. The earlier age

at diagnosis in successive generations of these families appears to result from a secular trend in cancer occurrence and not from a biological feature of HNPCC.

Risk of cancer in HNPCC families

We found a 40% CI of colorectal cancer at age 75 in first-degree HNPCC family members, theoretically consisting of 50% gene carriers and 50% non-carriers. Vasen *et al.* assessed cancer risk in a large series of gene carriers and estimated the lifetime risk of colorectal cancer to be 80% [99]. Aarnio *et al.* observed a similar cumulative lifetime risk of 78% in putative HNPCC gene carriers [88].

Due to the sources of the incidence data, differences in the selection of subjects might have led to biases in the CIR, due to either over- or underestimation of the CI in HNPCC families and/or the general population. The HNPCC families are a fixed cohort, showing an increasing trend in the risk of cancer over several decades, whereas the incidence figures in the general population were based on cross-sectional data (1989-1992) of a dynamic population. Therefore, the time period of diagnosis in younger cohorts of HNPCC families largely corresponds to that of the NCR [94]. However, the older cohorts of HNPCC families have also been compared to recent incidence data of the general population. Data from the oldest regional cancer registry in The Netherlands, the Eindhoven Cancer Registry [2], show a marked gradual increase in incidence of colorectal cancer since the 1970s, especially in the higher age categories. Therefore, the true incidence in the older age categories of the general population may be overestimated. The CIR of 148 at age 40 is probably reasonably realistic, whereas the CIRs in the older age categories are underestimated.

Overestimation of risk in HNPCC family members may occur since families were only included in the study if they had the required minimum of 3 cases of colorectal cancer. Since the average HNPCC family has an average of 6 colorectal cancer cases, the CIR might theoretically be overestimated by a factor 2, but even this would still leave a very large risk difference between HNPCC families and the general population.

The use of the "Amsterdam criteria" may have resulted in false-positive families, based on chance aggregation of tumours, as well as false-negative families, due to the low probability of finding 3 cases of colorectal cancer within small families [100]. DNA analyses of the mismatch repair genes may resolve this problem in the future. Until now, DNA analysis has only been successfully performed in a limited number of families. Therefore, our results reflect colorectal cancer risk for all first-degree family members and not only for gene carriers.

Smaller biases may be related to the methods of calculation of CIs and of data analysis. First, we did not strictly adhere to the "Amsterdam Criteria" since we included 14 HNPCC families, in which some diagnoses were based on medical reports only, without histological verification. It is not likely that this has seriously affected the CIR, since the results of the

survival analysis were not markedly affected by excluding these families. Second, the incidence rates of the NCR include 1-5% HNPCC cases [86;87] in the rates' numerator, as well as observation time of prevalent cancer cases in the denominator. This may have had a small but unknown net effect on the incidence rate and, consequently, on the CIR.

Anticipation of cancer in HNPCC

As observed by others [23;27], the age at diagnosis decreases in successive generations within HNPCC families. This might reflect an earlier onset of disease in successive generations (*i.e.* anticipation), but it could also be due to ascertainment bias: the chance of finding HNPCC families with young cases in recent generations is larger than that of finding HNPCC families with young cases in older generations. Furthermore, follow-up of the younger birth cohorts does not exceed the first 5 decades of life, whereas it covers the entire life span in the older birth cohorts. Consequently, the mean age at diagnosis in the younger birth cohorts is necessarily restricted to the younger age range, whereas it can be much higher in older generations. When survival analysis was used to account for these differences in observation time, we did observe an increasing relative risk of colorectal cancer in successive generations of HNPCC families. However, RRs found for generations were similar to those found for birth cohorts in univariate analyses. Adjusting the effect of generation for the secular trend resulted in non-significant RRs close to unity, suggesting that the higher risk at younger ages in successive generations can be attributed to a secular trend in cancer rather than to a generation effect.

In other hereditary forms of cancer such as melanoma and breast cancer, similar findings of generation and birth cohort effects have been found [101;102].

In the general population, an increase in incidence of colorectal cancer, although less marked, has also been observed over the last decades, especially in the older age categories [2]. The survival analyses show that the increasing disease risk in successive HNPCC generations is not a disease characteristic *per se*, but is likely to reflect a secular trend of colorectal cancer risk within these families. This suggests that both hereditary and sporadic colorectal carcinoma have shared etiologic factors, *e.g.* in terms of dietary and environmental exposure patterns that changed during the past century.

However, it could also be postulated that part of the trend in HNPCC is due to progress in medicine. Diagnostic procedures have changed during the century, colonoscopy has become more widely available, and health awareness has increased, the latter two factors particularly in HNPCC families. First, HNPCC cases were included only when the diagnosis was histologically verified. Because of the smaller percentage of histologically verified diagnoses in the older generations in our HNPCC families, this may partially account for the generation effect observed. The number of ancestors was small, however, and we used the first generation as the reference category in the survival analysis. Second, screening of

HNPCC family members may have contributed to the younger age at diagnosis. However, there were only 11 screening-detected cases in the dataset, too few to justify separate analyses or to account for serious bias in the observed associations. On the other hand, HNPCC family members may have undergone colonoscopy and prophylactic polypectomy, which may have led to an underestimation of the true colorectal cancer risk.

In conclusion, our data confirm that HNPCC families have a very high risk of colorectal carcinoma as compared to the general population, especially before age 50, with a trend toward higher incidence in successive generations. This, however, is not necessarily a genetic characteristic of the disease, but may simply reflect the same secular trend as observed in sporadic colorectal carcinoma.

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NO MAJOR DIFFERENCE IN K-*RAS* AND P53 ABNORMALITIES IN SPORADIC AND HEREDITARY NON-POLYPOSIS COLORECTAL ADENOMAS

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CHAPTER 3 NO MAJOR DIFFERENCE IN K-RAS AND P53 ABNORMALITIES IN SPORADIC AND HEREDITARY NON-POLYPOSIS COLORECTAL ADENOMAS

K-*ras* and p53 gene mutations are known to occur in high frequencies in sporadic colorectal cancers, but findings are inconsistent in Hereditary Non Polyposis Colorectal Cancer. We compared K-*ras* and p53 abnormalities in colorectal adenomas from HNPCC and sporadic patients, to examine whether they may represent similar or different molecular pathways to cancer.

In 48 HNPCC and 59 sporadic colorectal adenomas codon 12 and 13 of the K-*ras* gene were examined for mutations using Mutant Allele Specific Amplification (MASA)-PCR, and p53 protein overexpression was assessed using immunohistochemical methods.

In sporadic adenomas K-*ras* mutations were detected in 32% and p53 overexpression in 31% of the cases. Similarly, K-*ras* mutations and p53 overexpression were both found in 25% of HNPCC adenomas. The frequencies of these abnormalities were not significantly different between HNPCC and sporadic adenomas. When taking differences in adenoma size into account, the frequencies were even more similar. The type of mutations in K-*ras* (transversion vs transition) did differ between HNPCC and sporadic adenomas.

These results provide evidence for a similar molecular pathway to adenomas in HNPCC and sporadic carcinogenesis, with respect to involvement of K-*ras* and p53.

INTRODUCTION

Hereditary as well as environmental factors play an important role in the etiology of colorectal adenomas and subsequently colorectal cancer. During the adenoma-carcinoma sequence several mutations in oncogenes and tumor-suppressor-genes are acquired. In sporadic colorectal cancer the Adenomatous Polyposis Coli (APC)-"gatekeeper"-gene, underlying Familial Adenomatous Polyposis, is one of the first targets for a somatic mutation [9]; subsequently mutations in other genes (e.g. K-*ras*, p53) may occur [7]. Hereditary Non Polyposis Colorectal Cancer (HNPCC), is a distinct hereditary disease leading to colorectal cancer. HNPCC is caused by germline mutation in one of at least four mismatch repair genes [85]. Mismatches in microsatellite sequences, which often occur during normal DNA replication, are not repaired if one of the mismatch repair genes is mutated [103]. This microsatellite instability gives rise to a mutator phenotype and occurs in about 80-90% of HNPCC tumors and also in a small proportion (10-15%) of sporadic

colorectal cancers [16;17;18]. The genetic instability reflected in replication errors (RER), the frequent mutations in the Transforming Growth Factor β type II receptor (TGFβ-RII) [104], and the more favorable prognosis of HNPCC patients versus sporadic patients [105], suggest different molecular pathways of sporadic and HNPCC carcinogenesis. On the other hand, the initiating mutation in the APC gene in sporadic carcinogenesis is also frequent in RER positive carcinomas [28]. Moreover, RER positive carcinomas are also reported to harbor somatic mutations in the K-*ras* and p53 genes [16;106]. In sporadic colorectal cancer K-*ras* and p53 are both involved in about 50% of the tumors, K-*ras* also occurring in about half of the large adenomas and p53 in a lower percentage of adenomas [7].
Recently a few studies have addressed the issue of K-*ras* and p53 mutation frequencies in HNPCC or mismatch repair related carcinomas [29;106;107]. However, these studies were often relatively small (18-27 HNPCC or RER+ cases) and may therefore have resulted in inconsistent findings as to the frequency of these mutations (varying from 6-61% for K-*ras* mutations and 12-64% for p53 mutations or overexpression). Frequencies of K-*ras* and p53 abnormalities in earlier stages of HNPCC carcinogenesis have not been studied systematically. We have conducted a study of HNPCC as well as sporadic colorectal adenomas and examined whether these adenomas develop by distinctly different molecular pathways, with respect to acquired abnormalities in the K-*ras* and p53 genes.

MATERIALS AND METHODS

Study population

A case control study on dietary factors, genetic susceptibility and DNA-damage in colorectal adenomas was conducted between December 1995 and February 1998. Histological material was obtained from 59 sporadic and 48 HNPCC colorectal adenomas resected at endoscopy. Sporadic cases were recruited by the Departments of Gastroenterology of the Nijmegen University Hospital and two regional hospitals (Rivierenland, Tiel; Gelderse Vallei, Ede-Wageningen-Bennekom). The families of all HNPCC cases fulfilled the Amsterdam criteria for HNPCC [25]: at least three members in at least two successive generations must have colorectal cancer, with at least one case diagnosed before the age of 50 years; one of the affected members should be a first-degree relative of the other two; Familial Adenomatous Polyposis should be ruled out. HNPCC cases were first-degree relatives of colorectal or endometrium cancer cases, and were recruited in a similar fashion, either by the Nijmegen University Hospital or the Netherlands Foundation for the Detection of Hereditary Tumors which keeps a registry of HNPCC families in The Netherlands. For most HNPCC participants no information was available on their gene carrier status for the mismatch repair genes.

All participants were Dutch-speaking and of Western European origin, diagnosed before the age of 75, without a history of colorectal cancer, colon resection, polyposis coli, or inflammatory bowel disease. Sporadic cases were excluded if they had had previous adenomas more than 3 years before entrance to the study. Eligible patients were informed about the study by the gastroenterologist participating in the conduct of this study or by the Netherlands Foundation for the Detection of Hereditary Tumors, after approval of their 'physician in attendance'. Of the 319 subjects who initially agreed to participate in the study 22, (7%) did not return questionnaires (on dietary and lifestyle factors, family history of cancer, bowel complaints, etc.) and 21 (6%) appeared not to meet the eligibility criteria retrospectively, resulting in 122 cases and 154 controls available for further study. Only adenoma cases were used in the analyses described in this paper. From 15 of the 122 cases no tumor material could be retrieved and they were therefore excluded from the current analyses.

Tumors

Clinical information, regarding the part of the colon inspected, the location of adenoma, and possible other abnormalities, was retrieved at endoscopy. In each subject who had adenomas at more than one endoscopy, the adenomas diagnosed closest to the date of entrance to the study were used. Characteristics of these adenomas were retrieved from histology reports. Adenoma characteristics and information on K-*ras* and p53 abnormalities were recorded for the largest adenoma diagnosed during that endoscopy.

K-ras mutation analysis

DNA was isolated from paraffin sections. After deparaffination and rehydration, tissue was incubated for 18 hours at 56°C with 0.5 mg/ml proteinase K (Boehringer Mannheim, Almere, The Netherlands) in 500 µl cell lysis solution (DNA-isolation kit; PureGene Gentra Systems Inc., Landgraaf, The Netherlands) and additionally 72 hours at 37°C. After cooling the samples to 4°C, protein was precipitated. To the supernatant, 500 µl isopropanol was added for DNA precipitation. After washing the pellet with 70% ethanol at 4°C and centrifugation, DNA was air-dried and rehydrated.

Codon 12 and 13 of the K-*ras* gene were examined for mutations by Mutant Allele Specific Amplification (MASA)-PCR. A mixture of primers corresponds to variants of the first (set A) and second (set B) nucleotide of codon 12, or the first (set C) or the second (set D) nucleotide of codon 13. The following primers were used:

for wild-type Ki-ras: 5'TGTGGTAGTTGGAGCTG;

Set A 5'TGTGGTAGTTGGAGCTC, 5'TGTGGTAGTTGGAGCTA, and

5'TGTGGTAGTTGGAGCTT;

Set B 5'GTGGTAGTTGGAGCTGT, 5'GTGGTAGTTGGAGCTGA, and 5'GTGGTAGTTGGAGCTGC;
Set C 5'GGTAGTTGGAGCTGGTC, 5'GGTAGTTGGAGCTGGTA, and 5'GGTAGTTGGAGCTGGTT;
Set D 5'GTAGTTGGAGCTGGTGC, 5'GTAGTTGGAGCTGGTGA, and 5'GTAGTTGGAGCTGGTGT.
For all PCRs the same 3'-primer was used: 3'TCAAAAGAATGGTCCTGGACC.
MASA-PCR was performed in 35 cycles (0.5', 94°C; 0.5', 60°C; 1', 72°C) using 300 ng DNA, 200 μM of each dNTP, 20 pmol of each primer, 0.3 U of Thermoperfect Plus DNA polymerase (Integro BV, Zaandam, The Netherlands), 20 mM $(NH_4)SO_4$, 75 mM Tris-HCL pH 9.0, 0.01% Tween and 2.5 mM $MgCl_2$. Following amplification, 15 μl of each reaction mixture was loaded on a 2% agarose gel, electrophoresed and stained with ethidium bromide.
On those samples that gave negative results with one single round of PCR, a nested PCR was performed. In the first round of this nested PCR (35 cycles; 0.5', 94ºC; 0.5', 55ºC; 1', 72ºC) we added 100 ng of DNA and used ACTCATGAAAATGGTCAGAG (3'-primer) and GTACTGGTGGAGTATTTGATAG (5'-primer) as primers. For the second round (25 cycles; 0.5', 94ºC; 0.5', 60ºC; 1', 72ºC) primers sets and reaction conditions were the same as described above for the MASA-PCR. The only difference was that 1 μl of a 1:100 dilution of the first PCR product was used instead of 300 ng DNA.

P53 immunohistochemical analysis

As many adenomas were too small to provide sufficient amounts of DNA for performing p53 mutation analysis, immunohistochemistry methods were used to assess p53 overexpression. Paraffin sections were mounted on charged glass slides (Superfrost) and dried overnight at 60°C. Sections were deparaffinized and rehydrated. Microwave antigen retrieval was performed by boiling the sections in 10 mM citrate-buffer pH 6,0 for 5 min. Additional buffer was added and the sections were boiled for another 5 min. and then allowed to cool to room temperature for 45 min. Sections were rinsed in PBS. Endogenous avidin/biotin was blocked with an Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame CA, USA). Endogenous peroxidase activity was blocked by incubation in 0,6 % H_2O_2 for 30 min. Sections were rinsed in PBS. Aspecific antibody binding was blocked by pre-incubation of the sections with 10% normal horse serum in PBS/BSA for 10 min. Sections were incubated overnight at 4°C with a cocktail of two anti-p53 antibodies: 0,4 μg/ml DO-7 (Neomarkers, Fremont, USA) which recognizes both mutant and wild forms of p53, and 1 μg/ml 240 (Neomarkers, Fremont, USA) which recognizes only the mutant form of p53. Negative control sections were treated with PBS/BSA alone. Sections were rinsed in PBS, and antibody binding was visualized using

the Vector ABC Elite kit (Vector Laboratories, Burlingame CA, USA) using 3,3'-diaminobenzidine as substrate and copper enhancement according to the manufacturer's protocol. Finally, sections were dehydrated through graded concentrations of ethanol, incubated in xylol and mounted with permount.
Stained sections were scored independently by a pathologist and one of the investigators (G.N.P. v.M.). Inconsistencies (<10%) were discussed together to mutual agreement. We have used two cut-off points for determining p53 overexpression. Tumors were scored as p53 positive if more than 20% of the cells displayed nuclear positivity. As p53 overexpression in adenomas often takes a focal pattern [108;109], adenomas with strong staining in 5-20% of cells were also considered positive.

Data analysis

To test differences in tumor characteristics and frequencies of molecular abnormalities (K-*ras* mutations and p53 overexpression) between the sporadic and the HNPCC groups, Chi-square tests were used for categorical variables, or t-tests in case of a continuous variable. Logistic regression was used to estimate the relative risk of a K-*ras* mutation or p53 overexpression in HNPCC vs sporadic adenomas and to adjust for possible confounding by tumor characteristics. As the indications for having an endoscopy may differ for sporadic and HNPCC cases, factors related to the indication for endoscopy (bowel complaints versus routine screening), such as presence of bowel complaints (assessed by questionnaire), and part of colon visualized (type of colonic examination), were also taken into account.

RESULTS

Table 3.1 shows the general characteristics of the study population. As expected, the HNPCC cases were younger than the sporadic cases, while gender distribution was similar in both groups. Sporadic cases more often reported having bowel complaints prior to diagnosis of the adenoma(s). HNPCC cases often had small solitary adenomas, whereas the sporadic cases harbored larger adenomas accompanied by one or more other adenomas. The adenomas in HNPCC patients were more often localized in the proximal colon, while on the contrary sporadic adenomas were mainly found in the distal colon and rectum. As some of these characteristics may be related to frequency of endoscopy and the part of the colon visualized, we adjusted these analyses for 'type of colonic examination' (full colonoscopy including coecum versus sigmoidoscopy or partial colonoscopy combined with barium enema) and presence of bowel complaints. This did not markedly change the association between adenoma characteristics and hereditary (HNPCC) versus sporadic status of the cases (data not shown).

K-*ras* mutations and p53 overexpression occur in similar frequencies in these adenomas and do not significantly differ between sporadic and HNPCC adenomas (Table 1). In HNPCC patients K-*ras* mutations were detected in 12 of 48 (25%) of adenomas. Of 59 adenomas in the sporadic group, 19 (32%) harbor at least one mutation in the K-*ras* gene. The frequency of p53 protein overexpression was also similar in HNPCC (25%) and sporadic adenomas (31%).

Table 3.1. *General characteristics of the study population and frequencies of K-ras gene mutations and p53 protein overexpression; associations with sporadic vs HNPCC adenomas.*

		Sporadic (n=59) n (%)		HNPCC (n=48) n (%)		OR (95% CI)* HNPCC vs sporadic	
age (mean ± SD)		55	± 11	46	± 10†	0.93	(0.89-0.97)
gender	men	34	(58)	25	(52)	1.0	
	women	25	(42)	23	(48)	1.2	(0.6-2.7)
bowel complaints¶	no	20	(53)	32	(78)	1.0	
	yes	18	(47)	9	(22)	0.3	(0.1-0.8)
full colonoscopy¶	yes	50	(85)	43	(91)	1.0	
	no	9	(15)	4	(8)	0.5	(0.1-1.8)
adenoma characteristics‡							
adenoma size	1-4 mm	16	(27)	27	(57)†	1.0	
	5-9 mm	18	(30)	11	(23)	0.4	(0.1-1.0)
	≥10 mm	25	(42)	9	(19)	0.2	(0.1-0.6)
single adenoma		34	(58)	35	(73)	1.0	
multiple adenomas		25	(42)	13	(27)	0.5	(0.2-1.1)
localization¶	distal colon + rectum	43	(80)	26	(60)†	1.0	
	proximal colon	11	(20)	17	(40)	2.6	(1.0-6.3)
molecular abnormalities‡							
K-ras gene mutations							
	no	40	(68)	36	(75)	1.0	
	yes	19	(32)	12	(25)	0.7	(0.3-1.6)
p53 protein overexpression							
	no	41	(69)	36	(75)	1.0	
	yes	18	(31)	12	(25)	0.8	(0.3-1.8)

* OR=Odds Ratio, 95% CI=95% Confidence Interval

† $p<0.05$, T-test for continuous, and Chi-square test for categorial variables

¶ presence of bowel complaints (as reported by questionnaire) was unknown for 21 sporadic and 7 HNPCC cases; the type of endoscopy (full colonoscopy: yes, including coecum; no, sigmoidoscopy or partial colonoscopy combined with barium enema) was unknown for 1 HNPCC case; exact location of the adenoma (proximal: coecum, ascendens, hepatic flexure, transverse; distal: splenic flexure, descendens, sigmoid, rectosigmoid, rectum) was unknown for 5 sporadic and 5 HNPCC adenomas

‡ characteristics and molecular abnormalities of the largest adenoma (in case of multiple adenomas)

Crude logistic models showed slightly lower non significant odds ratios for the association between family history (HNPCC versus sporadic) and molecular abnormalities (K-*ras* mutation and p53 overexpression).

As shown in Table 3.2, increasing adenoma size is positively associated with harboring K-*ras* or p53 abnormalities in the sporadic group. In the HNPCC group the association with adenoma size was of similar magnitude but not statistically significant. Having multiple adenomas was positively associated with p53 overexpression in the HNPCC cases. The location of the adenoma in the colon was not associated with differences in K-*ras* and p53 abnormalities, neither in HNPCC nor in sporadic cases.

Table 3.2. *Association between molecular and clinical tumor characteristics in HNPCC and sporadic adenomas.*

clinical tumor and case characteristics	contrast	OR (95% CI)* of molecular tumor characteristics			
		K-ras mutation		p53 overexpression	
		sporadic	HNPCC	sporadic	HNPCC
age	per 10 years	1.2 (0.7-1.9)	1.0 (0.5-1.9)	0.8 (0.5-1.4)	1.8 (0.9-3.6)
gender	female vs male	1.0 (0.3-3.0)	0.4 (0.1-1.8)	0.6 (0.2-1.8)	2.8 (0.7-11.0)
adenoma size	per 5 mm	1.5 (1.1-2.2)	1.5 (0.7-3.1)	1.4 (1.0-2.0)	1.5 (0.7-3.1)
No. of adenomas	multiple vs single	1.4 (0.4-4.0)	2.5(0.6-10.0)	1.6 (0.5-4.8)	4.1 (1.0-16.0)
location in colon	Proximal vs distal	0.6 (0.1-2.7)	1.4 (0.3-5.6)	0.4 (0.1-2.0)	0.4 (0.1-1.8)

* OR=Odds Ratio; 95% CI=95% Confidence Interval

Table 3.3. *Association between molecular adenoma characteristics and HNPCC vs sporadic colorectal adenomas.*

Model including adjustments	OR (95% CI) HNPCC vs sporadic			
	K-ras	p53	K-ras or p53	K-ras and p53
crude model of HNPCC vs sporadic adenomas	0.7 (0.3-1.6)	0.8 (0.3-1.8)	1.1 (0.5-2.5)	0.4 (0.1-1.6)
model of HNPCC vs sporadic adenomas adjusted for:				
adenoma size	0.9 (0.3-2.2)	1.2 (0.5-3.1)	1.4 (0.5-3.4)	0.8 (0.2-3.3)
location adenoma	0.6 (0.2-1.6)	0.9 (0.4-2.2)	1.0 (0.4-2.6)	0.5 (0.1-1.9)
multiple adenomas	0.8 (0.3-1.8)	0.9 (0.4-2.1)	1.2 (0.5-2.7)	0.5 (0.1-2.0)
age	0.8 (0.3-1.9)	0.8 (0.3-2.0)	1.1 (0.4-2.7)	0.6 (0.1-2.1)
gender	0.7 (0.3-1.7)	0.8 (0.3-1.8)	1.1 (0.5-2.5)	0.5 (0.1-1.6)
bowel complaints	0.5 (0.2-1.4)	0.4 (0.1-1.1)	0.5 (0.2-1.5)	0.2 (0.0-1.1)
type of endoscopy	0.7 (0.3-1.8)	0.8 (0.3-1.8)	1.1 (0.4-2.5)	0.5 (0.1-1.8)
size + complaints	0.9 (0.4-2.4)	1.2 (0.5-3.2)	1.4 (0.5-3.6)	0.9 (0.2-3.6)

We further examined whether differences in adenoma characteristics contributed to the slightly lower frequencies of K-*ras* mutations and p53 overexpression in HNPCC adenomas as compared to sporadic adenomas (Table 3.3). When taking differences in adenoma size into account the frequencies of K-*ras* mutation, p53 overexpression, or both, were even more similar between HNPCC and sporadic adenomas. Other characteristics of the adenomas and age and gender of the patients changed the odds ratios only slightly. Adjustment for the presence of bowel complaints slightly and non-significantly lowered the odds ratios. Adjustment for type of endoscopy did not markedly change the odds ratios. When adenoma size and presence of bowel complaints were added to the models simultaneously, the odds ratios showed no difference in frequencies of K-*ras* and p53 abnormalities, but only adenoma size remained significant in the model.

Details about the type of K-*ras* mutations in the 19 sporadic and 12 HNPCC adenomas with K-*ras* mutations, are shown in Table 3.4. Multiple mutations in the K-*ras* gene were detected in 9 (29%) of these 31 adenomas. The majority of adenomas with K-*ras* mutations (94%) harbor a mutation in codon 12. Mutations in codon 13 occur in about 25% of adenomas with K-*ras* mutations, both in the sporadic and the HNPCC group. Mutations in the second nucleotide of codon 12 and 13 were most common: GAT in codon 12 (Gly-Asp), GTT in codon 12 (Gly-Val), and GAC in codon 13 (Gly-Asp), consisting of 31%, 24% and 19% of all 42 mutations respectively. Sporadic adenomas harbored transversion mutations more often (63%) than HNPCC adenomas (33%), whereas transition mutations occurred more often in HNPCC (75%) than sporadic adenomas (58%). Interestingly, all eight adenomas with K-*ras* mutations which were located in the proximal colon, harbored a transition mutation, whereas only about half of the distal adenomas with K-*ras* mutations harbored a transition.

Discussion

In this study similar frequencies of both K-*ras* and p53 abnormalities could be observed in HNPCC and sporadic colorectal adenomas. If the size of the adenomas was taken into account, frequencies of K-*ras* and p53 abnormalities were even more similar between HNPCC and sporadic adenomas. These results do not provide evidence for a different molecular pathway to colorectal carcinogenesis in HNPCC, as far as involvement of K-*ras* and p53 is concerned.

Table 3.4. Specific K-ras mutations in sporadic and HNPCC colorectal adenomas*.

case	age yrs	gender M/F	no. of adenomas	size in mm	location in colon	type of mutation	K-ras-12 (GGT= wildtype)	K-ras-13 (GGC= wildtype)
					sporadic adenomas			
1	74	M	7	20	ascending	transition	GAT	-
2	54	M	3	15	sigmoid	transition+transversion	TGT/GAT	-
3	46	M	1	25	rectum	transversion	GCT	-
4	70	F	1	3	sigmoid	transversion	CGT	-
5	40	M	1	2	rectum	transition	GAT	-
6	51	F	1	20	rectosigmoid	transversion	GTT	-
7	64	F	1	2	ascending	transition	-	GAC
8	53	F	2	20	splenic flexure	transversion	GTT	-
9	64	M	6	10	sigmoid	transversion	TGT	-
10	50	M	2	25	rectosigmoid	transition+transversion	GTT/GAT	GAC
11	58	F	3	5	rectum	transversion	GTT	-
12	50	F	1	10	sigmoid	transition	GAT	-
13	62	M	3	13	sigmoid	transition	GAT	-
14	74	M	1	7	sigmoid	transition	-	GAC
15	61	M	3	20	rectum	transition+transversion	GTT/GAT	GAC
16	56	F	3	30	rectum	transition	GAT	-
17	53	F	1	8	ascending	transition+transversion	GTT	GAC
18	41	M	1	20	sigmoid	transversion	CGT/GCT	-
19	47	M	1	5	sigmoid	transversion	GTT	-
					HNPCC adenomas			
20	56	M	1	3	hepatic flexure	transition	GAT	-
21	43	F	6	15	ascending	transition	AGT	-
22	53	M	2	10	coecum	transition	GAT	GAC
23	64	M	1	2	transverse	transition	GAT	GAC
24	28	M	1	2	overlapping	transition	GAT	-
25	51	F	6	4	coecum	transition	AGT	-
26	52	F	1	10	descending	transition+transversion	CGT/AGT	-
27	29	M	1	-	rectosigmoid	transversion	GTT	-
28	47	F	3	8	descending	transversion	GTT	-
29	57	M	1	3	sigmoid	transversion	GTT	-
30	38	M	9	4	sigmoid	transition	AGT	GAC
31	37	M	1	10	rectum	transition	GAT	-

* adenoma characteristics and K-ras mutations are described for the largest adenoma. Regarding the location in the colon: coecum, ascending colon, hepatic flexure and transverse colon are considered proximal colon; splenic flexure, sigmoid, rectosigmoid and rectum are considered distal colon.

We examined whether HNPCC and sporadic tumors develop by different molecular pathways in the early stages of carcinogenesis. If HNPCC adenomas develop by a K-*ras* and p53 independent pathway, one would expect a very low frequency of mutations in these tumors. Our study of 59 sporadic and 48 HNPCC adenoma patients provides us with a power of 80% (with 95% CI) to detect a difference, if the frequency of either abnormality would be 35% in sporadic adenomas and 10% or less in HNPCC adenomas.

The HNPCC status of the cases in this study was determined by family history (Amsterdam criteria) [25]. Occurrence of the RER phenotype and mutations in the mismatch repair genes are only known in a small number of cases, and were therefore not taken into account in the results presented. In a recent study by Brown *et al.* no correlation was seen between RER status and strength of family history except in HNPCC families [110]. Moreover, in a recent study consisting mainly of HNPCC families from The Netherlands, fulfillment of the Amsterdam criteria was strongly associated with germ-line mutations in MSH2 and MLH1 [111]. However, our group of HNPCC cases may include some cases without a germline mutation in one of the mismatch repair genes. In addition, our group of sporadic cases may include some cases with the RER phenotype. Therefore, some bias resulting from misclassification may have diluted the differences between sporadic and HNPCC adenomas.

K-*ras* mutations were assessed using the MASA method, a method more sensitive than the SSCP method which is often used [112]. We evaluated mutations in codon 12 and 13, and thereby may have missed a small number of potential mutations in codon 61, as they account for up to 5% of the total number of mutations in colorectal cancer [8]. Of the sporadic adenomas in this study 32% harbored a K-*ras* mutation, which is within the range of frequencies (15-75%) found in other studies [113;114;115]. The spectrum of mutations is also similar to that observed in other studies of colorectal adenomas and carcinomas. In a large multi-center study of 2721 colorectal carcinomas the most frequently occurring mutations were the GAT transition (31%) and the GTT transversion (23%) in codon 12, and the GAC transition (17%) in codon 13 [12]. Frequencies in our study of adenomas were 31%, 24% and 19% of the 42 detected mutations, respectively. Noticeably, Andreyev *et al.* reported 81% mutations in codon 12 and 19% in codon 13, and only rarely multiple mutations in one carcinoma [12]. However, of the adenomas with K-*ras* mutations in our study 94% harbor mutations in codon 12 and 26% in codon 13, with 9 of 31 adenomas (29%) harboring multiple mutations.

To assess overexpression of the p53 protein we used standard immunohistochemical methods. In a study of colon carcinomas we found the concordance between p53 mutation analysis (SSCP of exon 5 to 8, followed by sequencing) and immunohistochemical detection of p53 overexpression (same method as in this study, using 20% cut-off point) to be 72%. In studies of carcinomas a cut-off point of 20% of cells positively stained is often used to categorize overexpression [116;117]. However, Yao *et al.* and Shan *et al.* described that adenomas often have a more focal pattern of staining, with only a small percentage of cells with strong staining [108;109]. We have therefore used two cut-off points for scoring p53 overexpression: 5-20% of cells with strong staining,

which contributed about 25% to the total number of adenomas with overexpression, and more than 20% of cells staining, which contributed about 75% to positive adenomas. In total, we observed p53 overexpression in 31% of sporadic colorectal adenomas, which is within the wide range of 9-46% found in smaller studies of adenomas [109;118;119;120;121;122]. In colorectal carcinomas, overexpression of the p53 protein is found in 40-50% [116;123;124], which is somewhat higher than observed in adenomas, agreeing with the concept that p53 is mutated in a later phase of colorectal carcinogenesis [7].

In carcinomas marked differences in mutation frequencies of K-*ras* and p53 have been reported in HNPCC subjects. Two studies have reported low frequencies (6-17%) of K-*ras* and p53 abnormalities in HNPCC carcinomas [29;107]. In contrast, others reported relatively high frequencies (25-64%) of these abnormalities [12;106;125]. Inconsistencies between studies may be due to selection of patients and laboratory methods used, as well as to the limited size of most studies.
This study of adenomas, as early stages in hereditary as well as sporadic colorectal carcinogenesis, provides further clues to molecular pathways. We observed 25% of HNPCC adenomas to harbor a K-*ras* mutation and also 25% with p53 overexpression, frequencies similar to those observed in the sporadic adenomas. As harboring either of these molecular abnormalities is significantly associated with the size of the adenoma, frequencies were even more similar between these two groups if adenoma size was taken into account. Not accounted for in these analyses is the grade of dysplasia of the adenomas, which may also be associated with molecular abnormalities [115;118;126]. Part of the similarity in frequencies of K-*ras* mutations and p53 overexpression may therefore be explained by the generally higher grade of dysplasia of HNPCC adenomas [127]. However, taking this into consideration, the results still point towards similar pathways in the adenomatous stage of carcinogenesis, with respect to K-*ras* and p53 abnormalities.

Some differences in the type of K-ras mutations were observed between HNPCC and sporadic adenomas. Transversion mutations occurred slightly more often in sporadic adenomas, whereas transition mutations occurred more often in HNPCC adenomas especially in adenomas in the proximal colon. This might point at a difference in the cause of mutations between HNPCC and sporadic adenomas.
The similar frequencies of K-*ras* and p53 abnormalities in HNPCC and sporadic adenomas may seem at variance with the differences in mutation frequencies found in some studies in sporadic and HNPCC carcinomas, and the distinct clinical and prognostic differences in HNPCC adenomas and carcinomas. We therefore postulate that, with respect to K-*ras* and p53, the molecular pathways of HNPCC and sporadic colorectal carcinogenesis diverge only in the development from large adenoma to carcinoma.

Sporadic and HNPCC colorectal adenomas may develop through similar molecular pathways, both starting with an initiating event like an APC mutation, which may occur at an earlier age in HNPCC subjects who are mismatch repair deficient. Both types of adenomas accumulate mutations in K-*ras* and p53 at similar rates; due to their mutator phenotype, HNPCC adenomas also accumulate other mutations like those in TGFβ-RII leading to a growth advantage, and from that point onwards they progress to malignancy much faster. It may be postulated that during this rapid progression to malignancy there is less time to accumulate a higher percentage of additional mutations in K-*ras* and p53, leading to larger differences in mutation frequencies between HNPCC and sporadic tumors in later stages of carcinogenesis.

In conclusion, the results of this study do not provide evidence for differences between sporadic and HNPCC subjects in the molecular pathway to the early stage of colorectal carcinogenesis, with respect to K-*ras* and p53 abnormalities. Possibly, similar endogenous or exogenous factors may play a role in the etiology of sporadic and HNPCC colorectal carcinogenesis. More carcinomas as well as adenomas of different size and grade of dysplasia need to be evaluated before any definite conclusions about the molecular pathways in sporadic and hereditary colorectal cancer can be drawn.

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ASSESSING THE HUMAN INTAKE OF HETEROCYCLIC AMINES: LIMITED LOSS OF INFORMATION USING REDUCED SETS OF QUESTIONS

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CHAPTER 4 ASSESSING THE HUMAN INTAKE OF HETEROCYCLIC AMINES: LIMITED LOSS OF INFORMATION USING REDUCED SETS OF QUESTIONS

The aim of this study was to evaluate loss of information of a reduced food frequency questionnaire, as compared to an extensive reference method, developed to assess the intake of heterocyclic amines (HCAs). Food frequency data were linked to concentrations of HCAs in cooked foods to estimate the individual daily exposure to a combination of five HCAs.

The number of food items in the questionnaire was reduced and selected in three ways: according to the contribution to the estimated total intake, the between-person variance, or dishes included in other studies. The effect on sensitivity, specificity, concordance, the correlation coefficient, kappa, and simulated relative risks was determined using information from a population-based study conducted in Stockholm.

Only a limited amount of misclassification was introduced when the number of dishes was reduced from 39 to 15 or 20, and no major difference was seen when dishes were selected according to the total intake or to the between-person variance.

Our data indicate that for a specific exposure, such as HCAs, the loss of accuracy in an analytical epidemiological study is small, and may not be relevant, when the number of dishes in a food frequency questionnaire is decreased - if the initially chosen dishes are carefully selected, and cover a reasonable part of the total intake or between-person variance.

INTRODUCTION

High consumption of meat has been associated with an increased risk of colorectal cancer [36;37;58;70;72]. Such a risk may be due to substances naturally occurring in the meat per se, agents added to the meat, or compounds formed during cooking. Mutagenic heterocyclic amines (HCAs) are formed from amino acids, creatine/creatinine and sugar [128;129] and were first measured in the charred parts of cooked meat and fish [130]. In order to assess the intake of HCAs, factors to be considered include not only the type of dish ingested, portion size, and frequency of consumption, but also cooking methods, cooking temperature, intake of gravy, and the concentration of HCAs in each dish. The validity of assessing cancer risks in relation to a dietary intake, such as HCAs, in an observational study can be distorted by systematic errors such as misclassification, misrepresentation, and confounding [131;132]. The extent of misclassification depends on the sensitivity and

specificity of the dietary assessment method. In epidemiological research, the trade-off between an extensive, accurate, and time consuming method for a shorter version is often guided by practical and financial considerations. When reducing the number of items in a questionnaire, identifying a method to select the most informative food items may reduce the resulting systematic error. The magnitude of misclassification introduced when making these compromises, and the effect on risk estimates, must be considered for different reduction methods. There are alternative means of identifying informative food items in order to design reduced sets of questions. One may choose food items that contribute most to the total intake of the nutrient of interest in the study population, or select food items that vary most between subjects. The latter way is thought to be an effective method when the aim is to rank people according to their intake, and when the absolute level of intake is of less importance, as often is the case in epidemiological analytical studies [133].

In a Swedish population-based study, the intake of HCAs was estimated by linking consumption data collected by means of an extensive food frequency questionnaire to concentrations of HCAs in cooked meat and fish dishes [134]. In the present analysis, we simulated three different reduced sets of questions, and assessed the degree of misclassification as compared to the original questionnaire. In addition, to elucidate the effect on risk estimates, we simulated relative risks using the reduced sets of questions.

SUBJECTS AND METHODS

Study population

In this study, data were used from a previously conducted case control study on exposure to food mutagens and cancer risk. Subjects were born in Sweden between 1918 and 1942, having a permanent address in a demarcated geographical region in and around Stockholm for at least one month during the observation period from November 1, 1992, to December 31, 1994. Information about cases was retrieved from the population-based cancer register in Stockholm. Notification to the registry is mandatory by law both for attending physicians and pathologists. Controls were randomly selected from a population register during the observation period and frequency-matched according to age (five-year intervals) and gender distribution among the cases of colon cancer.

A questionnaire was mailed to the subjects after they had received an introductory letter. After returning the questionnaire, missing information was completed by telephone interviews. Altogether, information was retrieved from 548 controls and 1056 cases of cancer of the colon (ICD=153), rectum (ICD=154), bladder (ICD=188), or kidney (ICD=189), according to the International Classification of Diseases, Injuries and Causes of Death [135].

The referent dietary assessment method

The extensive dietary assessment method is described in detail elsewhere [134]. In brief, diet was assessed by means of a semiquantitative food frequency questionnaire including a total of 188 food items, 27 of which were fried meat dishes, 16 oven-roasted meat dishes, 11 boiled meat dishes, 4 grilled meat dishes, 7 fish dishes, 2 egg dishes and 1 blood pudding, as well as 4 different types of gravy/sauce. All questions took account of eating habits five years previously. Ten categories of intake frequencies ranged from '2-3 times per day' to 'never'. Variables used to assess the intake of HCAs from meat and fish were type of meat/fish ingested, frequency of consumption, portion size, cooking method, degree of surface browning, and concentration of HCAs. Color photos showed 6 dishes, each fried at 4 different temperatures, giving varying degrees of surface browning. Each photo corresponds to a known frying temperature.

To assess concentrations of HCAs, 22 dishes with pan residues were cooked and analyzed, 15 of them being fried meat dishes, 3 baked/roasted meat dishes, 2 fried fish dishes, 1 fried eggs, and 1 blood pudding. Nineteen dishes were fried in a standardized manner at 150, 175, 200 and 225°C, and 3 were roasted/baked in a normal household oven at 150 and 200°C. Chemical analyses and the obtained concentrations of HCAs (2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (DiMeIQx), and 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine (PhIP)) in the cooked foods, have been reported in detail elsewhere [136-138]. The classification of surface browning and concentration of HCAs for the remaining fried dishes in the questionnaire were linked to the 6 photos and the 22 analyzed dishes, respectively. The intake of HCAs from pan residue and gravy was calculated using a method previously described, which gives the concentration in one individually calculated standard serving [134].

The reduced dietary assessment methods

In order to reduce the set of questions on intake frequency of fried/baked/grilled dishes, we computed the mean contribution of each dish to the total intake of HCAs among controls. After this, sets of informative dishes were generated using three alternative principles. First, all meat and fish dishes were ordered according to their contribution to the total mean daily intake of heterocyclic amines [139]. This ranking was done for the meat/fish dishes and the corresponding gravy separately and combined. Secondly, the dishes were ordered according to their contribution to the between-person variance, using stepwise regression [133]. Thirdly, groupings of dishes of special interest were formed, i.e. dishes used in another Swedish study (the 6 photographed dishes) and in Dutch studies (a set of 18 dishes that approximate habitual meat dishes in The Netherlands, and

are part of a 22-dish questionnaire used in a case control study on dietary factors and colorectal adenomas).

Data analysis

To explore misclassification by the reduced sets of questions, controls and cases were categorized into quintiles, based on the distribution of the intake of HCAs in controls. The ability of the reduced sets of questions to categorize subjects into the same quintile as the reference method was estimated in terms of sensitivity and specificity. Sensitivity is defined as the probability of a positive test result (e.g. quintile 1 according to the reduced method), provided the exposure is truly present (i.e. quintile 1 according to the reference method). Analogously, specificity is the proportion of truly non exposed subjects (quintiles 2 to 5 according to reference method), who are correctly categorized as non exposed by the test (same quintiles according to reduced method). Furthermore, Pearson correlation coefficients for quintiles were calculated, as well as Kappa, a measure of concordance in contingency tables, taking into account agreement on the basis of chance alone [133]. In order to explore the effect of misclassification on the range of relative risks that may be anticipated in epidemiological studies, relative risks of magnitude RR=2 and RR=3 were simulated. Using the reference method, the exposure to HCAs was categorized into quintiles, based on the distribution among controls, resulting in equal numbers of controls in each category of intake. The case distribution over the quintiles was selected to simulate "true" relative risks of 2.0 and 3.0 for the highest versus the lowest quintile of intake. These selected cases, and all controls, were subsequently categorized into new quintiles, based on the distribution of the intake of HCAs, using the reduced methods. Logistic regression was used to model the odds ratio [98], estimating the incidence rate ratio (relative risk) between the exposed and unexposed in the study population.

RESULTS

Table 4.1 shows the mean amount of heterocyclic amines (IQ, MeIQ, MeIQx, DiMeIQx and PhIP), contributed by each dish to the total mean intake among controls. Considering the contribution stemming from meat and gravy combined, the 10 dishes contributing the most were fried bacon, ground beef patties, pork filet, chicken/turkey, meat balls, pork belly, loin of pork, entrecote and minced meat sauce. These dishes contributed 77 percent of the total intake of HCAs among controls, as obtained using the extensive method. The top 15 and 20 dishes contributed 88 and 94 percent of the total intake, respectively. The remaining 6 percent of the intake was generated by an additional 20 dishes. The total mean intake of HCAs from all 39 meat and fish dishes was 157 ng per day. MeIQx and PhIP each comprised of 45%, DiMeIQx 10% and IQ and MeIQ both less than 1% of the total intake of HCAs (data not shown).

Table 4.1. *Contribution of meat and fish dishes and gravy to the total intake of heterocyclic amines in an elderly population in Stockholm.*

		meat intake		gravy intake		total intake		
		ng	%meat	ng	%gravy	ng	%tot	cum%
1	bacon, fried	26.1	24.6	0.5	1.0	26.6	16.9	16.9
2	minced beef patties, fried	6.8	6.5	10.4	20.4	17.3	11.0	27.9
3	pork fillet, fried	5.3	5.0	7.1	13.9	12.4	7.9	35.8
4	chicken/turkey, fried	11.1	10.5	0.5	1.0	11.6	7.4	43.2
5	meat balls, fried	8.8	8.3	2.3	4.5	11.1	7.1	50.3
6	pork belly, fried	8.9	8.3	1.5	2.9	10.3	6.6	56.8
7	loin of pork, fried	4.1	3.9	6.0	11.8	10.1	6.4	63.3
8	pork chops, fried	4.9	4.6	5.2	10.2	10.1	6.4	69.7
9	entrecote, fried	3.9	3.7	3.1	6.1	7.0	4.4	74.1
10	minced meat sauce	5.3	5.0	0.0	0.0	5.3	3.3	77.5
11	beef steak, fried	2.9	2.7	2.2	4.3	5.1	3.2	80.7
12	beef fillet, fried	2.1	1.9	1.6	3.1	3.6	2.3	83.0
13	smoked pork, fried	1.5	1.4	1.3	2.5	2.7	1.7	84.8
14	stew, browned	1.1	1.0	1.6	3.1	2.7	1.7	86.5
15	fish, fried (breaded)	2.2	2.0	0.2	0.4	2.4	1.5	88.0
16	lamb, fried	1.8	1.7	0.3	0.6	2.1	1.3	89.4
17	minute beef, fried	0.9	0.8	1.1	2.2	1.9	1.2	90.6
18	game, fried	1.2	1.1	0.6	1.2	1.8	1.1	91.8
19	beef, grilled	1.2	1.1	0.6	1.2	1.8	1.1	92.9
20	ham, fried	0.7	0.7	0.9	1.8	1.6	1.0	93.9
21	pork stew, browned	0.6	0.5	0.9	1.7	1.4	0.9	94.8
22	pork, grilled	0.7	0.7	0.5	1.0	1.2	0.8	95.6
23	minced meat dish, grilled	0.6	0.5	0.4	0.8	1.0	0.6	96.3
24	meat loaf, roasted	0.9	0.9	0.0	0.1	1.0	0.6	96.9
25	black pudding, fried	0.8	0.8	0.0	0.0	0.8	0.5	97.4
26	beef patties, roasted	0.3	0.3	0.5	1.0	0.8	0.5	97.9
27	meat balls, roasted	0.6	0.5	0.1	0.2	0.7	0.4	98.4
28	'falu'sausage, fried	0.0	0.0	0.5	1.0	0.5	0.3	98.7
29	fish sticks, fried	0.4	0.4	0.1	0.1	0.5	0.3	99.0
30	chicken/turkey, roasted	0.1	0.1	0.4	0.8	0.5	0.3	99.3
31	'wiener'sausage, fried	0.0	0.0	0.2	0.4	0.2	0.1	99.5
32	'chipolata'sausage, fried	0.2	0.2	0.0	0.0	0.2	0.1	99.6
33	smoked sausage, fried	0.0	0.0	0.2	0.3	0.2	0.1	99.7
34	fish, fried (not breaded)	0.2	0.2	0.0	0.0	0.2	0.1	99.8
35	'stång'sausage, fried	0.0	0.0	0.1	0.2	0.1	0.1	99.9
36	sausage, grilled	0.0	0.0	0.0	0.1	0.1	0.0	100.0
37	sausage, fried	0.0	0.0	0.1	0.1	0.1	0.0	100.0
38	'falu'sausage, roasted	0.0	0.0	0.0	0.0	0.0	0.0	100.0
39	'wiener'sausage, roasted	0.0	0.0	0.0	0.0	0.0	0.0	100.0
	total	106.2		50.9		157.1		

In this population gravy is very often prepared from pan-residues and eaten with most meat dishes. Therefore, gravy contributes substantially to the total intake of HCAs. The dishes contributing the most were ground beef patties, pork filet, loin of pork, pork chops, entrecote, meat stew, beef steak, and, finally, pork belly.

When ranking the top 10 dishes according to the explained percentage of the between-person variance in the total intake (Table 4.2), 9 out of 10 were the same dishes as when ranking according to the total intake, but the dishes appeared in an alternative order. Considering the contribution from both meat and gravy, the 10 dishes contributing the most, in descending order, were fried pork chop, bacon, ground beef patties, entrecote, loin of pork, pork belly, pork filet, chicken, breaded fish, and minced meat sauce. These dishes accounted for 98 percent of the variance and 72 percent of the total intake of HCAs. A daily mean intake of HCAs based on these dishes would be 113 ng. The cumulative R^2 (percentage of the between-person variance) and the percentage of the daily intake of HCAs accounted for by varying numbers of dishes, as selected by these two principles, is shown in Figure 4.1.

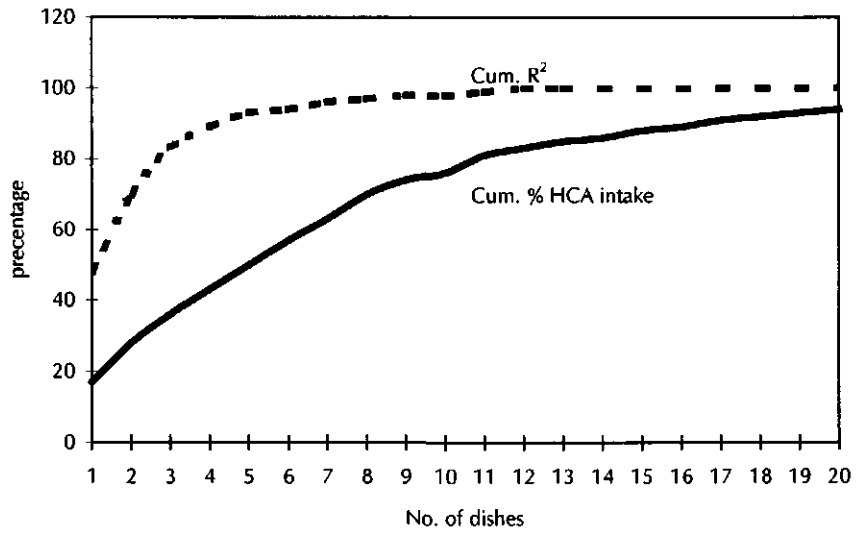

Figure 4.1. *Cumulative R^2 and percentage of heterocyclic amines accounted for according to number of meat dishes included, in controls.*

Table 4.2. *Ranking of most informative dishes according to between-person variance in intake of heterocyclic amines using stepwise regression analysis.*

no.	meat dish	cumulative R^2	correlation coefficient*	cum% total intake
1	pork chops, fried	0.48	0.70	6.4
2	bacon, fried	0.70	0.63	23.3
3	minced beef patties, fried	0.83	0.60	34.3
4	entrecote, fried	0.89	0.35	38.7
5	loin of pork, fried	0.93	0.44	45.1
6	pork belly, fried	0.94	0.56	51.7
7	pork fillet, fried	0.96	0.60	59.6
8	chicken, fried	0.97	0.22	67.0
9	fish, fried (breaded)	0.98	0.22	68.5
10	minced meat sauce	0.98	0.39	71.8

* simple Pearson coefficient of correlation for each dish to the total daily intake

Sensitivity and specificity for different reduced sets of questions compared to the reference method are shown in Table 4.3. For sets of questions including 20 and 15 dishes, sensitivity and specificity were equally good, regardless of the selection principles. A reduced questionnaire including 10 dishes performed less well, but the sensitivity was somewhat better for dishes selected according to their contribution to the total intake than for dishes selected according to variance. When only 5 dishes were used, the reduced methods performed even worse, and again, sensitivity was better for dishes selected according to contribution to the total intake than for dishes selected according to the amount of between-person variance explained. Concordance was measured as the ability of a reduced set of questions to categorize each person into the same quintile as the reference method. The concordance increased rapidly from 5 to 15 dishes, however, only small improvements were seen for a total of 20 dishes. Dishes selected according to the total intake generally performed better than dishes chosen by variance. The Pearson correlation coefficient increased with an increasing number of dishes, but performed equally well in the two methods of selecting dishes. Kappa, a measure of concordance accounting for chance, increased rapidly with the number of dishes, but dishes selected according to the total intake gave higher values. The sensitivity and specificity of the "Dutch module" including 18 dishes, were higher than 0.85 in all quintiles. More than 90 percent of the subjects were classified into the same quintile as when using the reference method, 9 percent were classified one quintile higher or lower than in the reference method, and none were classified more than one quintile higher or lower. The correlation between the "Dutch module" and the reference method was also high. The short Swedish method comprising 6 dishes performed less well.

Table 4.3. *Performance of truncated methods compared to the reference method.*

Truncated methods	No. of dishes	Sensitivity (%)					Specificity (%)					Concordance*				
		Q1	Q2	Q3	Q4	Q5	Q1	Q2	Q3	Q4	Q5	% same	% 1Q off	% >1Q off	r†	K¶
total intake																
	5	82	67	68	65	86	97	91	89	94	96	74	26	1	0.92	0.67
	10	88	85	78	85	93	99	94	95	96	98	86	14	0	0.96	0.82
	15	90	90	88	92	96	99	96	97	98	99	91	8	0	0.98	0.89
	20	94	93	95	95	98	100	98	98	99	100	95	5	0	0.99	0.94
between-person variance																
	5	72	51	59	60	85	95	88	86	92	96	65	33	2	0.89	0.57
	10	85	76	70	80	93	98	93	93	94	98	81	19	0	0.94	0.76
	15	88	88	88	91	95	99	95	96	97	99	90	10	0	0.98	0.87
	20	92	91	91	96	97	99	97	98	98	100	93	7	0	0.98	0.92
Swedish	6	74	49	50	58	79	96	88	85	88	95	62	34	3	0.87	0.52
Dutch	18	90	90	86	90	97	99	96	97	98	99	90	9	0	0.97	0.88

* the percentage of subjects categorized on the basis of a reduced method, in the same quintile, one quintile higher or lower, and more than one quintile away from the same quintile

† Pearsons coefficient of correlation for categorization into quintiles

¶ Kappa is a measure of concordance taking chance into account $(p^o-p^e)/(1-p^e)$

Table 4.4. *The effect of misclassification, for reduced sets of questions, on relative risk estimates for each quintile (Q) of exposure.*

methods	dishes	Q1	Q2	Q3	Q4	Q5	Q1	Q2	Q3	Q4	Q5
simulated "true" RR											
	39	1.00	1.24	1.50	1.72	2.00	1.00	1.50	2.02	2.48	3.03
total intake											
	5	1.00	1.41	1.85	1.62	2.15	1.00	1.49	2.35	2.22	3.02
	10	1.00	1.57	1.64	1.96	2.22	1.00	1.84	2.12	2.83	3.26
	15	1.00	1.49	1.74	2.00	2.18	1.00	1.72	2.30	2.87	3.25
	20	1.00	1.30	1.68	1.83	2.06	1.00	1.47	2.19	2.56	3.02
between-person variance											
	5	1.00	1.34	2.11	1.67	2.20	1.00	1.52	2.68	2.25	3.14
	10	1.00	1.40	1.56	1.84	2.14	1.00	1.56	1.99	2.43	3.04
	15	1.00	1.52	1.62	1.93	2.13	1.00	1.83	2.12	2.74	3.18
	20	1.00	1.32	1.50	1.95	2.00	1.00	1.55	1.97	2.75	2.97
Swedish method											
	6	1.00	1.45	2.08	1.95	2.33	1.00	1.61	2.70	2.61	3.33
Dutch method											
	18	1.00	1.50	1.47	1.89	2.18	1.00	1.81	1.95	2.72	3.29

The effect of decreased sensitivity and specificity on the estimated relative risks is shown in Table 4.4. Only small effects on the relative risks were seen for the reduced methods comprising 10, 15 or 20 dishes, regardless of the selection criteria. Relative risks based on truncated modules comprising only 5 dishes deviated markedly from the simulated "true" relative risks, especially for the 5 dishes selected according to the between-person variance.

Discussion

We studied the loss of information when reducing an extensive food frequency questionnaire designed to estimate human intake of HCAs, as part of a population-based study of cancer risk. The findings of this study indicate that the loss of information is limited when the number of enumerated dishes was reduced from 39 to 15 or 20. Results were similar for the two principles of selection, when using 15 or 20 dishes. Using 5 or 10 dishes, estimates of intake based on dishes selected according to the total intake of HCAs were more accurate than those based on dishes selected according to the between-person variance. The reference method in this study was an extensive semi-quantitative food frequency questionnaire, designed to estimate human cancer risks as related to HCAs in a Swedish population [134]. The selection of dishes was based on previous studies of frequently eaten fried foods in an elderly population in Stockholm. The total intake of HCAs was estimated using questionnaire-data on the cooking method, frying temperature, portion size, intake frequency, intake of gravy, and data on the

concentration of HCAs in various dishes from chemical analyses. The extensive food frequency questionnaire was used as a reference method, but cannot be considered as a "gold standard". Validation of HCA intake by identifying DNA adducts [140-143], excretion of HCAs in urine [144], or collecting double portions of food for chemical analysis [133] may be possible. However, these approaches have their own methodological and analytical limitations. To compose a database of HCA content in cooked meat, 22 dishes and corresponding gravies were cooked in a standardized manner at different temperatures, resulting in 152 samples. Subsequently, these were chemically analyzed for the five heterocyclic amines, IQ, MeIQ, MeIQx, DiMeIQx and PhIP. If the chemical analysis of these samples suffered from systematic errors, this would result in a systematic shift in the magnitude of the estimated HCA intake in both cases and controls. However, random analytical errors may result in nondifferential misclassification, diluting the association and therefore increasing the chance of missing a possible real carcinogenic effect of HCAs (ß-error).

The 22 analyzed dishes, of a total of 39 dishes in the questionnaire, account for 79 percent of the total intake of HCAs among controls, as estimated when using the reference method. The remaining 17 dishes were not cooked and analyzed, but data on HCA content were taken from similar dishes, which may have introduced some additional measurement errors. Considering the large number of dishes cooked and analyzed, the magnitude of these errors is probably relatively small.

As shown by others [139;145] using the percentage contribution to the total intake, or the percentage explained of the between-person variance derived by stepwise regression, are legitimate methods to reduce the number of items in a questionnaire. Of course, subjects may answer differently to *n* items in an extensive 188-item questionnaire, than to a short questionnaire including the *n* items only. It is difficult to argue which of those approaches would lead to the most valid estimate.

In this study the reduced methods biased some of the 'true' relative risks upward, instead of towards the null. This may be caused by the fact that a continuous variable is categorized into five categories, which is known to sometimes result in bias away from the null [146;147]. Furthermore, the selection of dishes is based on the controls, which may lead to some bias if patterns of consumption of meat dishes are substantially different in cases.

When studying the effect of exposure to HCAs on cancer risk, one should study individual HCAs, because they occur in varying concentrations and may have different mutagenic and carcinogenic effects. In this study we summed the intake of five HCAs to estimate total intake. When a specific HCA is the relevant exposure in studies of cancer risk, an estimation of the total intake of HCAs may be a suboptimal surrogate variable,

especially if the amine occurs in relatively low concentrations compared to other HCAs. Indeed, intake of individual HCAs may be better estimated by different sets of dishes, as was recently shown by Byrne *et al.* [148].

In conclusion, a limited amount of misclassification was introduced when the number of fried/baked/grilled meat and fish dishes was reduced. This may imply that food frequency questionnaires focused on intake of HCAs can be relatively short. One reason to shorten a questionnaire is to increase the participation rate, which may improve the accuracy of the findings more than the decrease introduced by the additional measurement error.

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MEAT CONSUMPTION AND PREPARATION IS RELATED TO SPORADIC BUT NOT TO HEREDITARY COLORECTAL ADENOMAS

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CHAPTER 5 MEAT CONSUMPTION AND PREPARATION IS RELATED TO SPORADIC BUT NOT TO HEREDITARY COLORECTAL ADENOMAS

Meat consumption and preparation are thought to be associated with the risk of colorectal cancer, and possibly also to adenomas. It remains to be elucidated whether environmental factors also play a role in hereditary non-polyposis colorectal cancer (HNPCC). We examined whether meat consumption and preparation are similarly associated with sporadic and HNPCC colorectal adenomas.

A case control study was conducted among 57 sporadic and 62 HNPCC adenoma cases. Controls for both groups (65 sporadic and 83 HNPCC controls) also underwent full colonic examination to exclude adenomas. To assess meat consumption and preparation we used a questionnaire designed especially for this purpose.

Frequency of red meat consumption was positively, although not significantly, associated with risk, only in the sporadic group (OR daily vs < 4x /wk, 95% CI: sporadic 4.1, 0.7-23.0; HNPCC 0.4, 0.1-2.2). Interestingly, preferred color of the meat surface was significantly associated with risk, independent of red meat intake, but again only in the sporadic group (OR dark vs light, 95% CI: sporadic 3.0, 1.2-7.5; HNPCC 0.7, 0.3-1.4).

Despite limited numbers, our results suggest that meat consumption and preparation may only be relevant in sporadic colorectal adenomas.

INTRODUCTION

Colorectal cancer is the fourth most common incident cancer throughout the world [1]. It is thought to develop through an adenoma-carcinoma sequence, by accumulation of alterations in genes that control cell growth and differentiation [7]. This accumulation of mutations and the progression to cancer is thought to be promoted by diets high in fat and low in fibre [149].

Several cohort studies [36;150] and case-control studies [68;70;72;74] have suggested that a high intake of red meat may be associated with the risk of colorectal cancer independent of the fat content of the diet. Some studies have found this increased risk to be specifically associated with fried meat [72], high temperature cooking of meat [58], meat "well done" [81] or meat with heavily browned surface [58]. An increased risk of colorectal adenomas with high consumption of red meat was also found in some studies [57;64;151], but not in others [44;49]. Probst-Hensch *et al.* reported an increased risk of colorectal adenomas (OR=2.2, 95%CI=1.1-4.3) with consumption of red meat more than once a week, of which more

than 10% was fried, and a preference for a darkly browned surface, as compared to consumption of meat less than once a week, of which less than 10% was fried, and a preference for a lightly browned surface [82].
During the preparation of meat at high temperatures, heterocyclic amines may be formed from amino acids, creatine/creatinine and sugars [152]. Heterocyclic amines have been found to be mutagenic and cause tumors in the colon and various other organs in animal models.

Apart from environmental factors, hereditary factors are known to be important in the etiology of colorectal adenomas and carcinomas. Two genetic syndromes leading to colorectal cancer develop by an autosomal dominant inheritance pattern. Familial Adenomatous Polyposis is caused by a germline mutation in the Adenomatous Polyposis Coli (APC) gene, and is characterized by the development of many (>100) colorectal adenomas at an early age [153]. The second hereditary form of colorectal cancer is Hereditary Non-Polyposis Colorectal Cancer (HNPCC), which is caused by a germline mutation in one of at least five mismatch repair genes, resulting in microsatellite instability (MSI) [18]. HNPCC patients develop colorectal adenomas in a similar fashion as sporadic patients, however, one of the characteristic features of HNPCC is the very rapid progression from adenoma to cancer, due to a defect in the mismatch repair system. Besides colorectal cancer HNPCC subjects have a higher risk of developing malignancies at several other sites [24]. Interestingly, the tumor spectrum in HNPCC has changed over generations [26], suggesting a role for environmental factors in HNPCC carcinogenesis. Other characteristics of HNPCC (e.g. incomplete penetrance [22], earlier onset of disease in successive generations [23;27], somatic mutations in APC, K-ras and p53 genes [12;28;29]), point in a similar direction. Possibly, similar environmental factors to those in sporadic colorectal carcinogenesis, play a role especially in the early stages of colorectal carcinogenesis in HNPCC.

This case-control study of sporadic and HNPCC colorectal adenomas examines whether meat consumption and preparation are related to adenoma risk in these two types of populations in a similar fashion.

MATERIALS AND METHODS

Study population and data collection

This case-control study on dietary factors and genetic susceptibility in colorectal adenomas was conducted in The Netherlands between December 1995 and February 1998, and approved of by the medical ethical committies of the Wageningen Agricultural

University and the Nijmegen University Hospital. The study population included sporadic as well as HNPCC cases with adenomas removed by polypectomy, and control-groups for each case group. Sporadic controls were patients examined at the hospital for various reasons such as bowel complaints (abdominal pain, rectal blood loss, constipation, diarrhoea) and HNPCC controls for screening purposes. All controls had a full colonic examination (colonoscopy or sigmoidoscopy combined with barium enema) to exclude presence of adenomas, and were not previously diagnosed with colorectal adenomas or cancer.

All participants were Dutch-speaking and of Western European origin, diagnosed before the age of 75, without a history of colorectal cancer, colon resection, polyposis coli, or inflammatory bowel disease. Sporadic cases were excluded if they had previous adenomas more than 3 years before entrance to the study, because of possible changes in dietary habits with a long history of repeated endoscopies and colorectal adenomas. The possible dietary effects of regular screening are expected to be similar in HNPCC cases and controls. Based on this assumption and for reasons of efficiency HNPCC cases were allowed to have had adenomas more than 3 years prior to the most recent adenoma. The pedigrees of all HNPCC cases and controls fulfilled the Amsterdam criteria [72]: at least three members in at least two successive generations had colorectal cancer, with at least one case diagnosed before the age of 50 years; one of the affected members was a first-degree relative of the other two; familial adenomatous polyposis was ruled out. HNPCC cases and controls were first-degree relatives of colorectal or endometrium cancer cases, all with a theoretical 50% risk of being a gene-carrier.

Sporadic cases and controls were recruited by the Departments of Gastroenterology of the Nijmegen University Hospital and two regional hospitals (Rivierenland, Tiel; Gelderse Vallei, Ede-Wageningen-Bennekom). HNPCC cases and controls were recruited in a similar fashion, either by the Nijmegen University Hospital or by the Netherlands Foundation for the Detection of Hereditary Tumors which keeps a registry of HNPCC families in The Netherlands. Eligible subjects were informed about the study by the gastroenterologist participating in the study or by the Netherlands Foundation for the Detection of Hereditary Tumors, after approval of their 'physician in attendance'.

After receiving information about the study, subjects decided whether they wanted to participate in the study and subsequently signed for informed consent. Nine percent of subjects invited by the Nijmegen University Hospital, and 12% of subjects invited by the Netherlands Foundation for the Detection of Hereditary Tumors, were not willing to participate in the study. In total, 319 eligible subjects signed for informed consent. Subsequently, those who were willing to participate received a set of questionnaires at endoscopy (in case of recruitment by one of the hospitals) or by mail (in case of

recruitment by the Foundation for the Detection of Hereditary Tumors). The set of questionnaires consisted of a general questionnaire on lifestyle and socio-economic factors, a semi-quantitative food frequency questionnaire and a questionnaire on meat consumption and preparation. The questionnaires were filled out at home and returned by mail.
Clinical information regarding case/control status, endoscopy reports, adenoma characteristics, and HNPCC/sporadic status was retrieved from hospital records retrospectively, after we received the filled-out questionnaires. Of the 319 patients who initially agreed to participate in the study 22 (7%) did not return the questionnaires, and 21 (6%) did not meet the eligibility criteria retrospectively. Additionally, 9 participants incompletely filled out the meat consumption and preparation questionnaire and were therefore excluded from the current analyses. This resulted in 119 cases and 148 controls available for further study.

Dietary assessment
To quantify energy and nutrient intake, a validated semi-quantitiative food frequency questionnaire was used, which was developed for the Dutch cohort of the EPIC study (European Prospective Investigation into Cancer and Nutrition) [154;155]. Frequency of consumption of food groups was based on the habitual consumption of 178 food items during the year before endoscopy. Nutrient intake was quantified for each individual using an extended version of the 1993 computerised Dutch food composition table. In the present study this EPIC questionnaire was used for information on nutrient intake and frequency of meat consumption.

To obtain additional information on the consumption and preparation of red meat and poultry, a questionnaire was developed especially for this purpose. The questionnaire consists of four parts, starting with some general questions regarding frequency of eating a hot meal, meat with the hot meal, grilled or barbecued meat, and gravy, as well as a question on preference with respect to color of the meat surface. The second part focuses on frequency and portion size of 16 types of meat. Intensity of surface browning of four types of meat (beef patties, pork chops, steak and bacon) was assessed using four photos for each type of meat showing the meat with varying degrees of surface browning (from very dark to very light, prepared at 225, 200, 175 and 150°C, respectively). The photos originate from a Swedish questionnaire used in a case control study on intake of heterocyclic amines in relation to cancer risk [134]. In the last part of our questionnaire, preparation methods for each of six meat categories were assessed using multiple choice questions on factors relevant to the temperature during meat preparation: type of pan used, temperature of cooking fat when meat is added, height of the heat source, addition

of water, use of a lid, color of surface just after browning compared to the end stage of cooking.

Data analysis

Differences in general characteristics between groups were tested using χ^2-tests for categorical and t-tests for continuous variables. All analyses were stratified for HNPCC and sporadic groups. Multivariate logistic regression analyses were used to estimate the relative risk for adenomas and simultaneously adjust for possible confounding factors. Nutrients were adjusted for energy intake by using residuals from linear regression modelling of energy intake.

Odds ratios for nutrients were calculated for tertiles based on the distribution in controls, as well as using the continuous variable and expressing the odds ratio for quartile ranges. Associations with total meat, red meat, white meat (including fish), and gravy were assessed for frequency categories as well as using a test for trend. To group types of meat according to the general method of preparation, the 16 types of meat in the frequency-part of the questionnaire were categorized in 3 groups: 'low risk cooking types' (i.e. simmered meat), 'medium risk cooking types' (i.e. meat shortly browned then cooked), and 'high risk cooking types' (i.e. meat fried at high temperatures). To take habitual meat preparation practices into account, subjects were categorized based on multiple choice questions (for 6 types of meat) regarding height of the heat source, use of a lid, and addition of water. Usual color of the meat surface after preparation was assessed using an average score for the four photo-questions. The minimum score of one means the person scored the lightest surface color in each of the four photo-questions, whereas the maximum score of four means the person scored the darkest surface color in each of the four photo-questions. If people did not fill out all four photo-questions on meat surface, because some types of meat were not eaten, the average score was based on the mean of the remaining photo-questions.

To control for potential confounding by energy intake, all nutrients have been adjusted for energy using the residuals from linear regression of energy intake, and additionally, both nutrients and food groups are adjusted for total energy intake by including total energy intake in the logistic regression models. As the age and gender distribution differs between cases and controls and between both populations, all analyses are adjusted for age and gender. As the cases in this study may have a generally 'less healthy' pattern of other lifestyle related risk factors (i.e. higher BMI, less never-smokers, less physically active, more regular alcohol drinkers and less regular aspirin users), these factors could act as possible confounders, and were each separately taken into account by including them in the logistic regression models (in combination with energy, age, gender). None of the

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for total meat and red meat separately, but this did not markedly change the odds ratios (data not shown).

Table 5.2. *"Meat related" nutrient intakes and the risk of colorectal adenomas in sporadic and HNPCC groups separately.*

	Sporadic				HNPCC			
	Controls		(n= 65, 57)		Controls		(n= 83, 62)	
Nutrient intakes*	/Cases		OR	(95% CI)†	/Cases		OR	(95% CI)†
Total protein								
Low < 76 g	17	9	1.0		31	18	1.0	
Medium 76-91 g	27	23	1.3	(0.4-3.8)	24	24	0.9	(0.4-2.4)
High ≥ 91 g	21	25	1.2	(0.3-4.1)	28	20	0.5	(0.2-1.6)
Continuous¶ / 18 g			0.9	(0.5-1.6)			0.7	(0.4-1.2)
Animal protein								
Low < 45 g	18	9	1.0		30	15	1.0	
Medium 45-59 g	19	31	4.0	(1.3-12.1)	32	31	1.3	(0.5-3.3)
High ≥ 59 g	28	17	0.5	(0.2-1.6)	21	16	1.0	(0.4-2.9)
Continuous¶ / 19 g			0.7	(0.4-1.2)			0.9	(0.5-1.5)
Total fat								
Low < 83 g	20	18	1.0		28	15	1.0	
Medium 83-97 g	24	16	0.5	(0.2-1.3)	27	20	1.3	(0.5-3.3)
High ≥ 97 g	21	23	0.4	(0.1-1.6)	28	27	1.4	(0.4-4.4)
Continuous¶ / 22 g			0.7	(0.4-1.5)			1.3	(0.7-2.4)
Saturated fatty acids								
Low < 32 g	21	19	1.0		27	18	1.0	
Medium 32-38 g	22	13	0.4	(0.1-1.2)	29	23	1.0	(0.4-2.4)
High ≥ 38 g	22	25	0.4	(0.1-1.4)	27	21	0.8	(0.2-2.4)
Continuous¶ / 10 g			0.8	(0.4-1.5)			1.0	(0.5-1.8)
N-3 long chain fish fatty acids								
Low < 0.07 g	17	22	1.0		31	19	1.0	
Medium 0.07-0.19 g	24	16	0.4	(0.1-1.0)	27	22	1.2	(0.5-2.9)
High ≥ 0.19 g	24	19	0.4	(0.2-1.1)	25	21	1.1	(0.4-2.6)
Continuous¶ / 0.14 g			0.9	(0.6-1.4)			0.8	(0.6-1.2)

* nutrient intakes are energy adjusted using residual regression analysis, tertile cut-off points are based on the distribution in the "pooled" control group

† all models are adjusted for age and gender as well as energy intake

¶ the odds ratio for the continuous variables of nutrient intakes are expressed for an exposure contrast representing the difference between the first and third quartile

Table 5.3. *Meat consumption frequency and the risk of colorectal adenomas in the sporadic and HNPCC group.*

		Sporadic				HNPCC			
		n=65 Controls	n=57 Cases	OR	(95% CI)*	n=83 Controls	n=62 Cases	OR	(95% CI)*
Consumption frequency									
Total meat (excl. fish)									
Low	≤ 4x /wk	8	9	1.0		23	18	1.0	
Medium	5-6x /wk	36	24	0.6	(0.2-2.1)	34	26	0.9	(0.4-2.2)
High	7x /wk	21	24	1.0	(0.3-3.6)	26	18	0.6	(0.2-1.6)
Trend	p-value			p=0.63				p=0.33	
Red meat									
Low	≤ 4x /wk	19	14	1.0		29	22	1.0	
Medium	5-6x /wk	38	29	1.1	(0.3-3.9)	38	33	1.1	(0.3-3.7)
High	7x /wk	8	14	4.1	(0.7-23.0)	16	7	0.4	(0.1-2.2)
Trend	p-value			p=0.08				p=0.26	
White meat (incl. fish)									
Low	≤ 0.5x /wk	4	9	1.0		15	10	1.0	
Medium	0.5-2x /wk	45	33	0.2	(0.0-0.9)	48	37	1.2	(0.4-3.6)
High	>2x /wk	16	15	0.3	(0.1-1.4)	20	15	1.3	(0.4-4.3)
Trend	p-value			p=0.36				p=0.68	
Gravy									
Low	≤ 2x /wk	19	18	1.0		36	22	1.0	
Medium	≤ 5x /wk	25	17	0.5	(0.2-1.4)	35	23	1.1	(0.5-2.5)
High	> 5x /wk	21	22	0.6	(0.2-1.9)	12	17	1.9	(0.7-5.4)
Trend	p-value			p=0.38				p=0.26	

* all models are adjusted for age, gender, energy intake and total meat consumption.

For each of the 3 variables representing "habitual meat preparation", every possible answer was scored as either 'high risk method' or 'low risk method', according to its assumed effect on heterocyclic amine formation. Subjects were then categorized based on the frequency with which they usually prepare their meat, i.e. always using a 'high risk method' or a 'low risk method', or using both methods depending on the type of meat (intermediate category). None of the 3 variables regarding preparation methods were significantly associated with risk of adenomas in either group.

Table 5.4. *Meat preparation and the risk of colorectal adenomas in the sporadic and HNPCC group.*

		Sporadic				HNPCC			
		Controls /Cases		OR	(95% CI)*	Controls /Cases		OR	(95% CI)*
Meat categorized by usual mode of preparation									
Frequency 'low risk meat' (simmered)									
Low	<1x /wk	53	46	1.0		68	53	1.0	
High	≥1x /wk	12	11	1.1	(0.4-2.9)	15	9	0.5	(0.2-1.4)
Frequency 'medium risk meat' (shortly browned then cooked)									
Low	<4x /wk	49	36	1.0		60	43	1.0	
High	≥4x /wk	16	21	2.4	(0.9-6.3)	23	19	1.6	(0.6-3.8)
Frequency 'high risk meat' (fried at high temperatures)									
Low	<2x /wk	46	39	1.0		66	49	1.0	
High	≥2x /wk	19	18	0.8	(0.3-2.0)	17	13	0.6	(0.2-1.5)
Habitual meat preparation†									
Height of the heat source during browning of meat									
low with every meat type		32	31	1.0		32	24	1.0	
low/high depending on meat type		14	13	0.8	(0.3-2.1)	29	19	0.8	(0.3-1.8)
high with every meat type		17	13	0.9	(0.3-2.3)	17	16	1.0	(0.4-2.7)
Addition of water									
added directly after browning		16	9	1.0		11	12	1.0	
added directly/afterwards		32	32	1.9	(0.7-5.2)	52	28	0.5	(0.2-1.4)
added afterwards		16	16	2.0	(0.6-6.5)	15	20	1.2	(0.4-3.8)
Use of a lid									
closed lid with every meat type		10	16	1.0		15	8	1.0	
lid/no lid depending on meat type		18	10	0.3	(0.1-1.0)	24	13	0.8	(0.2-2.6)
no lid with every meat type		35	31	0.4	(0.1-1.1)	39	39	1.5	(0.5-4.6)
Categories by preferred color of the meat surface									
Color meat surface¶									
Light	score ≤ 2	27	10	1.0		32	29	1.0	
Dark	score > 2	38	47	3.0	(1.2-7.5)	51	33	0.7	(0.3-1.4)

* models are adjusted for age, gender, energy intake and total meat consumption.

† categories ordered according to increasing risk

¶ 1= very light, 4=very dark

Preferred color of the meat surface, as assessed using photos, was significantly associated with increased risk of colorectal adenomas, only in the sporadic group. Those with an average score of more than two (1= very light, 4= very dark), had a three-fold increase in risk as compared to those with an average score of two or lower (OR, 95% CI: 3.0, 1.2-7.5). This odds ratio did not markedly change when adjusting the analysis separately for total meat, red meat, or 'medium risk' type of meat.

Discussion

In this study of colorectal adenomas a general preference for darkly browned meat surface appeared to be associated with an increased risk of sporadic adenomas, but not of adenomas among first degree HNPCC family members. Apart from this association and a non-significant increase in risk with high consumption of red meat (≥7x/week vs ≤ 4x/week) in the sporadic group, no other factors related to meat consumption and preparation were found to be associated with the risk of sporadic and HNPCC adenomas.

The size of our study is relatively small, especially since strata of the sporadic and HNPCC groups were analyzed separately. However, this is one of the first studies to examine possible differences or similarities in risk factors for sporadic and HNPCC colorectal carcinogenesis. For known risk factors, such as Body Mass Index, smoking, alcohol consumption and aspirin use, we found non-significant case-control differences in the expected direction. Our results suggests that red meat consumption and especially darkly browned meat may be associated to sporadic adenoma risk, but not to HNPCC adenoma risk. This does not rule out that other risk factors may be similarly associated to sporadic and HNPCC adenoma formation or other phases of colorectal carcinogenesis.

Selection and recruitment of study subjects and methods of data collection may have affected our results. In sporadic cases, the adenomas were diagnosed during the study period, and cases were excluded if they had earlier adenomas more than 3 years prior to entrance of the study. HNPCC cases however, were allowed to have had adenomas previously and may have been diagnosed before the study period. Controls are defined in both populations as subjects who have never had any adenomas. Selection of controls was very similar to that of cases in both groups and both recruitment procedures. As HNPCC cases were allowed to have a longer adenoma history than sporadic cases, they may have changed their diet more. However, as HNPCC family members are generally considered to be relatively health conscious, irrespective of their adenoma status, this is not expected to influence the results to a large extend. There were no marked differences between the sporadic and HNPCC populations in several lifestyle characteristics, and energy and macro nutrient intakes. Therefore, we do not expect that any small differences will have biased the results to a relevant extent.
The population recruited directly by the gastroenterologist in the hospital (both sporadic and HNPCC subjects) may have had different reasons for non-participation than those recruited by the Netherlands Foundation for the Detection of Hereditary Tumors (HNPCC subjects only). However, since participation rates were high for both recruitment procedures, this is thought not to have materially affected the results.

At the time of filling out the questionnaires, at home shortly after endoscopy, most sporadic patients are unaware of their diagnosis with respect to adenomas. Recall of dietary habits is therefore expected to be similar in sporadic cases and controls. HNPCC cases and controls are generally more aware of their risk. As HNPCC family members, irrespective of their adenoma history, are generally considered to be relatively health conscious due to the very strong family history of cancer, this is not expected to have lead to differences between cases and controls.

The meat consumption and preparation questionnaire is designed especially to estimate meat consumption and preparation with respect to formation of heterocyclic amines. It is adapted to Dutch meat preparation methods and uses photos from a very extensive Swedish questionnaire. If linked to a database on contents of specific heterocyclic amines in meat, a questionnaire with this limited number of items and type of questions, could in theory explain a large part of the variation in heterocyclic amine intake. Byrne *et al.* showed that in 3 prospective cohort studies in the United States, estimates of HCA intake and determination of associations with disease risk are feasible[148], if additional information on meat cooking methods is obtained [156]. Meat consumption frequencies were assessed using the validated EPIC questionnaire [154] as well as the meat consumption and preparation questionnaire. Estimated intakes for total meat, red meat and poultry based on these two questionnaires were highly correlated (Spearman correlation coefficients ranged between 0.69 and 0.77). With respect to meat preparation we observed in an experimental setting (data not shown) that the level of the heat source, addition of water, and use of a lid were all related to the temperature at the meat surface, which is a determinant of heterocyclic amine formation [157]. Those specific aspects of meat preparation were however inconsistent in their association with adenoma risk in our study. Possibly the color of the meat surface provides an overall estimate of habitual meat preparation.

With respect to the exposure of interest, our hypothesis derives from epidemiologic observations in sporadic colorectal cancer populations. However, one may question whether associations with dietary factors would be similar in a HNPCC population, which is known for its hereditary background risk. Interestingly, there is some evidence that the molecular pathways leading to cancer involve steps that are similar in these two groups. These steps involve somatic mutations in regulatory genes, which may be caused by dietary carcinogens such as heterocyclic amines [158]. The APC gene is known to be involved in both sporadic colorectal cancer and HNPCC colorectal cancer [9;28]. Abnormalities in the K-ras and p53 genes, known to be highly frequent in sporadic colorectal cancer, also occur in HNPCC carcinomas, but reported frequencies vary

between studies [12;29;106;107;125]. We have previously shown that in the adenomas in this study there are no major differences in gene involvement of k-ras and p53 in the sporadic and HNPCC adenomas.

Despite the similarities regarding the genes involved, our results suggest that there may be relevant differences in the etiology of sporadic and HNPCC colorectal cancer, already in the early adenoma stage. As APC is considered to be the first gene to become mutated, it may be postulated that meat consumption and preparation could be related to APC gene involvement in sporadic adenomas, whereas in HNPCC different APC mutations of endogenous nature may originate from the mismatch repair phenotype. In studies in rats it is shown that heterocyclic amines may induce specific mutations in the APC gene [35].

In conclusion, despite limited numbers, our results suggest that red meat consumption and color of the meat surface may be relevant to adenoma formation in sporadic colorectal carcinogenesis. These factors appear to be of less importance to risk of HNPCC adenomas, or are not detectable among the high background risk in a HNPCC population. This does not exclude meat consumption and preparation to be relevant to later stages, both in sporadic and HNPCC populations.

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ANIMAL PRODUCTS AND K-RAS CODON 12 AND 13 MUTATIONS IN COLON CARCINOMAS

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CHAPTER 6 ANIMAL PRODUCTS AND K-RAS CODON 12 AND 13 MUTATIONS IN COLON CARCINOMAS

It is hypothesized that the involvement of the K-ras oncogene and its specific mutations in colon tumors may be influenced by the consumption of animal products and their constituents.

Based on a previously conducted case-control study on diet and colon cancer, we collected paraffine-embedded carcinoma tissue of the cases to study the potential association between K-ras mutational status and the consumption of foods of animal origin. K-ras mutations in codons 12 and 13 were determined by PCR-based mutant allele specific amplification (MASA) in tumor tissue of 185 colon cancer patients: 36% harbored mutations, of which 82% were located in codon 12.

Within cases, K-ras mutational status was not markedly associated with the consumption of most animal products and their constituents; for fish a slight association was observed (Odds Ratio (OR) K-ras mutant versus K-ras wildtype per 19 grams = 1.2, 95% Confidence Intervals (CI) = 1.0-1.5). Comparing cases to controls showed that this association was confined to those harboring K-ras mutations. Interestingly, high intakes of animal protein, calcium and poultry were differently associated with codon 12 and codon 13 mutations: OR and 95% CI codon 12 versus codon 13 were 9.0 (2.0-42), 4.1 (1.4-12) and 15 (1.4-160), respectively. In case-control comparisons, high intakes of animal protein and calcium were positively associated with colon tumors harboring codon 12 mutations: for protein per 17 g OR (95%CI) =1.5 (1.0-2.1); for calcium per 459 mg 1.2 (0.9-1.6), while inverse associations were observed for tumors with K-ras mutations in codon 13 (for animal protein 0.4 (0.2-1.0); for calcium 0.6 (0.3-1.2)). Transition and transversion mutations were not differently associated with these dietary factors.

Although no overall association between animal products and K-ras mutations was observed, these data do suggest involvement of their consituents in the etiology of colon tumors harboring K-ras codon 12 and codon 13 mutations.

INTRODUCTION

Epidemiological studies suggest that diet contributes to over 60 percent of colon cancer: diets rich in meat, saturated fat and animal protein are considered to increase risk; vegetables, dietary fiber and other plant-related compounds may prevent colon cancer [159]. Molecular studies have shown that during colon carcinogenesis somatic mutations accumulate [7], but the role of diet in their etiology is still under debate.

About 50 percent of colon tumors harbor single point mutations in the Kirsten ras (K-ras) oncogene [160]. G to A transitions at the second base of codons 12 or 13 of K-ras are common in human colon tumors and are characteristic effects of alkylating compounds, such as N-nitrosocompounds [160]. It has been demonstrated in humans that endogenous formation of N-nitrosocompounds is raised when red meat intake is increased [32]. Other potential meat-related carcinogens, such as heterocyclic amines present in meat cooked at high temperatures, have been shown to induce K-ras mutations at guanine bases in aberrant crypt foci, putative preneoplastic lesions [34], pointing to an early effect of these compounds in the carcinogenic process. Dietary factors may also affect clonal selection by modifying growth of colon tumors harboring K-ras mutations. Possibly as a result of a direct antiproliferation action on hyperplasia, supplemental calcium reduces the incidence of K-ras mutations in dimethylhydrazine-induced colon tumors [161]. This is in line with the results of an epidemiological retrospective study by Bautista and colleagues [162], which showed that the intake of calcium was inversely associated with colon tumors harboring K-ras mutations.

About 80 percent of K-ras mutations in colon tumors are located in codon 12, while mutations in codon 13 are observed in about 10 percent [8;160]. Mutations in codon 12 are either more likely to occur or they are more likely to exert a selective growth advantage when they occur [160]. Most mutations in codon 13 are transition mutations, while in codon 12 the G-A transition mutation as well as the G-T transversion mutation are most common [12].

We explored whether the consumption of products from animal origin and their constituents are associated with the occurrence of K-ras mutations in colon tumors and whether these dietary factors may be differently associated with the prevalence of transition and transversion mutations in codons 12 and 13 of the K-ras gene. We explore these hypotheses in a previously conducted case-control study, in which meat consumption and calcium intake increased risk of colon cancer [68;163].

MATERIALS AND METHODS

Study population

A population-based case-control study on diet and colon cancer was conducted in the Netherlands between 1989 and 1993. Methods have been published in detail previously [68]. In total, 232 incident colon cancer cases and 259 population-based controls were included. Cases were men and women with colon cancer, newly diagnosed at surgery. Population-based controls, frequency matched to the cases by age (5-year intervals), gender, region, and degree of urbanization, were recruited randomly by general practitioners of the cases. All subjects were of Western-European origin, spoke Dutch,

were up to 75 years old at time of diagnosis and did not have previous cancer, polyposis coli, or inflammatory bowel disease. Cases were invited by their surgeons within three months of diagnosis in regional hospitals from the east and central parts of the Netherlands; of all eligible cases 47% was actually invited, which was due to technical and administrative reasons. Cancer registries in the study areas were used as a check of completeness and provided additional clinical and pathological information. Except for a more advanced Dukes' stage, non-invited cases did not importantly differ from those invited. The response of cases and controls invited to be interviewed was about 60%. Except for Dukes' stage, the distribution of age, gender, tumor site and therapies of the interviewed cases was comparable to the distribution among all eligible cases. Among controls, participation was higher among younger subjects and those living in urban areas. Cases and controls who participated did not differ significantly in the consumption of nutrients and food groups of interest from non-participants who filled out a non-response questionnaire.

For 28 of the 232 cases, the cancer registry defined the location of the tumor as rectosigmoid in contrast to the surgeon who located the tumor in the colon. Since the etiology of rectal tumors might differ from those situated in the colon, the present analyses are restricted to colon cancer cases (ICD-O 153). Paraffin-embedded tissue from primary colon tumors resected before chemo- or radiotherapy, was collected between 1995 and 1997 from 185 of the colon cancer cases. Nineteen tumor specimens could not be collected for administrative reasons. These cases did not differ materially in demographic or dietary factors from those of whom archival material could be collected.

Dietary assessment

To assess usual dietary habits, a detailed, structured, interview-based dietary history questionnaire was used, referring to the previous full year (in cases before symptoms occurred). The consumption frequency per month, number of months during which the item was used, number of portions per consumption and portion size of 289 food items of 19 food groups were inquired. To be able to estimate portion sizes, the contents of frequently used household utensils and cups were measured. To minimize errors, the dietary questionnaire was entered in the computer using a specially designed computer program which included range-checks and consistency checks with other foods.

Average daily intake of nutrients was calculated using the Dutch National Food Table [164]. Nutrient intake was adjusted for energy by the residual method [165] for men and women separately.

In addition to the dietary part, the interview included questions on previous and current smoking habits, aspirin and NSAIDs use, family history of colorectal cancer, and medical history.

DNA-isolation

DNA was isolated from nine 8 μm paraffin sections. Guided by an haematoxylin /eosin stained section, tumor tissue was scraped off. After deparaffination and rehydration, tissue was incubated for 18 hours at 56°C with 0.5 mg/ml proteinase K (Boehringer Mannheim, Almere, The Netherlands) in 500 μl cell lysis solution (DNA-isolation kit; PureGene Gentra Systems Inc, Landgraaf, The Netherlands) and additional 72 hours at 37°C. After cooling the samples to 4°C, protein was precipitated. To the supernatant, 500 μl isopropanol was added for DNA precipitation. After washing the pellet with 70% ethanol at 4°C and centrifugation, DNA was air-dried and rehydrated.

Mutant Allele Specific Amplification (MASA)-PCR

Codon 12 and 13 of K-ras were examined by MASA [112]. A mixture of primers for MASA corresponds to variants of the first (set A) or the second (set B) nucleotide of codon 12, or the first (set C) or the second (set D) nucleotide of codon 13. The following primers were used:

for wild-type Ki-ras: 5'TGTGGTAGTTGGAGCTG3';

Set A 5'TGTGGTAGTTGGAGCTC3', 5'TGTGGTAGTTGGAGCTA3', and 5'TGTGGTAGTTGGAGCTT3';

Set B 5'GTGGTAGTTGGAGCTGT3', 5'GTGGTAGTTGGAGCTGA3', and 5'GTGGTAGTTGGAGCTGC3';

Set C 5'GGTAGTTGGAGCTGGTC3', 5'GGTAGTTGGAGCTGGTA3', and 5'GGTAGTTGGAGCTGGTT3';

Set D 5'GTAGTTGGAGCTGGTGC3', 5'GTAGTTGGAGCTGGTGA3', and 5'GTAGTTGGAGCTGGTGT3'.

For all PCR experiments the same reverse primer was used:

5'CCAGGTCCTGGTAAGAAAACT3'.

MASA-PCR was performed as follows: PCR reaction mixture 1 (40 µl) consisted of 300 ng of purified DNA, 20 mmol/l $(NH_4)_2SO_4$, 75 mmol/l Tris-HCl (pH 9.0), 0.01% Tween, 200 µmol/l of dNTPs, 0.4 µmol/l of reverse primer, 2.5 mmol/l $MgCl_2$. After an initial denaturation step of 3 min. at 94°C, mixture 2 (10µl), which consisted of 0.3 units of Thermoperfect Plus DNA polymerase (Integro BV, Zaandam, The Netherlands), and 20 pmol of each of the forward primers, was added immediately to mixture 1. PCR was performed in 35 cycles of 30 sec. at 94°C, 30 sec at 60°C and 1 min. at 72°, followed by a final extension of 72°C for 5 min., in a Mastercycler 5330 (Eppendorf Geratebau GmbH, Hamburg, Germany). Following amplification, 15 μl of each reaction mixture was loaded on a 2% agarose gel containing 100 ng/ml ethidium bromide and electrophoresed.

A second PCR was performed on those DNA samples that were found positive. In this new PCR single primers that correspond to each of the variant nucleotides of the positive first PCR set were added per incubation. Conditions were the same as in the PCR described above.

Since all samples studied contain wildtype K-ras DNA due to the presence of tumor stroma in all lesions tested, we used wildtype K-ras DNA as an internal control for the quality of the DNA isolated form paraffin-embedded tissue. For each sample we first amplified wildtype K-ras by PCR using a primer combination that amplifies the same fragment as in the MASA. This wildtype PCR had to be positive, otherwise the sample was excluded from mutation analysis by MASA. In our series of lesions we found that in 98% of the samples wildtype K-ras could easily be amplified.

To validate the robustness of MASA we performed sequence analysis on the PCR products of a number of samples, each with a different mutation in codon 12 or 13. In all cases analyzed we found the same mutation by sequencing as was detected by MASA.

Data analysis

The association between diet and K-ras gene mutations was evaluated by comparing the mutation prevalence among cases using logistic regression models [166]. In addition, odds ratios (OR) and 95% confidence intervals (CI) were calculated separately for mutant cases and wildtype cases versus the population-based control group. First, quartile analyses of dietary intake were conducted. To quantify the associations on a continuous scale, all ORs and 95%CIs are expressed for the distance between the first and third quartile. All analyses were adjusted for age, sex and total energy intake. Additional adjustment for Dukes' stage (in case-case comparisons only), smoking habits, BMI, and other dietary factors, such as the consumption of vegetables and fruits, did not change the estimates importantly. Data analyses were conducted for all K-ras mutations combined. Moreover, analyses were conducted separately for codon 12 and codon 13 and for transition and transversion mutations.

RESULTS

Of the 185 colon tumors included in this study, 66 (36%) harbored K-ras mutations in codons 12 or 13: 55 tumors (82%) showed a mutation in codon 12, and 12 (18%) in codon 13. In one tumor, mutations were found in both codons. Of the 55 mutations in codon 12 (wildtype GGT), 23 (42%) were transition mutations, of which GAT (96%) was most common, and 32 (58%) were transversion mutations of which the GTT mutation (53%) was most frequent. In codon 13 (wildtype GGC), GAC transitions (92%) were most frequent.

Table 6.1 shows characteristics of the study population; cases are categorized by the absence or presence of K-ras mutations. The occurrence of K-ras mutations was related to advanced tumor stage. There were no marked differences between K-ras mutant and K-ras wildtype tumors with respect to age, sex, tumor-site and total energy intake. Poultry and fish consumption was slightly higher among those with K-ras mutations as compared to those without ($p<0.1$). Fish consumption was also slightly higher as compared to controls ($p<0.1$). The consumption of dairy products was higher among both case-groups as compared to controls. Energy intake was significantly higher among both case groups as compared to controls, while energy-adjusted total fat intake was significantly higher among cases harboring K-ras mutations as compared to controls.

Table 6.1. *Characteristics of the study population; cases are categorized by the absence (wildtype) or presence (mutant) of K-ras mutations in codons 12 and 13.*

Variable	Controls	Colon cancer cases (n=185)	
	n=259	wildtype (n=119)	mutant (n=66)
Age (years; mean ± SD)	61.8 ± 10.0	61.7 ± 10.2	61.7 ± 10.6
Sex (% men)	52.2	53.8	56.1
Smoking status (% never smoked)	31.3	35.3	25.8
Tumor characteristics			
Tumor site (% proximal)	n.a.*	46.4	43.5
Dukes' stage (% C and D)	n.a.	32.1	43.6¶
Food groups (mean ± SD)			
Red meat (g/day)	73.9 ± 34.1	67.3 ± 37.0	80.6 ± 34.7
Poultry (g/day)	13.8 ± 13.7	13.6 ± 15.6¶	14.7 ± 14.3
Fish (g/day)	18.2 ± 21.2	18.4 ± 21.4¶	28.1 ± 45.4‡
Dairy products (g/day)	268.2 ± 244.3	325.6 ± 339.1‡	322.7 ± 305.4
Nutrients (mean±SD)			
Energy (kJ/day)	9,362 ± 2,844	10,276 ± 3,307§	10,511 ±3,140§
Total fat (g/day)†	100.4 ± 24.9	102.7 ± 23.8	106.3 ± 28.6§
Saturated fat (g/day)†	42.0 ± 10.7	43.0 ± 11.4	44.2 ± 13.0
Total protein (g/day)†	83.2 ± 14.9	83.5 ± 17.1	85.5 ± 15.7
Animal protein (g/day)†	56.8 ± 13.6	57.5 ± 14.5	59.2 ± 16.0
Calcium (mg/day)†	1,248.5 ± 406.0	1,273.0 ± 425.9	1,245.0 ± 418.4

* not applicable

† adjusted for total energy intake by regression analysis

¶ wildtype vs mutant $p<0.1$

‡ wildtype or mutant vs controls $p<0.1$

§ wildtype or mutant vs controls $p<0.05$

Table 6.2 presents odds ratios and 95% confidence intervals for case-case as well as case-control comparisons evaluating the association between animal products and K-ras mutations. Fish consumption increased colon cancer risk only among those patients

harboring K-ras mutated tumors. K-ras mutational status did not materially alter the associations between other animal food groups, nutrients and colon cancer risk. (Table 6.2).

Table 6.2. *Animal foods and nutrients as related to K-ras mutations in colon tumors.*

Foods/Nutrients		Odds ratios and 95% Confidence intervals*		
		mutant vs wildtype n=66 vs 119	mutant vs controls n=66 vs 259	wildtype vs controls n=119 vs 259
Food groups				
Meat	per 43 g†	1.1 (0.8-1.6)	1.1 (0.7-1.6)	1.0 (0.7-1.3)
Beef	per 26 g	1.0 (0.7-1.4)	0.8 (0.6-1.1)	0.8 (0.6-1.1)
Processed meat	per 23 g	1.0 (0.7-1.5)	1.1 (0.8-1.5)	1.2 (0.9-1.6)
Poultry	per 17 g	1.1 (0.8-1.5)	1.0 (0.7-1.4)	1.0 (0.8-1.3)
Fish	per 20 g	1.2 (1.0-1.5)	1.2 (1.0-1.4)	1.0 (0.7-1.2)
Dairy products	per 287 g	1.0 (0.7-1.3)	1.1 (0.8-1.5)	1.1 (0.9-1.4)
Nutrients				
Total fat	per 20 g	1.2 (0.9-1.6)	1.2 (0.9-1.6)	1.1 (0.9-1.4)
Saturated fat	per 16 g	1.1 (0.8-1.4)	1.2 (0.9-1.5)	1.1 (0.9-1.4)
Cholesterol	per 118 g	1.0 (0.7-1.3)	1.1 (0.8-1.4)	1.1 (0.9-1.4)
Total protein	per 19 g	1.1 (0.8-1.6)	1.2 (0.8-1.6)	1.0 (0.8-1.4)
Animal protein	per 17 g	1.1 (0.8-1.6)	1.2 (0.9-1.6)	1.1 (0.8-1.4)
Calcium	per 459 g	0.9 (0.6-1.3)	1.0 (0.7-1.4)	1.1 (0.8-1.4)

* adjusted for age, gender and total energy intake

† continuous variable as distance between third and first quartile

Table 6.3 shows the odds ratios and 95% confidence intervals for case-case comparisons of cases with codon 12 mutations versus cases with codon 13 mutations and cases with transition mutations versus transversion mutations in codon 12 . Substantial differences were observed according to the codon affected. A high intake of protein, especially animal protein, showed a nearly 10-fold risk of K-ras mutations in codon 12 compared to mutations in codon 13. For calcium, poultry and dairy products important differences were also observed between mutations in codons 12 and 13. Comparing transition mutations with transversion mutations in codon 12, showed no important animal product-related differences.

To further evaluate the observed etiologic heterogeneity between codons 12 and 13, Table 6.4 presents comparisons with the population-based control group for both case groups. For (animal) protein, calcium and poultry consumption, positive associations were observed with mutations in codon 12, while mutations in codon 13 appeared to be inversely associated with these dietary factors.

Table 6.3. *Animal foods and nutrients as related to specific K-ras mutations in colon tumors: case-case comparisons.*

Food/nutrient		Odds ratios and 95% confidence intervals*	
		codon 12 mutations vs codon 13 mutations n=54 vs 11†	transitions mutations vs transversions mutations n=23 vs 32
food groups			
total red meat	per 43 g‡	1.3 (0.5-3.4)	1.4 (0.7-2.9)
beef	per 26 g	1.7 (0.7-4.2)	0.9 (0.5-1.8)
poultry	per 17 g	15.0 (1.4-160)	0.9 (0.5-1.8)
fish	per 20 g	1.8 (0.8-4.4)	0.9 (0.7-1.1)
processed meat	per 23 g	1.0 (0.5-2.2)	1.1 (0.6-1.8)
dairy products	per 287 g	2.7 (0.8-9.5)	1.2 (0.7-2.0)
Nutrients			
total fat	per 20 g	1.2 (0.6-2.3)	0.9 (0.6-1.4)
saturated fat	per 16 g	1.4 (0.7-2.6)	1.1 (0.7-1.6)
cholesterol	per 118 g	1.4 (0.6-3.3)	0.9 (0.5-1.5)
total protein	per 19 g	9.3 (1.9-45)	1.3 (0.7-2.5)
animal protein	per 17 g	9.0 (2.0-42)	1.0 (0.6-1.8)
calcium	per 459 mg	4.1 (1.4-12)	1.4 (0.7-2.7)

* adjusted for age, sex and total energy intake

† one person harboring mutations in codon 12 and codon 13 was excluded from the analyses

‡ continuous variable as distance between first and third quartile

Table 6.4. *Animal foods and nutrients as related to K-ras codon 12 and 13 mutations in colon tumors: case-control comparisons.*

Foods/nutrients		Odds ratios and 95% confidence Intervals*	
		codon 12 mutations vs controls (n=55 vs. 259)	codon 13 mutations vs controls (n=12 vs. 259)
food groups			
total red meat	per 43 g†	1.1 (0.8-1.7)	0.9 (0.4-1.9)
beef	per 26 g	0.9 (0.6-1.3)	0.5 (0.2-1.3)
poultry	per 17 g	1.2 (0.8-1.6)	0.4 (0.1-1.2)
fish	per 20 g	1.2 (1.0-1.5)	0.8 (0.4-1.6)
processed meat	per 23 g	1.1 (0.8-1.5)	0.9 (0.4-2.0)
dairy products	per 287 g	1.2 (0.9-1.7)	0.7 (0.3-1.6)
Nutrients			
total fat	per 20 g	1.2 (0.9-1.6)	1.0 (0.6-1.1)
saturated fat	per 16 g	1.2 (0.9-1.6)	0.9 (0.5-1.7)
cholesterol	per 118 g	1.1 (0.8-1.4)	0.9 (0.5-1.6)
total protein	per 19 g	1.5 (1.0-2.1)	0.4 (0.2-1.0)
animal protein	per 17 g	1.5 (1.0-2.1)	0.4 (0.2-1.0)
calcium	per 459 g	1.2 (0.9-1.6)	0.6 (0.3-1.2)

* adjusted for age, sex and total energy intake

† continuous variable as distance between first and third quartile

Discussion

Overall, these data do not support the hypothesis that the occurrence of K-ras mutations in colon tumors are importantly influenced by the consumption of animal products. However, these data do suggest that colon tumors with codon 12 and codon 13 K-ras mutations are related differently to the intake of protein and calcium and the consumption of poultry. No diet-related differences were observed for transition versus transversion mutations.

Although this is the largest study on diet and specific K-ras mutations published to date, it is still of limited size, since collection of both dietary data and tissue blocks from a large number of colon cancer cases is extremely labor intensive. Moreover, it could be argued that multiple comparisons increase the possibility of chance findings, especially for the codon and mutation specific analyses. However, the differences between the two codons appear to be substantial, warranting further investigations.

In any retrospective case-control study, selection bias and information bias may affect internal validity. Our investigation focused primarily on etiological differences between cases with mutations in codon 12 or 13 of the K-ras gene compared to cases without these mutations. Since cases are unaware of the mutational status of their tumors, in these case-case comparisons, systematic errors in dietary recall are less likely to bias study results than in traditional case-control comparisons. However, recall of dietary habits can still be influenced by (knowledge of) a more severe tumor stage or medical treatment influencing appetite. Adjusting all case-case comparisons for Dukes' stage did not change the estimates importantly, however.

The prevalence of K-ras gene mutations (36%) is at the low end of the range reported in other studies: from 35% to 65% [12;113;160]. This may be partially explained by a lower participation rate among cases with Dukes C and D tumors, who tend to have higher mutation prevalences. In addition, we evaluated only codons 12 and 13, thereby missing potential mutations in codon 61, which may account for up to 5% of K-ras mutations in colorectal cancer [8]. On the other hand, we assessed K-ras mutations using the MASA method, which is more sensitive than a SSCP method commonly used, or direct sequencing. MASA detects less than 1% mutated cells while sequencing needs at least 10 to 20% mutated cells [167].

The spectrum of specific K-ras mutations is similar to that observed in a large multi-center study evaluating the prognostic significance of K-ras mutations among 2214 colorectal cancer patients: 835 (38%) harbored a mutation, of which 81 percent were located in codon 12 [12]. As in our study, the G to A transitions and G to T transversions at the second

position of codon 12 were most common, while the GAC mutation occurred most frequently in codon 13 [12].

The results obtained from our 185 colon cancer cases do not correspond to a similar study by Bautista and associates in Majorca, that included 106 colorectal cancer (62 colon) cases, 37% harboring a K-ras mutation [162]. Among this Spanish population, the authors observed an inverse association between high calcium intake and risk of developing a K-ras mutation. In the Dutch population described here, in contrast to Spain, there is a high level and wide range of calcium intake. We did not observe an inverse association between calcium and K-ras gene mutations overall; we did observe, however, an inverse association for tumors with codon 13 mutations.
Our data suggest a different dietary etiology of K-ras codon 12 and 13 mutations: animal protein, calcium and poultry, and to a lesser extent dairy products and fish, are differently related to codon 12 and 13 mutations. A diet high in meat, animal protein and low in dairy products and calcium might optimize the exposure to arylamines, N-nitroso compounds and other carcinogens which could directly interact with genomic DNA to form bulky adducts ultimately leading to mutations in growth regulating genes [159]. It might be that certain carcinogenic compounds preferably form adducts on codon 12, while codon 13 is not a significant target. Methylnitrosourea (MNU) is known to induce G-A transition mutations [168]. However, these transition mutations are common in both codons and no important differences between transition and transversion mutations were observed in this study. Limited by the size of the study, we could not evaluate specific transition and transversion mutations. Whether the G-T (glycine to valine) transversion mutation in codon 12, which is observed to be associated with a more severe prognosis [12], is associated with specific exposures would be of considerable interest.
Another explanation for the differences between codons might be that mutations occur at a similar rate in both codons, but that dietary factors, such as protein and calcium, provide an environment with a specific growth advantage for tumors harboring codon 12 mutations or an environment which prevents the outgrowth of tumors with K-ras mutations in codon 13.

If our findings can be reproduced in larger studies, the diet-related differences between the two main targets of mutations in the K-ras oncogene, may provide further insight in the etiology of colon cancer.

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P53 OVEREXPRESSION AND P53 MUTATIONS IN COLON CARCINOMAS: RELATION TO DIETARY RISK FACTORS

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CHAPTER 7 P53 OVEREXPRESSION AND P53 MUTATIONS IN COLON CARCINOMAS: RELATION TO DIETARY RISK FACTORS

Epidemiological studies have suggested that dietary factors may differently affect p53-dependent and p53-independent pathways to colon cancer. Results of such studies may depend on the method used to assess p53 status. This case-control study of 185 colon cancer cases and 259 controls examines this relation, using both immunohistochemistry and SSCP(exon 5-8) / sequencing to detect p53 abnormalities.

Of 185 carcinomas analyzed using immunohistochemistry, 81 (44%) were categorized as p53 overexpression. P53 mutations were detected in 59 tumors (32%). A slight increase in risk observed for intake of saturated fat was largely due to an increased risk in cases without p53 overexpression (OR per 16.1 g/day: 1.46; 95% CI, 1.08-1.97), and no association in cases with p53 overexpression (OR: 1.07; 95% CI, 0.78-1.47). However, findings were less pronounced when cases were classified by mutation analysis (wildtype OR: 1.33; 95% CI, 1.01-1.75; mutated OR: 1.16; 95% CI, 0.81-1.65). Similar results were observed for total fat intake. For other nutrients and for vegetable and meat food groups no differences in risk for both p53 pathways were observed, independent of the laboratory technique used. Interestingly, in cases with transversion mutations in the p53 gene, an increased risk was observed for saturated fat (OR: 2.00; 95% CI, 0.97-4.14), in contrast to those with mutations at CpG sites (OR: 0.93; 95% CI, 0.55-1.57).

In conclusion, an increase in colon cancer risk for the p53-independent pathway due to fat intake, is more pronounced when using immunohistochemistry. However, mutation analysis is needed to study the possible association with a small group of tumors with transversion mutations.

INTRODUCTION

Cancer of the colon and rectum is the fourth most common type of cancer and cause of death from cancer throughout the world [1]. The transformation from normal colonic mucosa to carcinoma is accompanied by accumulation of genetic alterations, involving activation of oncogenes and inactivation of tumor suppressor genes [8]. Alteration of the p53 tumor suppressor gene is one of the most frequent molecular abnormalities observed in human cancer and is associated with the transition from adenoma to carcinoma in colorectal cancer [169]. Mutations in the p53 gene are mostly missense mutations, which result in loss of tumor suppressor function and lead to increased half-life and quantity of

the p53 protein [14]. This protein overexpression can be visualized by nuclear staining using immunohistochemistry (IHC).

Epidemiological studies have implicated dietary habits in the etiology of colorectal cancer. It has been estimated that over 50% of colorectal cancer cases can be prevented by diet [1]. High intake of red meat and animal fat may increase the risk whereas a high intake of fruit and vegetables may decrease risk of colon cancer [3]. Some dietary factors may be related to specific genetic alterations, such as mutations in the p53 tumor suppressor gene. Recently, epidemiological studies have addressed risk factor patterns of colorectal carcinomas with and without p53 overexpression [116;117;124;170;171]. Interestingly, Freedman *et al.* found cruciferous vegetables to be protective among cases with p53 positive tumors, but not in p53 negative cases, as compared with controls [117]. In the same study, beef consumption increased risk in p53 negative cases, but not in p53 positive cases, as compared with controls. So far, studies on risk factors of colorectal cancer and p53 status all used p53 overexpression and not mutation analysis.

The objective of the present analysis is to examine whether the diet-cancer association depends on p53 abnormalities in the tumor, and whether mutation analysis using single stranded conformational polymorphism (SSCP) of exon 5-8 followed by sequencing, as compared to immunohistochemical methods, leads to different associations or yields an added value.

MATERIALS AND METHODS

Study population and data collection

Between 1989 and 1993, a population-based case-control study has been conducted in The Netherlands to examine the relationship between dietary factors and the risk of colon cancer. Details about the design and conduct of this study are described previously [163]. In short, cases were defined as newly diagnosed patients with histologically confirmed adenocarcinoma of the colon (ICD-O 153), as determined at surgery. All Dutch-speaking men and women of Western European origin, up to the age of 75 years old at diagnosis, without a history of cancer, polyposis coli or inflammatory bowel disease were eligible. Of all eligible cases diagnosed, 47% were invited to participate by their surgeon, and of those invited 60% agreed to participate. In order to explore potential selection bias, participants and non-participants were asked to fill out a short questionnaire on diet and lifestyle habits. Although non-participants did have a worse prognosis according to Dukes' status, they were similar with respect to the consumption of food groups. A total of 232 cases participated in the initial study, including 28 cases whose tumor was classified as rectosigmoid according to the cancer registry. To minimize misclassification, resulting from potential differences in etiology and p53 mutation spectrum between colon and

rectum cancer [172], the latter group was excluded from the present analysis. The control group (n=259) was recruited randomly by the general practitioners of the participating cases, and was frequency matched by age and sex to the cases. Of the 520 controls invited, 57% agreed to participate. Dietary and lifestyle patterns were similar for those participating and those who refused. Trained dietitians conducted personal interviews at the participants homes. The dietary part of the questionnaire requested information on frequency and amount of foods consumed in the year before the interview (for cases before diagnosis and complaints) and consisted of a detailed, structured dietary history questionnaire.

Tumor material

For p53 analysis we obtained formalin-fixed, paraffin-embedded tumor material from 185 of the initial 204 cases of colon cancer in the case-control study. Of the remaining 19 cases no tumor material could be retrieved, due to administrative reasons. Tumor characteristics and dietary habits of these cases did not differ from the cases whose tumor material was available for analysis. The tumor samples were analyzed for accumulation of the p53 protein by immunohistochemistry, and for mutations in exons 5-8 of the p53 gene by SSCP followed by sequencing.

Immunohistochemistry

From formalin-fixed, paraffin-embedded specimens 4 μm sections were mounted on charged glass slides (Superfrost) and dried overnight at 60°C. Sections were deparaffinized in xylol for 10 min. and rehydrated through graded concentrations of ethanol and water. Microwave antigen retrieval was performed by boiling the sections in 10 mM citrate-buffer pH 6,0 for 5 min. at full power. Additional buffer was added and the sections were boiled for another 5 min. and then allowed to cool to room temperature for 45 min. Sections were rinsed in PBS. Endogenous peroxidase activity was blocked by incubation in 0,6 % H_2O_2 for 30 min. Sections were rinsed in PBS. Aspecific antibody binding was blocked by pre-incubation of the sections with 10% normal horse serum in PBS/BSA for 10 min. Sections were incubated overnight at 4°C with a cocktail of two anti-p53 antibodies: 0,4 μg/ml DO-7 (Neomarkers, Fremont, USA) which recognizes both mutant and wild forms of p53, and 1 μg/ml 240 (Neomarkers, Fremont, USA) which recognizes only the mutant form of p53. Negative control sections were treated with PBS/BSA alone. Sections were rinsed in PBS, and antibody binding was visualized using the Vector ABC Elite kit (Vector laboratories, Burlingame CA, USA) using 3,3'-diaminobenzidine as substrate and copper enhancement according to the manufacturer's protocol. Finally, sections were dehydrated through graded concentrations of ethanol, incubated in xylol and mounted with permount.

Stained sections were scored independently by two investigators (A.A. v. K., G.N.P. v. M.). As the occurrence of only very few strongly positive cells in a tumor does not seem to correlate with p53 mutations [173;174], we used a cut-off point of 20% of tumor cells stained, like in other epidemiological studies [116;117]. Tumors were scored as p53 negative if less than 20% of the cells displayed nuclear positivity and positive if otherwise.

DNA isolation

DNA isolation was performed using the Puregene DNA isolation kit (Gentrasystems, Triangle Park, USA). From the formalin-fixed, paraffin-embedded specimens six to nine 8 μm sections were cut for DNA isolation and one 4 μm section was cut for Hematoxylin and Eosin (H&E) staining. Guided by the H&E-stained section, tumor tissue was scraped from the 8 μm thick sections. After deparaffination with xylol and rehydration with ethanol the tumor tissue was incubated for 18 hours at 56°C with 0,5 mg/ml proteinase K (Boehringer Mannheim GmbH, Almere, The Netherlands) in 500 μl cell lysis solution according to the recommendations of the manufacturer and for additional 72 hours at 37°C . After cooling-down the samples to 4°C, 150 μl of protein precipitation solution was added, followed by mixing for 20 sec. and centrifugation for 10 min. at 14,000xg, 4°C. To the supernatant 500 μl isopropanol (100%) was added followed by gentle mixing and centrifugation for 10 min. at 14,000xg, 4°C. After washing the pellet with 500 μl 70% ethanol at 4°C, DNA was air-dried and rehydrated in 100 μl hydratation solution for 60 min. at 65°C. DNA was stored at 4°C until use. Analysis of the quality of the isolated DNA by agarose gel electrophoresis showed that the size of the DNA fragments was 200-400 bp.

SSCP-analysis

The highly conserved regions of the p53 gene, exons 5-8 were each amplified separately using the following intronic oligonucleotide primers:

Exon 5: 5F: 5'TCACTTGTGCCCTGACTT3'
5R: 5'GAGGAATCAGAGGCCTGG3'

Exon 6: 6F: 5'GAGACGACAGGGCTGGTT3'
6R: 5'GAGACCCCAGTTGCAAAC3'

Exon 7: 7F: 5'CCAAGGCGCACTGGCCTC3'
7R: 5'GCGGCAAGCAGAGGCTGG3'

Exon 8: 8F: 5'CCTTACTGCCTCTTGCTTC3'

8R: 5'TGAATCTGAGGCATAACT3'

PCR reaction mixture 1 (40 μl) consisted of 20 mmol/l $(NH_4)_2SO_4$, 75 mmol/l Tris-HCl (pH 9.0), 0.01%Tween, 200 μmol/l of dNTPs, 0.2 μmol/l of the forward primer, 1.0 mmol/l $MgCl_2$ (exon 5,7) or 1.5 mmol/l $MgCl_2$ (exon 6) or 2.5 mmol/l $MgCl_2$ (exon 8). After an initial denaturation step of 3 min. at 94°C, mixture 2 (10 μl), which consisted of 0.3 units of thermoperfect plus DNA polymerase (Integro BV, Zaandam, The Netherlands) and 10 pmol of the reverse primer, was added immediately to mixture 1. PCR was performed for 40 cycles as follows: 94°C for 30 sec., 55°C (exon 5) or 60°C (exon 6,7,8) for 45 sec. and 72°C for 1 min., followed by a final extension at 72°C for 5 min. in a Mastercycler 5330 (Eppendorf Geratebau GmbH, Hamburg, Germany). A negative control without DNA was included in each PCR experiment. After amplification 10μl of the product was subjected to electrophoresis on a 2% agarose gel containing 100 ng/ml ethidium bromide to confirm success of DNA amplification. The sizes of the PCR products were as follows: exon 5, 292 bp; exon 6, 197 bp; exon 7, 212 bp and exon 8, 237 bp.

For SSCP-analysis 3 μl of the PCR-product was diluted in 3 μl loading buffer (95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.5% xylene cyanol), heated at 95°C for 3 min. and cooled on ice before loading. The electrophoretic analysis was carried out on a Genephor Electrophoresis Unit (Amersham Pharmacia, Uppsala, Sweden) using GeneGel Excel 12.5/24 kit (Amersham Pharmacia, Uppsala, Sweden). Electrophoresis was performed at 7°C (exon 7) or 18°C (exon 5,6,8) for 2 hours at 25mA, 15W and 600V. Finally the gels were stained in a Hoefer Automated Gel Stainer (Amersham Pharmacia, Uppsala, Sweden) with the PlusOne DNA Silver Staining Kit (Amersham Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

For reasons of efficiency not all 4 exons were analyzed in each tumor. As mutations in more than one exon are exceptional [175;176], we decided that, if a mutation was detected in one exon, the tumor would be excluded for analysis of the subsequent exons. According to the frequency of mutations in each exon reported in other studies [123;172,177;178], the exons were analyzed in the following order: exon 7, 8, 5 and 6.

Sequence analysis

Sequencing was performed on original PCR products of exon 5-8 of p53 for all SSCP-positive tumors. Where SSCP indicated the presence of a mutation not detected by sequencing, the variant band was selected from the gel and reamplified. In 5 tumors a mutation still could not be confirmed and were therefore scored as not harboring a p53 mutation. Sequencing primers were the same as those used for PCR. The PCR product was purified using QIAquick purification columns (Qiagen GmbH, Düsseldorf, Germany) and followed by Taq cycle sequencing using Dye terminator cycle sequencing ready reaction kit (ABI Prism, Perkin Elmer, Branchburg, USA) according to the manufacturer's

instructions. Sequence analysis was performed by the Applied Biosystems Model 370A DNA sequencer (Perkin Elmer, Branchburg, USA). To avoid false positive results due to polymerase errors, all samples showing a mutation after SSCP/sequencing, were tested again by performing a new PCR, SSCP and sequence analysis.

Data analysis

Results of immunohistochemical analysis were compared with the actual mutation spectrum of exons 5-8 of the p53 gene in these colon carcinomas. Sensitivity of IHC is defined as the probability of a positive result (20% or more overexpression), given an actual mutation in exon 5-8 was determined by SSCP/sequencing. Specificity is defined as the probability of a negative result in IHC (<20% overexpression), given no mutation was found by SSCP/sequencing of exon 5-8. The proportion of carcinomas with a mutation detected by SSCP (exon 5-8)/sequencing was calculated in each category of p53 overexpression. This was repeated, excluding carcinomas with a silent mutation or a mutation leading to a stopcodon, because these are not expected to result in p53 overexpression.

Both p53 overexpression and p53 mutation, were used to study the relationship with dietary factors. Intake of nutrients and food groups was categorized using quartiles, according to the distribution among controls. To test for trend and to quantify the associations on a continuous scale, quartile medians were used as an additional exposure variable [179]. All odds ratios (OR) and 95% confidence intervals (95% CI) are expressed for the distance between the first and the third quartile cut-off points. To simultaneously account for confounding factors, multiple logistic regression models were used. Nutrient intake values were adjusted for energy intake by the residual method for men and women separately [165], and by adding energy intake to the models. Additionally, to adjust for differences in distribution, age and gender were included in all models. Potential risk factors such as body mass index, family history, smoking and alcohol intake did not change the OR's more than 10% when added individually to the models, and were therefore not included in the final models.

Data analysis was performed using the SAS-system and consisted of several phases. First, to identify relevant etiological heterogeneity for specific dietary risk factors, case-case comparisons were performed. The resulting OR can be interpreted as the ratio of the relative risk for developing p53-positive disease to the relative risk for developing p53-negative disease. These case-case comparisons were conducted separately for immunohistochemistry and SSCP (exon 5-8)/sequencing. Subsequently, it was evaluated whether dietary factors were associated with colon cancer in specific subgroups of cases, i.e. p53 overexpression, any p53 mutation, or specific p53 mutations. The ORs for these

subgroup specific case-control analyses were expressed using all 259 controls as the reference group.

RESULTS

Results of immunohistochemical detection of p53 overexpression and SSCP/sequencing analysis of p53 mutations in exon 5-8 are summarized in Table 7.1. A total of 185 tumors were analyzed, of which 81 (44%) were categorized as IHC-positive. Using SSCP analysis of exons 5-8 followed by sequencing, a p53 mutation was detected in 59 tumors (32%). In total 133 tumors (72%) were consistent for IHC and SSCP/sequencing, 44 (24%) were positive in both and 89 (48%) were negative in both.

Table 7.1. *Number of colon carcinomas with p53 protein overexpression and mutation of exon 5-8 of the p53 gene.*

	IHC % of cells positive					
	negative (n=104)*		Positive (n=81)			total (n=185)
	0%	1-19%	20-49%	50-79%	80-100%	
SSCP/sequencing						
mutation†						
no	66	23	12	16	9	126
yes	13	2	5	16	23	59
stopcodon¶	9	1	0	1	1	12
silent‡	2	0	0	0	1	3
exon 5	3	0	1	4	9	17
exon 6	3	1	0	2	2	8
exon 7	4	0	2	5	5	16
exon 8	3	1	2	5	7	18
total	79	25	17	32	32	185

* <20% positive nuclei of tumor cells was scored 'negative', ≥20% positive nuclei of tumor cells was scored 'positive'

† SSCP/sequencing analysis was done in the following order of exons: 7, 8, 5, 6; if a mutation was found in an exon the tumor was excluded for analysis of the subsequent exons

¶ in 12 of 59 carcinomas with a mutation a mutation resulting in a stopcodon was found, which is not expected to lead to overexpression of the protein

‡ in 3 of 59 carcinomas with a mutation a silent mutation was found, which does not lead to an amino acid change and thus not to overexpression of the protein

In 15 of 59 tumors in which a mutation was detected by SSCP/sequencing, no p53 overexpression was found. In 12 of these the discrepancy could be explained: 10 mutations resulted in stopcodons (Table 7.1), and 2 mutations did not lead to an amino acid change. IHC was positive in 37 tumors (20%) in which no mutation could be

detected by SSCP and sequencing of exons 5-8. Thus, taking SSCP (exon 5-8) /sequencing as the reference, sensitivity and specificity of IHC were 74% and 71% respectively.

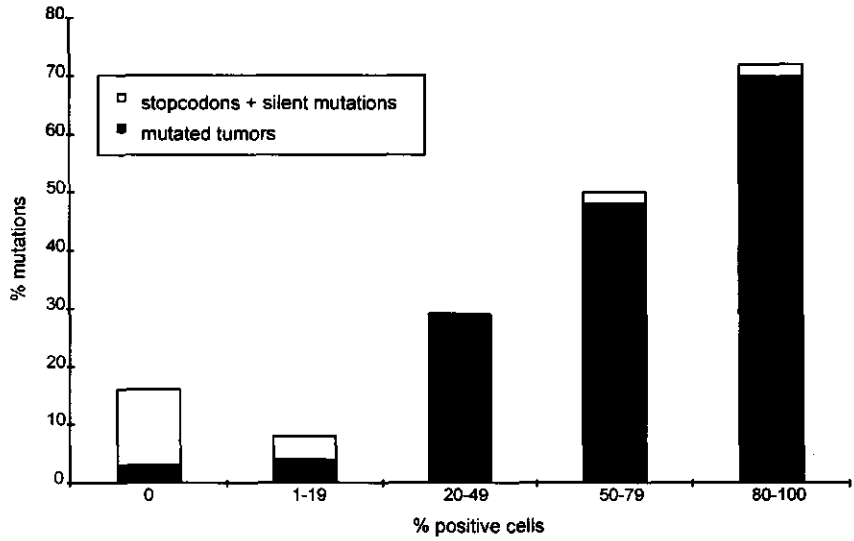

Figure 7.1. *The proportion of mutations in categories of p53 overexpression.*

Figure 7.1 shows the probability of a mutation in exon 5-8 in different categories of p53 overexpression. The probability increased with an increasing number of cells with positive staining. This was especially clear, when not taking into account silent mutations and mutations leading to a stopcodon, which are not expected to result in overexpression.
Table 7.2 shows potential risk factors in the control group and in the colon carcinoma patients stratified by p53 status based on IHC and SSCP (exon 5-8)/sequencing (SEQ). Age was similar in all groups. Gender was not significantly different in all case groups as compared with the controls, but the proportion of women was higher in those classified as IHC negative. The percentage of carcinomas with Dukes' C or D was similar in all case groups. No statistically significant differences in mean intake of nutrients and food groups were found between the 4 case groups. Energy intake was higher in all case groups as compared to controls. Intake of vegetables was significantly lower in all case groups as compared to controls. The total red meat intake was highest in cases with a positive result in SSCP (exon 5-8)/sequencing, however not significantly different from controls or other case groups. In the cases which were negative for both IHC and SSCP (exon 5-8)/ sequencing, intake of beef was significantly lower than in controls ($p<0.10$). Intake of processed meat was significantly higher in cases both positive in IHC and SSCP (exon 5-8)/ sequencing as compared with controls.

When comparing risks between cases with and without overexpression, we found ORs markedly deviating from 1 for total fat (positive vs negative OR per quartile range of 37.3 g/day, 0.61; 95% CI, 0.38, 0.98) and for saturated fat (positive vs negative OR per quartile range of 16.1 g/day, 0.76; 95% CI, 0.52, 1.09). No statistically significant ORs were found for any of the case-case comparisons for other nutrients or food groups (data not shown). When comparing mutated and wildtype cases no statistically significant ORs were found either, the OR was 1.07 for total fat (95% CI= 0.65, 1.75) and 0.90 for saturated fat (95% CI= 0.62, 1.32). The ORs did not markedly change when the case-case analyses were adjusted for Dukes' stage.

Table 7.3 presents the ORs and 95% CIs for the risk estimates for nutrient and food group intake according to p53 overexpression and according to p53 mutation status, as compared with controls. Total fat and saturated fat intake increased the risk of colon carcinomas without p53 overexpression (OR, 1.94; 95% CI, 1.32, 2.85, and OR, 1.46; 95% CI, 1.08, 1.97, respectively), but not of carcinomas with p53 overexpression (OR, 1.20; 95% CI, 0.81, 1.77, and OR, 1.07; 95%CI, 0.78, 1.47, respectively). When p53 status was evaluated using mutation analysis, total fat intake was associated with an increase in risk in both mutated and wildtype cases (OR, 1.57; 95% CI, 1.00, 2.45, and OR, 1.56; 95% CI, 1.10, 2.20, respectively), whereas the OR for saturated fat was slightly higher in wildtype cases than in mutated cases (OR, 1.33; 95% CI, 1.01, 1.75, and OR, 1.16; 95%CI, 0.81, 1.65, respectively).

The ORs for the remaining nutrients and food groups did not markedly differ between the case groups or between methods of assessing p53 abnormalities. The decrease in risk associated with vegetables was similar in specific case groups and also comparable to the risk in all cases combined. Intake of cruciferous vegetables was only significantly associated with a decrease in risk in cases without overexpression (OR, 0.62; 95% CI, 0.41, 0.95) and in wildtype cases (OR, 0.61; 95% CI, 0.41, 0.91), and the difference in ORs between cases with and without p53 abnormalities was more pronounced when using mutation analysis. When the analyses for cruciferous vegetables were adjusted for total intake of vegetables, the ORs no longer indicated a decrease in risk (data not shown), suggesting the inverse association is not specific for cruciferous vegetables. We found no significant associations with intake of any of the meat product groups in either of the case groups.

Table 7.2. *Characteristics of the study population categorised by p53 status as assessed by IHC and SSCP (exon 5-8) / sequencing.*

			cases (n=185)			
			consistently negative*	inconsistently classified		consistently positive*
		controls (n=259)	IHC-/SEQ- (n=89)	IHC+/SEQ- (n=37)	IHC-/SEQ+ (n=15)	IHC+/SEQ+ (n=44)
age (yrs)		61.8 ± 10.0	62.4 ± 10.2	60.9 ± 10.6	60.5 ± 11.8	61.4 ± 10.1
gender (% women)		47.5	49.4	40.5	60.0	36.4
Dukes stage (% C,D)			32.9	40.6	33.3	39.5
energy intake (kJ)		9,362 ± 2,844†	10,324 ± 3,550¶	10,087 ± 2.779	10,484 ± 2,313	10,619 ± 3,290¶
fat (g/d)		100 ± 25	104 ± 25	101 ± 20	103 ± 29	106 ± 30
saturated fat (g/d)		42 ± 11	44 ± 11	42 ± 10	43 ± 15	44 ± 14
protein (g/d)		83 ± 15	84 ± 16	84 ± 16	78 ± 17	86 ± 17
animal protein (g/d)		57 ± 14	57 ± 15	58 ± 15	56 ± 11	60 ± 16
dietary fiber (g/d)		29 ± 8	28 ± 10	26 ± 8§	26 ± 7	27 ± 7
calcium (mg/d)		1264 ± 406	1275 ± 422	1305 ± 443	1169 ± 335	1233 ± 437
alcohol (g/d)		14 ± 18	16 ± 19	16 ± 22	11 ± 15	21 ± 37
vegetables,	total (g/d)	208 ± 124†	176 ± 90¶	171 ± 76‡	144 ± 54¶	171 ± 77¶
	cruciferous (g/d)	34 ± 21	33 ± 23	29 ± 24	28 ± 16	38 ± 29
fruit (g/d)		231 ± 181	219 ± 177	224 ± 159	202 ± 110	212 ± 160
meat,	red meat (g/d)	74 ± 34	76 ± 41	76 ± 25	81 ± 33	81 ± 35
	beef only (g/d)	35 ± 22	30 ± 19§	33 ± 22	29 ± 22	33 ± 25
	chicken (g/d)	14 ± 14	14 ± 17	12 ± 12	14 ± 14	15 ± 13
fish (g/d)		18 ± 21	20 ± 24	18 ± 16	23 ± 17	28 ± 54
processed meat (g/d)		22 ± 19†	23 ± 17	26 ± 24	23 ± 16	31 ± 22¶

* IHC+:overexpression, 20% or more cells stained, IHC-: 0-19% cells stained, SEQ+: SSCP(exon 5-8)/sequencing, mutated, SEQ-: wildtype

† ($p<0.05$) t-test between the control group (n=259) and the total case group (n=185)

¶ ($p<0.01$) or ‡ ($p<0.05$) or § ($p<0.10$) t-test between specific case group and the controls

Table 7.3. *Odds Ratios of colon cancer risk for nutrients and food group consumption, using quartile medians as continuous variable, expressed per quartile range (Q3-Q1, g/day), stratified by p53 status using immunohistochemistry and SSCP/sequencing.*

		cases (185) vs controls (259)		immunohistochemistry cases positive (n=81) vs. controls		cases negative (n=104) vs. controls		SSCP(exon 5-8)/sequencing cases mutated (n=59) vs. controls		cases wildtype (n=126) vs. controls	
Dietary factor	(/ quartile range)	OR*	(95% CI)	OR*	(95% CI)	OR*	(95% CI)	OR*	(95% CI)	OR*	(95% CI)
Energy	(/ 3566 kJ)	1.89	(1.32-2.72)	1.72	(1.06-2.80)	2.05	(1.33-3.15)	1.99	(1.15-3.44)	1.87	(1.25-2.81)
Fat	(/ 37.3 g)	1.58	(1.16-2.15)	1.20	(0.81-1.77)	1.94	(1.32-2.85)	1.57	(1.00-2.45)	1.56	(1.10-2.20)
Saturated fat	(/ 16.1 g)	1.28	(1.01-1.64)	1.07	(0.78-1.47)	1.46	(1.08-1.97)	1.16	(0.81-1.65)	1.33	(1.01-1.75)
Protein	(/ 19.2 g)	1.02	(0.75-1.39)	0.92	(0.61-1.37)	1.13	(0.78-1.63)	1.00	(0.63-1.58)	1.04	(0.74-1.46)
Animal protein	(/ 17.0 g)	1.11	(0.82-1.51)	1.14	(0.76-1.71)	1.08	(0.74-1.58)	1.12	(0.71-1.78)	1.10	(0.78-1.56)
Dietary fiber	(/10.4 g)	0.76	(0.56-1.04)	0.69	(0.46-1.05)	0.85	(0.58-1.24)	0.68	(0.42-1.11)	0.81	(0.57-1.14)
Calcium	(/459 mg)	1.15	(0.85-1.56)	1.07	(0.72-1.60)	1.24	(0.86-1.80)	1.03	(0.65-1.63)	1.22	(0.87-1.72)
Alcohol	(/18.2 g)	1.07	(0.84-1.36)	0.99	(0.72-1.36)	1.15	(0.86-1.56)	0.98	(0.67-1.41)	1.13	(0.86-1.48)
Vegetables,											
total	(/ 106 g)	0.59	(0.44-0.79)	0.55	(0.36-0.82)	0.61	(0.43-0.87)	0.48	(0.30-0.77)	0.63	(0.45-0.88)
cruciferous	(/ 28 g)	0.69	(0.48-0.97)	0.79	(0.50-1.25)	0.62	(0.41-0.95)	0.90	(0.54-1.49)	0.61	(0.41-0.91)
Fruits	(/ 164 g)	0.87	(0.67-1.14)	0.97	(0.68-1.38)	0.81	(0.59-1.12)	0.89	(0.60-1.32)	0.86	(0.64-1.16)
Meat,											
red meat	(/ 43 g)	1.12	(0.82-1.53)	1.24	(0.81-1.88)	1.03	(0.70-1.52)	1.23	(0.76-2.02)	1.07	(0.75-1.52)
beef only	(/ 26 g)	0.81	(0.61-1.07)	0.83	(0.58-1.20)	0.77	(0.54-1.09)	0.83	(0.55-1.27)	0.78	(0.57-1.08)
poultry	(/ 17 g)	0.93	(0.70-1.24)	0.90	(0.62-1.30)	0.94	(0.66-1.33)	1.13	(0.75-1.71)	0.82	(0.59-1.14)
Fish	(/ 20 g)	1.09	(0.86-1.37)	1.15	(0.84-1.57)	1.05	(0.79-1.39)	1.21	(0.85-1.73)	1.04	(0.80-1.36)
Processed meat	(/ 22 g)	1.25	(0.87-1.79)	1.37	(0.84-2.22)	1.17	(0.76-1.80)	1.51	(0.86-2.66)	1.15	(0.77-1.71)

* Odds ratios (OR) and 95% confidence intervals (95% CI), all models are adjusted for age, gender, and energy intake.

Of the 59 mutations detected by SSCP/sequencing, 7 mutations (12%) consisted of 5 deletion mutations (1-17 bp) and 2 one base pair insertions, which all lead to stopcodons, and 52 (88%) were point mutations (data not shown). Twenty-three G:C to A:T mutations at CpG sites (39%) were found in known hotspots for colorectal cancer: 4 at codon 175, 5 at codon 248, 9 at codon 273, and 5 at codon 282. Of the point mutations 5 led to a stopcodon, 4 were mutated in codon 306 or codon 213, also at CpG sites. Thirteen tumors (22%) harbored a transversion mutation, which is the type of mutation often related to smoking in lung cancers.

Table 7.4. *Risk of colon carcinomas with specific p-53 mutations, for nutrients and food group consumption, using quartile medians as continuous variable, expressed per quartile range (Q3-Q1, g/day).*

Dietary factor		(/ quartile range)	mutations at CpG-islands vs controls (27 vs 259) OR (95% CI)*		transversion mutations vs controls (13 vs 259 OR (95% CI)*	
Energy		(/ 3566 kJ)	1.36	(0.64-2.88)	2.48	(0.78-7.93)
Total fat		(/ 37.3 g)	1.52	(0.80-2.89)	1.89	(0.78-4.56)
Saturated fat		(/ 16.1 g)	0.93	(0.55-1.57)	2.00	(0.97-4.14)
Protein		(/ 19.2 g)	0.78	(0.40-1.48)	2.24	(0.88-5.70)
Animal protein		(/ 17.0 g)	0.96	(0.50-1.85)	2.57	(0.98-6.76)
Dietary fiber		(/ 10.4 g)	0.63	(0.32-1.26)	0.63	(0.24-1.66)
Calcium		(/ 459 mg)	1.15	(0.60-2.19)	1.12	(0.47-2.65)
Alcohol		(/ 18.2 g)	0.89	(0.51-1.55)	0.82	(0.40-1.70)
Vegetables,	total	(/ 106 g)	0.42	(0.21-0.83)	0.38	(0.14-1.03)
	cruciferous	(/ 28 g)	0.76	(0.37-1.54)	0.59	(0.21-1.65)
Fruits		(/ 164 g)	0.78	(0.44-1.36)	0.93	(0.41-2.12)
Meat,	red meat	(/ 43 g)	1.41	(0.71-2.80)	1.08	(0.40-2.90)
	beef only	(/ 26 g)	0.68	(0.37-1.27)	0.58	(0.24-1.42)
	poultry	(/ 17 g)	1.34	(0.76-2.37)	1.12	(0.50-2.50)
Fish		(/ 20 g)	1.26	(0.78-2.05)	1.32	(0.66-2.62)
Processed meat		(/ 22 g)	1.36	(0.62-2.98)	1.98	(0.63-6.22)

* Odds ratios (OR) and 95% confidence intervals (95% CI), all models are adjusted for age, gender and energy intake.

In Table 7.4 ORs and 95% CIs are shown for intake of nutrients and food groups for cases with mutations at CpG islands and transversion mutations. Except for vegetable intake, which is inversely associated with mutations at CpG islands as well as transversion mutations, none of the nutrients and food groups were significantly associated with mutations at CpG islands. However, transversion mutations were positively associated with intake of energy, total fat, saturated fat, total protein, and animal protein. Although

these nutrients are all significantly correlated with total red meat intake (Pearson r = 0.32, 0.33, 0.29, 0.33, 0.39 respectively), no positive associations were found with meat product groups.

Discussion

In this case-control study including 185 colon cancer cases, we evaluated the association between dietary factors and p53 abnormalities using both p53 protein overexpression and specific p53 mutations as the endpoints of interest. For most dietary factors similar observations were found for tumors with and without p53 abnormalities. However, total fat and saturated fat were positively associated with colon cancer risk in tumors that did not show p53 abnormalities. Although the percentage of cases consistently positive or consistently negative for both endpoints was relatively high (72%), differences in ORs were more pronounced when p53 abnormalities were determined by immunohistochemistry as compared to mutation analysis. Interestingly, when examining specific types of mutations, an increased risk with fat and saturated fat was observed for transversion mutations but not for mutations at CpG sites. Although the number of cases was small, especially for the evaluation of the specific p53 mutations, this study suggests a role for (saturated) fat in the p53-independent pathway to colon cancer (normal protein expression), as well as in the p53-dependent pathway (transversion mutations).

This study was initially designed to address the role of dietary risk factors in the etiology of colon cancer [163], and the results were in line with those reported by others [3]. In case control studies, selection bias and information bias limit the interpretation. In our study, however, responders and non-responders, both among cases and controls, had essentially similar dietary patterns. In addition, the disease may indirectly have affected reported food intake of cases. In this respect, it is relevant that 64% of the responding cases had Dukes' stage A or B tumors, whereas in the Dutch Cancer Registry this is 51% [2]. Therefore it is less likely that advanced disease has severely influenced the reported food intake. Advanced disease, and therefore presence of symptoms and information bias, correlates with p53 abnormalities. In our study we found p53 overexpression in 41% of cases with Dukes' A or B, and in 48% of cases with Dukes C or D. Moreover, we observed lower consumption of vegetables in cases with Dukes' C or D as compared to cases with Dukes' A or B. Results of the case-case analyses were not materially changed however, if Dukes' stage was taken into account. In conclusion, it seems unlikely that inherent pitfalls in design and conduct of case-control studies have seriously affected our results.

In our data, the percentage of p53 overexpression is similar, but the percentage of p53 mutations is slightly lower than reported in the literature. This may be due to the

overrepresentation of early stage tumors (Dukes' A or B, see above), as well as to our restriction to analysis of exon 5-8.

We have used standard IHC methods to detect p53 overexpression. Like others we used 20% of cells with nuclear staining as the cut-off point for scoring a tumor as being positive in p53 overexpression. Using this method we found 44% of the tumors to show p53 overexpression. Others have found 40-50% of colorectal cancer cases to show p53 overexpression [116;117;123;124]. To detect mutations, we used SSCP of exons 5-8 of the p53 gene as a screening method, after which mutations were confirmed and specified by sequencing. To maximize sensitivity, sequencing was performed even if SSCP was only marginally positive. Where SSCP suggested the presence of a mutation not confirmed by sequencing, the variant band was selected from the gel and reamplified. We found 32% of colon carcinomas to harbor a mutation in exons 5-8 of the p53 gene, which is somewhat low. In a review on p53 mutations in cancer Greenblatt et al. reported 50% of colon cancers to harbor a mutation in the p53 gene, based on studies using PCR-based techniques, screening at least exon 5-8 [14]. This difference may be due to our restriction to exons 5-8, the sensitivity and specificity of SSCP, or other factors. Mutations outside exons 5-8 are expected to occur in 5-20% of tumors, depending on the tumor site [176], and are usually frameshift or null mutations which do not lead to protein overexpression and therefore cannot be detected by IHC [14]. SSCP is thought to have a sensitivity and specificity of 90% for detecting a mutation in the p53 gene [14]. Therefore, our 32% mutations as assessed by SSCP (exon 5-8)/sequencing, should represent about 72-85% of all mutations in our cases, the estimated true percentage thus being 38-44%. Furthermore, our method is not able to discriminate between one or more mutations in exons 5-8, since we stopped analysis of other exons when a mutation was found in one exon. Mutations in more than one exon are, however, exceptional [175;176]. In our study the percentage of mutations in each exon was similar as found by others [123;172;177;178]. Thus the apparently low percentage of mutations (32%) can be partly attributed to the methods used. Possible misclassification resulting from this, may explain the weaker associations observed with mutation analysis as compared with immunohistochemistry methods. Furthermore, the relatively high percentage of Dukes' A & B tumors in our study may harbor less p53 mutations than Dukes C & D tumors.

We found sensitivity and specificity of detecting p53 overexpression using IHC with SSCP (exon 5-8)/sequencing as the reference method to be 74% and 71% respectively. In 84 studies identified by Greenblatt et *al.* comparing IHC with sequencing, positive staining was found in 44% of tumors by IHC, while 36% actually contained mutations [14]. The sensitivity in these 84 studies was on average 75% (range 36-100%), and the positive predictive value was 63% (range 8-100%), with considerable variation among tumors at different sites [14], no details were given for specific sites. However, in an evaluation of

antibodies for immunohistochemistry, Baas et al observed a sensitivity of 67% and a specificity of 90% in 19 colorectal neoplasms in The Netherlands, using information on mutations from sequencing analysis as reference [174]. There are several possible explanations for the inconsistencies between the two methods for detecting p53 abnormalities. P53 overexpression in tumors where no mutation was detected may be due to: the ratio of tumor cells and normal cells in the sample used for PCR being too low to detect a mutation; binding to viral or cellular proteins which may lead to overexpression without the presence of a mutation; mutations outside exon 5-8 (these consist mainly of frameshift or null mutations which are not detectable by IHC) or in introns. Nonsense mutations in exon 5-8 which do not lead to overexpression, explained most of the cases where no overexpression was detected in the presence of a mutation.
The moderate sensitivity and specificity, and the fundamental difference between cellular and molecular processes related to protein expression and gene mutation may also suggest that both markers of p53 involvement in colon tumorigenesis represent different entities, with only partially shared etiological determinants.

To our knowledge we are among the first to report p53 abnormalities in colon cancer not only in relation to food groups but also in relation to nutrient intake. When comparing cases without p53 abnormalities with controls we observed a positive association with total fat and saturated fat intake, which was more pronounced when using immunohistochemistry methods instead of mutation analysis. No increased risk was observed in cases with p53 abnormalities as compared with controls, except for those with transversion mutations in the p53 gene. These latter cases showed a positive association with total fat and saturated fat intake.

Freedman *et al.*, in their case control study on dietary risk factors and p53 overexpression in 163 colorectal cancer cases and 326 controls, found beef intake to be positively associated with colorectal cancer when comparing cases without overexpression with controls [117]. In our data, total fat as well as saturated fat, protein and animal protein were correlated with intake of meat. However, no positive associations with (specific) colon carcinomas were observed for red meat. As Freedman *et al.* did not present results on nutrient intakes, we can only speculate that nutrients correlated with beef intake are associated with a p53 independent pathway in their study as well. Whether intake of specific fatty acids or other substances more specifically associated with meat intake (e.g. heterocyclic amines) are the underlying etiologic factors remains to be elucidated.
Our results are only partially in line with the inverse association between cruciferous vegetables and colon cancer in cases with p53 overexpression, as found by Freedman *et al.* [117] We found cruciferous vegetables to be protective both in cases with and without

p53 abnormalities; in contrast to Freedman's findings only significantly so in cases without overexpression or mutation. In The Netherlands cruciferous vegetables account for a large proportion of the total intake of vegetables. When the analyses for cruciferous vegetables were adjusted for total intake of vegetables, there was no longer a marked association between cruciferous vegetables and cancer risk in any of the case groups, suggesting that the inverse association cannot be explained by consumption of cruciferous vegetables only. Freedman *et al.* found smoking and family history to be associated with p53 status as well [117]. In our study these factors were also associated with colon cancer risk, however the OR's for dietary factors did not markedly change after adjustment for smoking status and family history (data not shown).
In contrast to the study by Freedman *et al.*, which included both colon and rectum tumors [117], our study only included colon cancer cases, which may be a subgroup of cancers with a different etiology than rectal cancer. Additionally, Freedman *et al.* used different antibodies, which could result in differences in sensitivity.

Although no important effect of dietary factors on overall presence of mutations was found, looking at specific mutations showed that intake of total fat, saturated fat, total protein and animal protein were all associated with an increased risk of colon tumors with a transversion mutation. None of the dietary factors were associated with colon cases with mutations at CpG-islands, which are thought to be of endogenous origin. In cancer of the lung, transversion mutations, and more specifically G:C to T:A transversions, are strongly associated with smoking [15]. Adjusting our analyses on transversion mutations for smoking did not markedly change the ORs. If transversion mutations can be generally seen as mutations caused by exogenous carcinogens, our results suggest that diet may also be relevant for a small subgroup of tumors that harbor such a mutation.

In conclusion, for most dietary factors, results were similar for both p53 pathways in colon cancer, both when using p53 overexpression and when using p53 mutation as endpoint. However, we observed a positive association between (saturated) fat and colon cancer, limited to the p53 independent pathway, which was stronger when using p53 overexpression as the endpoint. Interestingly, for the subgroup of p53 transversion mutations, fat consumption appeared to be important as well. Although this is the largest study to date, it obviously is of limited size, requiring confirmation in larger studies.

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GENERAL DISCUSSION

CHAPTER 8 GENERAL DISCUSSION

INTRODUCTION

Although diet is assumed to play an important role in the etiology of colorectal carcinogenesis, the specific effect of dietary factors remains to be elucidated. As cancer is a "disease of the genes", additional clues to the etiology will be provided by studying diet-gene interactions in colorectal carcinogenesis. The studies in this thesis were conducted with the objective to gain more insight in the etiology of both sporadic and HNPCC colorectal carcinogenesis.

The first section of this general discussion serves to integrate the main findings as summarized in Table 8.1. Secondly, methodological considerations regarding statistical power and several types of bias are discussed. In this part the methodological study on questionnaires to assess meat consumption and preparation (CHAPTER 4) is addressed. In the third section the biological plausibility of the examined hypotheses is outlined. Finally, conclusions are drawn about the study results, and possible lines of future research into diet-gene interactions are presented.

Table 8.1. *Overview of the main findings of the studies described in this thesis.*

Ch.	Topic	Study population	Main findings
2	Anticipation	Successive generations in HNPCC families	Birth cohort effect explains earlier age of onset of colorectal cancer
3	Frequency K-ras and p53 abnormalities	Sporadic and HNPCC colorectal adenomas	Similar frequencies of K-ras gene mutation and p53 protein overexpression
4	Questionnaire on meat consumption and preparation	Swedish case-control study on several types of cancer	Limited loss of information for heterocyclic amine intake with reduced questionnaire (methodological study)
5	Meat consumption and preparation	Sporadic and HNPCC adenoma cases and controls	Red meat and darkly browned meat surface associated with risk in sporadic group; no associations in HNPCC group
6	Diet in relation to (specific) K-ras mutations	Colon carcinomas	Positive association with animal protein and calcium for codon 12 mutations; inverse for codon 13 mutations
7	Diet in relation to p53 overexpression and mutations	Colon carcinomas	Positive association with (saturated) fat for p53-negative (no overexpression) and -positive (transversion mutation) cancers

MAIN FINDINGS INTEGRATED

Dietary factors in HNPCC colorectal carcinogenesis

AIM 1 of this thesis (CHAPTERS 2, 3 & 5) was: *to examine evidence for a role of dietary risk factors in the etiology of HNPCC colorectal carcinogenesis; are some aspects of the etiology similar to sporadic colorectal carcinogenesis?*

In a descriptive epidemiological study (CHAPTER 2) we found the cumulative incidence of colorectal cancer to increase in successive generations of HNPCC families, which could be attributed to a birth cohort effect. In the general population, both incidence and mean age at diagnosis have gradually increased since the 1960s [2]. These increasing trends in incidence might suggest that environmental factors similarly affect the etiology of sporadic and HNPCC colorectal carcinogenesis.

The similar frequencies of mutations in the K-ras gene and overexpression of the p53 protein in HNPCC and sporadic colorectal adenomas (CHAPTER 3), suggest similar molecular pathways in early stages of colorectal carcinogenesis with regard to K-ras and p53. Possibly, dietary factors are associated with these molecular abnormalities (as studied in CHAPTERS 6 & 7) both in sporadic and HNPCC colorectal carcinogenesis.

Subsequently, we studied directly whether diet (meat consumption and preparation) is similarly associated with sporadic and HNPCC adenomas (CHAPTER 5). However, we found meat consumption and preparation to be differently associated with risk of sporadic and HNPCC colorectal adenomas. Red meat consumption and darkly browned meat surface were associated only with an increased risk of sporadic adenomas but not with HNPCC adenomas. Thus, meat consumption and preparation may not be relevant in the etiology of HNPCC adenomas. These results, although not in line with the trend of findings in the previous chapters, do not rule out a possible role for (other) dietary factors in these or other stages of HNPCC colorectal carcinogenesis.

Diet in relation to K-ras and p53 genes in sporadic colon cancer

AIM 2 of this thesis (CHAPTERS 6 & 7) was: *to examine evidence for a differential effect of dietary risk factors on K-ras/p53 dependent and independent pathways to colorectal carcinogenesis.*

In the studies described in the previous section (AIM 1) we studied trends in cancer incidence, molecular abnormalities in K-ras and p53, and dietary factors, to support our hypothesis of a role for environmental factors in HNPCC colorectal carcinogenesis. In the following section we directly studied the interaction between the latter two, in sporadic colon cancer.

With respect to diet in relation to K-ras mutations (CHAPTER 6), we observed no differences in dietary factors between carcinomas with and without a K-ras mutation.

Examining specific K-ras mutations, we found high intakes of animal protein and calcium to be positively associated with carcinomas harboring codon 12 mutations, and to be inversely associated with carcinomas harboring K-ras codon 13 mutations. Transition and transversion mutations were not differently associated with dietary factors.
We studied p53 overexpression and p53 mutations in relation to diet (CHAPTER 7), and found intake of total fat and saturated fat to be positively associated with risk of carcinomas, especially of the p53 independent pathway. Differences in associations were more pronounced with p53 overexpression than with p53 mutations. Interestingly, when studying specific p53 mutations, we did observe similar associations of diet with risk of carcinomas harboring p53 transversion mutations, but not with carcinomas harboring mutations at CpG islands.
If our findings with regard to K-ras and p53 abnormalities would be confirmed by others, this would suggest that especially dietary factors of animal origin could be associated with specific mutations in K-ras (codon 12) or p53 (transversions) but also with overexpression of p53 protein.

Secondary objectives of our studies

Regarding the aims (AIM 1 & AIM 2), the studies in this thesis are among the first to examine diet-gene interactions in colorectal carcinogenesis, in the etiology of HNPCC and mutations in K-ras and p53 genes, using an epidemiological approach. Such "early" studies into a new area, tend to be of small size, and they are therefore not sufficient to provide "proof" of diet-gene interactions. Nevertheless, they can suggest directions for future research. A secondary objective of our studies was to develop and expand research infrastructures relevant to the study of diet-gene interactions in human populations. In this way our studies have provided a basis to evaluate methods and study designs.

METHODOLOGICAL CONSIDERATIONS

In any case-control study selection of subjects and assessment of exposure may result in biases. In the following section the design, conduct and analysis of our studies will be discussed in the light of these biases and their effect on the interpretation of our study results. Sections are ordered with respect to selection of subjects, assessment of molecular abnormalities, and assessment of exposure, addressing both non-differential and differential misclassification. Potential confounding of the associations by disease characteristics and several lifestyle factors will be discussed. Subsequently, some remarks about power considerations will be made.

Selection of adenoma/carcinoma cases and controls

In the studies described in this thesis both colon carcinomas and colorectal adenomas are used as endpoints, and control groups are derived from the general population and a hospital population (i.e. endoscopy patients), respectively. Assessment of endpoints may have been subject to misclassification. The choice of these case groups and their respective control groups may have led to selection bias, if differentially associated with exposures under study.

Colon carcinoma cases have been verified by histology and registered by the Netherlands Cancer Registry, reducing possibility of false positive cases (CHAPTERS 6 & 7). The control group consists of "healthy" population subjects randomly selected from the files of the general practitioners of the cases. As cumulative incidence at age 75 (the maximum age in this population) is only about 5% [94], the number of undetected colon carcinomas in our control group is expected to be very low.

Non-participation (or non-response) of both cases and controls may lead to selection bias if differentially associated with dietary factors. As participating controls (participation rate 50%) did not markedly differ from non-participating controls with respect to age, gender, and dietary habits, no relevant selection bias is to be expected with respect to the relevant exposure among controls. The case group in this study (participation rate 60%) consisted of relatively more Dukes' A and B carcinomas than overall in colon carcinoma patients. As dietary habits might be associated with severity of the disease, selection bias could have been introduced. However, this higher percentage of Dukes' A and B cases may actually benefit the quality of the dietary data, as these cases are less likely to have changed their diets as a result of the disease. Moreover, adjustment of the analyses for Dukes' stage did not markedly change the results.

Colorectal adenomas are highly prevalent (35-60%), especially with increasing age, in populations at high risk for colorectal cancer [180], and frequently occur without presenting symptoms. Therefore, the adenoma control group (CHAPTER 5) underwent full colonic examination to exclude adenomas. The adenomatous component in polyps of all cases has been verified by histology. Because of full colonic examination and histopathologic review, misclassification of disease status is expected not to have markedly influenced the results. Subjects harboring hyperplastic polyps, verified by histopathology, have been included in the control group, as hyperplastic polyps are generally not considered to be precancerous lesions. However, hyperplastic polyps may also be associated with dietary factors; this may therefore have led to a dilution of associations. As this study involves only few controls with hyperplastic polyps (10%), this bias is expected to be small.

In both the sporadic and the HNPCC case groups the participation rates were high (90%). Sporadic adenoma cases and controls both underwent endoscopy, came to hospital for similar indications, and fulfilled the same eligibility criteria. Indications for endoscopy, e.g. abdominal pain, diarrhea, and constipation, may be related to dietary factors, but are usually not related to having adenomas. Rectal blood loss, another frequent indication for endoscopy, may be related to having adenomas, but is usually not related to dietary habits. Therefore, these indications for endoscopy are unlikely to have affected our results. With respect to the HNPCC group, both adenoma cases and controls are generally thought to be relatively health conscious, with more favorable dietary habits than the general population. This health consciousness, however, is thought to be a characteristic of HNPCC family members, irrespective of whether they have been diagnosed with adenomas, and is not related to screening, participation or enrollment in our study. These assumptions suggest selection bias will not have seriously influenced our results.

The HNPCC population Both the 'true' HNPCC status of the families included in these studies, as well as the gene carrier status of adenoma cases and controls may affect interpretation of the results.

The HNPCC families are ascertained as such using the "Amsterdam criteria" [25]. These are based on family history data and have been designed to establish uniformity in studies of HNPCC. This approach leads to both false positive families (when chance aggregation leads to high numbers of cases in a family) and to false negative families (when families are too small to include enough cases). Since 1993 five genes have been found responsible for the defects in the mismatch repair system which cause HNPCC [18]. Only a small percentage of the HNPCC families included in our studies have been examined for germline mutations in these genes, and therefore these mutations could not be used to confirm HNPCC-status. However, in a similar Dutch population of HNPCC families it was found that the Amsterdam criteria strongly correlate with mutations in mismatch repair genes [111].

Gene carrier status in the source population of HNPCC family members is unknown. The underlying assumption in the studies among first degree HNPCC family members (CHAPTERS 2, 3 & 5) is that the first degree relatives of colorectal cancer cases have a 50% chance of harboring a germline mutation in one of the mismatch repair genes. The proportion of non-carriers in both adenoma cases and the control group may influence interpretation of the observed associations. Adenoma cases are assumed to be gene-carriers. However, if they are diagnosed at a relatively old age, they may also be non-carriers, i.e. sporadic adenoma cases. If HNPCC adenoma cases partly consist of non-carriers and if risk associated with diet is actually higher in a sporadic population, this

could have led to an upwards biased estimate of the relative risk in the HNPCC population, by making the groups more similar. Thus, with respect to the relative risk associated with meat consumption and preparation, we can safely assume that if such a bias occurred, the true association in the HNPCC group would be even less. In contrast to our assumption in the adenoma cases, we expect the controls to consist of both gene-carriers and non-carriers. As gene-carriers and non-carriers among HNPCC controls are assumed to have similar dietary patterns, no biased estimates are expected to result from incorporating both types of controls.

Assessment of K-ras and p53 abnormalities

Molecular abnormalities of the K-ras and p53 genes are studied both with respect to their frequency in HNPCC and sporadic adenomas, and with respect to their relation to dietary risk factors in sporadic colon carcinomas. As in any laboratory method, detection of these abnormalities may be accompanied by false positive as well as false negative results. The sensitivity and specificity of the methods are discussed in detail in CHAPTERS 3, 6 & 7.

K-ras mutation frequency in the colon carcinomas (36%) seems relatively low in comparison with findings of others (see Figure 8.1), but can be explained by the relatively early Dukes stage of the carcinomas in our study and our restriction to analysis of codons 12 and 13. We have used the same method on colorectal adenomas and found a similar percentage of mutations (32%), which is within the range that could be expected in relatively small adenomas. As the distribution of different types of mutations in both carcinomas and adenomas was very similar to those found by others, we do not expect to have missed a large percentage of mutations using this method.

P53 abnormalities have been detected using both immunohistochemical methods and PCR-based methods. Concordance between the two methods was 72% in our study of colon carcinomas, similar to other studies of colorectal cancer (68-82%) [181]. Frequencies of mutation (32%) and overexpression (44%) in colon carcinomas and overexpression (31%) in sporadic colorectal adenomas are also within the range found by others (see Figure 8.1). The use of both methods to detect p53 abnormalities showed that use of either method may lead to somewhat different results in epidemiological studies of dietary risk factors in relation to p53. Depending on the aim of the study, a choice should be made between p53 mutation analysis (i.e. when aiming to find dietary causes for specific mutations) and assessment of p53 overexpression (i.e. when aiming to evaluate overall involvement of p53 in relation to diet).

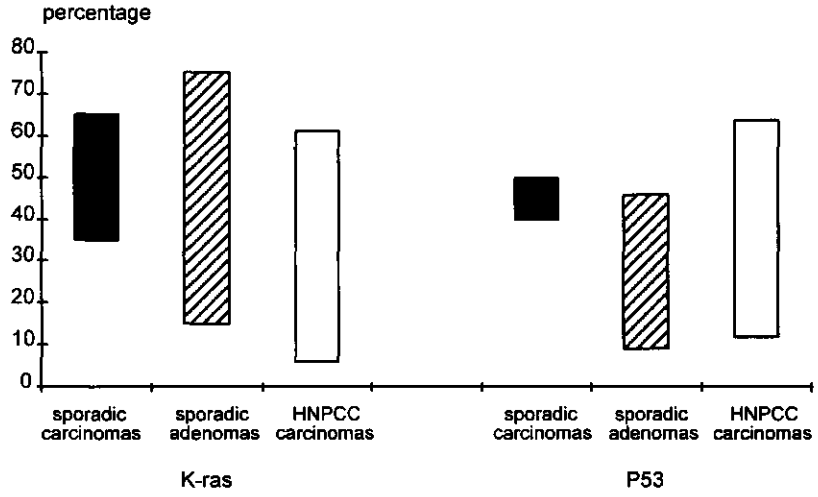

Figure 8.1. *The range of frequencies of K-ras mutations and p53 mutations or overexpression, reported in studies of K-ras and p53 abnormalities in sporadic and HNPCC colorectal adenomas and carcinomas.*

Assessment of dietary exposures

Measurement errors in assessment of dietary factors, and more specifically meat consumption and preparation, may have occurred due to limited validity and reproducibility of the instruments used to assess dietary intake. As far as random measurement errors are concerned, we assume this may have led to minor bias towards the null hypothesis. If differential for cases and controls, misclassification will have caused information bias in our studies. The studies in CHAPTERS 5, 6 & 7 may be especially prone to information bias due to differential measurement errors, as these studies involve the retrospective assessment of diet in a case-control setting.

In the adenoma study on meat consumption and preparation (CHAPTER 5) we used a validated semi-quantitative food-frequency questionnaire designed for the European Prospective Investigation into Cancer to assess dietary intake of macro-nutrients and food groups [154;155]. To assess meat preparation a questionnaire was developed especially for this purpose. The photos used in this questionnaire are derived from a very extensive Swedish questionnaire designed to assess the intake of heterocyclic amines. The food frequency questionnaire and the meat preparation questionnaire correlated well with each other with respect to the consumption of total meat, red meat and poultry.

The methodological study In this study (CHAPTER 4) we examined whether a reduced set of questions on meat consumption and preparation provides sufficient information to correctly categorize subjects with regard to exposure to heterocyclic amines. The analyses were performed on data from a Swedish case control study which used a very extensive questionnaire to assess the intake of heterocyclic amines in a population of Stockholm elderly [134]. Reduction of the number of items in the questionnaire did not substantially change the estimated relative risks in that case control study. Thus, the number of items and the type of questions used in our meat preparation questionnaire should in theory be sufficient to give a relatively good estimate of heterocyclic amine intake. Ideally, one would use a valid biomarker for the exposure to heterocyclic amines. However, methods such as measurement of DNA-adducts in colon tissue [140;141;143] or metabolites in urine [144] have their own methodological and analytical limitations.

In this adenoma case control study (CHAPTER 5) information bias may have occurred if cases and controls report their past diet differently. Differences in recall between cases and controls in the HNPCC group are thought to be minimal, as this population is generally thought to be relatively health conscious, and very aware of their risk of colorectal cancer, irrespective of whether they had adenomas at their most recent colonic examination. In contrast to HNPCC family members, the sporadic population frequently does not know if they have adenomas shortly after endoscopy, which is the time of filling out the questionnaires in the majority of our study population. Moreover, controls in the sporadic group reported having bowel complaints more often than did the cases. All analyses have been adjusted for bowel complaints, but this did not markedly affect the results.

In the case control study on colon cancer (CHAPTERS 6 & 7) an interview-based dietary-history questionnaire was used. It was an adapted version of a validated questionnaire used in a study on breast cancer [182]. When studying K-ras and p53 abnormalities as endpoints, cases are generally not aware of their K-ras or p53 status. However, molecular abnormalities may be related to severity of disease, which may cause differences in recall or changes in dietary habits. Therefore, all case-case analyses were adjusted for Dukes' stage of the tumors, but again, this did not markedly alter the associations.

Confounding

Several dietary and other lifestyle factors may be related to the endpoints as well as to the exposures, especially in the studies focusing on dietary risk factors (CHAPTERS 5, 6 & 7). In each of these studies, total energy intake is taken into account as a possible confounder

by using energy adjusted nutrient intakes [165] and by including energy intake in the logistic regression models. Other potential risk factors in colorectal carcinogenesis are:

- high Body Mass Index, as a measure of long term energy imbalance [149]
- low physical activity, a sedentary lifestyle is thought to increase risk [149]
- smoking, may increase risk especially of adenomas [183;184]
- alcohol intake, may increase risk especially in combination with low folate intake [185;186]
- regular aspirin or NSAIDs use, has been shown to be protective in both adenomas and carcinomas [187;188].

As these risk factors may be associated with dietary risk factors such as meat consumption and preparation, they were all included in the logistic regression models separately, but none markedly changed the associations with the exposure under study.

Molecular abnormalities were studied as endpoints in CHAPTERS 3, 6 & 7. Clinical characteristics of the adenomas or carcinomas, such as size and localization of the tumor, multiplicity, stage, may all be associated with these molecular abnormalities and, where possible, have therefore been taken into account in the analyses.

Power considerations

The studies described in this thesis are among the first epidemiological studies to investigate diet-gene interactions in colorectal carcinogenesis, with respect to environmental factors in the etiology of HNPCC and molecular abnormalities in the K-ras and p53 genes. As little knowledge is available on these diet-gene interactions in humans, very large and expensive studies are not justifyable at this time. Therefore, the objective is to identify directions for future studies, by conducting studies with smaller sample sizes. In the interpretation of the results, larger confidence intervals are acceptable, to serve identification of major factors or generating new hypotheses.

Originally the case control study on colorectal adenomas also aimed to examine whether meat consumption and preparation are differently associated with adenomas with and without abnormalities in the K-ras and p53 genes. However, as the frequency of K-ras and p53 abnormalities was lower than anticipated in these adenomas, and the variation in variables regarding meat intake and preparation is relatively small in a Dutch population, the estimates obtained appeared to be very imprecise and therefore not informative.

BIOLOGICAL PLAUSIBILITY

In the following paragraph the plausibility of the diet-gene interactions that we have observed is discussed, both with respect to the etiology of HNPCC colorectal

carcinogenesis, and to molecular abnormalities in the K-ras and p53 genes in colon cancer. This will be done by comparison with other studies and by providing a biological rationale.

Molecular pathways and environmental factors in HNPCC

The high cumulative incidence of colorectal cancer and the relatively young age at diagnosis as described in HNPCC families (CHAPTER 2), are well known characteristics of HNPCC [22]. They are due to the genetic defect in the mismatch repair system, which leads to genetic instability and therefore to high frequencies of mutations in regulatory genes [18]. Anticipation (i.e. earlier onset of disease in successive generations) however, is a far more difficult phenomenon to explain. We have provided evidence that the younger age at diagnosis in successive generations of HNPCC families is mainly due to a birth-cohort effect, which resembles the secular trend in cancer incidence in the general population. Similar features have also been described in other hereditary diseases [101;102]. The comparability of these trends in sporadic and HNPCC cancer incidence indicate that the inherited genes may not be solely responsible for the incidence of cancer in HNPCC families. This hypothesis is further supported by findings of changes in the tumor spectrum of HNPCC over time [26]. Experimental evidence from studies in mice also suggest that the HNPCC tumor spectrum is determined by exposure to exogenous factors [189].

If environmental factors are postulated to play a similar role in HNPCC and sporadic colorectal cancer, one might expect the same genes to be involved. Mutations in K-ras and p53 may both be related to environmental factors, but reports on small series of HNPCC colorectal carcinomas have resulted in inconsistent findings with regard to mutation frequencies of K-ras and p53 (Figure 8.1). We report similar frequencies of K-ras and p53 abnormalities in HNPCC and sporadic colorectal adenomas (CHAPTER 3).

Distinctly different molecular pathways to sporadic and hereditary colorectal cancer have been suggested, because of the underlying germline defect in the mismatch repair system. The resulting microsatellite instability occurs in about 80-90% of HNPCC colorectal cancers and only 10-15% of sporadic colorectal cancers [16-18]. Mutations in the Transforming Growth Factor β type II receptor (TGFβ-RII) are found almost exclusively in tumors with microsatellite instability [104]. However, the detection of high frequencies of somatic APC mutations in both sporadic and HNPCC tumors [28], has cast some doubt on the hypothesis of distinctly different pathways to sporadic and HNPCC colorectal cancer. With respect to K-ras and p53, the few studies with relatively small series of cases, as mentioned earlier, provided a wide range of reported frequencies in HNPCC colorectal cancer (Figure 8.1).

Our results suggest that K-ras and p53 genes may be involved in a similar fashion in the early stages of sporadic and HNPCC carcinogenesis. Do the similar frequencies of mutations in K-ras and p53 in sporadic and HNPCC adenomas imply that environmental factors play a similar role in the etiology of HNPCC carcinogenesis?
We did not find similar associations with meat consumption and preparation for HNPCC and sporadic colorectal adenomas (CHAPTER 5). High red meat consumption and darkly browned meat surface were positively associated with sporadic adenomas but not with HNPCC adenomas. Possibly meat consumption and preparation are associated with inducing early molecular alterations, such as somatic APC mutations, in sporadic adenomas. In HNPCC adenomas however, mutations in APC might be largely due to the defect mismatch repair system. To our knowledge no published papers have yet reported on dietary risk factors in HNPCC colorectal carcinogenesis.

In conclusion, it appears likely that dietary factors play a role in the etiology of HNPCC colorectal carcinogenesis. However, it remains to be elucidated to which genes diet is related and in which phase of the carcinogenic process.

Association of diet with K-ras and p53 genes

Diet-related carcinogens such as alkylating agents have been suggested to cause point mutations in the K-ras gene, especially at the second nucleotide of codons 12 and 13 [160]. Possibly, dietary factors may also affect clonal selection by modifying growth of colon tumors harboring K-ras mutations. Some studies suggest that aggressiveness, prognosis and survival may differ for cancers with or without K-ras mutations and for cancers with specific K-ras mutations [126,190-192]. These results have been rather inconsistent, but recently a very large multi-center study showed that some specific mutations (i.e. G to T transversions at codon 12) are associated with poorer prognosis [12].
We found high intakes of calcium and protein to be inversely associated only with carcinomas with K-ras codon 13 mutations, but positively associated with carcinomas with K-ras codon 12 mutations (CHAPTER 6). In contrast to our study, Bautista *et al.* reported an inverse association for calcium in the K-ras-mutated pathway and for mono-unsaturated fatty acids in the K-ras-wildtype pathway only [162]. We did not find total fat, saturated or unsaturated fatty acids to be associated with K-ras mutations. These apparent inconsistencies may in part be due to large differences in dietary patterns between the Spanish population in the study by Bautista *et al.* [162] (low calcium intake, high olive oil consumption) and the Dutch population in our study (high calcium intake, low olive oil consumption).

Environmental factors such as smoking cause mutational fingerprints in the p53 gene (i.e. G:C to T:A transversion mutations) in lung cancer. This type of mutation also occurs in colorectal carcinomas, although in lower frequencies, and might also be caused by smoking or other exogenous factors. The type of p53 mutation which occurs most frequently in colorectal carcinomas (i.e. G:C to A:T transitions at CpG-dinucleotides) is thought to be endogenous of origin, resulting from spontaneous deamination of methylated cytosine [14]. Unknown is whether dietary factors might be involved in the methylation process, e.g. folate, vitamin B12, alcohol.
In our study, high intakes of nutrients of animal origin, such as total fat and saturated fat, were associated with an increased risk of colon carcinomas, especially of the p53 independent pathway (CHAPTER 7). These differences between the p53 dependent and independent pathways were more pronounced when studying overexpression as compared to mutations. Although total and saturated fat intake are highly correlated with meat consumption, we did not find total meat and beef to be associated with risk. In contrast to this, Freedman *et al.* did report beef consumption to be associated with the p53 independent pathway only, but did not report associations with nutrients [117]. Interestingly, when examining specific mutations we found that the same dietary risk factors (i.e. total fat, saturated fat) were associated with an increased risk for carcinomas with transversion mutations, but not with mutations at CpG islands.

It appears from the combined results of our own studies as well as those of others, that the dietary factors that interact with K-ras and p53 abnormalities still have to be identified. However, the results prompt to further research on the overall involvement of p53 (possibly using p53 overexpression as endpoint) in the association between dietary factors and colon cancer risk. Additionally, the role of nutrients and food groups of animal origin in the etiology of both specific K-ras (codon 12) and p53 (transversion) mutations should be subject to further study.

CONCLUSIONS AND PERSPECTIVES

General remarks

Cancer is known as a "disease of the genes". Simultaneously, environmental factors such as diet are held responsible for causing 90% of colorectal cancer. Therefore, it is merely rational to postulate that these two, genes and environment, may interact in causing the disease. In this thesis two types of diet-gene interaction have been studied: (1) genetic susceptibility as expressed in a dominant monogenetic trait (HNPCC) and the relevance of dietary factors in its etiology; (2) dietary factors in relation to somatic mutations which

may lead to cancer, i.e. dietary factors causing these mutations or differential effect of environmental factors depending on these mutations.
In our studies we found suggestive evidence for both types of diet-gene interaction. Although meat consumption and preparation appear not to be associated with risk of HNPCC adenomas, the rationale for an association with (other) dietary factors in all phases of HNPCC colorectal carcinogenesis remains. Our finding of associations between dietary factors of animal origin and specific K-ras and p53 mutations needs confirmation.
As our studies are of limited size, they have not provided scientific proof of these associations, in terms of statistical significance at $p<0.05$. Therefore, no definite conclusions will be drawn from these studies with respect to the studied diet-gene interactions. The limited body of empirical knowledge in this field however, together with our own results suggest that further research may indeed provide clues to the etiology of colorectal carcinogenesis. Moreover, our results may serve as a basis for new hypothesis to be tested in future, larger, studies.

Future studies

- A substantial role for dietary factors in the etiology of sporadic colorectal carcinogenesis is already well established. However, we not only need to know what the attributable risk in the general population is. We may also want to know whether a relevant proportion of the colorectal carcinomas in a high risk group such as HNPCC families, is preventable by dietary changes.
- Evidence for a possible role of environmental factors in the etiology of HNPCC, will be gained from currently conducted and future studies addressing the spectrum of genes, such as TGFβ-RII, IGF-RII, BAX, APC, K-ras, p53. The frequencies and type of mutations in these genes and the stage in HNPCC carcinogenesis in which they occur, may provide additional links to which environmental factors are relevant.
- Most promising, in directly studying dietary risk factors in HNPCC, may prove to be dietary intervention studies. A very large contrast of exposure to a specific dietary factor will probably be needed to discern the risk associated with this dietary factor from the high background risk due to mismatch repair deficiency. Studies on colorectal adenomas are likely to be the most appropriate stage to study, as the effects of the genetic instability may increase from there onwards. Controlled trials are currently being initiated in Europe, to study the protective effect of resistant starch and aspirin in HNPCC families, i.e. the Concerted Action Polyposis Prevention (CAPP)-studies [193].

- A potential source of evidence regarding effects of a specific dietary factor on the molecular level, lies in analysis of mutations in multiple genes in archival tissue of

ongoing or already conducted large cohort and intervention studies with primary or recurrent adenomas as endpoints. With new rapid and efficient methods for mutation analysis in view (e.g. gene chips), multiple genes may be scanned for mutations in large series of tissue samples. Our case control studies into colorectal adenomas and carcinomas have already been extended to include mutations in the APC gene.

- The use of early biomarkers of cancer risk in such studies may help to disentangle the "molecular effect" of a specific dietary factor. With respect to potential early endpoints of both sporadic and HNPCC colorectal carcinogenesis, processes that occur even before gene mutation may be relevant. (Epi)genetic changes such as methylation of specific genes (e.g. mismatch repair genes, oestrogen receptor, insulin growth factor) and DNA damage due to oxidative stress, might be influenced by diet. However, their predictive value should first be evaluated.
- Such studies on a molecular level should be conducted in sporadic colorectal cancer, however, studies in HNPCC colorectal adenomas and carcinomas may also provide evidence which could be extended to sporadic colorectal carcinogenesis.

- Meat consumption and preparation was the main dietary exposure of interest in part of this thesis. Future studies with the aim to investigate the causal agent(s) in meat with respect to the etiology of colorectal cancer, need to assess: (1) preparation methods using specially designed questionnaires, (2) exposure to specific heterocyclic amines, if possible using biomarkers such as DNA adducts or urine metabolites, and (3) exposure to other possible (pro)carcinogens related to meat consumption, e.g. N-nitroso-compounds and iron.
- Accounting for genetic polymorphisms of metabolizing enzymes, may serve to discriminate between groups with a genetic susceptibility to DNA damage caused by environmental factors such as meat consumption and preparation. Our case control study on sporadic colorectal cancer has already been extended into this direction.

CONCLUDING, future studies may clarify the role of environmental factors in the etiology of monogenetic traits such as HNPCC. Based on our results, it should be doubted whether HNPCC is a purely hereditary disease, and the search for environmental causes should be continued. Moreover, further studies in the field of gene-environment interactions in colorectal cancer are needed to disentangle the effects of diet in all molecular phases of the adenoma carcinoma sequence.

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SUMMARY

Cancer is a genetic disease, requiring accumulation of alterations in regulatory genes for a tumor to develop. A familial component in colorectal carcinogenesis is suggested by an increased risk for persons with a positive family history of colorectal cancer. Hereditary factors are clearly present in two autosomal dominantly inherited syndromes (i.e. Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC)), for which the genetic basis has now been discovered.

Although cancer is known as a disease of the genes, environmental factors are also suggested to play a major role. For many decades diet is suggested to be an important risk factor for colorectal cancer and for colorectal adenoma as its precursor lesion. However, the mechanisms by which specific dietary components affect carcinogenesis remain unclear. Studies of diet-gene interactions may provide further clues to the etiology of colorectal carcinogenesis.

Diet-gene interactions may not only be relevant in sporadic but also in hereditary forms of colorectal cancer. Since the development of adenomas in HNPCC patients has characteristics in common with adonoma development in sporadic patients, adenomas in HNPCC patients may also be related to dietary factors. Additionally, these adenomas may serve as a model in studies of sporadic colorectal carcinogenesis. HNPCC adenomas are usually diagnosed at a younger age and recurrences occur over a shorter time interval than in sporadic adenoma patients. Therefore, diet-gene interactions may be more efficiently studied in HNPCC family members.

This thesis examines diet-gene interactions in the etiology of colorectal cancer from two different epidemiological perspectives (as in AIM 1 & 2).

CHAPTER 1 introduces some of the basic concepts in the etiology of colorectal cancer. Colorectal cancer is described as a genetic disease, which develops through the adenoma-carcinoma sequence, by accumulating alterations in regulatory genes such as APC, K-ras, p53, and others. These alterations may be caused by exogenous factors as well as endogenous processes. Hereditary Non-Polyposis Colorectal Cancer (HNPCC), has its genetic basis in a germline defect in the mismatch repair system, but also has characteristics in common with sporadic colorectal carcinogenesis and may well share similar molecular pathways with sporadic colorectal cancer. Some of the acquired genetic alterations may be induced by dietary factors and alternatively, altered gene products may function differently depending on diet. Diets high in meat and fat have long been thought to increase the risk of both colorectal adenomas and carcinomas, but the evidence is inconsistent. Some experimental studies in animals suggest that factors relating to meat consumption and preparation, such as heterocyclic amines, may cause

mutations in regulatory genes. The fact that such mutations may occur both in sporadic and HNPCC colorectal carcinogenesis, was an important consideration to study diet-gene interactions in the general population as well as in HNPCC families.

AIM 1: *To examine evidence for a role of dietary risk factors in the etiology of hereditary non-polyposis colorectal carcinogenesis; are some aspects similar to the etiology of sporadic colorectal cancer?*

In CHAPTER 2 we assessed the cumulative incidence of colorectal cancer in different age categories of family members from 51 HNPCC families selected according to the "Amsterdam criteria", in order to quantify lifetime risk. In this descriptive epidemiological study the cumulative incidence at age 75 increased from 19% in the ancestors, to 32% in the first generation and 55% in the second generation, which is largely attributable to a birth-cohort effect. The earlier age of onset in successive HNPCC generations thus appears not to be a biological feature of HNPCC, but reflects a time trend in cancer occurrence which is similar to trends in the general population.

To examine whether HNPCC and sporadic adenomas may represent similar molecular pathways to cancer, we investigated the frequency of mutations in codons 12 and 13 of the K-ras gene, and overexpression of the p53 protein in these adenomas (CHAPTER 3). The K-ras and p53 genes are both commonly mutated genes in colorectal carcinogenesis, and may be influenced by exogenous or endogenous factors. In 48 HNPCC adenomas and 59 sporadic adenomas the frequency of abnormalities in these genes were similar: 25% and 32% respectively for K-ras, and 25% and 31% respectively for p53. When taking adenoma size into account, the frequencies of abnormalities in HNPCC and sporadic adenomas were even more similar.

In a methodological study performed on data from a Swedish case-control study (CHAPTER 4), we reported limited loss of information regarding heterocyclic amine intake (concordance was 90% and 95% with 15 and 20 of 39 items respectively) when reducing an extensive questionnaire on meat consumption and preparation. This supports the use of relatively short questionnaires on meat consumption and preparation (including photos to assess color of meat surface).

In CHAPTER 5 we focus on differences in the associations of meat consumption and preparation with risk of sporadic and HNPCC colorectal adenomas respectively, using such a questionnaire. In this case-control study of adenoma cases and adenoma-free controls, red meat consumption was positively, but not significantly, associated with the risk of colorectal adenomas in the sporadic population (Odds ratio (OR) 4.1, 95% Confidence Interval (95% CI) 0.7-23.0), but not in the HNPCC population (OR 0.4, 95% CI 0.1-2.2). A preference for a darkly browned meat surface significantly increased the risk of sporadic colorectal adenomas (OR, 95% CI: 3.0, 1.2-7.5). Again this increased risk

was not observed for HNPCC adenomas (0.7, 0.3-1.4). Possibly, factors related to meat consumption and preparation such as heterocyclic amine intake may be relevant only to the etiology of sporadic colorectal adenomas. However, this does not rule out a potential role for these or other dietary factors in other stages of HNPCC colorectal carcinogenesis. Concluding, similar time trends in incidence and similar involvement of K-ras and p53 suggest a role for environmental factors in the etiology of HNPCC colorectal carcinogenesis. However, meat consumption and preparation were not similarly associated with risk of HNPCC and sporadic adenomas.

AIM 2: *To examine evidence for a differential effect of dietary risk factors on K-ras/p53 dependent and independent pathways to colorectal carcinogenesis.*

In CHAPTER 6 we examine whether dietary factors of animal origin are associated with (specific) mutations in the K-ras gene. K-ras codon 12 and 13 mutations were detected in 36% of 185 colon cancers (82% in codon 12) from a previously conducted case-control study on diet and colon cancer. Food groups and nutrients of animal origin were not differently associated with colon cancers with or without K-ras mutations. However, high intakes of (animal) protein and calcium were positively associated with tumours harboring K-ras codon 12 mutations (for protein per 19 g: OR 1.5, 95% CI 1.0-2.1; for calcium per 459 mg: OR 1.2, 95% CI 0.9-1.6), while inversely associated with tumors harboring with codon 13 mutations (for protein: OR 0.4, 95% CI 0.2-1.0; for calcium: OR 0.6, 95% CI 0.3-1.2). Transition and transversion mutations were not differently associated with these dietary factors. These data suggest a different dietary etiology of colon tumors harboring K-ras codon 12 and codon 13 mutations. In combination with a suggested difference in prognosis for cancers with specific K-ras mutations, these results warrants further research. In CHAPTER 7 we concentrated on the question whether dietary risk factors may be differently associated with colon tumours with or without p53 abnormalities, and whether these associations depend on the endpoint used (i.e. mutation vs overexpression). Of 185 colon carcinomas 81 (44%) were categorized as p53 overexpression, whereas 59 of 185 (32%) were found to harbor a mutation in exon 5-8 of the p53 gene. Concordance between these two methods was 72%. Using p53 overexpression as endpoint, an increased risk with high intakes of total fat and saturated fat was observed for colon carcinomas without p53 overexpression only (OR for saturated fat per 16 g, 95% CI: 1.1, 0.8-1.5 with overexpression; 1.5, 1.1-2.0 without overexpression). When cases were classified by mutation analysis, the difference between cases with and without p53 abnormalities was less pronounced (1.2, 0.8-1.6 mutation; 1.3, 1.0-1.8 wildtype). Interestingly, for cases with transversion mutations in the p53 gene, we observed an increased risk for similar dietary factors, in contrast to cases with mutations at CpG sites.

Concluding, both studies on K-ras and p53 in colon cancer suggest dietary factors may be differently associated with tumors harboring specific mutations in these genes. Whether diet has a role in inducing mutations or acts differently depending on the (altered) function of the gene remains to be elucidated.

In CHAPTER 8 we summarize the results of our studies and discuss these in the light of possible biases. The biological plausibility of the studied diet-gene interactions is discussed, endorsed by results from experimental research.
Our studies are among the first to examine diet-gene interactions in colorectal carcinogenesis, with respect to environmental factors in the etiology of HNPCC and molecular abnormalities in the K-ras and p53 genes. The results could therefore be compared to only few other published studies. As there is still little knowledge available on these diet-gene interactions, statistical testing in our studies was accompanied by the acceptance of a larger range of type α-errors. Consequently, our studies have mainly served as a basis for new hypotheses and to expand research infrastructures.
Future studies should continue to aim at clarifying a possible role of environmental factors in HNPCC. Any proportion of cancers preventable by diet is relevant to HNPCC families. Furthermore, HNPCC adenomas may serve as a model to study colorectal carcinogenesis, which may be extended to colorectal carcinogenesis in the general population. To disentangle the effects of diet in all molecular phases of the adenoma-carcinoma sequence, several intermediate molecular biomarkers of cancer risk should be evaluated.

SAMENVATTING

Voor een ieder die geinteresseerd is in het onderzoek beschreven in dit proefschrift.....

Kanker is een ziekte van de genen (ons erfelijk materiaal). Voor het ontstaan van kanker zijn meerdere veranderingen in regulerende genen nodig.
Dikke darmkanker is na long-, maag- en borstkanker, de meest voorkomende vorm van kanker in de wereld. In Nederland wordt ieder jaar bij ongeveer 8000 personen dikke darmkanker vastgesteld. Hoewel dikke darmkanker in bepaalde families vaker lijkt voor te komen, is slechts in ca. 5% van alle gevallen van dikke darmkanker echt sprake van erfelijkheid. HNPCC (Hereditair Non-Polyposis Colorectaal Carcinoom) is zo'n erfelijke vorm van dikke darmkanker. Hoewel kanker dus een ziekte van de genen is, neemt men aan dat leefstijlfactoren een prominente rol spelen. Gedurende tientallen jaren van onderzoek zijn aanwijzingen verkregen dat voedingsgewoonten een belangrijke risicofactor zijn voor het ontstaan van dikke darmkanker en adenomateuze poliepen (adenomen, goedaardige voorlopers van kanker). Het is nog niet bekend via welke mechanismen specifieke voedingscomponenten invloed hebben op het ontstaan van kanker. Bestudering van het samenspel tussen voeding en genen (voeding-gen interacties) kan mogelijk verdere aanwijzingen leveren over het onstaan van dikke darmkanker.
Voeding-gen interacties zijn mogelijk niet alleen relevant voor het onstaan van "gewone" vormen van dikke darmkanker (in het vervolg sporadische dikke darmkanker genoemd), maar ook voor erfelijke vormen van dikke darmkanker (zoals HNPCC). De vorming van adenomen bij patienten uit HNPCC-families vertoont vergelijkbare eigenschappen met de vorming van sporadische adenomen, en wordt daarom misschien ook wel beinvloed door voedingsgewoonten. Adenomen bij HNPCC-familieleden worden gewoonlijk op jongere leeftijd ontdekt en komen eerder terug dan bij sporadische patienten. Om die reden kunnen voeding-gen interacties mogelijk efficienter bestudeerd worden in HNPCC families.
Dit proefschrift onderzoekt voeding-gen interacties tijdens het ontstaan van dikke darmkanker vanuit twee verschillende epidemiologische invalshoeken.

In HOOFDSTUK 1 worden een aantal van de basisbegrippen over het ontstaan van dikke darmkanker geintroduceerd. Dikke darmkanker wordt beschreven als een genetische ziekte. De kanker ontwikkeld zich vanuit een adenoom, doordat een aantal opeenvolgende veranderingen plaatsvindt in regulerende genen zoals APC, K-ras, p53. Deze veranderingen kunnen veroorzaakt worden door factoren van buitenaf (bv. voeding), alsook door processen in het lichaam. HNPCC wordt veroorzaakt door een aangeboren verandering in een reparatiesysteem van het erfelijke materiaal. Ondanks

deze duidelijke aangeboren oorzaak, vertoont de vorming van adenomen in HNPCC en sporadische patienten een aantal overeenkomsten. Mogelijk ontstaan beide vormen van dikke darmkanker op gen-nivo gedeeltelijk op dezelfde manier. Sommige van de verworven (niet aangeboren) beschadigingen aan de genen worden mogelijk veroorzaakt of beinvloedt door voedingsfactoren. Van een voedingspatroon met veel vlees en vet wordt al heel lang gedacht dat dit het risico op het ontstaan van dikke darm adenomen en kanker verhoogd, maar de aanwijzingen zijn niet éénduidig. Een aantal experimentele dierstudies suggereert dat factoren die samenhangen met vleesconsumptie en -bereiding, mogelijk mutaties (beschadigingen) in regulerende genen veroorzaken. Het feit dat dergelijke mutaties ook bij mensen voorkomen, zowel tijdens het ontstaan van sporadische als HNPCC dikke darmkanker, was een belangrijke overweging om voeding-gen interacties te bestuderen in zowel de algemene bevolking als ook in HNPCC families. We zijn bij ons onderzoek naar voeding-gen interacties tijdens het onstaan van dikke darmkanker uitgegaan van twee doelstellingen:

DOELSTELLING 1: *Het onderzoeken van aanwijzingen voor een mogelijke rol van voedingsfactoren in het ontstaan van HNPCC; zijn bepaalde aspecten in de ontstaanswijze vergelijkbaar met het ontstaan van sporadische dikke darmkanker?*

In HOOFDSTUK 2 hebben we het optreden van dikke darmkanker in verschillende leeftijdscategorien van familieleden van 51 HNPCC families berekend. Op de leeftijd van 75 jaar had 19% van de stamoudsten in deze families dikke darmkanker ontwikkeld, terwijl dit 32% was in de eerstvolgende generatie en 55% in de daaropvolgende generatie. Dit verschil tussen generaties was toe te schrijven aan de periode waarin deze personen geboren waren. De jongere leeftijd waarop dikke darmkanker ontstaat in de opeenvolgende generaties van deze HNPCC families, is daarom niet zozeer een biologische eigenschap van HNPCC. Het laat een trend zien in het optreden van kanker die vergelijkbaar is met de tijdtrend die men waarneemt in de algemene bevolking.

Om te onderzoeken of HNPCC en sporadische adenomen op gen-nivo gedeeltelijk op eenzelfde manier ontstaan, hebben we onderzocht hoe vaak afwijkingen in de K-ras en p53 genen voorkomen (HOOFDSTUK 3). Mutaties in de K-ras en p53 genen zijn gebruikelijk bij dikke darmkanker, en worden mogelijk veroorzaakt door factoren van buitenaf of door processen in het lichaam. In de 48 HNPCC adenomen en 59 sporadische adenomen vonden we een vergelijkbaar aantal afwijkingen. Afwijkingen aan het K-ras gen vonden we bij 25% van de HNPCC en 32% van de sporadische adenomen. Bij respectievelijk 25% en 31% vonden we p53 afwijkingen.

In een methodologische studie (HOOFDSTUK 4) hebben we het gebruik van een vragenlijst over vleesconsumptie en -bereiding onderzocht. Hiermee probeert men in te schatten hoeveel de inneming is aan heterocyclische amines (stoffen die onstaan bij de bereiding

van vlees bij hoge temperatuur). We rapporteren een beperkt verlies aan informatie over de inneming van heterocyclische amines, wanneer het aantal vragen in de vragenlijst wordt gehalveerd (van 39 naar 15 of 20). Dit ondersteunt het gebruik van een relatief korte vragenlijst over vleesconsumptie en –bereiding, inclusief foto's om de kleur van het vlees vast te stellen, zoals wij die gebruikt hebben in ons verdere onderzoek bij patienten met dikke darm adenomen.

In HOOFDSTUK 5 richten we ons op de relatie tussen vleesconsumptie en –bereiding en het risico op het ontstaan van respectievelijk sporadische en HNPCC adenomen, gebruikmakend van een dergelijke vragenlijst. In dit zogenaamde patient-controle onderzoek vergelijken we de voedingsgewoonten van personen met dikke darm adenomen en controle-personen zonder adenomen. De consumptie van rood vlees (rund- en varkensvlees, geen kip) leek geassocieerd met het risico op dikke darm adenomen in de sporadische groep, maar niet in de HNPCC groep. Personen met een voorkeur voor donkerbruin vlees hadden vaker dikke darm adenomen, maar weer alleen in de sporadische groep en niet in de HNPCC groep. Mogelijk zijn factoren die gerelateerd zijn aan de consumptie en bereiding van vlees, alleen relevant voor het ontstaan van sporadische adenomen en niet voor HNPCC adenomen. Echter, het blijft mogelijk dat vleesbereiding of andere voedingsfactoren in eerdere of latere fasen van het onstaan van HNPCC dikke darmkanker een rol spelen.

Concluderend, vergelijkbare tijdtrends in het voorkomen van dikke darmkanker en een vergelijkbare betrokkenheid van de K-ras en p53 genen suggereren een rol voor omgevingsfactoren in het ontstaan van HNPCC dikke darmkanker. Echter, vleesconsumptie en –bereiding lijken geen vergelijkbaar effect op het risico op HNPCC en sporadische dikke darm adenomen te hebben.

DOELSTELLING 2: *Het onderzoeken van aanwijzingen voor een mogelijk verschillend effect van voedingsfactoren op het ontstaan van dikke darmkanker met en zonder mutaties in de K-ras en p53 genen.*

We hebben hierbij gebruik gemaakt van eerder uitgevoerd patient-controle onderzoek met dikke darmkanker patienten, van wie de voedingsgegevens al verzameld waren.

In HOOFDSTUK 6 hebben we onderzocht of voedingsfactoren van dierlijke oorsprong geassocieerd zijn met (specifieke) mutaties in het K-ras gen. Mutaties in codon 12 en 13 (verschillende delen van het K-ras gen) werden gevonden in 36% van de 185 dikke darmkankers (82% in codon 12). Bij deze dikke-darmkankerpatienten waren voedselgroepen en voedingsstoffen van dierlijke oorsprong niet verschillend geassocieerd met dikke darmkankers met of zonder een mutatie in het K-ras gen. Echter, we vonden wel dat een hoge inneming van (dierlijk) eiwit en calcium geassocieerd was met een hoger risico op kanker met een mutatie in codon 12 van het K-ras gen. Voor mutaties in

codon 13 van het K-ras gen vonden we het tegenovergestelde. Deze gegevens suggereren een verschillende rol van voeding in het onstaan kanker met mutaties in codon 12 en codon 13 van het K-ras gen.

In HOOFDSTUK 7 hebben we ons geconcentreerd op de vraag of voedingsfactoren verschillend geassocieerd zijn met dikke darmkanker met of zonder afwijkingen aan het p53 gen. Verder wilden we bestuderen of de gevonden associaties afhankelijk zijn van de methode die gebruikt wordt om de afwijkingen waar te nemen (mutatie of overexpressie). We vonden p53 overexpressie in 44% van de 185 dikke darmkankers en een p53 mutatie in 32%. Als overexpressie werd bestudeerd, werd voor personen met een hogere inneming van totaal vet en verzadigd vet een hoger risico op dikke darmkankers zonder overexpressie gevonden. Als de patienten werden ingedeeld op basis van mutaties, was het verschil tussen patienten met en zonder een mutatie minder duidelijk. Alleen zeer specifieke mutaties (transversies) leken wel geassocieerd met dezelfde voedingsfactoren.

Concluderend, deze studies over K-ras en p53 in dikke darmkanker suggereren dat voedingsfactoren mogelijk verschillend geassocieerd zijn met kankers met specifieke mutaties in deze genen. Of voeding een rol speelt in het veroorzaken van mutaties of verschillend werkt afhankelijk van de (eventueel veranderde) functie van het gen moet onderzocht worden. Tevens zou de invloed van vleesconsumptie en –bereiding op het ontstaan van specifieke mutaties verder onderzocht moeten worden.

In HOOFDSTUK 8 vatten we de resultaten van onze studies samen en bediscuseren deze in het licht van mogelijke methodologische beperkingen. De biologische plausibiliteit wordt besproken binnen het kader van de huidige kennis, aangevuld met resultaten uit experimenteel onderzoek.

Aangezien zo weinig bekend is over voeding-gen interacties bij het ontstaan van dikke darmkanker, zijn deze eerste studies van beperkte omvang en daardoor statistisch minder overtuigend. Als gevolg hiervan zijn de studies vooral gebruikt om nieuwe hypothesen te ontwikkelen en de infrastructuur voor verder onderzoek uit te breiden.

Toekomstige studies moeten bevestigen of HNPCC adenomen mogelijk als model kunnen dienen voor het bestuderen van dikke darmkanker, zowel in HNPCC families als de algemene bevolking. De vraag blijft of voeding mogelijk een rol speelt in het ontstaan van erfelijke dikke darmkanker, want zelfs als een klein aantal kankers voorkomen kan worden door voeding, is dat relevant voor HNPCC families. Om de rol van voeding te ontrafelen in alle fasen van de vorming van adenomen en kanker, moet het effect van specifieke voedingsfactoren vaker op gen-nivo geevalueerd worden. Interventie studies, waarbij voor bepaalde tijd de voeding door de onderzoekers wordt vastgesteld en gecontroleerd, bieden hiervoor de beste mogelijkheid.

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