MEAT, SMOKING, ALCOHOL, AND COLORECTAL TUMORS:

THE ROLE OF GENETIC SUSCEPTIBILITY

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MEAT, SMOKING, ALCOHOL, AND COLORECTAL TUMORS:

THE ROLE OF GENETIC SUSCEPTIBILITY

E. W. Tiemersma

Proefschrift

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Stellingen

1. Zoals veel wetenschappelijke bevindingen, is het effect van gen-omgevingsinteracties voor het ontstaan van chronische ziekten aanvankelijk overschat.
   Dit proefschrift.

2. De risicofactoren voor dikke darmpoliepen zijn in belangrijke mate gelijk aan die voor dikke darmkanker.
   Dit proefschrift.

3. Het gezegde 'wie het kleine niet eert, is het grote niet weerd' is ook van toepassing op goed uitgevoerde kleine wetenschappelijke studies ten opzichte van epidemiologische mega-studies met oppervlakkige blootstellingsdata.
   N.a.v. García-Closas et al., *1999; American Journal of Epidemiology 149: 689-692.*

4. De behandeling van patiënten in buitenlandse ziekenhuizen heeft, met het oog op antibiotica-resistentie, ongewenste neveneffecten.
   N.a.v. Toenbreker et al., *1998; Infectieziekten Bulletin 9 (2).*

5. Het internationaal recht wordt onrecht aangedaan doordat rijke landen hun verplichtingen ten aanzien van gezondheidsonderzoek in arme landen niet nakomen.
   N.a.v. Attaran, 1999; Health and Human Rights 4: 26-58.

6. Scouting is in verschillende opzichten goed voor een kind: voor de sociale ontwikkeling en tegen het ontwikkelen van allergieën.
   N.a.v. o.a. Martinez & Holt, 1999; Lancet 354 (Suppl II): 12-15.

7. Vooroordelen tegen asielzoekers bevestigen dat vijf eeuwen na de kettervervolgingen nog steeds geldt dat de inquisiteur de ketter maakt.
   Naar Umberto Eco in "De naam van de roos".

8. Zeker voor schaatsen geldt dat men nooit is uitgeleerd.

Stellingen behorend bij het proefschrift
'Meat, smoking, alcohol, and colorectal tumors: the role of genetic susceptibility'.
Edine Tiemersma
Wageningen, 12 april 2002

Wat je ziet

bepaalt hoe je het ziet,

Hoe je het ziet

bepaalt wat je dan ziet.

Abstract

Colorectal cancer is the second most common cancer in the Western world and is thought to arise mainly from colorectal adenomas. Red meat and alcohol intake and (long-term) cigarette smoking probably increase colorectal tumor risk. Although risk increase was found to be weak, certain subgroups might be more susceptible to these carcinogens because of inherited polymorphisms resulting in increased activation of potential carcinogens. In this thesis, we investigated whether meat consumption, cigarette smoking, and alcohol intake, in combination with such genetic polymorphisms, increase the risk of colorectal adenomas and colorectal cancer.

For this purpose, we used two different study populations. One was an adenoma case-control study with 440 adenoma cases and 447 polyp-free controls recruited among those undergoing endoscopy at eight Dutch outpatient clinics between June 1997 and June 2000. The other was a Dutch prospective cohort enrolled between January 1987 and December 1991. Follow-up for 8.5 years resulted in 102 colorectal cancer cases. We compared these cases with a random sample of 537 controls frequency-matched on age, sex and town. Information on dietary and lifestyle habits was collected through self-administered questionnaires. DNA was isolated from whole blood and genetic polymorphisms were subsequently determined by use of standardized methods.

We found that colorectal *adenoma* risk was not increased with high meat consumption (OR 1.2, 95% CI 0.8-1.9) or unfavorable meat preparation methods. These null-associations were not modified by genetic polymorphisms affecting metabolism of heterocyclic amines that may be formed during preparation of meat at high temperatures (*N*-acetyltransferases (*NAT*) 1 and 2, sulfotransferase (*SULT*) *1A1*, and glutathione *S*-transferases (*GST*) *M1* and *T1*). Long-term cigarette smoking increased adenoma risk (OR 2.4, 95% CI 1.4-4.1 for smoking for more than 25 years compared to never smokers). Although most pronounced in those with fast *SULT1A1* (OR 4.3, 95% CI 1.6-11.8) and slow *NAT2* variants (OR 3.5, 95% CI 1.9-6.4), there was no statistically significant effect modification by genetic polymorphisms involved in metabolism of arylamines and polycyclic aromatic hydrocarbons from cigarette smoke (*NAT1*, *NAT2*, *SULT1A1*, *GSTM1*, *GSTT1*, and epoxide hydrolase). Alcohol consumption increased colorectal adenoma risk especially among women (OR 1.8, 95% CI 1.0-3.2 for 10 or more drinks weekly versus less than one drink per week). Among men, adenoma risk increased only with consumption of more than 21 drinks per week (OR 1.8, 95% CI 0.9-3.8). Alcohol is metabolized to carcinogenic acetaldehyde by alcohol dehydrogenase (ADH3). The association between alcohol and adenomas was weakly - but not statistically significantly - stronger among those with the fast *ADH3* variant compared to those with imputed slow phenotypes.

Colorectal *cancer* risk increased slightly with frequent red meat consumption (OR 1.6, 95% CI 0.9-2.9, highest *vs.* lowest intake). Genetic polymorphisms in *NAT1*, *NAT2*, and *GSTM1* did not importantly modify this association. Risk of colorectal cancer was increased with smoking duration, but only among former smokers having smoked for more than 15 years (OR 2.7, 95% CI 1.0-7.4) compared to former smokers having smoked for shorter time. *NAT1*, *NAT2*, and *GSTM1* polymorphisms did not influence this association.

In summary, the results of our studies do not point toward strong modifying effects of genetic polymorphisms of enzymes involved in carcinogen metabolism, which is in accordance with results of similar studies on colorectal tumors. Such effects may however be present, but possibly, we were not able to demonstrate them. To elucidate the potential role of genetic susceptibility in colorectal carcinogenesis, alternative epidemiologic study designs and statistical methods should be considered. These observational studies should be conducted simoultaneously with experimental studies aiming to generate more biological knowledge on the diverse processes leading to colorectal tumorigenesis in humans.

Contents

List of abbreviations

AαC	2-amino-9H-pyrido[2,2-*b*]indoline
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
ASO	allele specific oligo hybridization
BMI	body mass index
bp	base pairs
CI	confidence interval
DiMeIQx	2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline
EPHX	microsomal epoxide hydrolase (gene)
GST	glutathione *S*-transferase
HCA	heterocyclic aromatic amine
IQ	2-amino-3-methylimidazo[4,5-*f*]quinoline
mEH	microsomal epoxide hydrolase (enzyme)
MeIQx	2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
NAT	*N*-acetyltransferase
NSAID	non-steroidal anti-inflammatory drugs
OLA	oligonucleotide ligation assay
OR	odds ratio
PAH	polycyclic aromatic hydrocarbon
PCR	polymerase chain reaction
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
RERI	excess risk due to interaction
RFLP	restriction fragment length polymorphism
RLB	reverse line blot
RR	relative risk
SULT	sulfotransferase
TriMeIQx	2-amino-3,4,7,8-tetramethyl-3H-imidazo[4,5-*f*]quinoxaline
UV	ultraviolet

1.

General introduction

All over the world, cancer is a major cause of death and in most Western societies, it is only exceeded by cardiovascular diseases. In 1996, 10 million new cases and more than 7 million deaths from cancer were estimated globally [1]. The prevalence rates of specific cancers, among which colorectal cancer, vary highly over the world [2]. This variation is to a large extent caused by variation in environmental exposure to dietary and lifestyle factors [1].

Colorectal cancer is after prostate cancer in men and breast cancer in women the second most common cancer in developed countries [2] and is thought to arise almost uniquely from colorectal adenomas [3]. It is estimated that less than 10% of all colorectal cancers can be attributed to inheritance of rare and highly penetrant genetic mutations causing cancer in about 90% of affected offspring at a relatively young age [4]. In the majority of colorectal cancers, however, genetic factors probably only determine the impact of the exposure to carcinogenic and protective environmental substances on cancer risk [5].

Environmental exposure

The colorectal epithelium is exposed to many substances from the environment, of which most originate from the diet. Some of these substances may increase colorectal cancer risk, whereas others might be protective. So far, it is largely unknown which factors of this complex mix of factors influencing carcinogenesis at different stages, are important [6,7].

Thus, dietary factors are thought to be important in the etiology of colorectal cancer, although only weak associations were found for all factors that possibly influence colorectal cancer risk [1]. In this respect, there is most evidence that vegetables decrease the risk of colorectal neoplasm, possibly through their high content of potential anti-carcinogenic substances such as dietary fiber, folate and other vitamins, or their association with a healthy lifestyle [1]. Other factors probably decreasing colorectal cancer risk are a high level of physical activity and intake of non-steroidal anti-inflammatory drugs [7]. In this thesis, however, we focus on risk factors of colorectal cancer rather than on protective factors.

Factors that probably increase the risk of colorectal cancer are the consumption of red meat and of alcohol [1]. Further, evidence is accumulating that (long-term) cigarette smoking may be a risk factor of colorectal cancer [8].

To date, it is not known which substances in meat may cause an increase in colorectal neoplasm risk. Animal fat [9] and heme [10] are candidate substances. Potential carcinogens formed during meat processing and preparation may also be responsible, as both processed and well-done meats were found to increase colorectal neoplasm risk in epidemiological studies [11-17]. The preparation of meat at temperatures of above 150°C leads to formation of heterocyclic aromatic amines (HCAs) by pyrolysis of proteins [18,19].

HCAs were found to be potent animal carcinogens and are possibly carcinogenic to humans even at low doses [20].

Cigarette smoke contains many potential carcinogens, among which polycyclic aromatic hydrocarbons (PAHs) and arylamines [21]. The colon and rectum can become exposed when smoke is ingested, or more indirectly, through transport of potential carcinogens by blood or bile via the liver or otherwise, to the colon [22,23].

Alcohol may stimulate tumor promotion and/or progression via co-carcinogenesis, and may also induce DNA hypomethylation [24]. The main evidence for ethanol as risk factor of colorectal carcinogenesis points to its main metabolite, acetaldehyde, a probable carcinogen found to cause various types of DNA damage in metabolic and in animal experiments [25].

Although these factors have been identified as probable risk factors of colorectal cancer, estimated relative risks generally vary between one and two [1,8,12]. This indicates that if (red) meat consumption, cigarette smoking and alcohol intake increase cancer risk, their effect is expected to be small. However, the risk of colorectal cancer resulting from exposure to these substances may be higher within certain subgroups being more susceptible to specific carcinogens than the general population.

Metabolism of carcinogens

Most carcinogens need metabolic activation in the human body before they can cause DNA damage, and thus, possibly increase cancer risk. This implicates that the metabolism of these potential carcinogens is crucial with respect to cancer risk. The metabolism of many carcinogens involves a primary modification step catalyzed by so-called phase I enzymes. This first step results in metabolites with functional chemical groups, determining the further pathway of the metabolite. Some of these intermediate metabolites can form DNA and protein adducts. After the phase I reaction, conjugation reactions catalyzed by phase II enzymes take place, in which the metabolite is inactivated, or alternatively, is further activated.

The extent to which potential carcinogens become activated or detoxified depends on the (genetically determined) properties of metabolic enzymes and determines the individuals' susceptibility to environmentally induced cancer. This concept is illustrated in figure 1.1.

HCA metabolism starts with activation by cytochrome P450 enzymes (mainly by CYP1A2), after which HCA metabolites are further activated or are inactivated through phase II reactions. Both activation and detoxification can be catalyzed by *N*-acetyltransferases (NATs) and sulfotransferases (SULTs) [26]. Glutathione *S*-transferases (GSTs) may directly inactivate HCA metabolites [27] but may also play a more indirect role in HCA metabolism, via the induction of the CYP1A2 [28].

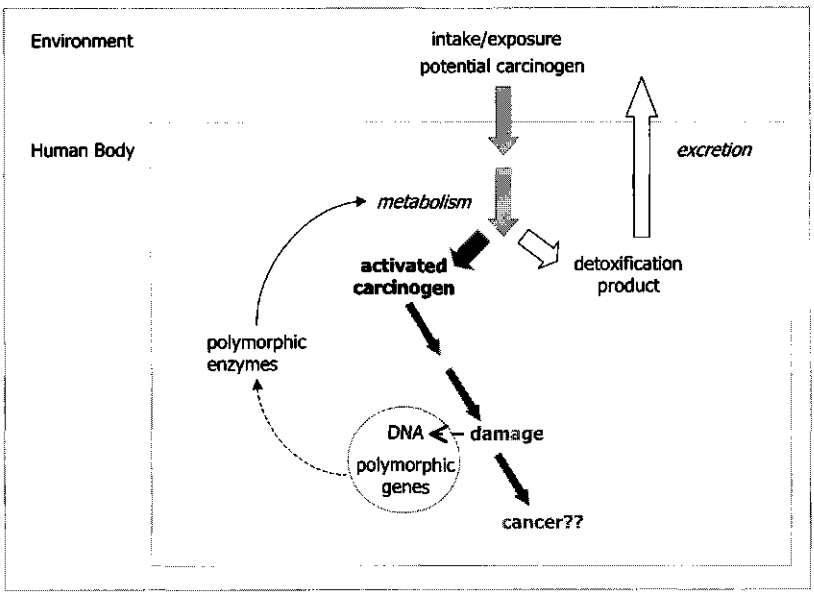

Figure 1.1. Simplified metabolism of environmental carcinogens in relation to genetic susceptibility by polymorphisms genes encoding metabolic enzymes. Gray arrows indicate events that potentially increase risk of neoplasm.

Arylamines from cigarette smoke are metabolized via similar pathways as HCAs, although the metabolites produced from arylamines may differ from those formed from HCAs [29]. The metabolism of arylamines and HCAs is, in simplified form, depicted in figure 1.2. PAH metabolism occurs in a similar way as HCA and arylamine metabolism and involves a first activation step catalyzed by CYP enzymes, after which further activation or detoxification is catalyzed by several phase II enzymes such as GSTs and SULTs. Microsomal epoxide hydrolase (encoded by the *EPHX* gene) catalyzes phase I and phase II reactions and may activate or inactivate PAH metabolites [30,31].

Acetaldehyde is formed through dehydrogenation of ethanol by alcohol dehydrogenase enzymes, of which ADH-γ is encoded by *ADH3*. Acetaldehyde can subsequently be detoxified by aldehyde dehydrogenase.

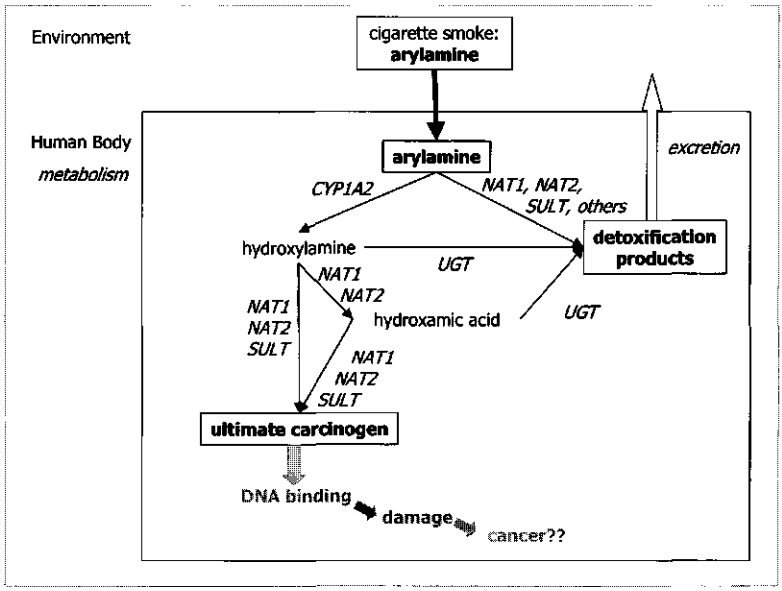

Figure 1.2. Metabolism of arylamines. Simplified from Grant et al. (1997) [26]. UGT: UDP-glucuronosyltransferase.

Genetic susceptibility

In the context of this thesis, the term 'genetic susceptibility' is used for an underlying genetic polymorphism causing carcinogens from the environment to be metabolized at reduced or increased rate, thereby altering exposure and thus formation of DNA damage and risk of neoplasm. Genetic polymorphisms result in altered gene expression or in protein variants with different activity, stability or substrate affinity. Polymorphisms occur at high frequency in populations and are associated with a low absolute cancer risk, but because they are highly prevalent, they could involve a high population attributable risk [5].

Many of the enzymes involved in the metabolism of HCAs, arylamines, PAHs, and ethanol are encoded by polymorphic genes. For most of these genes, tight correlations between the specific genotypes and their corresponding phenotypes were found.

N-acetyltransferase enzymes are encoded by *NAT1* and *NAT2* genes. For *NAT2*, the correlation between genotype and phenotype is well established [32]. In comparison with *NAT2*4* and *NAT2*12* alleles, *in vitro* studies showed that several other allelic variants code for enzymes with reduced stability or affinity, or do not result in a protein at

all [26,32,33]. Most of these so-called 'slow' alleles occur at relatively high frequencies [26,34], so that slow acetylation occurs in more than 50% of Caucasians [35].

In contrast to *NAT2*, there has been debate about the correlation between *NAT1* genotype and the corresponding phenotype [33]. Compared to *NAT1*4* encoded enzymes, it was initially thought that the *NAT1*10* allele was associated with increased acetylation activity [36]. However, more recent studies indicate that the activity of the *NAT1*10* encoded enzyme is similar to 'normal' NAT1 acetylation, whereas *NAT1*11* is related to decreased enzyme activity [37]. The frequency of the *NAT1*11* allele – which occurs most frequently of all slow acetylation alleles, is, however, lower (5%) than that of the **10* allele (20%) [38,38], and this has consequences for the sample size required to be able to detect effects of *NAT1* polymorphism.

Sulfotransferases are encoded by various genes, of which *SULT1A1* is highly expressed in the liver. This gene was found to harbor a polymorphism leading to variation in enzyme activity [39]. The *SULT1A1*2* allele leads to substantially decreased enzyme activity and thermostability and occurs with a frequency of about 35% in Caucasian populations [40].

The *GSTM1* polymorphism is determined by a gene deletion, and the GST-μ enzyme is not expressed in subjects who are homozygous for the null allele [41]. The *GSTM1* null genotype occurs in about half of Caucasians [42].

As *GSTM1*, a polymorphism in the *GSTT1* gene also leads to absence of the encoded enzyme. *GSTT1* gene deletion occurs at lower frequencies of about 15-20% in Caucasian populations [42].

Lifestyle and colorectal neoplasm: possible role of genetic susceptibility

There are three main reasons why information about metabolic polymorphisms should be incorporated into cancer epidemiology [43]. First, the identification of subpopulations susceptible to a certain environmental factor known to increase cancer risk would increase the power of epidemiological studies. Second, incorporation of polymorphisms in genes involved in the metabolism of a certain agent thought to increase cancer risk may strenghten the evidence for that agent, if these polymorphisms indeed modify the association between the agent and cancer. Third, the study of metabolic polymorphisms may help in setting tolerance limits for theoretically risky low-level exposures, for which individual susceptibility should be considered.

The impact of genetic susceptibility on the association between environmental exposure and cancer can only be studied if there is some evidence that 1) the genetic polymorphism is related to altered enzyme expression or function; 2) the gene codes for an enzyme that is relevant in activation or deactivation pathways; 3) the enzyme

catalyzes biotransformation of (a component of) the studied exposure factor; and 4) the studied exposure factor is associated with increased risk of neoplasm [43].

The interplay between genetic susceptibility and environmental exposure is often referred to as gene-environment interaction. However, we prefer not to use this term since this might incorrectly suggest presence of statistical interaction, whereas the term refers to biological co-action.

As can be concluded from the above, genetic polymorphisms influencing the metabolism of potential carcinogens from the diet or from cigarette smoke may be important factors in environmentally determined colorectal neoplasm. Table 1.1 summarizes published studies in which the impact of genetic susceptibility to HCAs, arylamines, PAHs and ethanol on the association of meat consumption, smoking and alcohol intake with colorectal neoplasm were investigated.

Only few studies incorporating metabolic polymorphisms had been published by 1995 (table 1.1), when the studies described in this thesis were initiated. Most of those studies included only small populations, but were nevertheless indicative for a role of polymorphisms in metabolic genes in environmentally induced colorectal neoplasm [44,45], thereby warranting the initiation of larger studies. In later years, both study size and the number of metabolic polymorphisms that was included increased.

However, we conclude from table 1.1 that the studies incorporating metabolic polymorphisms have yielded inconsistent results so far. Results from studies on meat consumption and preparation did not give evidence for a role of *GSTM1* genotype. The effect of *NAT2* polymorphism, if any, was only small, indicating that high meat consumption and/or high-risk meat preparation in combination with fast *NAT2* acetylation might modestly increase colorectal neoplasm risk. Only one study incorporated *NAT1* and the two studies incorporating *EPHX* polymorphisms yielded opposite results. Indications for *GSTM1* genotype to weakly influence smoking-associated colorectal neoplasm risk were found in three out of six studies. *GSTT1* genotype was not found to be important. *NAT2* polymorphism might modestly influence the association between smoking and colorectal neoplasm, but the studies conducted so far provide no consistence about which of the imputed phenotypes increases colorectal neoplasm risk. Again, results on *EPHX* polymorphisms as presented from two studies, were contradictory (table 1.1). The only study investigating the potential role of *ADH3* in the association of alcohol consumption with colorectal cancer found a weak indication that those with the *ADH3* slow imputed phenotype might be at increased risk of colorectal cancer [46].

Table 1.1. Environmental exposure and genetic susceptibility in colorectal neoplasm: overview of epidemiological studies investigating the combinations also investigated in our studies and included in this thesis.

Reference	end point	environmental exposure	genetic susceptibility	high-risk group	reference group	risk estimate (95% CI)	P(int) [a]
Meat consumption and preparation (Chapters 4 and 7)							
Lang [44]	adenomas & cancer	well-done meat preference (y/n)	NAT2 [b] CYP1A2 [b]	well-done meat & fast NAT2 + fast CYP1A2	no well-done meat & slow NAT2 + slow CYP1A2	OR 6.5 (CI not given)	n.g. [c]
Roberts-Thomson 1996 [47]	adenomas & cancer	total meat (amount)	NAT2 [b]	high meat intake & fast NAT2	low meat intake & fast NAT2 [d]	OR 3.5 (1.0-13.1)	n.g.
Welfare 1997 [48]	cancer	fried meat (frequency)	*NAT2*	frequent fried meat intake & fast *NAT2*	unclear (all other combinations of fried meat and *NAT2*?)	OR 6.0 (1.6-26)	n.g.
Chen 1998 [49]	cancer	red meat (frequency)	*NAT1*	high red meat intake & fast *NAT1* [e]	low intake & fast *NAT1* [d]	RR 1.7 (0.8-3.8)	0.19
			NAT2	high red meat intake & fast *NAT2*	low intake & fast *NAT2* [d]	RR 1.5 (0.6-3.6)	0.56
				high red meat intake & fast *NAT1* + fast *NAT2*	low intake & fast *NAT1* + fast *NAT2* combinations [d]	RR 2.4 (0.8-7.1)	0.16
Gertig 1998 [50]	cancer	red meat (frequency)	*GSTM1*	frequent red meat intake & *GSTM1* null	low red meat intake & *GSTM1* present	no effect	n.a. [f]
			GSTT1	frequent red meat intake & *GSTT1* null	low red meat intake & *GSTT1* present		
Kampman 1999 [51]	cancer	mutagen index processed meat (frequency)	*NAT2*	high mutagen index & *NAT2* intermediate/fast	low mutagen index & slow *NAT2*	OR 1.3 (1.0-1.7)	n.s. [g]
			GSTM1	high processed meat intake & intermediate/fast *NAT2*	low processed meat intake & slow *NAT2*	OR 1.5 (1.1-2.0) no effect of *GSTM1*	n.s.
Le Marchand 2001 [52]	cancer	well-done meat (preference)	*NAT2*, CYP1A2 [b]	well-done meat intake & *NAT2* fast + CYP1A2 fast	no well-done meat intake & *NAT2* intermediate/slow + CYP1A2 slow	OR 3.3 (1.3-8.1)	0.12
				ever smokers & well-done meat intake & *NAT2* fast + CYP1A2 fast	never smokers & no well-done meat intake & *NAT2* intermediate/slow + CYP1A2 slow	OR 8.8 (1.7-44.9) no effect of *NAT1*, *NAT2*	0.01 n.s
Ulrich 2001 [53]	adenomas	fried meat (frequency)	*EPHX* exon 3 + 4	high fried meat intake & very slow *EPHX* exon 3+4	low fried meat intake & 'normal' *EPHX* exon 3+4	OR 2.6 (1.1-6.2)	n.s.
				high fried meat intake & slow *EPHX* exon 3	low fried meat intake & fast *EPHX* exon 3	OR 3.3 (1.4-7.9) no effect of *EPHX* ex4	n.s.
Cortessis 2001 [54]	adenomas	well-done meat (frequent intake y/n)	*EPHX* exon 3 + 4	frequent well done meat intake & high stability *EPHX* exon 3+4 (~fast) among never smokers	no well done meat & low stability *EPHX* exon 3+4 (~slow) among never smokers	OR 2.5 (0.99-6.2)	0.03

Cigarette smoking (Chapters 5 and 7)							
Lin 1995 [55]	adenomas	smoking status	*GSTM1*	current smoking & *GSTM1* null	never smoking & *GSTM1* present	OR 2.1 (1.1-3.8)	0.30
Probst-Hensch 1995 [56]	adenomas	smoking status	*NAT2* *GSTM1*	current smoking & fast *NAT2* current smoking & fast *NAT2* + *GSTM1* null	never smoking & slow *NAT2* current smoking & slow *NAT2* + *GSTM1* present	OR 2.3 (1.0-5.1) OR 10.3 (1.9-55)	n.g.
Katoh 1996 [57]	cancer	smoking status pack-years	*GSTM1* *GSTT1*	cigarette exposure level & *GSTM1* null cigarette exposure level & *GSTT1* null	same exposure level & *GSTM1* present same exposure level & *GSTT1* present	no effect	n.a.
Welfare 1997 [48]	cancer	smoking status	*NAT2*	smoking in past 5 years & slow *NAT2*	unclear (all other combinations of exposure and *NAT2*?)	OR 1.9 (1.0-3.5)	n.g.
Gertig 1998 [50]	cancer	smoking status pack-years age start smoking	*GSTM1* *GSTT1*	current smoking/ high dose & *GSTM1* null current smoking/ high dose & *GSTT1* null	never/non smoking/ low dose & *GSTM1* present never/non smoking/ low dose & *GSTT1* present	no effect	n.a.
Slattery 1998 [58]	cancer	smoking status dose duration time since quit	*NAT2* *GSTM1*	current smoking / high dose / quit recently & intermediate/fast *NAT2* women quit smoking since 5-14 yrs & intermediate/ fast *NAT2*	never smoking & slow *NAT2* never smoking women & slow *NAT2*	no effect of most combinations OR 2.5 (1.4-4.4) no effect of *GSTM1*	 n.g.
Potter 1999 [59]	adenomas	smoking status	*NAT2*	current smoking & *NAT2* intermediate/fast	never smoking & slow *NAT2*	OR 2.3 (1.4-3.9)	0.87
Katoh 2000 [60]	cancer	smoking status pack-years	*NAT1* *NAT2*	high dose & fast *NAT1* † high dose & slow *NAT2*	never smoking & slow *NAT1* never smoking & fast *NAT2*	no effect	n.a.
Inoue 2001 [61]	adenomas	smoke years [h]	*GSTM1*	high dose & *GSTM1* null	never smoking & *GSTM1* present	OR 4.0 (1.0-10.7)	n.g.
Ulrich 2001 [53]	adenomas	pack-years	*EPHX* exon 3 + 4	high dose & very slow *EPHX* exon 3+4 high dose & slow *EPHX* exon 3	never smoking & 'normal' *EPHX* exon 3+4 never smoking & fast *EPHX* exon 3	OR 7.7 (1.9-30.9) OR 4.9 (1.9-12.8) no effect *EPHX* exon 4	n.s. n.s.
Cortessis 2001 [54]	adenomas	smoking status	*EPHX* exon 3 + 4	current smoking & high stability *EPHX* exon 3+4 (~fast)	never smoking & low stability *EPHX* exon 3+4 (~slow)	OR 4.3 (1.7-10.8)	0.11
Alcohol consumption (Chapter 6)							
Chen 2001 [46]	cancer	alcohol (drinks/wk)	*ADH3*	high alcohol intake & *ADH3* slow	low alcohol intake & *ADH3* fast	OR 1.6 (0.8-3.5)	0.06

[a] p(int), p-value for interaction between environmental exposure and genetic susceptibility; [b] phenotype; [c] n.g., not given; [d] stratification by imputed phenotype, i.e., combined effect of imputed phenotype and exposure not examined; [e] fast imputed phenotype for *NAT1*: at least one *NAT1*10* allele present; [f] n.a., not applicable; [g] n.s., exact p-value not given, but not statistically significant; [h] defined as the number of cigarettes smoked per year x the number of years smoked.

Rationale and aim of thesis

Our studies are focused on exposure to potential carcinogens, especially from meat and cigarette smoke, but also from alcohol. Of these potential carcinogens, relatively much is known about metabolic pathways, polymorphisms in genes coding for important metabolizing enzymes, and phenotype-genotype correlations. As the exposure to HCAs, arylamines, and PAHs is largely determined by cigarette smoking and possibly, the consumption of heavily browned meat, we developed questionnaires to assess cigarette smoking and meat consumption and preparation in detail. To estimate exposure to the main metabolite of ethanol, acetaldehyde, alcohol consumption was also assessed. Genetic susceptibility factors discussed are major genetically polymorphic biotransformation enzymes such as glutathione *S*-transferases, *N*-acetyltransferases, and sulfotransferase. Environmental exposure and genetic susceptibility might both be important during tumor initiation, promotion and progression. Therefore, this thesis discusses the interplay between genetic susceptibility and environmental exposure in several types and stages of neoplasm, i.e. colorectal adenomas and colorectal carcinomas.

The main purpose of the studies described in this thesis was to evaluate whether there is interplay between common genetic polymorphisms encoding metabolic enzymes, and exposure to environmental carcinogens in the etiology of colorectal neoplasm.

Specific questions were:

- Are HCAs present in meat prepared according to Dutch habits, and if so, do metabolic polymorphisms of genes encoding enzymes that may be important in the metabolism of HCAs (i.e., *NAT1*, *NAT2*, *SULT1A1*, *GSTM1*, *GSTT1*) influence the associations of high meat consumption and/or assumed high-risk meat preparation methods with colorectal adenomas (Chapter 4) and/or cancer (Chapter 7)?
- Do polymorphisms in genes that encode enzymes important in the metabolism of carcinogens from cigarette smoke further increase the risk of smoking-associated adenomas (chapter 5) and/or smoking-associated colorectal cancer (Chapter 7)?
- Is there evidence for modification of the association between alcohol consumption and colorectal adenomas by the *ADH3* gene polymorphism (Chapter 6)?

Outline of thesis

Chapter 2 describes the recruitment of cases and controls for the studies on colorectal adenomas. Because the recruitment of such a study population is prone to selection and information bias, methodological and practical issues are discussed.

Chapter 3 illustrates how genetic polymorphisms can be studied efficiently in large epidemiological studies, taking detection of *N*-acetyltransferase 1 and 2 polymorphism as an example.

Chapters 4 through 6 describe the impact of several metabolic polymorphisms on associations of meat consumption and preparation (chapter 4), cigarette smoking (chapter 5), and alcohol intake (chapter 6), with colorectal adenomas (see also the appendix to this thesis for additional analyses). The interplay between genetic polymorphisms and meat consumption and cigarette smoking in association with colorectal carcinomas is discussed in Chapter 7.

Finally, in Chapter 8, the results of the research described in this thesis are critically reviewed and integrated into the current knowledge from epidemiological studies investigating the potential interplay between environmental exposure and metabolic polymorphisms in cancer. Further, the potential for these types of studies in the future, and other possibilities for future research are discussed.

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2.

Case-control study on colorectal adenomas

Recruitment of the study population and methodological considerations

Colorectal adenomas are benign tumors of glandular colorectal epithelium [1] and are generally regarded as precursors of colorectal cancer [2]. Adenomas are highly prevalent in the general population. In European populations, prevalences of around 30% have been reported from the general population, increasing to 50% in populations older than 70 [3]. The main study described in this thesis is a case-control study including colorectal adenoma cases and polyp-free controls (see chapters 3-6). In this chapter, we discuss the advantages and disadvantages of this case-control study.

SAMPLE SIZE

The main aim of this study was to investigate the joint effect of commonly occurring genetic polymorphisms and environmental exposures on the risk of adenomas. Required sample sizes were calculated using the expected frequencies of the co-occurrence of combinations of genetic susceptibility and environmental exposure (in tertiles, Table 2.1).

Table 2.1. Examples of the required number of cases and equal numbers of controls to detect specific odds ratio with a power (1-β) of 90% (two-sided α=0.05).

Determinant	Expected frequency/ prevalence	Detectable odds ratio		
		1.5	2.0	2.5
Genetic polymorphism	0.40	520	178	104
Environmental exposure (tertiles)	0.33	570	190	108
Genetic polymorphism and environmental exposure	0.12	1048	329	177

With a sample size of about 435 cases and an equal number of controls, we were able to detect an odds ratio of 1.84 with a power of 90% [4] for risk of colorectal adenomas among exposed subjects with high genetic susceptibility (see Figure 2.1).

Recruitment of the study population

Our adenoma case-control study was conducted at outpatient clinics of eight Dutch hospitals in the central region of the Netherlands. In the analyses described in this thesis, cases and controls recruited between June 1997 and June 2000 are included. New cases and controls are currently enrolled for additional analyses. The study logo (Figure 2.2) includes the main components of the study: adenomas, genetic susceptibility (incorporated in the polyp's 'hair' as DNA strands), and environmental exposure (i.e. preparation of meat and cigarette smoking).

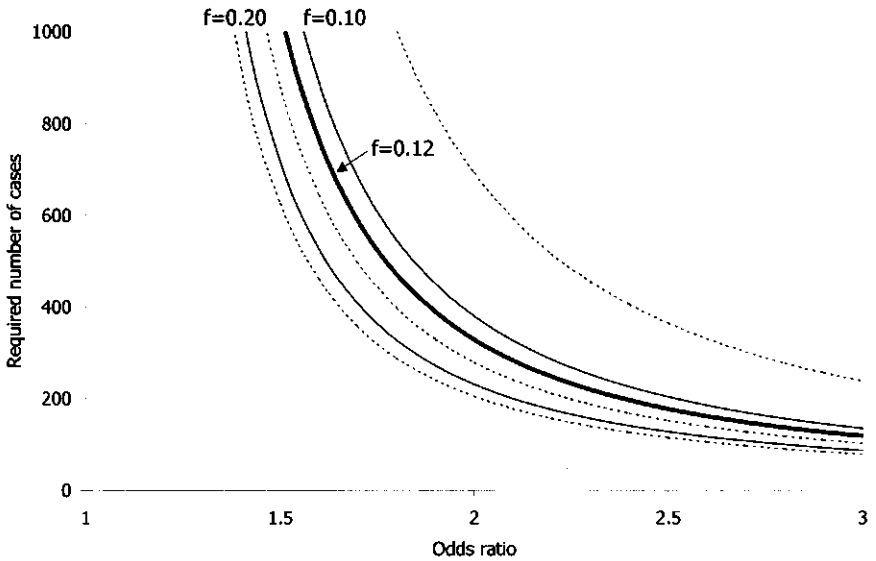

Figure 2.1. Required number of cases (assuming equal numbers of controls) to detect specific odds ratios with a power (1-β) of 90% and a two-sided α of 0.05 at different frequencies (f) of combinations of exposure and genetic susceptibility. Concrete lines depict frequencies of 0.20 and 0.10; dotted lines illustrate frequencies of 0.25, 0.15, and 0.05, respectively. The bold line illustrates required numbers of cases at a combined frequency of 0.12 occurring when exposure is divided in tertiles and a genetic factor is prevalent at a frequency of 0.4.

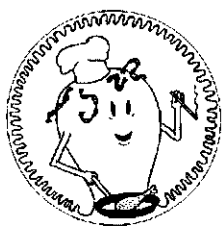

Figure 2.2. Logo of the adenoma case-control study (Dutch name: *POLIEP*-studie)

Definition of cases and controls

We defined cases as those diagnosed with at least one histologically confirmed colorectal adenomatous polyp ever in their life. Controls had no history of any type of polyps, including hyperplastic and metaplastic polyps. Cases and controls were Dutch speaking, of European origin, aged 18 to 75 years at time of endoscopy, were not suspected to have hereditary colorectal cancer syndromes (such as hereditary non-polyposis colorectal cancer, familial adenomatous polyposis coli, Gardners syndrome), did not suffer from chronic inflammatory bowel diseases, and did not have a history of colorectal cancer or (partial) bowel resection.

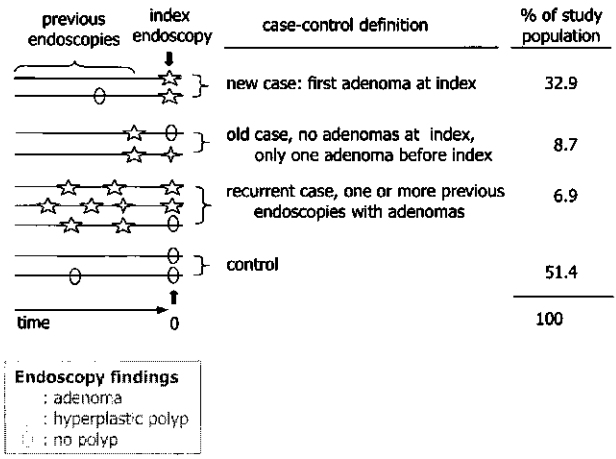

Figure 2.3. Schematic overview of examples of typical cases and controls included in the adenoma study, based on 823 subjects (see text for explanation).

In Figure 2.3, several typical examples of cases and controls included in the study are depicted. The endoscopy leading to invitation is referred to as the index endoscopy, indicated with black vertical arrows in Figure 2.3. Note that results of endoscopies conducted in the year previous to the index endoscopy were considered as if these were the result of the index endoscopy. Thus, if an adenoma was found at one of these occasions the index endoscopy was considered positive and the subject was classified as new a case. This one-year interval between the index endoscopy and possible previous endoscopies was taken because endoscopies conducted within one year mainly serve to check for adequate removal of the initial adenoma(s) rather than to check for recurrent adenomas, for which a control colonoscopy is indicated only after three to six years [5]. Events that happened in the past, thus before the index endoscopy, are depicted left of the index endoscopy. Because negative endoscopies conducted in the past were not recorded, we could not differentiate between 'incident' and 'prevalent' cases, but rather refer to 'new', 'old' and 'recurrent' cases as a summary definition of several subtypes of cases and controls (see Figure 2.3). The definitions shown in Figure 2.3 specify all cases and controls enrolled in our study population. So-called 'new' cases were diagnosed with a first adenoma at most one year before the index endoscopy. 'Old' cases were diagnosed with adenomas more than one year before the index endoscopy and were adenoma-negative at index endoscopy. Recurrent cases, diagnosed with adenomas at

least twice, were also included. Controls could have had polyp-negative endoscopies before, but we did not include subjects who were diagnosed with hyperplastic polyps only. In contrast, cases could have had hyperplastic polyps if they had been diagnosed at least once with an adenoma (Figure 2.3).

RECRUITMENT PROCEDURES

Cases and controls were enrolled at the outpatient clinics of eight hospitals (henceforth numbered 1-8). Recruitment started in June 1997 in two hospitals, and was subsequently extended to the other hospitals. The last hospital entering the study was hospital 8 in December 1998. Cases and controls for our study were recruited among those undergoing endoscopy. The recruitment was conducted in close cooperation with staff of the outpatient endoscopy units of the eight hospitals participating in our study. Therefore, the recruitment procedures slightly differed between hospitals, depending on the preference of the endoscopy staff. The used procedures can roughly be divided into two different methods: a *direct* method, involving recruitment by endoscopy staff, and an *indirect* method, according to which recruitment was carried out by a research nurse reviewing endoscopy reports at three-month intervals. Details of the recruitment procedures in each of the eight hospitals are given in the appendix to this Chapter.

In general, according to the *direct* method, eligible subjects were informed about the study and were invited by endoscopy staff upon endoscopy. If potential participants agreed to participate, they received an information package containing an information brochure, an informed consent form, a short questionnaire (hereafter referred to as the *short questionnaire*), and a stamped addressed envelope. Blood for DNA analyses was subsequently drawn by endoscopy staff or at the hospitals' laboratory. Depending on the hospital-specific procedure (see appendix to this Chapter) the participants either directly were given three detailed self-administered questionnaires, or these were sent to them by mail once the informed consent was received at the research center. The questionnaires were a semi-quantitative food frequency questionnaire, a meat consumption and preparation questionnaire, and a general questionnaire on medical history, family history of colorectal cancer, and several lifestyle factors such as smoking and drug use. Participants were requested to complete the self-administered questionnaires at home and to return these by mail in stamped addressed envelopes.

For the *indirect* method, at three-month intervals, a research nurse reviewed the reports of all endoscopies performed during the previous period. Eligible subjects were invited by a letter from the endoscopist who had conducted the examination. With this letter, they also received the information package (see direct method). After receiving the participants' informed consent and the short questionnaire, an appointment was made for the collection of blood. At this appointment, dietary and lifestyle questionnaires were provided to the participant. The questionnaires were completed at home and were returned to the investigators by mail.

In four hospitals, subjects were recruited according to the direct method, whereas the indirect method was applied in three hospitals. In one hospital, direct was changed to indirect recruitment after approximately a year and a half, when a research nurse took over the recruitment. Of a total of 1140 subjects included by 1. June 2000, 53% was recruited via the direct method.

RESPONSE RATES

After three years of recruitment, 1140 subjects who had undergone endoscopy agreed to participate. The average response for the direct procedure was 83%, whereas it was 44% for the indirect method (Table 2.4). The overall response was 54%. After exclusion of those who did not complete the questionnaires (n=68), who participated twice (n=4; first record was used), for whom medical files were not available (n=37), whom, by retrospection, proved not to meet the inclusion criteria (n=69), and those who were diagnosed with non-adenomatous (n=46) or unknown type of polyps (n=55) only, our study population counted 861 subjects. The study population used for the analyses described in this thesis additionally included 64 subjects meeting our criteria from a preceding study, for which sporadic adenoma cases and polyp-free controls were enrolled in one of the eight hospitals [6] (hospital 1, see appendix to this Chapter for details), so that our final population counted 925 subjects. For the analyses, 38 subjects of whom dietary information was judged insufficient (because of too many blanks or serious inconsistencies in answers) were excluded, reducing the study population to 887 subjects, 440 cases and 447 controls, as well subjects of whom specific information (genotypes, meat preparation methods, etc.) was not available, depending on the specific analyses (see chapters 4-6).

CHARACTERISTICS OF THE STUDY POPULATION

As shown in Table 2.2, cases and controls differed in several respects. The case group consisted of significantly more men than the control group, and cases were older than controls. Consequently, the mean body mass index was higher for cases than for controls. Moreover, the main reasons for endoscopy differed between cases and controls; bowel complaints were more common among controls than among cases, whereas cases more often suffered from rectal bleeding. Rectal bleeding is regarded as an indication for colorectal adenomas as is a history of adenomas (which was the major reason (>90%) for screening among cases). Apart from these two indications, all other indications (i.e., bowel complaints, defecation problems, iron deficiency anemia, and other/unknown) are not considered to be indicative for adenomas [7,8]. By subtraction of the proportion of cases undergoing endoscopy for reasons indicative of adenomas (i.e., rectal bleeding (27%) and screening (39%)) from the total case population, we estimated that for about 44% of the cases, adenomas should be regarded as an incidental finding.

More controls than cases indicated that they had changed their diet because of bowel complaints. Self-reported constipation occurred more frequent among controls than among cases (Table 2.2).

Table 2.2. Background characteristics and medical history of the study population.

Characteristic	Cases (N=440)	Controls (N=447)	p-value [a]
Background			
Female, n (%)	199 (45)	282 (63)	< 0.001
Age (years), mean ± SD	58.9 ± 10.6	50.4 ± 14.1	< 0.001
Low education level, n (%) [b]	148 (33.6)	131 (29.3)	0.17
Body mass index (kg/m2), mean ± SD	26.1 ± 3.9	25.4± 4.1	0.02
Medical history, n (%)			
Endoscopy indication			< 0.001
Rectal bleeding	120 (27.3)	77 (17.2)	
bowel complaints	64 (14.6)	170 (38.0)	
defecation problems	44 (10.0)	95 (21.3)	
screening [c]	173 (39.3)	32 (7.2)	
iron deficiency anemia	21 (4.8)	42 (9.4)	
other or unknown	18 (4.1)	31 (6.9)	
Dietary changes for bowel complaints	80 (18.2)	127 (28.4)	< 0.001
Frequent constipation (>once/month)	43 (9.8)	83 (18.6)	< 0.001
Family history of colorectal cancer	102 (23.2)	85 (19.0)	0.13
Previous diagnosis of polyp [d]	135 (30.7)	0	< 0.001

[a] p-values are calculated by t-test for continuous variables and by chi-square test for categorical variables; [b] primary school and lower vocational training; [c] screening for recurrent adenomas and family history (less than 10% of those screened among cases); [d] including old cases, recurrent cases, non-adenomatous polyps, and 'recurrence' within one year.

Methodological considerations

RATIONALE OF ADENOMA CASE-CONTROL STUDY

We studied colorectal adenomas because colorectal adenoma studies have several advantages above those on colorectal cancer. First, as adenomas are considered precursors of colorectal cancer, epidemiological studies on adenomas might indicate the risk factors important in the early stages of carcinogenesis [3]. Second, in view of the relatively high prevalence of adenomas in the general population [3], the recruitment period of case-control studies can be shorter than for studies considering cancer. Third, dietary and lifestyle habits may be more adequately recalled by adenoma cases than by cancer cases, as these are inquired relatively shortly after the initiation of the disease. Moreover, in contrast to colorectal cancer [9], colorectal adenomas do probably not directly affect dietary and other lifestyle habits. This reduces the chance of recall bias [3].

However, studying adenomas instead of cancer also involves disadvantages. First, not all adenomas will develop into cancerous lesions [10,11]. It is possible that some specific risk factors are determinants only of adenomas that do not develop into carcinomas, as has in the past been proposed for smoking [12,13]. However, colorectal adenoma risk factors are very similar to risk factors of colorectal cancer [14,15]. Second, the choice of an appropriate control group may be debated. Controls can be sampled randomly from the general population, but adenomas are highly prevalent in the general population and often remain asymptomatic [3]. Thus, a population-based control group would include subjects with undetected adenomas. If one wants to exclude those with polyps from the control group, it is required that all controls undergo colonoscopy, resulting in an 'endoscopy-based' control population. Some case-control studies including both a population-based control group and a control group recruited at endoscopy showed no major differences between these two groups [16,17], but another study showed that alcohol consumption and smoking were risk factors for adenomas when cases were compared with endoscopy controls, but not when compared with population-controls [18]. Controls should be sampled from the same population in which the cases arise [19]. This population is called the 'source' or 'base' population [19,20]. In some studies, an endoscopy control group might better reflect this source population than the general population [21,22], provided that the controls seek medical care for similar reasons as the cases and that these reasons are unrelated to the exposures under study [3]. We observed that in the hospitals participating in our study, patients mainly undergo endoscopy because of gastrointestinal complaints, defecation problems, anal bleeding, screening because of adenoma history or a family history of colorectal cancer, or iron deficiency anemia. This means that cases and controls are comparable in the sense that both groups have a history of complaints, and in the sense that their theoretical possibility to be diagnosed with adenomas is equal, which does not apply for the general population.

Precision

Based on the results of power calculations given in Table 2.1, with a study size of 435 cases and an equal number of controls, the power of this study was large enough (i.e., > 90%) to detect potential effects of combinations of genetic polymorphisms and environmental factors. To detect statistical interactions between exposure and genetic susceptibility, however, no main effect of the genetic and the environmental factor is assumed. This assumption was valid for almost all of the genetic polymorphisms, but not for the exposure factors under study, for which risk estimates ranged between 1.2 for daily meat consumption and 2.4 for smoking during more than 25 years (see chapter 4-6). Thus, the power to detect statistically significant interactions between exposure and genetic susceptibility may have been overestimated [23].

Table 2.3. Retrospectively calculated power for the detection of the multiplicative interactions observed between exposure and genetic susceptibility.

Determinant	Exposure frequency/ prevalence	OR for high-risk combination	OR (int) [a]	Power for detection of OR (int) [b]	Detectable OR(int) [c]
Long-term smoking	0.33				
and SULT1A1 fast	0.33*0.74=0.24	4.32	1.25	0.14	2.89
and NAT2 slow	0.33*0.59=0.19	3.43	1.80	0.52	2.43
High alcohol intake	0.33				
and ADH3 fast	0.33*0.36=0.12	1.76	1.53	0.34	2.38
High meat intake	0.33				
and NAT2 slow	0.33*0.59=0.19	1.57	1.41	0.27	2.34

[a] OR(int), odds ratio for interaction; [b] the truly achieved power within our study population for the detection of a statistically significant interaction term of the same magnitude as observed in our study, one-sided test with $\alpha=0.05$; [c] given an achieved sample size of 440 cases (equal number of controls), detectable OR(int) are calculated at $\alpha=0.05$ (one-sided) with a power of 80%.

Consequently, the study power was large enough to evaluate the effect of 'high-risk combinations'. However, as shown in Table 2.3, to study gene-environment interactions, larger populations are needed [24,25]. The power was sufficient (i.e., > 80%) for the detection of relatively strong interactions (i.e., OR for interaction of 3 or 4). However, the strength of the interaction is probably small and decreases in the presence of (non-)differential misclassification [26].

Selection bias

Selection bias refers to situations in which the relation between exposure and disease is different for participants in comparison with those who are in theory eligible, and may be introduced by differential selection procedures or by differential participation rates [19,27]. Here, we discuss these possibilities.

Our study population was recruited via two different methods, as has been explained (see also the appendix to this Chapter). To decrease the possibility of differential selection, the study protocol required cases and controls to be enrolled according to preset criteria both via the direct and the indirect method. It is not likely that exposure-related invitation occurred, since the level of the exposure under study was not known at time of endoscopy. An important advantage of the direct method was that the obtained response rates were relatively high (Table 2.4). However, the investigators could not closely monitor recruitment so that exact calculation of response rates was hampered and the possibility of selection bias was increased. In contrast, the indirect recruitment procedure enabled the investigators to select the eligibles according to the preset criteria,

and to monitor response. However, response rates were considerably lower than those obtained via the direct method (see Table 2.4).

Table 2.4. Response rates per hospital.

Hospital	Recruitment method	Estimated response in % [a]	Number of participants in study (%)
1	direct + indirect	35	168 (18.9)
2	direct	77	124 (14.0)
3	direct	91	141 (15.9)
4	direct	69	39 (4.4)
5	indirect	48	178 (20.1)
6	direct	87	22 (2.5)
7	indirect	38	56 (6.3)
8	indirect	52	159 (17.9)
Total		54	887 (100)

[a] Response rates were not exactly known since endoscopy staff did not report non-responders (direct method), and of the invited subjects, some might have responded after the closing date of the recruitment period, 1 June 2000 (both methods).

Inclusion of cases with recurrent adenomas

We included cases with recurrent adenomas in our study (see Figure 2.3), which possibly resulted in overrepresentation of cases with a history of adenomas in our study population since these had a higher probability of being invited. This might have introduced bias. Subjects suffering from recurrent adenomas may be at higher risk of adenomas [8,28], and hereditary factors may play a more important role in these subjects than in persons being diagnosed with adenomas once in their lives. However, 57% of the recurrent cases in our study were found to have adenomas only once before the index endoscopy, and in 28% of the recurrent cases, an adenoma had been detected twice before the index endoscopy, indicating that genetic factors might not be of major importance. Besides, risk factors for those cases previously diagnosed with adenomas were similar to the risk factors found for those diagnosed with primary adenomas (see also Table 2.7). The results did not change after exclusion of recurrent cases from the analyses for all of the potential risk factors under study (see Chapters 4-6). We could probably adequately control for this higher chance for recurrent cases to be invited by conducting analyses adjusted for indication of endoscopy (classified as complaints, screening, and other/unknown). More important in this respect is the potential role of lifestyle changes, which will be discussed below.

Differences between hospitals

There were large differences in case-control ratios between hospitals, as is shown in Figure 2.4. When considering the hospital-specific populations, the percentage of cases

ranged from 16% to 100% (Figure 2.4). This variation can be due to differences in recruitment procedures as applied by endoscopy staff (hospital 4), to the relatively short recruitment period in the hospital (hospital 7), or to small sample sizes (hospital 6). Alternatively, these differences might reflect differences between hospitals with respect to endoscopy guidelines determining which patients get an endoscopy, or with respect to differences between the populations served.

In spite of the large differences between hospitals, we did not include 'hospital of endoscopy' as a covariate in the multivariate analyses presented in this thesis, because of the small cell numbers obtained for some hospitals (Table 2.4). With the proportion of cases, indication of endoscopy tended to differ over hospitals. Indication of endoscopy was included in the multivariate models described in chapters 4-6 to control for differences between cases and controls. However, our results might suffer from residual confounding by uncontrolled differences between hospitals. As recruitment proceeds, with increasing hospital-specific sample sizes, this issue can be more adequately addressed in future studies.

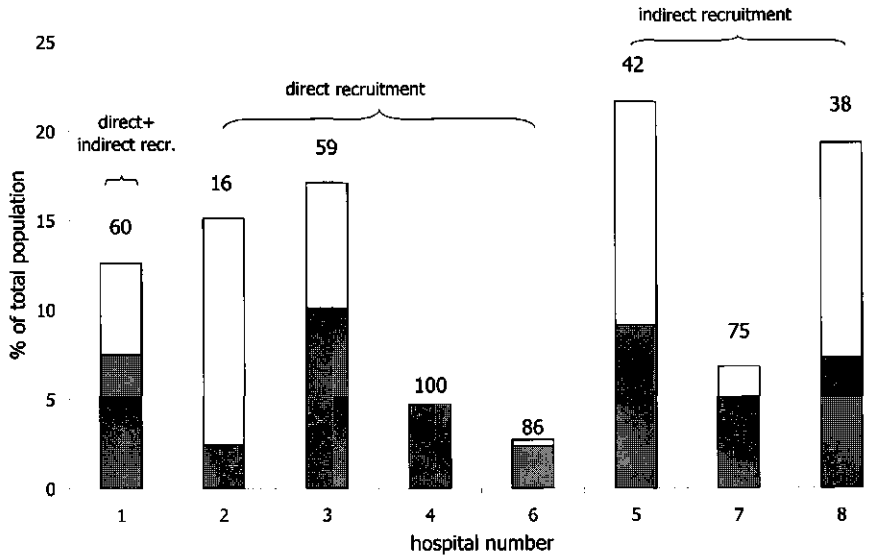

Figure 2.4. Recruitment of the study population per hospital. Total bars give the contribution of hospital-specific samples to the study population; the percentage of cases per hospital-specific sample is given by the gray bars and indicated by the figures above the bars (see also text).

Response bias

The observed differences in the proportion of cases between hospitals did apparently not depend on response rates, although these also varied over hospitals (Table 2.4). The

direct method resulted in a high response rate probably because all potential participants were personally approached by a member of the endoscopy staff. Comparison of responses to the short questionnaire as given by participants with the answers from those refusing participation but completing the short questionnaire (Table 2.5) revealed not much difference between participants and non-participants, although non-participants were somewhat older and consumed less meat. However, the results of this non-response analysis are difficult to interpret. Only about one third of non-participants completed the short questionnaire and, similar to the participants, these could have been more health-conscious or healthier than the total of non-participants [29,30]. Moreover, for ethical reasons, we had no information on the disease status of the non-participants, which hinders investigation of exposure-disease relationships for non-participants.

Table 2.5. Characteristics of participants of the adenoma case-control study in comparison with invited subjects who did not participate, but completed the short questionnaire.

Characteristic	Participants to adenoma study N=1114 [a]	Non-participants N=238
Male, %	46.5	45.6
Age (yr.), mean ± SD	56 ± 12	60 ± 12 [b]
BMI (kg/m^2), median (25th ; 75th percentile)	25 (23 ; 28)	25 (22 ; 28) [b]
High education level, % [c]	21.1	21.4
Ever smoked, %	69.9	69.9
Alcohol intake (drinks/wk), median (25th ; 75th percentile)	4 (0 ; 12)	4 (0 ; 12)
Meat consumption (frequency/week), mean ± SD	5.4 ± 1.5	5.1 ± 1.6 [b]

[a] Including all those who had agreed to participate and filled out the short questionnaire at 1 May 2000; [b] significantly different from participants ($p < 0.05$); [c] at least B.Sc. degree.

POTENTIAL BIAS BY AGE AND SEX DIFFERENCES BETWEEN CASES AND CONTROLS

As shown in Table 2.2, the control group consisted of significantly more women, possibly because women were more likely than men to undergo endoscopy for major bowel complaints such as irritable bowel syndrome (IBS). IBS was found to be more prevalent in Dutch women than in men [31]. Also, cases were older than controls. Such differences may introduce several types of bias, but not if gender and age are properly monitored and adjusted for [19]. Like others who were confronted with similar gender and age differences [22,32,33], we therefore adjusted all analyses presented in this thesis for sex and age.

Information bias

Information bias may occur when the exposure of interest is differentially reported by cases and controls, due to differential recall or to under- or over-reporting. In this respect, several potential sources of bias need to be discussed, such as the effect of prior diagnosis of adenomas on exposure, and of the inclusion of subjects with bowel complaints. Differential recall of dietary and smoking habits could have occurred, though controls had an equal 'recall stimulus' [19] because, like cases, they underwent endoscopy. However, indication of the endoscopy was different for cases and controls and might have influenced both recall and behavior. Here, we will discuss possible sources of bias, such as the effect of time interval between enrollment and endoscopy, prior diagnosis of adenomas, and the potential influence of bowel complaints.

Influence of time interval between endoscopy and invitation

The chance of recall bias was possibly higher for subjects recruited via the indirect methods than for those recruited via the direct method, as the time-interval between endoscopy and invitation was longer (Table 2.6), increasing the probability for the invited subjects to be aware of their disease-status. If the factors under study would have been known as a risk factor for colorectal adenomas, cases might have reported lower or higher exposure than their true level of exposure. However, smoking and alcohol consumption are generally believed to increase risk of several cancers, but probably not of colorectal adenomas. As shown in Table 2.6, relatively more cases were recruited by the direct method than by the indirect method. Moreover, subjects recruited by the direct method were less likely to be female or to suffer from bowel complaints, and more likely to have ever smoked, but these latter two differences could also result from the observed sex differences.

Table 2.6. Statistically significant differences by recruitment procedure.

Characteristic	Direct recruitment N=326 (45.3 %)	Indirect recruitment N=393 (54.7 %)
Interval between blood collection and index endoscopy (months), mean ± SD	0.4 ± 1.8	3.7 ± 2.2
Cases, %	49.4	45.0
Colon complaints, %	23.9	31.3
Females, %	49.4	58.8
Ever smokers, %	58.6	50.1

Cases with recurrent adenomas

We included recurrent adenoma cases. Those being diagnosed with colorectal adenomas may have been advised to change their diet or to increase their physical activity. However, none of those previously being diagnosed with adenomas indicated to have

changed alcohol consumption because of colorectal adenomas, whereas smoking rates did not differ between cases with primary and cases with recurrent adenomas. Meat consumption was not different for recurrent and non-recurrent cases. Except for differences in endoscopy indication, the only difference observed between the two types of cases was that recurrent cases were slightly older than non-recurrent cases (Table 2.7). Therefore, adjustment for age, sex and indication of endoscopy seemed adequate. Indeed, adjustment for dietary changes did not markedly change observed associations between exposure factors and adenomas. Moreover, the results did not change after exclusion of recurrent cases from the analyses for all of the potential risk factors under study (see Chapters 4-6).

Table 2.7. Statistically significant differences between recurrent cases and those diagnosed with adenomas only once.

Characteristic	Recurrent cases N=61 (13.9 %)	Non-recurrent cases N=379 (86.1 %)
Indication of endoscopy, %		
complaints	4.9	59.4
screening	93.4	30.6
other/unknown	1.6	10.0
Age, mean ± SD	61.6 ± 9.4	58.4 ± 10.7

Bowel complaints

Large bowel complaints seemed to be more common among controls than among cases. Inclusion of controls with bowel complaints might in theory lead to overestimation of the studied associations between e.g. alcohol and adenomas, as bowel complaints might cause patients to reduce their alcohol intake. However, habitual alcohol use did not differ between subjects suspected from irritable bowel syndrome (IBS) or diverticular disease and apparently healthy subjects in other studies [31,34]. Underestimation of the studied associations is also possible, as excess alcohol intake might lead to bowel complaints [35]. Under- or overestimation of the odds ratio did probably not occur, as those undergoing endoscopy because of large bowel complaints differed from other subjects with respect to fiber intake and coffee consumption, but not on any of the exposures under study (Table 2.8). Indeed, exclusion of those undergoing endoscopy for bowel complaints did not change any of the results described. In our study population, two controls reported to have stopped drinking alcohol because of bowel complaints, whereas none of the cases and controls changed their meat consumption.

Non-differential misclassification of exposure

Except differential misclassification, non-differential misclassification may also have occurred and might in theory have lead to bias toward, but also to bias away from the null when categorizing subjects into tertiles of exposure [19]. However, this topic is not so

much related to our study population as to the nature of the questionnaires, and will therefore not be discussed here.

Table 2.8. Statistically significant differences between subjects undergoing endoscopy for bowel complaints and subjects undergoing endoscopy for other reasons.

Characteristic	Bowel complaints as indication N=234 (26.4 %)	Other indications for endoscopy N=653 (73.6 %)
Cases, %	27.4	57.6
History of polyps, %	2.6	19.8
Family history of colorectal cancer, %	16.2	22.8
Frequency of constipation, %	22.2	11.3
Female, %	61.5	51.6
Age (yr.), mean ± SD	51.2 ± 14.5	55.8 ± 12.5
Fiber intake (g/d), mean ± SD	22.9 ± 6.4	24.0 ± 6.7
Coffee (cups/d), mean ± SD	4.0 ± 2.7	4.4 ± 2.7

MISCLASSIFICATION OF CASES AND CONTROLS

Some of the controls might truly have been cases, as previous studies showed that at single colonoscopies, 10-15% of polyps may be missed, depending on polyp size [10,36]. Moreover, not all controls in our study underwent complete colonoscopy. The inclusion of controls with incomplete endoscopy (23% of controls) could in theory have resulted in misclassification leading to bias toward the null, since these controls could have undetected adenomas in the proximal colon. However, controls who did not undergo complete colonoscopy only differed with respect to the reason for endoscopy (mostly complaints) whereas those undergoing complete colonoscopy more often sought medical care for screening because of a positive family history (Table 2.9). Exclusion of controls with incomplete colonoscopy did not strengthen or attenuate our results.

Table 2.9. Statistically significant differences between control subjects undergoing complete and incomplete endoscopy.

Characteristic	Complete endoscopy N=345 (77.1 %)	Incomplete endoscopy N=102 (22.8 %)
Indication for endoscopy, %		
complaints	73.6	86.3
screening	9.3	0
other/unknown	17.1	13.7
Family history of colorectal cancer, %	22.0	8.8

EXTERNAL VALIDITY OF RESULTS DESCRIBED IN THIS THESIS

With respect to external validity, a limitation of our study might be the choice of the control group, as not all controls were asymptomatic, average-risk individuals. Most of

the controls had some bowel-related complaints or a family history of colorectal cancer. Moreover, our study population might have been more health-conscious than the general population, which might be reflected by the lower smoking rates observed in our study population (23.4%) in comparison with rates in the general Dutch population of the same age (30.6% †). This might affect the generalizability of our results. To be able to compare our case-control population with the general population on important determinants of colorectal adenomas, such as meat consumption (Chapter 4), smoking (Chapter 5), and alcohol consumption (Chapter 6), we conducted a separate study.

In this study, 4000 subjects were randomly selected from three district council registries. These subjects inhabited three different districts in the central parts of the Netherlands (which was also the region where our case-control population originated from), and the sample had the same age and sex distribution as our case-control population. All selected subjects received an invitation from the district council and the same short questionnaire as was completed by our cases and controls (see before). After three months, 1987 subjects had completed and returned the questionnaire (response rate 49%), of which 1935 were included in preliminary analyses. Early interim analyses including 66 controls from the adenoma case-control study did not reveal differences between the two populations. However, later analyses showed some clear differences. As shown in Table 2.10, compared to the endoscopy control group, the sample from the general population was somewhat younger, counted less men, more well-educated subjects, and less subjects with a family history of colorectal cancer. Alcohol consumption was lower in the population-based sample than in the case-control population, although this difference might be attributable to sex and age differences between the two populations (Table 2.10).

Table 2.10. Comparison of the adenoma case-control study with a population-based sample as estimated from the short questionnaire.

Characteristic	Population-based sample N=1935	Endoscopy control group N=423	Adenoma cases N=400
Male, %	32	37 [a]	55 [a]
Age, mean ± SD	45.7 ± 12.5	50.6 ± 14.1 [a]	59.3 ± 10.3 [a]
Body mass index, mean ± SD	24.8 ± 4.3	25.4 ± 4.1	26.1 ± 4.0 [a]
High education level [b], %	42	25 [a]	25 [a]
Family history of colorectal cancer, %	5	19 [a]	24 [a]
Ever smoked cigarettes, %	62	51 [a]	64
Alcoholic drinks per week, mean ± SD	5.4 ± 7.3	6.3 ± 8.9	8.6 ± 10.8 [a]
Meat consumption in g/d, mean ± SD	98.4 ± 51.6	98.3 ± 51.0	97.9 ± 45.9

[a] $p \leq 0.05$ for statistically significant differences between the populations-based sample and the other two groups; [b] subject or partner of subject obtained at least B.Sc. degree.

† see http://www.cbs.nl; Centraal Bureau voor de Statistiek, 2001.

Conclusion and recommendations

We conclude that our case-control population seems to have acceptable validity, although rather large differences between hospitals were observed. These differences possibly reflect differences in the populations served, but could also reflect hospital guidelines with respect to who should get an endoscopy, or problems with adequate recruitment of study subjects. We controlled for these differences by adjusting all analyses for indication of endoscopy, although some residual confounding due to these hospital-differences may have remained. Our findings may not be applicable to the general population as our control group mostly underwent endoscopy for complaints. Consequently, we should be prudent in generalizing our results. Although the results presented in chapter 4-6 may not apply to the general population, they are comparable to results reported from other adenoma case-control studies [3,14].

To investigate the characteristics of our control group in relation to the general population in more detail, the information we collected from the population-based sample (see Table 2.10) should be more carefully compared with information from our case-control group, and more data from this population-based sample should preferably be collected. Also, the hospital-specific differences between case-control populations should be studied in more detail. This will be possible with the enlargement of the study population in the coming years. In the future, the case-population of our adenoma study could also be used to conduct case-only analyses to evaluate the potential joint effect of environmental exposures and genetic susceptibility [37] (see Chapter 8).

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Appendix to Chapter 2. Details of recruitment methods within adenoma case-control study per hospital.

Hospital	Method [a]	Recruited by	Informed consent	Blood collection	Questionnaires	Months in study
1	Direct + indirect	Research nurse	Written consent mailed to investigators	By appointment, after receiving informed consent	Given at blood collection	36
2	Direct	Charge nurse of endoscopy unit	Oral, at later stage written consent mailed to investigators	At hospital laboratory, after endoscopy	Directly given with invitation brochure	36
3	Direct	Gastroenterologist	Oral, at later stage written consent mailed to investigators	At time of endoscopy	Directly given with invitation brochure	33
4	Direct	Charge nurse endoscopy unit / gastroenterologist	Oral, at later stage written consent mailed to investigators	At hospital laboratory, after endoscopy	Directly given with invitation brochure	29
5	Indirect	Research nurse	Written consent mailed to investigators	By appointment, after receiving informed consent	Given at blood collection	29
6	Direct	Endoscopy nurse	Written consent mailed to investigators	By appointment, after receiving informed consent	Given at blood collection	22
7	Indirect	Research nurse	Written consent mailed to investigators	By appointment, after receiving informed consent	Given at blood collection	16
8	Indirect	Research nurse	Written consent mailed to investigators	By appointment, after receiving informed consent	Given at blood collection	18

[a] A definition of the direct and indirect method is given in the text.

3.

Simultaneous determination of polymorphism in *N*-acetyltransferase 1 and 2 genes by reverse line blot hybridization

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Abstract

Polymorphism in *N*-acetyltransferases *NAT1* and *NAT2* may contribute to differences in cancer susceptibility of subjects exposed to alkylating compounds. We developed a robust method for simultaneous determination of these *NAT* polymorphisms: Reverse Line Blot (RLB) hybridization, based on PCR followed by allele-specific oligo-hybridization. On a membrane, allele-specific oligonucleotide probes of the *NAT* genes (*NAT1*4, *3, *10, *11* and *NAT2*4, *5, *6, *7 *12*) were applied in lines. After separate amplification of the *NAT* genes, simultaneous hybridization of these products in lines perpendicular to the lines with oligonucleotide probes was performed, followed by non-radioactive detection. This resulted in hybridization patterns, representing the *NAT* genotype of an individual. RLB hybridizations were conducted on DNA from 240 Dutch Caucasian participants in an ongoing case-control study on colorectal adenoma (including 126 polyp-free control subject). Results were in complete agreement to those obtained by commonly used methods, i.e. allele-specific PCR and PCR-RFLP. Allele-frequencies in the polyp-free control group were similar to those described in the literature. RLB hybridization is, however, considerably faster and cheaper than the common assays. Moreover, expansion with allelic variants of other genes is relatively easy, which makes RLB hybridization very useful for multiplex analysis of numerous polymorphisms in epidemiological studies.

Introduction

N-acetyltransferases 1 and 2 (*NAT1*, *NAT2*) are important enzymes in the biotransformation of various xenobiotics with a primary aromatic amine or hydrazine structure, such as heterocyclic aromatic amines (HCAs), which may play an important role in the etiology of colorectal, breast and bladder cancer [1]. Genes coding for the NAT enzymes are observed to be polymorphic and specific variants may be related to increased risk of cancer in subjects exposed to HCAs [1].

Commonly used methods for determination of polymorphism in *NAT1* and *NAT2* genes [2-5] are respectively based on allele specific PCR methods and PCR followed by restriction fragment length polymorphism analyses (PCR-RFLP), both of which are time consuming and relatively expensive. For epidemiological studies, efficient, less labor intensive and less contamination prone methods are preferable. Recently, new methods for *NAT2* genotyping based on oligonucleotide ligation (OLA) [6] or using allele specific oligo (ASO) hybridization with fluorescent probes and melting curve analysis [7] were developed. These approaches can easily be used in large epidemiological studies because of their simplicity and high sample throughput. So far, simultaneous detection of the allelic variants in both *NAT1* and *NAT2* genes was thought to be impossible because of the high degree of similarity of the genes [8,9]. Labuda *et al.* recently developed a method in which dot blotting of in multiplex amplified products of both *NAT* genes was followed by ASO hybridization [10]. Although this method enabled simultaneous amplification of both *NAT* genes, detection was relatively labor intensive.

In this paper, we describe a rapid Reverse Line Blot (RLB) hybridization method, previously used for genotyping human bacterial pathogens [11,12], enabling simultaneous determination of *NAT1* and *NAT2* allelic variants potentially relevant to cancer risk. Results and performance of this method are compared with commonly used allele-specific PCR and PCR-RFLP methods.

Materials and methods

Subjects, blood sampling and DNA isolation

NAT1 and *NAT2* genotyping was conducted on DNA samples isolated from blood of 240 participants in an ongoing Dutch case-control study on the etiology of colorectal adenomatous polyps. All participants were from Caucasian decent. Controls (n=126) had no (history of) polyps and underwent endoscopy for gastrointestinal complaints such as anal bleeding, pain or defecation problems. Cases (n=114) were diagnosed with adenomatous polyps at least once and visited the outpatient clinic because of gastrointestinal complaints or for follow-up of colorectal polyps.

DNA was isolated from 200 µl frozen whole blood and purified using the QIAamp blood Kit (QIAGEN Inc. USA). The eluted DNA was diluted to a concentration of about 20 ng/µl and stored at 4°C in deep-well microtiterplates.

Genotyping methods

Genotyping was performed by RLB hybridization and by the reference methods. To test the efficiency of the method, all laboratory analyses were done only once and in case of negative results (because of, for example, the quality of DNA or pipetting errors during the procedure), amplifications were not repeated. One well in every column of a microtiterplate did not contain a DNA sample but water as negative PCR control instead (n=36) to check for cross contamination. Twelve duplicate DNA samples were randomly distributed over the three microtiterplates.

In order to evaluate the RLB method, we compared the outcome with the outcome of the commonly used methods. Moreover, genotype distributions and allele frequencies were calculated for the 126 polyp-free controls only, and compared with the frequencies of other population-based studies.

Reverse Line Blot (RLB) hybridization for simultaneous *NAT1* and *NAT2* genotyping

The principle of RLB hybridization is based on non-radioactive hybridization of 43 DNA samples with a maximum of 43 different oligonucleotide probes in one single assay and was first described by Kaufhold *et al.* [12]. In short, PCR products of both *NAT* genes are hybridized to a set of gene specific oligonucleotide probes which are bound to

a membrane, followed by chemiluminescent detection of the hybridization and exposure of the membrane to a light sensitive film. The principle of this method is depicted in Figure 3.1.

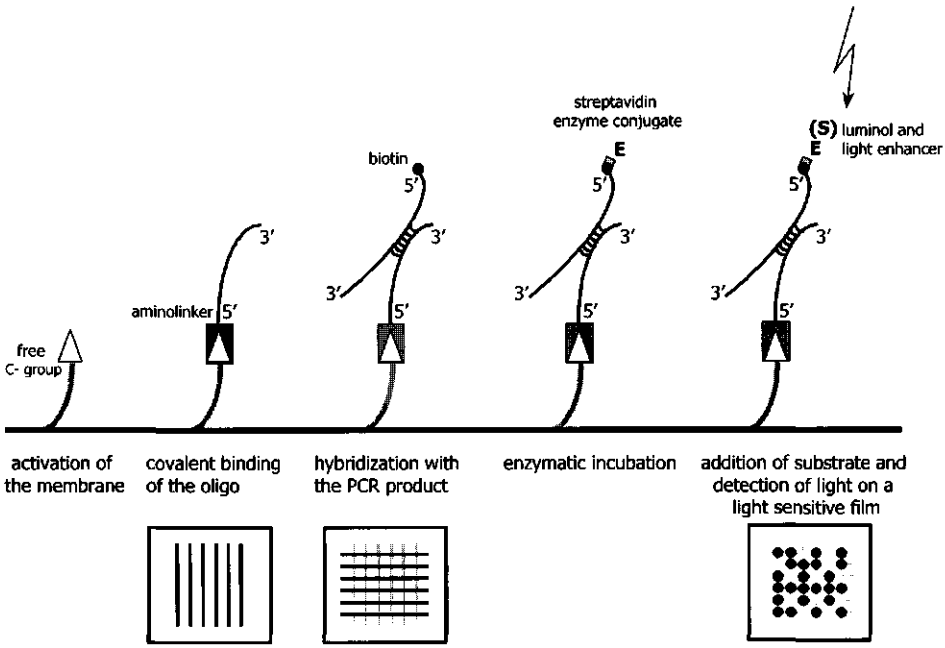

Figure 3.1. Principle of the Reverse Line Blot hybridization method.

Preceding the hybridization, amplification of *NAT1* and *NAT2* was performed using primer NAT1-1 in combination with primer NAT1-2b, and primer NAT2-1 in combination with primer NAT2-2b respectively (Table 3.1). To the different PCR mixes (25 µl: 10 mM Tris-HCL pH 9.0, 2.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.1% Triton X100, 0.2 mM dNTPs, 0.5 unit Super TAQ polymerase (SphaeroQ), 15 pmol of both primers) 100 ng of DNA was added. After 4 min of denaturation at 94°C, the mixture for *NAT1* amplification was subjected to 35 cycles of 30 s at 94°C, 30 s at 50°C and 1 min at 72°C, followed by a final elongation step of 5 min at 72°C. For amplification of *NAT2* the same temperatures were used but the duration of denaturation, annealing and elongation were doubled. All PCR were performed in a Programmable Thermal Controller (PTC-100; MJ Research). Contamination was prevented using filter-tips and mineral oil covering the amplification mixtures. The amplified products were visualized under UV light after DNA electrophoresis on an ethidium bromide stained 2% agarose gel. The *NAT1* fragment was 285 bp whereas the *NAT2* fragment was 1093 bp in size.

Table 3.1. Primers and oligonucleotide-probes used for RLB hybridization and the reference methods[a].

	Primer/ probe	Detection of polymorphic position(s)	Detection of allele/ gene	Sequence (5' → 3')	Reference
NAT1 RLB					
Amplification	NAT1-1			TAAAACAATCTTGTCTATTTG	3
	NAT1-2b			biotin-CAATAAACCAACATTAAAAG	this study
Hybridization	NAT1-RLB1	1088T/1095C	*NAT1*4*	amino-AATAATAATAAATGTCTTTTAAAGATGGC	this study
	NAT1-RLB2	1088T/1095A	*NAT1*3*	amino-TAATAATAAATGTATTTTAAAGAT GGCCT	this study
	NAT1-RLB3	1088A/1095A	*NAT1*10*	amino-ATAATAAAAAATGTATTTTAAAGA TGGCC	this study
	NAT1-RLB4	1095A + 9bp deletion	*NAT1*11*	amino-CCTTTTCAAATAATAATAATAATA AATGTATTTT	this study
NAT2 RLB					
Amplification	NAT2-1			GGAACAAATTGGACTTGG	3
	NAT2-2b			biotin-TCTAGCATGAATCACTCTGC	3
Hybridization	NAT2-RLB1	341T		amino-GACCATTGACGGCAGGAA	this study
	NAT2-RLB2	341C	*NAT2*5A* or **5BC*	amino-ACCACTGACGGCAGGAAT	this study
	NAT2-RLB3	590G		amino-GCTTGAACCTCGAACAATTGA	this study
	NAT2-RLB4	590A	*NAT2*6*	amino-CTTGAACCTCAAACAATTGAAGA	this study
	NAT2-RLB5	803A		amino-GGTTGAAGAAGTGCTGAAAAATA	this study
	NAT2-RLB6	803G	*NAT2*5B C* or **12*	amino-GTTGAAGAAGTGCTGAGAAATAT	this study
	NAT2-RLB7	857G		amino-CCTGGTGATGGATCCCTT	this study
	NAT2-RLB8	857A	*NAT2*7*	amino-AACCTGGTGATGAATCCCTTA	this study
NAT1 Reference method					
	NAT1-3	1088T	*NAT1*4*	GCCATCTTTAAAAGACATTTA	2
	NAT1-4	1088A	*NAT1*10*	GCCATCTTTAAAAGACATTTT	2
	NAT1-5			TATTTGTCATCCAGCTCACC	b
	NAT1-6	1095A	*NAT1*3* or **4*	CCACAGGCCATCTTTAAAAT	b
	NAT1-7	1095C	*NAT1*4*	CCACAGGCCATCTTTAAAAG	b
	His1		Histone	TGGAAATGAACGACTTTCGG	b
	His2		Histone	TGACGAAGGAGTTCATGATG	b
	His3		Histone	AATCTCCTTTTTACAAATGAG	b
	His4		Histone	CTGTTAATTTCATTCATTGAG	b
NAT2 Reference method					
	NAT2-3	341	*NAT2*5*	CACCTTCTCCTGCAGGTGACCG	4
	NAT2-4	341	*NAT2*5*	TGTCAAGCAGAAAATGCAAGGC	4
	NAT2-5	590	*NAT2*6*	GGCTGTTCCCTTTGAGAACC	c
	NAT2-6	590, 803, 857	*NAT2*6* or **7* or **12*	ACACAAGGGTTTATTTTGTTCC	5
	NAT2-7	803, 857	*NAT2*7* or **12*	GTGGGCTTCATCCTCACCTA	5

[a] Underlined bases denote the positions of the bases, which vary between the alleles. [b] Potter *et al.*, manuscript in preparation; [c] Bigler *et al.*, unpublished data.

For the hybridization experiments we designed the *NAT1* oligonucleotide probes derived from alleles **4, *3, *10* and **11* to detect polymorphism at position 1088 and 1095 and the 9-bp poly-A deletion in that region (Table 3.1, oligonucleotide probes NAT1-RLB1 to NAT1-RLB4). Probe NAT1-RLB4 (allele *NAT1*11*) also detects allele *NAT1*3* (the probe derived from allele *NAT1*3* is on the other hand not detecting *NAT1*11*). For *NAT2*, we designed oligonucleotide probes to establish allelic variants *NAT2*5, *6, *7, *12* (NAT2-RLB2, 4, 6, 8) as well as to detect the wild-type allele represented by hybridization with probe NAT2-RLB1, 3, 5, 7 (Table 3.1). Allele *NAT2*5A* is represented by hybridization with probe NAT2-RLB2 alone, while in combination with a hybridization signal with probe NAT2-RLB6 the *NAT2*5BC* allele is determined. Oligonucleotide probes were synthesized with a 5'-terminal amino group, which was used to covalently link the probes to the activated membrane. The membrane containing the oligonucleotide probes used for hybridization with the PCR fragments was made as described by Kaufhold *et al.* in 1994 [12]. In short, a Biodyne C membrane (Pall, Pall BioSupport) was activated with 16% (wt/v) EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma) and placed in a miniblotter MN45 (Immunetics). After 150 µl of the probes was applied in a concentration which varied between 40 and 5400 pmol/µl, the membrane was inactivated with 0.1 N NaOH and washed with 2x SSPE/0.1% SDS (SSPE; Gibco BRL, SDS, BDH). The concentrations of the probes on the membrane were established by first applying the probes in three different concentrations on the membrane (e.g. 167, 333 and 667 pmol/µl,) followed by hybridization with PCR samples of known genotype. Depending on the result, the concentrations were increased or decreased. Eventual concentrations were 42, 42, 83 and 667 pmol/µl for probes NAT1-RLB1 to NAT1-RLB4, respectively, and 5333, 5333, 167, 333, 167, 167, 167, and 667 pmol/µl for probes NAT2-RLB1 to NAT2-RLB8, respectively. Note that in practice the optimal probe concentrations may differ from the ones described here, due to different laboratory and manufacturing conditions.

After binding of the probes, the membrane was taken from the miniblotter, washed and placed into the miniblotter again in a 90° rotated position. In a microtube containing 150 µl of 2x SSPE/0.1% SDS, 10 µl of the PCR products of both *NAT1* and *NAT2* were added and heat-denatured. As positive hybridization controls, a set of 4 PCR samples of known genotype was used. After rapid cooling on ice, the PCR products were applied into the slots of the miniblotter and hybridized for 60 min at 45°C. The membrane was removed from the miniblotter and washed twice for 10 minutes at 64°C in 2x SSPE/0.5% SDS solution followed by 1-h incubation at 42°C with streptavidin-peroxidase (Boehringer Mannheim GmbH) diluted 1:4000 in 2x SSPE/0.5% SDS. After washing twice at the same temperature for 10 min with 2x SSPE/0.5% SDS and twice with 2x SSPE at room temperature for 5 min, visualization of the hybridization was carried out with the ECL nucleic acid detection reagent (Amersham Pharmacia Biotech) followed by exposure of the membrane to a light-sensitive film (hyperfilm ECL; Amersham Pharmacia Biotech) for about 30 min. After successful hybridization, the

PCR products were stripped from the membrane by incubating the membrane twice for 30 min in 1% SDS solution at 80°C and then the membrane was stored moist at 4°C for a further reuse.

REFERENCE METHODS FOR *NAT1* AND *NAT2* GENOTYPING

NAT1 genotyping was performed by allele specific amplification as used by Bell [2] and Potter (Potter *et al.*, manuscript in preparation). The PCR to detect the allele specific nucleotide at position 1088 was carried out using primer NAT1-1 together with either primer NAT1-3 or primer NAT1-4. Both reactions were co-amplified with primers His1 and His2 derived from the human histone gene (Table 3.1). The PCR to detect the allele specific nucleotide at position 1095 was carried out using primer NAT1-5 with either primer NAT1-6 or primer NAT1-7 whereas the histone gene was co-amplified using primers His3 and His4 (Table 3.1). In contrast with RLB hybridization, we did not differentiate between allele *NAT1*3* and allele *NAT1*11*, both amplified with primer NAT1-5 and primer NAT1-6. Fragments of these alleles differ only 9 bp in size which makes differences between these infrequent alleles hardly observable.

Polymorphism in the *NAT2* gene was determined by a primary PCR performed with primer NAT2-1 and NAT2-2 [3] (Table 3.1) followed by three nested PCRs and RFLP analyses ([4,5], Bigler *et al.*, unpublished data). The region encompassing position 341 (for detection of allele *NAT2*5*) was amplified using primer NAT2-3 and NAT2-4 and the resulting fragments were digested with *Aci*I. The region covering position 590 (for detection of allele *NAT2*6*) was amplified by primers NAT2-5 and NAT2-6 and the PCR fragments were digested with *Taq*I. To determine the mutations at positions 803 (for discrimination between allele *NAT2*5A* and *NAT2*5B*, or detection of allele *NAT2*12*) and 857 (for detection of allele *NAT2*7*) we used primer NAT2-6 and NAT2-7 (Table 3.1) and the PCR was followed by RFLP analyses with respectively *Dde*I and *BamH*I. PCR fragments as well as restriction fragments were separated with DNA electrophoresis on agarose gels stained with ethidium bromide and visualized under UV light.

Results

All samples were subjected to genotyping by RLB hybridization and the reference methods. We were able to perform all genotyping by RLB hybridization in three days (96 samples can be genotyped in one single day). The allele specific amplification and PCR-RFLP methods genotyping took approximately fifteen days (96 samples can be determined in 5 days) because of the number of separate steps in these methods.

We were able to determine the *NAT1* genotype of 239 out of 240 samples by RLB hybridization and 235 out of 240 samples by allele specific PCR. In addition, *NAT2* genotyping resulted in 238 positive determinations by RLB hybridization compared to 224

obtained by PCR-RFLP analysis (data not shown). The 12 duplicate DNA samples gave identical outcomes and we did not see any genotyping discrepancies in outcome between RLB hybridization and the reference methods (data not shown). All of the 36 negative PCR controls remained negative by RLB hybridization whereas by PCR-RFLP analysis of *NAT2*, 10 out of 36 negative PCR controls were positive (data not shown). For data analysis, positive hybridization results were scored in a spreadsheet as plus, while we scored minus if no hybridization signal appeared.

Figure 3.2 shows an example of an RLB result of the four positive controls (row 1-4), a negative PCR control (row 5) and seven samples from the study population (row 6-12). Deduction of the genotype on the basis of the hybridization pattern was simple. As an example, the PCR sample from a subject participating in the study, depicted in row 6, shows hybridization with *NAT1* oligonucleotide probes 1 and 3, indicating the presence of allele *NAT1*4* and *NAT1*10*. For *NAT2*, hybridization with oligonucleotide probe 1, 3, 4, 5 and 7 is observed resulting in the *NAT2*4/*6* genotype (Figure 3.2, Table 3.1 and 3.2).

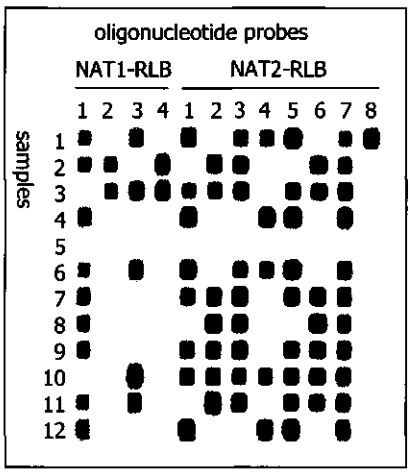

Figure 3.2. Reverse line blot hybridization patterns of amplified *NAT1* and *NAT2* genes. Columns 1 through 4 and columns 5 through 12 depict the oligonucleotide-probes specific for the various *NAT1* and *NAT2* alleles, respectively. Row 1 through 4 show hybridization patterns of a set of 4 PCR samples of known genotype, as a control to the hybridization. Rows 6 through 12 show genotyping patterns of samples from individuals from the study population. Row 5 carries a negative PCR control.

Table 3.2 shows an overview of all different RLB hybridization patterns of the 126 polyp-free individuals in this study and the corresponding frequencies of the *NAT1* and *NAT2* genotypes.

Allele frequencies as determined by RLB hybridization of these subjects were comparable with those obtained from previous studies, as shown in Table 3.3.

Table 3.2. *NAT1* and *NAT2* genotype of 126 polyp-free individuals of Caucasian descent: results from the RLB method.

Hybridization result per RLB oligonucleotide probe								Genotype	Number of individuals (%)
NAT1									
1	2	3	4						
●	-	-	-					**4/*4*	70 (55.6)
●	●	-	-					**4/*3*	3 (2.4)
●	-	●	-					**4/*10*	38 (30.2)
●	●	-	●					**4/*11*	4 (3.2)
-	●	●	-					**3/*10*	3 (2.4)
-	-	●	-					**10/*10*	6 (4.8)
-	●	●	●					**10/*11*	2 (1.6)
NAT2									
1	2	3	4	5	6	7	8		
●	-	●	-	●	-	●	-	**4/*4*	8 (6.3)
●	●	●	-	●	-	●	-	**4/*5A*	1 (0.8)
●	●	●	-	●	●	●	-	**4/*5BC*	26 (20.6)
●	-	●	●	●	-	●	-	**4/*6*	16 (12.7)
●	-	●	-	●	-	●	●	**4/*7*	1 (0.8)
-	●	●	-	●	●	●	-	**5A/*5BC*	2 (1.6)
●	●	●	●	●	-	●	-	**5A/*6*	1 (0.8)
-	●	●	-	-	●	●	-	**5BC/*5BC*	23 (18.3)
●	●	●	●	●	●	●	-	**5BC/*6*	36 (28.6)
●	●	●	-	●	●	●	●	*5BC/*7*	2 (1.6)
●	-	-	●	●	-	●	-	**6/*6*	6 (4.8)
●	-	●	●	●	-	●	●	**6/*7*	3 (2.4)
●	-	●	●	●	●	●	-	**6/*12*	1 (0.8)

Discussion

RLB hybridization is a rapid, robust and reliable method for genotyping human *NAT1* and *NAT2* alleles simultaneously. Within three days, 240 individuals were successfully genotyped for the *NAT1*4, NAT1*3, NAT1*10, NAT1*11, NAT2*4, NAT2*5A, NAT2*5BC, NAT2*6, NAT2*7* and *NAT2*12* alleles. The results of the assay were in complete agreement with the results of allele specific PCRs and PCR-RFLP methods.

Allele frequencies obtained by RLB hybridization of polyp-free subjects were comparable to those in the literature.

The commonly used methods for *NAT* genotyping have several disadvantages. The main drawback of allele specific methods used for *NAT1* genotyping is the requirement of four independent multiplex amplifications, which are difficult to optimize and often result in false positive bands. A disadvantage of *NAT2* genotyping by PCR-RFLP is that nested amplifications are conducted on the primary PCR product, which is a contamination prone procedure in large studies. Indeed, in our study ten out of 36 negative PCR controls gave a positive signal, probably due to well-to-well contamination with *NAT2* PCR products while preparing the primary PCR products for the nested PCR.

Table 3.3. *NAT1* and *NAT2* allele frequencies of the polyp-free population in this study; a comparison to other studies.

Study	*NAT1* allele frequencies					*NAT2* allele frequencies						
	alleles tested (n)	**4*	**3*	**10*	**11*	alleles tested (n)	**4*	**5A*	**5BC*	**6*	**7*	**12*
This paper	252	0.73	0.02	0.22	0.05	252	0.24	0.02	0.46	0.27	0.01	0.01
[13]	344	0.71	0.02	0.26[a]	0.01	344	0.26		0.40[b]	0.32	0.02	
[2]	224	0.77	0.03	0.16	0.08							
[3]						744	0.25		0.45[b]	0.28	0.02	
[14]						556	0.23	0.03	0.41[c]	0.31	0.01	
[15]						200				0.26	0.04	

[a] In paper differentiated in *NAT1*10* and *NAT1*14*; [b] allele *NAT*5ABC* and *NAT2*12*; [c] allele *NAT2*5BC* and *NAT2*12*.

In addition, the restriction enzymes used by RFLP analyses are relatively expensive. Both methods are time consuming because of the necessity of many amplifications and - for the RFLP analyses- the obligatory incubation and electrophoresis steps. Furthermore, interpretation of the gels after electrophoresis requires experience.

Although it is far less time consuming and less contamination prone, the RLB method has some drawbacks. Misclassification could occur by partial cross-hybridization of samples from subjects who possess a *NAT1*11* allele. These samples hybridize with the oligonucleotide probe derived from allele *NAT1*11* (NAT1-RLB4) as well as with the one derived from allele *NAT1*3* (NAT1-RLB2) because probe NAT1-RLB2 is 100% identical to a part of probe NAT1-RLB4. Therefore, we were unable to discriminate *NAT1*11* homozygous subjects from heterozygous *NAT1*3/*11* subjects. However,

none of the 240 samples were determined as *NAT1*3 /*11* since hybridization occurred only in combination with hybridization with probes derived from allele *NAT1*4* or *NAT1*10,* confirming that the homozygous *NAT1*11* genotype is very uncommon [2,13] and misinterpretation is hardly expected. For *NAT2*, misclassification between *NAT2*4/*5BC* and *NAT2*5A/*12* could occur because hybridization patterns belonging to these genotypes are identical: both genotypes include mutations at position 341 and 803. However, for *NAT2*4/*5BC* both mutations are on the same allele *(*5BC)*, whereas for *NAT2*5A/*12* the mutation at position 341 is positioned on allele **5A* and the other changed nucleotide is located at allele **12.* Nevertheless, *NAT2*12* is a very uncommon allele (Table 3.3) so only minor misinterpretation is expected.

RLB has important advantages over the usually applied methods. As mentioned earlier RLB hybridization results can easily be read and scored into a spreadsheet, which reduces the number of mistakes. The method is also less contamination prone, illustrated by the fact that all negative PCR controls remained negative by RLB analysis, whereas a substantial number of negative controls gave positive results by PCR-RFLP. The RLB hybridization method is faster than the described reference methods and one can expand the number of probes with oligonucleotide probes specific for other genes and allelic variants of interest up to 43. Moreover, it does not require expensive equipment or reagents and can easy be implemented in any laboratory that can perform PCR and hybridizations.

In conclusion, because of high throughput of samples, the ease of the procedure and the ability to enlarge the method with other allelic variants of different genes, the RLB hybridization method can easily be applied in large epidemiological studies and this will ultimately contribute to a better understanding of individual genetic susceptibility to cancer.

Acknowledgments

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4.

Risk of colorectal adenomas in relation to meat consumption, meat preparation, and genetic susceptibility

in a Dutch population

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Abstract

Heterocyclic aromatic amines (HCAs), formed during preparation of meat at high temperatures, may increase the risk of colorectal adenomas. Genetic susceptibility to HCAs possibly modifies this association. To study associations of meat consumption, meat preparation habits, and genetic susceptibility with risk of colorectal adenomas, we conducted a case-control study including 431 adenoma cases and 433 polyp-free controls, recruited among patients undergoing endoscopy. Participants completed a meat consumption and preparation questionnaire and provided blood samples for DNA isolation. Polymorphisms of *N*-actyltransferases (*NAT*) 1 and 2, sulfotransferase (*SULT*) 1A1, and glutathione-*S*-transferases (*GST*) M1 and T1 were determined. Although we detected HCAs in habitually prepared meat samples, high meat consumption did not importantly increase risk of colorectal adenomas (odds ratio (OR) 1.2, 95% confidence interval (CI) 0.8-1.9). Also, presumed unfavorable preparation habits such as the use of a lid and preference for darkly browned meat, did not increase adenoma risk (ORs and 95% CIs 0.8, 0.6-1.2 and 1.0, 0.6-1.5, respectively). There was no evidence for effect modification by *NAT1*, *SULT1A1*, *GSTM1*, and *GSTT1* polymorphisms. Only the *NAT2* slow phenotype slightly increased risk of adenomas in combination with high meat consumption (OR 1.6, 95% CI 1.1-2.3). Thus, in this Dutch population, unfavorable meat consumption and preparation habits did not increase colorectal adenoma risk, and these associations were not influenced by polymorphisms in genes involved in HCA biotransformation.

Introduction

Colorectal cancer is thought to arise from colorectal adenomas and is highly prevalent in the Western world [1,2]. Meat consumption probably increases the risk of colorectal cancer and adenomas [1-4]. This increased risk is possibly due to the exposure to heterocyclic aromatic amines (HCAs) predominantly formed during cooking of meat at high temperatures [5,6].

HCAs are potent mutagens *in vitro* and animal carcinogens [7-9] which are metabolized via various biotransformation pathways [10,11]. Many of the enzymes involved in activation and detoxification are encoded by polymorphic genes, for which several allelic variants exist that may increase or decrease enzyme expression, stability or activity. Such polymorphic genes are *N*-acetyltransferases (*NAT1* and *NAT2*) and sulfotransferase (*SULT1A1*). Glutathione-*S*-transferases (*GSTM1* and *GSTT1*) might inactivate some reactive HCA metabolites or may act more indirectly through induction of the cytochrome P450 1A2 enzyme [12,13]. Polymorphisms in all these genes are highly prevalent in Caucasian populations [14-16] and have been found to influence the association between meat preparation and colorectal neoplasm in several, but not all studies [17-19].

Besides differences in genotype frequencies over populations, these inconsistencies can be the result of differences in study and/or questionnaire design or meat consumption or preparation. In European countries, daily exposure to HCAs might be lower than in the US due to a lower meat intake and differences in meat preparation. However, the actual

exposure of the general population to these substances is unknown. In most previous studies, exposure has been estimated from HCA measurements in meat prepared under laboratory conditions [5,20-23] that do not necessarily reflect the habitual preparation methods of the general population. To investigate whether HCA exposure occurs in the Netherlands, we measured concentrations of six HCAs in beef patties prepared by volunteers according to their own preparation habits (henceforth referred to as the *'Meat preparation study'*).

To evaluate whether these preparation methods are associated with colorectal adenoma risk, we explored meat preparation methods as well as genetic polymorphisms in a case-control study on colorectal adenomas (henceforward referred to as the *'Case-control study'*).

Materials and Methods

MEAT PREPARATION STUDY

Study design

We recruited 63 volunteers out of a random sample of the general population with the same gender and age distribution as the control group of our case-control study (i.e., 66% women, mean age ± SD, 48.3±10.2 years). To maximize the variation in meat preparation methods, we selected 40 volunteers preferring meat with a darkly browned surface, and 23 subjects preferring a lightly browned surface. All volunteers prepared three beef patties (100 g per patty) at home in the way they habitually do this. After preparation, at least one patty was stored in the volunteers' fridge. If prepared from pan residues, the volunteers added a spoon of gravy. Samples were collected within 24 hr and stored at –20°C until further analyses. In addition, the volunteers completed a self-administered questionnaire on the preparation of beef patties several weeks before and during or shortly after preparation. At least 77% of volunteers answered questions on meat preparation similarly before and after baking indicating that most of them indeed prepared the meat as usual. In spite of our effort to maximize the variation in meat preparation, some preparation habits were quite uniform: only one volunteer prepared the meat at low temperature, and 10 volunteers added water during browning.

Determination of HCAs in beef patties

Beef patties were analyzed by a method based on the work described by Toribio and colleagues [24]. After homogenization, NaOH was added to 6-g aliquots. Samples were subsequently extracted with sonication. By three-step solid phase extraction, the analytes were transferred to dichloromethane and subsequently isolated using a cation exchange SPE cartridge. Further clean up of the samples was achieved using a C18 SPE cartridge. After elution of HCAs from the cartridge and evaporation, they were resolved in

methanol-water. Concentrations of six HCAs were assessed using HPLC: 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimi-dazo[4,5-*f*]quinoxaline (DiMeIQx), 2-amino-3,4,7,8-tetramethyl-3H-imida-zo[4,5-*f*]quinoxaline (TriMeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), and 2-amino-9H-pyrido[2,2-*b*]indoline (AαC). Part of the samples was analyzed in duplicate (10%) or in triplicate (8%). External calibration was applied using standard solutions of analytes. Recovery rates ranged between 36 and 57%. Detection limits, as calculated with Calwer 2.2 software using weighted regression models [25], were 1.0 ng/g for IQ, 3.7 ng/g for MeIQx, 3.9 ng/g for DiMeIQx and TriMeIQx, and 1.8 ng/g for PhIP and AαC.

CASE-CONTROL STUDY

Population

Cases and controls were recruited among patients undergoing endoscopy at the outpatient clinics of eight hospitals in the Netherlands between June 1997 and June 2000. Medical ethical committees of all participating hospitals and of Wageningen University approved the study protocol.

Potential participants were recruited at time of endoscopy by trained staff (47%), or were selected at regular intervals using endoscopy reports of all patients who had undergone endoscopy in the previous three months and invited by mail (53%). Eligible subjects were Dutch speaking, of European origin, aged 18 to 75 years at time of endoscopy, did not belong to families with hereditary colorectal cancer syndromes, did not suffer from chronic inflammatory bowel disease, and did not have a history of colorectal cancer or (partial) bowel resection. Overall response was 54%. After obtaining informed consent, blood samples were drawn for DNA analysis and questionnaires were administered. Cases had at least one histologically confirmed colorectal adenomatous polyp ever in their life. Controls had no history of any type of polyps. Complete visualization of the colon (i.e., full colonoscopy or sigmoidoscopy combined with X-ray) was achieved for 78% of controls and 89% of cases. Information on polyp recurrence, size, localization, and histology and the number of excised polyps was collected through medical files.

The study population counted 925 subjects, including 64 subjects who also met our criteria, but were recruited between December 1995 and June 1997 for a preceding study on somatic mutations in colorectal adenomas conducted in one of the eight hospitals [26].

Meat consumption and preparation assessment

Participants were requested to fill out self-administered dietary and lifestyle questionnaires according to habits in the year previous to their last endoscopy or bowel complaints.

To study meat consumption and preparation habits, a questionnaire inquiring habitual consumption of 16 meat types (frequency and portion sizes) and gravy was developed. The questionnaire also contained detailed questions on the preparation of several types of meat (e.g., height of heat source, addition of water, use of a lid) categorized into six groups according to similarities in preparation methods. These methods had been found to be determinants of the meat surface temperature in a pilot study (unpublished data). The color of the meat surface was assessed from color photographs ranging from very dark to very light (prepared at 225, 200, 175, and 150°C respectively) of four meat types (beef patties, pork chops, steak and bacon) that originate from a Swedish questionnaire [20].

The dietary questionnaire was a standardized and validated semi-quantitative food frequency questionnaire described in detail elsewhere [27] and was, in this study, used for estimation of total energy intake and intake of macro- and micronutrients, and of portion size of gravy.

Determination of genetic polymorphisms

DNA was isolated from 200 µl frozen whole blood using the QIAamp blood kit (Qiagen Inc., U.S.A.), diluted to a concentration of approximately 20 ng/µl, and stored at 4°C until analyzed. PCR was performed with internal negative and, where needed, positive controls. Laboratory personnel was blinded to case-control status.

NAT1 and NAT2. Allelic variants of *NAT1* and *NAT2* were determined by an allele specific oligo hybridization assay developed in our laboratory by Bunschoten and colleagues [28]. Using this method, we could identify *NAT1* alleles **4, *3, *10* and **11*, and *NAT2* alleles **4, *5, *6, *7, *12*. Validity and reproducibility of the method were extensively tested and proved to be 100%.

SULT1A1. The *SULT1A1* polymorphism (**1* and **2* alleles) was determined using a PCR-RFLP method described in detail by Engelke and colleagues [16]. The polymorphism was not determined in the 64 samples from the preceding study on somatic mutations (see under Population).

GSTM1 and GSTT1. *GSTM1* and *GSTT1* genotypes were simultaneously determined using a multiplex PCR procedure similar to the method of Arand and co-workers [29] with the inclusion of primers derived from β-globin instead of albumin as a positive PCR control [30]. To test reproducibility, approximately 10% of the samples were genotyped in duplicate; no differences were observed.

Data analysis

All beef patties (n=63) were analyzed for presence of HCA. Total HCA concentration was calculated by summation of the concentrations of all six HCAs. To study the probability of presence of at least one HCA in relation to preparation methods, logistic

regression was applied. Spearman rank correlations were calculated and, to study if specific preparation methods affect HCA concentrations, single classification ANOVA was applied.

From our case-control study, we excluded 61 subjects of whom dietary data was not complete, resulting in a final study population of 864 subjects: 431 cases and 433 controls. Variables describing the frequency of consumption of several meat types were divided in quartiles based on the distribution in the control group. Frequencies of red and white meat consumption were calculated by summing the frequencies of appropriate meat types, adjusted for the frequency of total meat consumption. Similarly, preparation characteristics were summarized per characteristic over the six meat categories and divided by the number of questions answered.

To study the associations of meat consumption and preparation characteristics with colorectal adenomas, odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated using unconditional logistic regression. Of potential confounders (i.e., anthropometry factors, physical activity, smoking status, history of large bowel complaints, family history of colorectal cancer, total energy intake, and intake of macro- and micronutrients and foods known to be possibly related to meat consumption), only age changed the ORs markedly. Therefore, we calculated ORs adjusted for age only, and adjusted for age, gender and indication of endoscopy to control for residual confounding by selection methods. We also considered the following variables for potential effect modification: gender, age, use of non-steroidal anti-inflammatory drugs (NSAIDs) and oral contraceptives, and family history, but none of these significantly modified the effect. When studying the association between specific types of meat or specific preparation methods and colorectal adenomas, we additionally considered inclusion of covariates describing other meat types or preparation methods in our multivariate models.

Case-case analyses were conducted to study the risk associated with adenoma recurrence (primary *vs.* recurrent), size (< 1 cm *vs.* ≥ 1 cm), localization (proximal *vs.* distal) and number (multiple *vs.* single) of adenomas. Furthermore, analyses were repeated after exclusion of cases who had been diagnosed with adenomas in the past (i.e., more than one year before the index endoscopy, n=132) and of controls without complete visualization of the colon (n=102). Also, the analyses were repeated without cases and controls who underwent endoscopy because of bowel complaints.

All analyses were conducted using Statistical Analysis Software (SAS version 6.12, SAS Institute, Cary, NC).

Results

MEAT PREPARATION STUDY

In Table 4.1, HCA concentrations in the 63 beef patty samples are linked to preparation methods. In 35% of beef patties one or more HCAs were determined, which was mostly MeIQx. Of the inquired preparation methods, use of a lid increased the probability of presence of MeIQx. Presence of IQ did not depend on preparation methods. The concentration of IQ and MeIQx seemed to be inversely related with preferred darkness of the meat surface, although not with statistical significance (r=-0.3, data not shown). The effect of cooking methods on the presence of other HCAs could not be studied due to the low number of samples in which these were observed.

Table 4.1. Results of the meat preparation study: concentration of heterocyclic amines in 63 beef patties, and correlation with preparation methods.

	Heterocyclic amine [a]					
	All six combined	IQ	MeIQx	Di-MeIQx	Tri-MeIQx	PhIP
N (%) of samples with ≥1 HCA	22 (35)	7 (11)	17 (27)	1 (2)	4 (6)	1 (2)
Median conc. [b] in positive samples (ng/g meat)	5.59	1.31	5.41	5.76	6.22	4.38
Range of conc. in positive samples (ng/g meat)	1.25-27.4	1.17-2.09	3.89-12.0	n.a. [c]	3.92-8.93	n.a. [c]
Probability of HCAs in meat in relation to preparation methods, RR (95% CI) [d]						
Preferred color of meat surface (very) dark	0.5 (0.2-1.4)	- [e]	0.5 (0.1-1.5)	-	-	-
Heat source high	1.1 (0.4-3.3)	-	0.7 (0.2-2.3)	-	-	-
Addition of water during browning	0.8 (0.2-3.3)	-	0.5 (0.1-2.1)	-	-	-
Use of a lid	3.2 (0.9-11.6)	-	5.5 (1.4-20.9)	-	-	-

[a] IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; DiMeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline; TriMeIQx, 2-amino-3,4,7,8-tetramethyl-3H-imidazo[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; 2-amino-9H-pyrido[2,2-*b*]indoline (AαC) was not detected in any of the samples and is therefore not included; [b] conc., concentration; [c] n.a., not applicable; [d] calculated from logistic regression analyses; [e] not enough data in subclasses.

CASE-CONTROL STUDY

Table 4.2 summarizes the characteristics of the case-control population. Cases were older than controls and the proportion of men was higher in the case than in the control

group. Moreover, cases had a higher body mass index, more frequently had a low educational level, more frequently (had) smoked, and less frequently underwent endoscopy because of complaints (i.e., pain, defecation problems, or rectal bleeding) than controls. Cases more frequently consumed gravy, including gravy from pan residues. Cases and controls did not differ in the way they used to prepare meat. There were no differences between cases and controls with respect to total daily energy intake (8776±2415 kJ among cases, 8677±2588 kJ among controls), or the intake of meat-related nutrients such as fat and animal proteins. However, cases consumed more alcohol and potatoes, and less grain (products) (i.e., cereals, rice, pasta) than controls (data not shown).

Table 4.2. Characteristics of the case-control study population.

Characteristic	Cases (n=431)	Controls (n=433)
General characteristics		
Age, mean ± SD	58.9±10.5	50.3±14.1[a]
Gender, % male	54.1	37.0[a]
Body mass index, mean ± SD	26.1±3.9	25.4±4.1[b]
Education level, % low	36.2	29.8[b]
Smoking, % ever smokers	61.1	49.0[a]
Low physical activity	34.8	31.9
Weekly use of NSAIDs	10.0	13.4
Endoscopy because of intestinal complaints	52.0	76.9[a]
Family history of colorectal cancer	23.4	19.2
Meat consumption, frequency/week, mean ± SD		
Total meat	5.5±1.5	5.4±1.6
Red meat	4.6±1.5	4.5±1.5
Poultry	0.9±0.8	0.9±0.7
Gravy	4.2±2.2	3.6±2.3[a]
Gravy from pan residues	3.8±2.4	3.3±2.5[b]
Meat preparation preference, %		
Never adds water during browning	27.5	26.5
Heat source always high during browning	17.9	23.9
Always uses lid	35.5	38.4

[a] $p < 0.05$ (chi-square test for categorical variables, t-test for continuous variables); [b] $p < 0.001$ (chi-square test for categorical variables, t-test for continuous variables).

In Table 4.3, the associations of meat and gravy consumption and of different preparation characteristics with colorectal adenomas are shown. As is concluded from this Table, frequent consumption of total meat was not markedly associated with colorectal adenomas. Although crude analysis suggested that gravy might be a risk factor for colorectal adenomas (OR 1.7, 95% CI 1.2-2.5), the association disappeared after adjustment for age. Risk of colorectal adenomas was not associated with frequent

consumption of red meat (Table 4.3) or white meat (multivariate OR and 95% CI 1.1, 0.7-1.6, highest vs. lowest quartile). None of the 16 meat types was associated with increased risk of colorectal adenomas, neither when included separately, nor when included all at once in the age adjusted or the multivariate model (data not shown).

Table 4.3. Risk of colorectal adenomas associated with habitual frequency of meat consumption and meat preparation methods[a].

	N cases/ controls	Age adjusted OR	Multivariate OR[b]
Total meat intake, times/week			
< 5	76/85	1 (REF)	1 (REF)
5	97/105	1.10 (0.71-1.71)	1.17 (0.72-1.89)
6	138/127	1.30 (0.86-1.97)	1.22 (0.77-1.94)
7	120/116	1.23 (0.81-1.88)	1.22 (0.76-1.94)
Red meat, times/week			
< 3.7	88/104	1 (REF)	1 (REF)
3.7 - 4.8	106/105	1.20 (0.79-1.81)	1.15 (0.73-1.81)
4.8 - 5.6	115/105	1.38 (0.92-2.08)	1.32 (0.84-2.06)
5.6+	115/105	1.22 (0.81-1.84)	1.11 (0.71-1.74)
Intake of gravy from pan residues, times/week			
0 - 0.24	93/113	1 (REF)	1 (REF)
0.25 - 4	109/136	0.98 (0.66-1.45)	0.98 (0.63-1.51)
5	95/82	1.21 (0.79-1.86)	1.22 (0.76-1.95)
6+	130/94	1.28 (0.85-1.93)	1.24 (0.79-1.95)
Temperature of heat source during browning			
Low - medium with every meat type	227/205	1 (REF)	1 (REF)
Depends on meat type	122/116	1.12 (0.80-1.57)	1.17 (0.81-1.68)
High with every meat type	76/101	0.89 (0.61-1.29)	0.83 (0.55-1.26)
Use of a lid			
Not with any meat type	152/152	1 (REF)	1 (REF)
Depends on meat type	122/108	1.16 (0.80-1.66)	1.19 (0.81-1.77)
With every meat type	151/162	0.78 (0.56-1.10)	0.81 (0.56-1.17)
Addition of water during browning			
With every meat type	139/138	1 (REF)	1 (REF)
Depends on meat type	169/173	1.02 (0.73-1.43)	1.09 (0.76-1.57)
Not with any meat type	117/112	0.99 (0.68-1.44)	0.95 (0.63-1.43)
Preferred color of meat surface			
≤ 1 of four meat types (very) dark	229/183	1 (REF)	1 (REF)
Two or three of four meat types (very) dark	130/172	0.81 (0.59-1.12)	0.83 (0.59-1.18)
All meat types (very) dark	72/78	0.94 (0.63-1.39)	0.96 (0.62-1.48)

[a] Numbers do not always add up to 431 (cases) or 433 (controls) because of missing data on some variables; [b] adjusted for age, gender, and indication of endoscopy.

When the amount of meat was taken into account, total meat increased the risk of colorectal adenomas (age adjusted OR per 100 g of meat per day 1.5, 95% CI 1.0-2.2, multivariate OR 1.4, 95% CI 0.9-2.1). The amount of gravy, as estimated from

photographs in the food frequency questionnaire, was positively – but not statistically significantly – associated with colorectal adenomas; the age adjusted OR per 100 g of gravy was 2.4 (95% CI, 0.94-6.4), and the multivariate OR 2.8 (95% CI, 0.96-7.9). If gravy not made from pan residues was excluded, the association weakened (multivariate OR 2.0, 95% CI 0.8-4.8). None of the meat preparation variables was associated with increased risk of adenomas (Table 4.3). The color of the meat surface as estimated from the photographs was not associated with adenomas (Table 4.3). None of the adenoma characteristics (i.e., histological type, size, location or number) was specifically associated with meat consumption and preparation (data not shown). Exclusion of recurrent cases yielded similar results as presented in Table 4.3 (data not shown).

Table 4.4. Genetic polymorphisms of *NAT1*, *NAT2*, *SULT1A1*, *GSTM1*, and *GSTT1* and risk of colorectal adenomas [a].

	Cases N (%)	Controls N (%)	OR (95% CI)
NAT1 [b]			
Slow (at least one **11* allele)	11 (2.6)	23 (5.3)	1 (REF)
Normal' (no **10* or **11* allele)	248 (58.1)	259 (60.1)	2.00 (0.96-4.19)
Fast (at least one **10*, no **11* allele)	168 (39.3)	149 (34.6)	2.36 (1.11-5.00)
NAT2			
Slow (no **4* or **12* alleles)	259 (60.7)	253 (58.6)	1 (REF)
Intermediate (one **4* or **12* allele)	144 (33.7)	146 (33.8)	0.96 (0.72-1.29)
Fast (*4/*4, *12/*12 or *4/*12)	24 (5.6)	33 (7.6)	0.71 (0.41-1.24)
SULT1A1			
Slow (**2/*2*)	40 (11.5)	58 (15.6)	1 (REF)
Intermediate (**1/*2*)	159 (45.7)	148 (39.7)	1.56 (0.98-2.47)
Fast (**1/*1*)	149 (42.8)	167 (44.8)	1.29 (0.82-2.05)
GSTM1			
Present	198 (46.3)	207 (47.9)	1 (REF)
Null	230 (53.7)	225 (52.1)	1.07 (0.82-1.40)
GSTT1			
Present	367 (85.8)	365 (84.5)	1 (REF)
Null	61 (14.3)	67 (15.5)	0.91 (0.62-1.32)

[a] Numbers do not always add up to 431 (cases) or 433 (controls) because of missing data on some polymorphisms; [b] some older studies suggest that the *NAT1*10* allele is associated with increased NAT1 activity [50,51] whereas no differences in activity were observed between **4/*4*, **4/*10* and **10/*10* genotypes in a recent study [52].

Table 4.4 gives the frequencies of imputed phenotypes of the genes under study, and of the associations of these genes with adenomatous polyps. As can be concluded from this Table, *NAT1* fast and 'normal' acetylator (i.e., no **10* or **11* allele) genotypes were positively associated with colorectal adenoma risk. *NAT2* imputed phenotypes were not

Table 4.5. Interplay between genetic susceptibility and frequency of consumption of total meat and gravy and meat preparation methods in the etiology of colorectal adenomas.

	NAT2 imputed phenotype [a]				*SULT1A1* imputed phenotype [b]			
	slow		intermediate/fast		slow		intermediate/fast	
	N ca/co [c]	OR (95% CI) [d]	N ca/co	OR (95% CI)	N ca/co	OR (95% CI)	N ca/co	OR (95% CI)
Total meat intake								
Low	100/121	1 (REF)	71/68	1.36 (0.87-2.14)	16/26	1 (REF)	126/137	1.49 (0.74-3.00)
High	159/132	1.57 (1.08-2.28)	97/111	1.11 (0.74-1.66)	24/32	1.45 (0.67-3.44)	182/178	1.64 (0.82-3.25)
Intake of gravy from pan residues								
Low	111/158	1 (REF)	88/90	1.37 (0.92-2.05)	20/35	1 (REF)	148/186	1.26 (0.67-2.34)
High	144/91	1.74 (1.19-2.54)	80/85	1.10 (0.73-1.67)	20/22	1.24 (0.53-2.93)	158/123	1.59 (0.84-3.00)
Heat source high with every meat type								
No	208/192	1 (REF)	137/128	1.02 (0.74-1.42)	30/40	1 (REF)	251/237	1.18 (0.71-1.98)
Yes	47/52	1.08 (0.67-1.72)	29/49	0.66 (0.39-1.12)	6/18	0.55 (0.19-1.61)	52/69	0.97 (0.52-1.78)
Use of a lid								
Never	83/81	1 (REF)	67/71	0.93 (0.58-1.51)	16/23	1 (REF)	104/111	1.13 (0.55-2.35)
Depends on meat type	80/62	1.30 (0.81-2.10)	42/45	0.93 (0.54-1.62)	10/13	1.13 (0.37-3.45)	94/76	1.51 (0.72-3.18)
Always	92/102	0.74 (0.47-1.15)	57/60	0.80 (0.48-1.33)	14/22	0.64 (0.24-1.71)	105/118	0.90 (0.43-1.86)
Preferred color of meat surface								
≤ 1 of 4 types dark	137/112	1 (REF)	90/70	1.04 (0.69-1.58)	21/23	1 (REF)	162/137	1.19 (0.61-2.32)
2 or 3 of 4 types dark	74/104	0.76 (0.50-1.15)	54/68	0.91 (0.57-1.45)	12/25	0.72 (0.28-1.88)	93/124	1.01 (0.51-2.01)
All types dark	48/37	1.39 (0.82-2.35)	24/41	0.59 (0.33-1.07)	7/10	0.99 (0.31-3.23)	53/54	1.24 (0.59-2.61)

[a] *NAT2* slow, no **4* or **12* alleles present; intermediate/ fast, at least one of the **4* or **12* alleles present; [b] *SULT1A1* slow, no **1* allele present; intermediate/fast, at least one **1* allele present; [c] N ca/co, number of cases over number of controls, numbers do not always add up to 431 (cases) or 433 (controls) because of missing data; [d] odds ratios and 95% confidence intervals are adjusted for age.

markedly associated with colorectal adenomas. Those with the *SULT1A1*1/*2* genotype had a borderline significantly increased risk of colorectal adenomas. *GSTM1* and *GSTT1* polymorphisms were not associated with adenomas (Table 4.4).

In Table 4.5, colorectal adenoma risk is shown for meat consumption and preparation in combination with imputed phenotypes of the genes that are considered most relevant for HCA detoxification and of which the phenotypic variants occur at sufficiently high frequencies, i.e., *NAT2* and *SULT1A1*. Multivariate ORs are not shown but are similar to the age-adjusted ORs. No specific combination clearly increased risk of adenomatous polyps. Interestingly, slow instead of fast *NAT2* acetylators had an increased risk of adenomas when consuming meat and gravy relatively often. Similarly, there was a suggestion for a positive association of the combination of *NAT2* slow acetylation and preference for heavily browned meat with colorectal adenomas, whereas fast acetylation appeared to decrease risk. Though subjects with intermediate/fast *SULT1A1* imputed phenotypes had a slightly higher risk of colorectal adenomas when consuming meat or gravy relatively frequently, the *SULT1A1* polymorphism did not modify the associations significantly (Table 4.5). Results for *NAT1* are not shown in Table 4.5 because of low counts in the subcategories, as stated above. Although the ORs for combinations of high meat and gravy consumption with *NAT1* fast acetylation were highest, there was no indication for an interaction between these factors (data not shown). There were no differences in risk of colorectal adenomas between combinations of *GSTM1* variants and meat or gravy consumption (data not shown). For those with the *GSTT1* null genotype, gravy consumption was not a risk factor, whereas those with other *GSTT1* genotypes had a borderline significantly increased risk of adenomas (OR 1.3, 95% CI 0.9-1.7).

Discussion

We found no strong indications for meat consumption to increase risk of colorectal adenomas. Also, risk of adenomas was not associated with specific meat preparation methods, including the preferred color of the meat surface. Only the *NAT2* slow phenotype slightly increased risk of adenomas in combination with high meat consumption. Polymorphisms of other genes (*NAT1*, *SULT1A1*, *GSTM1*, and *GSTT1*) did not markedly influence the associations of meat consumption and preparation with adenomas.

Participants in our case-control study underwent endoscopy because of bowel complaints (e.g. rectal bleeding, abdominal pain, defecation irregularities), or for screening because of previous adenomas or family history of colorectal cancer. This implies that we should be prudent in extrapolation of our results to the general population. However, our population was comparable with respect to risk factors such as meat consumption to a sample we randomly selected of the general population

(unpublished data). We do not expect selection bias to have occurred because cases and controls were selected using identical procedures and there were no differences between cases and controls with respect to energy intake and intake of macronutrients. Information bias is not likely to have occurred although most of the retrospectively recruited participants were aware of their case-control status at the time of completion of the questionnaires. As dietary advice, if provided, was to increase vegetable and/or fiber consumption only, our study population was probably not aware that meat consumption might be a risk factor for colorectal adenomas. Allele frequencies of the studied polymorphisms were similar to those reported from other studies [14-16,31].

Meat consumption as assessed by the meat consumption and preparation questionnaire correlated well with meat consumption as assessed by a semi-quantitative food frequency questionnaire validated for intake of energy, macro- and micronutrients, and all important food groups, including meat [27,32]. Correlation coefficients for the estimated frequencies of meat consumption ranged from 0.69 for beef to 0.88 for gravy. Portion sizes of the meats consumed were also inquired but were considered to be less precise than the frequencies. Portion sizes as calculated from the meat consumption and preparation questionnaire correlated moderately with those estimated from the food frequency questionnaire by photographs (correlation coefficients ranged from 0.58 for white meat to 0.70 for gravy) [27]. The questions on the preparation of meat referred to Dutch cooking methods determining the temperature at the meat surface, which is an important determinant of HCA concentration [33]. Our questionnaire included photos from an extensive Swedish questionnaire used to study the association between HCA intake and risk of several cancers [20]. Information on a limited number of meat dishes, comparable to the number and type of dishes in our Dutch questionnaire, estimated potential HCA exposure almost equally well as this extensive Swedish questionnaire, introducing only a very limited amount of misclassification [34].

Although the results of our case-control study are not in accordance with those of some studies [17,35-38], they correspond to those of others [19,39,40], and more importantly, to the observations in our meat preparation study. In the latter study, we found no associations of meat preparation methods (except use of a lid) and preferred color of the meat surface with HCA concentrations. There are several possible explanations for our results, concerning homogeneity of the study population, the method used for HCA determination, and the relation between the inquired meat preparation methods and HCA concentrations, as we explain below.

First, the populations we studied might have been too homogeneous with respect to meat consumption and preparation methods to observe effects. Populations studied by Lang, Sinha, and Probst-Hensch [17] included subjects from multiple ethnic groups and were therefore probably more heterogeneous [37,38]. Lack of variation in our meat preparation study could have been the result of the meat type used. We chose beef

patties because these are usually prepared at high temperature and were thus expected to contain at least low HCA concentrations. Moreover, beef patties are acceptable by most social, religious, and ethnic groups, ages and sexes, (i.e., no pork or white meat), their preparation is not time-consuming, and they are available at relatively low costs.

Second, the detection limits in our meat preparation study were relatively high and this could explain why HCAs were detected in only 35% of samples. These high limits were caused by background signals for which we adjusted using weighted regression [25]. Background signals were possibly high because whole meat samples including gravy were analyzed instead of meat crusts only. Recovery rates were comparable to those found in other studies [43]. High detection limits could be an explanation why PhIP was detected in only one sample, but it is more probable that PhIP levels were indeed low and more comparable to those found in Swedish studies [5], than to those reported from US studies [22,42,44,45]. Possibly, Dutch cooking methods are more similar to those applied by Swedish than by US populations. This is illustrated by results from a pilot study, in which Dutch volunteers judged the photographs developed by Sinha and colleagues (see e.g. [21,22]) too dark whereas those used in Sweden [20] were considered to be applicable to the Dutch situation.

Third, it is possible that the meat preparation methods we inquired are not the main determinants of HCA concentrations, although the color of the meat surface was found to be an important determinant of HCA concentrations in laboratory studies [5,21,22]. Laboratory conditions do not necessarily reflect domestic cooking conditions and this could explain our results as well as those from the study by Augustsson and colleagues [39]. According to Dutch cooking methods, meat is pan-fried, starting with a short frying phase to sear the meat, after which water is added (depending on the meat type) and the meat is then simmered until done. Meat prepared in this way can be well done without having a darkly browned surface and frying time might be an important determinant of HCA formation [41,44]. However, we had no information on frying time and it will be difficult to estimate this in large populations.

Even though preparation methods did not reflect HCA exposure in our study population, the consumption of meat might be an important determinant of colorectal adenoma risk, especially when genetic polymorphisms are taken into account. This was indeed found in several [17,18,46,47], but not in all studies [19]. Unexpectedly, we found that slow *NAT2* acetylators consuming meat and gravy relatively frequently were at highest risk of colorectal adenomas. Fast acetylators are generally considered to be the risk group because NAT-catalyzed activation of HCAs seems to be favored over detoxification [48]. However, the balance between these two may be determined by many factors. Our findings could, however, well be due to chance since we studied potential effects of gene-environment interactions that are now being regarded as having, at most, modest effects [49].

In conclusion, HCA exposure occurs in the general Dutch population but is not clearly related to inquired meat preparation methods or preferred meat color. This might explain why we found no association between meat preparation methods and colorectal adenomas and no obvious influence of genetic susceptibility to HCAs. Possibly, other factors or other substances in meat may explain the observed associations in other studies. However, as HCAs are potent carcinogens, they should still be considered as potential risk factors for colorectal neoplasm. A detailed study of the determinants of HCA formation in different countries and a large study in a heterogeneous population may help to elucidate the importance of HCA exposure with respect to colorectal neoplasm in Europe.

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5.

SULT1A1 and *NAT2* modify the association of cigarette smoking with colorectal adenomas

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Abstract

Cigarette smoke contains polycyclic hydrocarbons and arylamines that may both be activated by sulfotransferase, encoded by *SULT1A1*. A genetic polymorphism leads to an Arg213His substitution thereby decreasing enzyme activity and stability, and might thus modify the association between smoking and colorectal adenomas. We investigated this in a Dutch case-control study. Additionally, we evaluated potential roles of epoxide hydrolase (*EPHX*), *N*-acetyltransferases (*NAT1* and *NAT2*), and glutathione *S*-transferases (*GSTM1* and *GSTT1*). The data analysis included 431 adenoma cases and 432 polyp-free controls (54 % women, mean age 54.6 y) enrolled at endoscopy in eight Dutch hospitals between 1997 and 2000. All participants provided data on smoking habits and blood for DNA isolation. Genotyping was performed using appropriate PCR(-RFLP) procedures. Multivariate models included age, sex, endoscopy indication, consumption of snacks and alcohol, and, if appropriate, daily smoking dose or smoking duration. Smoking increased colorectal adenoma risk, most importantly with smoking duration. Smoking for more than 25 years more than doubled adenoma risk (OR 2.4, 95% CI 1.4-4.1) compared to never smokers. Combinations of *SULT1A1* fast sulfation (**1/*1*) and of *NAT2* slow acetylation with smoking, resulted in a four times higher risk of adenomas compared to never smokers with other inherited gene variants. Although variation over variants of *EPHX* was observed, there was no clear pattern. The other polymorphisms studied did not influence the association of smoking with adenomas. We conclude that smoking increases risk of colorectal adenomas and that *SULT1A1* and *NAT2* may modify in this association.

Introduction

Cigarette smoking is consistently found to be associated with the occurrence of colorectal adenomas [1], and long-term smoking might increase colorectal cancer risk (as recently reviewed by Giovannucci [2]). Tobacco smoke contains many potential carcinogens, among which polycyclic aromatic hydrocarbons (PAHs) and arylamines [3]. The metabolism of these substances is complex and involves activation and detoxification steps, catalyzed by many polymorphic enzymes, such as glutathione *S*-transferases Mu and Theta (encoded by *GSTM1* and *GSTT1*), *N*-acetyltransferases (encoded by *NAT1* and *NAT2*), microsomal epoxide hydrolase (encoded by *EPHX*), and thermo-stable phenol sulfotransferases (encoded by *SULT1A1*).

Although thermo-stable phenol sulfotransferase is a key-enzyme in the metabolism of arylamines and some PAHs [4-6], to our knowledge, genetic variants of *SULT1A1* have not yet been studied in combination with smoking and colorectal adenomas. The gene contains several polymorphic sites, of which a *G* to *A* transition leading to an Arg213His substitution is highly prevalent [7]. The His variant (**2* allele, occurring at a frequency of 0.3 [7]) was associated with decreased activity and thermal stability of the enzyme in platelets [8,9] as well as decreased bioactivation of various promutagens by cDNA-expressed enzymes [10]. From a study conducted on breast cancer, well-done meat consumption (which may contain heterocyclic aromatic amines and PAHs) was a risk factor only in women with the *SULT1A1*1/*1* and **1/*2* genotypes [11]. Considering the

role of *SULT1A1* in the activation of procarcinogens of cigarette smoke and similarities between determinants of breast and colorectal cancer, the *SULT1A1* polymorphism might importantly modify the association between smoking and colorectal adenomas.

EPHX polymorphisms were reported to be possibly important in the association of smoking with colorectal neoplasm. In line with previous research [12], Ulrich and colleagues found that subjects with slow or unstable *EPHX* variants were at higher risk of colorectal adenomas when exposed to cigarette smoke [13], whereas on the opposite, fast epoxide hydrolase increased the risk of smoking-associated adenomas in another study[14].

Several studies conducted on the role of *GSTs* and *NATs* in the association between smoking and colorectal neoplasm did not reveal consistent associations (reviewed by Cotton and co-workers [15] and Brockton and colleagues [16]).

Because *GSTs* may be involved in the inactivation of some reactive intermediates formed from arylamines and PAHs, *GSTM1* or *GSTT1* null genotypes might lead to higher risk of colorectal adenomas. The other enzymes play dual (activating and deactivating) roles in the metabolism of arylamines and PAHs [6,17,18]. We investigated the role of *SULT1A1* and *EPHX* genotypes, as well as potential roles of *NAT1*, *NAT2*, *GSTM1* and *GSTT1*, in smoking associated-colorectal adenomas in a Dutch case-control study.

Materials and Methods

Population

Cases and controls were recruited among subjects undergoing endoscopy at the outpatient clinics of eight hospitals in the Netherlands between June 1997 and June 2000. Medical Ethical committees of all participating hospitals and of Wageningen University approved the study protocol.

Potential participants were recruited at time of endoscopy by trained staff (47%), or were selected at three-month intervals – using endoscopy reports of all patients who had undergone endoscopy in the preceding months – and invited by mail (53%). Eligible subjects were Dutch speaking, of European origin, aged 18 to 75 years at time of endoscopy, had no hereditary colorectal cancer syndromes, chronic inflammatory bowel disease, or history of colorectal cancer or (partial) bowel resection. Overall response was 54%. After obtaining informed consent, blood samples were drawn for DNA analysis and questionnaires were administered. Cases had at least one histologically confirmed colorectal adenomatous polyp ever in their life. Controls had no history of any type of polyps. Complete visualization of the colon (i.e., full colonoscopy or sigmoidoscopy combined with X-ray) was achieved for 78% of controls and 89% of cases. Information on history, size, localization, histology, and number of polyps was collected through medical files.

We included information from 64 participants to a preceding and similar study that was conducted between December 1995 and June 1997 in one of the eight hospitals [19], resulting in a study population of 925 subjects.

QUESTIONNAIRES

Participants were requested to fill out self-administered questionnaires according to habits in the year previous to their last endoscopy or complaints.

Smoking habits were assessed inquiring current smoking status, smoking materials, amounts smoked per day (separately for cigarettes, cigars and pipe), total number of years smoked, and, if applicable, the age at which the participant stopped smoking. The questionnaire also included questions on potential confounders such as physical activity (assessed according to Baecke and colleagues [20]), frequency of constipation in the last three years, the number of first- and second-degree family members with colorectal cancer, and the highest completed level of education.

To assess dietary habits, we used a standardized semi-quantitative food frequency questionnaire, described by Ocké and co-workers [21]. This questionnaire was validated by comparison with dietary intake assessed by twelve 24-h recalls. For the nutrients considered in the present study, Pearson coefficients of correlation between these recalls and the questionnaire ranged from 0.85 for alcohol to 0.61 for fat and dietary fiber among men and from 0.87 (alcohol) to 0.63 (fat) among women. Of the foods considered as potential confounders, relative validity was lowest for vegetables among men and women (Spearman rank correlation coefficients 0.38 and 0.31, respectively) and highest for fruit among men (r=0.68) and for meat among women (r=0.70).

LABORATORY ANALYSES

DNA was isolated from 200 µl frozen whole blood using the QIAamp blood kit (Qiagen Inc., U.S.A.), diluted to a concentration of approximately 20 ng/µl, and stored at 4°C until analyzed.

SULT1A1. The *SULT1A1* polymorphism (**1* and **2* alleles) was determined by a PCR-RFLP method described in detail by Engelke and colleagues [22]. The polymorphism was not determined in the 64 samples from the preceding study on somatic mutations (see under Population).

EPHX. *EPHX* exon 3 and exon 4 allelic variants (*113Y* and *113H*, and *139H* and *139R* alleles) were determined by RFLP analysis as described elsewhere [23]. Reproducibility was tested by genotype determination of approximately 10% of the samples twice. The reproducibility of exon 3 and 4 genotyping was respectively 98% and 100%.

NAT1 and *NAT2.* Allelic variants of *NAT1* and *NAT2* were determined by an allele specific oligo hybridization assay developed in our laboratory [24] identifying *NAT1* alleles

**4, *3, *10* and **11*, and *NAT2* alleles **4, *5, *6, *7, *12*. Validity and reproducibility of the method were extensively tested and proved to be 100%.

GSTM1 and *GSTT1*. We determined the genotypes of *GSTM1* and *GSTT1* simultaneously using a multiplex PCR procedure adapted from Arand and co-workers [25]. To control PCR performance, primers derived from β-globin [26] were included. To test reproducibility, approximately 10% of the samples were genotyped in duplicate; no differences were observed.

PCR was performed with internal negative controls. Laboratory personnel was blinded to case-control status.

DATA ANALYSIS

We excluded 38 subjects with insufficient dietary data, ten subjects of whom cigarette smoking status was unknown, and 14 subjects of whom no information about any of the genotypes under study was available, resulting in a final study population of 863 subjects: 431 cases and 432 controls.

Since there were few cigar/pipe smokers in our study population and smoking of cigars and/or pipe was not a risk factor for colorectal adenomas, we only considered cigarette smoking habits, i.e., smoking status (never, former, and current smoking), total smoking duration (excluding intermediate periods without smoking), daily number of cigarettes smoked, and, if applicable, the time since giving up smoking. Categorical variables had a separate category for missing information. Non-categorical exposure variables were categorized so that each category contained approximately equal numbers of controls. The lowest exposure categories served as the reference.

GSTM1 and *GSTT1* homozygous deletion were considered as high-risk categories. *NAT1* genotypes were categorized as slow (at least one *NAT1*11* allele) and fast (all others) [27]. *NAT2* imputed phenotypes were fast (*NAT2*4/*4*, *NAT2*4/*12*, and *NAT2*12/*12*), intermediate (one *NAT2*4* or *NAT2*12* allele) and slow (all others). Phenotypes of *EPHX* were imputed for both exons separately and for the combination of exon 3 and exon 4 *113H* and *139H*, and their combination were considered to result in low enzyme activity. To facilitate comparison of our results with those of other studies, we compared *EPHX* phenotypes as we previously imputed [23] with the classification according to Cortessis and colleagues [14] and the classification used by Ulrich and colleagues [13]. *SULT1A1* was categorized as follows: **2/*2* as slow, **1/*2* as intermediate, and **1/*1* as fast sulfation.

Univariate analyses were conducted to test for potential confounders (i.e., gender, age, body mass index, physical activity, education level, indication of endoscopy, smoking of cigars or pipe (y/n), history of constipation (y/n), family history of colorectal cancer (y/n), consumption of vegetables, fruit, meat, alcohol, and snacks, and the intake of energy, fat, and fiber). These showed that gender, age, body mass index, indication of

endoscopy, dietary changes, and the consumption of total meat, alcohol and snacks were associated both with colorectal adenomas and with cigarette smoking. The multivariate models included the variables age, sex, and indication of endoscopy (three levels: gastrointestinal complaints, screening, or unknown/other). Additionally, we added consumption of snacks and alcohol (both in g/d) to the multivariate model, because these factors were found to influence univariate ORs for the association between smoking (both status and number of cigarettes per day) and colorectal cancer most importantly after age (>5% change in OR). ORs for smoking duration were additionally adjusted for the number of cigarette smoked per day, and vice versa.

To test whether the combinations of imputed phenotypes and smoking deviated from multiplicativity, we calculated p-values for interaction by inclusion of a term for imputed phenotype (as high-risk=2, intermediate-risk=1, and low-risk imputed phenotype=0) multiplied by smoking duration as a continuous variable into our multivariate models. To test whether combinations of imputed phenotypes and smoking were more or less than additive, we applied bootstrapping to calculate a 95% confidence interval with the calculated RERI (excess risk due to interaction) [28] according to the following formula:

$$RERI = RR_{(\text{'high-risk phenotype' and smoking})} - RR_{(\text{high-risk phenotype alone, no smoking})} - RR_{(\text{smoking, no 'high-risk phenotype'})} + 1.$$

A statistically significant confidence interval should not include the value 0.

To exclude the influence of previous adenomas (i.e., more than one year before the index endoscopy, n=129) among cases, and of undetected proximal polyps among controls (n=97), we repeated our analyses without these groups.

As smoking might be a risk factor in different stages of tumorigenesis [29-31], we studied primary *vs.* recurrent adenomas, adenomas smaller than 1 cm *vs.* those ≥ 1 cm), proximal *vs.* distal adenomas and multiple *vs.* single adenomas. These subgroup analyses, except the analyses on recurrent adenomas, were conducted for primary adenomas only.

All analyses were conducted using Statistical Analysis Software (SAS version 6.12, SAS Institute, Cary, NC).

Results

In Table 5.1, the characteristics of the study population are given by cigarette smoking status. Smokers had a higher probability to have ever been diagnosed with colorectal adenomas, had a higher intake of energy, fat, meat, and coffee, but consumed less fruit than never smokers. The group of past smokers was older than the never and current smokers, and counted more men and more alcohol drinkers than the other two groups. Current smokers were younger and less well educated, had a lower intake of fiber, vegetables, and fruit, and consumed more coffee than past and never smokers (Table 5.1). Strikingly, *GST* genotypes seemed to be associated with smoking status. The

frequency of the *GSTM1* null genotype was highest in the group of current smokers, whereas the *GSTT1* null genotype frequency was lowest in this group. Fast sulfation was present at highest frequency among never smokers (Table 5.1).

Table 5.1. Characteristics of the study population by cigarette smoking status.

Characteristic	Cigarette smoking status		
	Never smoker N=387	Past smoker N=274	Current smoker N=202
Age, years, mean ± SD	55.1 ± 13.5	57.5 ± 11.5 [a]	49.7 ± 13.5 [b,c]
Men, n (%)	151 (39.0)	167 (61.0) [a]	78 (38.6) [c]
Body mass index, kg/m^2, mean ± SD	25.9 ± 4.2	26.2 ± 3.6	24.8 ± 4.0 [b,c]
Cases, n (%)	166 (42.9)	161 (58.9) [a]	104 (51.5) [b]
Bowel complaints as indication, n (%)	245 (63.3)	172 (62.8)	139 (68.8)
Family history of colorectal cancer, n (%)	81 (20.9)	57 (20.8)	45 (22.3)
Low educational level, n (%) [d]	133 (34.4)	74 (27.0) [a]	81 (40.1) [b,c]
Low physical activity, n (%) [e]	128 (33.1)	103 (37.6)	61 (30.2)
Ever smoked pipe or cigars, n (%)	25 (6.5)	27 (9.9)	7 (3.5) [c]
Cigarette smoking duration, years, mean±SD	0 ± 0	23.6 ± 12.2 [a]	29.5 ± 12.4 [b,c]
Cigarettes per day, mean ± SD	0 ± 0	16.7 ± 11.4 [a]	15.6 ± 8.3 [b]
Alcohol drinkers, n (%)	302 (78.0)	243 (88.7) [a]	169 (83.7)
Energy intake, kJ/day, mean ± SD	8406 ± 2344	8951 ± 2407 [a]	9106 ± 2932 [b]
Fiber, g/day, mean ± SD	24.0 ± 6.6	24.1 ± 6.3	22.7 ± 7.1 [b,c]
Fat, g/day, mean ± SD	78.3 ± 27.3	84.7 ± 27.5 [a]	88.2 ± 34.5 [b]
Vegetables, g/day, mean ± SD	121.3 ± 43.4	121.2 ± 44.4	111.6 ± 45.0 [b,c]
Fruit, pieces/day, mean ± SD	1.6 ± 1.1	1.4 ± 1.0 [a]	1.1 ± 1.0 [b,c]
Meat, g/day, mean ± SD	97.5 ± 49.0	109.6 ± 53.4 [a]	109.9 ±57.4 [b]
Coffee, cups/day, mean ± SD	3.8 ± 2.3	4.5 ± 2.6 [a]	5.1 ± 3.2 [b,c]
Snacks, g/day, mean ± SD	30.6 ± 27.3	33.8 ± 29.6	36.9 ± 30.3 [b]
SULT1A1 fast, n (%) [f]	163 (49.1)	85 (38.8) [a]	70 (41.2)
EPHX exon 3 slow, n (%) [f]	48 (13.3)	34 (13.8)	19 (10.1)
EPHX exon 4 slow, n (%) [f]	234 (65.2)	167 (67.3)	111 (58.7)
NAT1 fast, n (%) [f]	368 (95.8)	265 (97.4)	190 (94.5)
NAT2 fast, n (%) [f]	164 (42.6)	112 (41.2)	71 (35.3)
GSTM1 null, n (%)	203 (52.6)	132 (48.5)	119 (59.2) [c]
GSTT1 null, n (%)	70 (18.1)	41 (15.1)	19 (9.5) [b]

[a] Significantly different between past and never smokers; [b] significantly different between current and never smokers; [c] significantly different between current and past smokers; [d] primary school or lower vocational training only; [e] scored according to Baecke [20] and divided in tertiles [f] fast *SULT1A1* sulfation is defined as presence of two **1* alleles, the *EPHX* slow imputed phenotypes are *HH* for exon 3 and *HH* for exon 4, fast *NAT1* acetylation is defined as absence of **11* alleles, fast *NAT2* acetylation includes the following combination of alleles: **4/*4*, **4/*12*, and **12/*12*.

Table 5.2. Cigarette smoking and risk of colorectal adenomas.

Characteristic	N cases/controls	OR (95% CI) [a]	p-trend
Cigarette smoking status			
Never	166/221	1 (REF)	0.0002
Former	161/113	1.62 (1.12-2.33)	
Current	104/98	2.10 (1.38-3.18)	
Duration of smoking (years)			
0	166/221	1 (REF)	0.0006
1-15	49/73	1.28 (0.70-2.33)	
16-25	63/55	2.19 (1.21-3.98)	
>25	153/83	2.42 (1.43-4.11)	
Cigarettes per day [b]			
0	166/221	1 (REF)	0.78
1-9	58/51	1.09 (0.56-2.13)	
10-19	91/77	0.89 (0.44-1.79)	
≥ 20	109/72	1.17 (0.59-2.33)	
Time since quitting (years) [c]			
Never smoked	166/221	1 (REF)	0.03
>18	52/39	1.23 (0.73-2.07)	
8.1-18	38/35	1.26 (0.70-2.27)	
1-8	57/33	2.06 (1.18-3.59)	
<1 [d]	113/104	2.13 (1.42-3.21)	

[a] Adjusted for age, sex, indication of endoscopy, and consumption of snacks and alcohol (in g/d). ORs for smoking duration were additionally adjusted for the number of cigarettes smoked per day (continuous), and ORs for cigarettes per day were also adjusted for smoking duration (years, continuous). [b] seven cases and 12 controls not included because of missing data. [c] six cases not included because of missing data. [d] includes nine cases and six controls who quit smoking less than one year ago, others are current smokers.

Table 5.2 shows the associations of cigarette smoking characteristics with risk of adenomas. Current smokers were at highest risk of colorectal adenomas, and although lower, risk of adenomas was still significantly higher among former than among never smokers. The risk of adenomas increased with cigarette smoking duration, also after adjustment for smoking dose. However, after adjustment of the number of cigarettes smoked per day by smoking duration, the number of cigarettes smoked per day no longer increased colorectal adenoma risk (Table 5.2). After giving up smoking, the risk of adenomas decreased and smoking was not associated with risk of adenomas anymore eight years after quitting. Adenoma risk was not different for different types of cigarettes smoked (i.e., filter, non-filter, or both types, data not shown). Although it was a risk factor for both sexes, smoking was a stronger risk factor for adenomas among men than it was among women (risk estimates for current *vs.* never smoking, OR 3.1, 95% CI 1.6-3.7, and OR 1.6, 95% CI 1.0-2.6, respectively). This was probably due to the longer

duration of cigarette smoking (mean ± SD, 18.6 ± 16.7) among men than among women (mean ± SD, 11.8 ± 14.7). The associations shown in Table 5.2 did not differ between former and current smokers. These associations did not change after restriction of the study population to cases with first adenoma diagnosed at the endoscopy at time of invitation and to controls whose colon was completely visualized, or to subjects undergoing an endoscopy for other reasons than gastrointestinal complaints or defecation problems.

The association of smoking duration with specific colorectal adenoma characteristics is shown in Table 5.3. We found that cigarette smoking was a stronger risk factor for small adenomas (equal to or smaller than 1 cm) compared to larger adenomas, and for (tubulo-)villous adenomas compared to adenomas without villous characteristics.

Table 5.3. Association of cigarette smoking with specific colorectal adenoma characteristics: odds ratios and 95% confidence intervals[a,b].

Adenoma characteristic		Duration of smoking (years)		
		0	1 - 25	> 25
	N controls	221	128	83
Size of largest adenoma				
≤ 1 cm	OR (95% CI)	1 (REF)	1.99 (1.04-3.82)	2.48 (1.28-4.81)
	N cases	43	44	45
> 1cm	OR (95% CI)	1 (REF)	1.14 (0.58-2.23)	2.10 (1.09-4.07)
	N cases	68	33	60
Most 'severe' histology				
tubular	OR (95% CI)	1 (REF)	1.58 (0.86-2.91)	2.40 (1.29-4.47)
	N cases	61	49	64
(tubulo-)villous	OR (95% CI)	1 (REF)	1.44 (0.70-2.87)	2.00 (1.00-3.98)
	N cases	52	29	45
Number of adenomas				
single	OR (95% CI)	1 (REF)	1.82 (1.02-3.24)	2.04 (1.11-3.75)
	N cases	69	53	55
multiple	OR (95% CI)	1 (REF)	1.05 (0.47-2.34)	2.57 (1.23-5.34)
	N cases	44	25	52
Location of adenomas				
all adenomas distal	OR (95% CI)	1 (REF)	1.73 (1.00-3.00)	2.21 (1.25-3.91)
	N cases	85	68	81
at least one proximal	OR (95% CI)	1 (REF)	0.68 (0.23-1.96)	2.37 (0.98-5.78)
	N cases	28	10	28

[a] Adjusted for age, sex, indication of endoscopy, consumption of snacks and alcohol (in g/d), and number of cigarettes smoked per day; [b] 6[1]/2 cases with first adenomas less than one year before the index endoscopy included, for about 2% of these, we had no detailed information on adenoma characteristics.

Table 5.4. Risk[a] of colorectal adenomas in association with combinations of smoking duration and inherited genetic susceptibility.

Imputed phenotype			Duration of smoking (years)		
			0	1 - 25	> 25
SULT1A1	Slow	OR (95% CI)	1 (REF)	1.51 (0.42-5.47)	3.47 (0.97-12.40)
		N ca/co[b]	13/27	13/20	13/9
	Intermediate	OR (95% CI)	1.94 (0.80-4.72)	3.07 (1.12-8.43)	3.94 (1.41-11.04)
		N ca/co	59/70	42/47	60/30
	Fast	OR (95% CI)	1.64 (0.69-3.90)	4.66 (1.66-13.11)	4.32 (1.59-11.77)
		N ca/co	66/97	32/28	51/34
EPHX exon 3	Fast	OR (95% CI)	1 (REF)	1.68 (0.84-3.34)	3.08 (1.59-5.97)
		N ca/co	72/101	42/57	76/39
	Intermediate	OR (95% CI)	0.93 (0.55-1.59)	2.35 (1.15-4.81)	1.39 (0.66-2.95)
		N ca/co	58/81	44/45	45/35
	Slow	OR (95% CI)	0.89 (0.43-1.86)	1.27 (0.88-8.52)	3.63 (1.22-10.83)
		N ca/co	22/26	11/15	20/7
EPHX exon 4	Fast	OR (95% CI)	1 (REF)	2.93 (0.48-17.80)	1.75 (0.33-9.27)
		N ca/co	8/16	5/4	5/5
	Intermediate	OR (95% CI)	0.88 (0.31-2.56)	2.74 (0.88-8.52)	2.06 (0.65-6.56)
		N ca/co	38/63	34/39	42/25
	Slow	OR (95% CI)	1.34 (0.50-3.62)	2.17 (0.73-6.44)	3.85 (1.29-11.50)
		N ca/co	105/129	58/75	94/51
EPHX exon 3 & exon 4	Fast	OR (95% CI)	1 (REF)	2.11 (1.22-3.65)	2.51 (1.44-4.36)
		N ca/co	136/196	88/108	128/78
	Slow	OR (95% CI)	1.51 (0.61-3.69)	1.66 (0.50-5.45)	6.93 (1.64-29.34)
		N ca/co	15/12	9/9	13/3
GSTM1	Present	OR (95% CI)	1 (REF)	2.01 (1.05-3.84)	2.45 (1.24-4.81)
		N ca/co	81/102	53/62	66/41
	Null	OR (95% CI)	1.18 (0.74-1.88)	1.88 (1.00-3.84)	2.89 (1.51-5.50)
		N ca/co	85/118	56/66	87/42
GSTT1	Present	OR (95% CI)	1 (REF)	1.63 (0.95-2.78)	2.30 (1.33-3.98)
		N ca/co	139/177	93/111	135/74
	Null	OR (95% CI)	0.73 (0.39-1.33)	2.02 (0.80-5.09)	2.38 (0.86-6.60)
		N ca/co	27/43	16/17	18/9
NAT2	Slow	OR (95% CI)	1 (REF)	1.97 (1.08-3.60)	3.48 (1.88-6.41)
		N ca/co	89/132	61/72	108/49
	Intermediate	OR (95% CI)	1.67 (1.01-2.76)	1.87 (0.93-3.76)	1.85 (0.88-3.90)
		N ca/co	66/67	39/48	40/29
	Fast	OR (95% CI)	0.58 (0.24-1.43)	5.34 (1.45-19.65)	2.57 (0.49-13.44)
		N ca/co	10/21	9/8	5/5

[a] Risk estimates are adjusted for age, sex, indication of endoscopy, consumption of snacks and alcohol (in g/d), and cigarettes (n/day). [b] N ca/co, number of cases over number of controls in specific category.

Genetic variants of *EPHX*, *GSTM1*, *GSTT1*, and *NAT2* were not associated with colorectal adenoma risk *per se* (data not shown), though the presence of slow imputed variants at both exons of the *EPHX* gene was more prevalent among adenoma cases (OR 1.67 95% CI 0.98-2.85). Our data indicate that the *SULT1A1*1* allele, coding for fast variants of the SULT enzyme, might predispose to adenomas (OR 1.41, 95% CI 0.91-2.18, homozygotes and heterozygotes included), whereas the *NAT1*11* allele, leading to slow acetylation, might protect against these (OR 0.47, 95% CI 0.23-0.97). The *SULT1A1* and *EPHX* polymorphisms were in Hardy-Weinberg equilibrium.

Table 5.4 shows the effect of the studied genetic variants on the association between smoking and colorectal adenomas. The frequency of slow variants of *NAT1* (≥1 **11* allele present) was too low to enable calculation of risk estimates and *NAT1* is therefore not included in the table. Of the studied genetic polymorphisms, variants of *SULT1A1*, *EPHX*, and *NAT2* seemed to increase the smoking-associated risk of colorectal adenomas, though not always via a clear pattern (see Figure 5.1). P-values for interaction were 0.03, 0.04 and <0.0001, respectively. This indicates that the combination of assumed high-risk variants with long smoking duration were lower than expected under the assumption of multiplicativity. Application of bootstrapping to calculate the OR and 95% CI for interaction under the assumption of additivity, as proposed by Assman *et al.* [28], revealed borderline significant ORs of 0.22 (95% CI –0.06-2.77) for fast *SULT1A1* in combination with smoking for more than 25 years, and of –0.35 (95% CI –1.83-0.14), for the combination of fast *NAT2* with long smoking duration. As can be concluded from Table 5.4 and Figure 5.1, presence of the *SULT1A1*1* allele increased the risk of smoking-associated colorectal adenomas. *NAT2* slow acetylation seemed to increase risk with smoking duration, although we observed a high OR among fast acetylators with a smoking history of 1-25 years. This finding might in part be the result of small numbers of subjects with fast NAT2 genotypes (Table 5.4). Because of these low counts, we combined fast and intermediate *NAT2* acetylators in Figure 5.1, which shows that risk of colorectal adenomas might be highest in slow acetylators. After combination of these two imputed phenotypes, the multivariately adjusted p-value for interaction remained highly statistically significant (p=0.003). For the variants of the *EPHX* polymorphic site in exon 3, the pattern was unclear. Highest risks were observed for those who had smoked for more than 25 years and had either the fast or slow variant, and for those who had smoked 1-25 years and had inherited the intermediate variant. The presence of slow variants at both exons seemed not to modify the association of smoking with adenomas. Use of the *EPHX* classifications described by Cortessis *et al.* [14] and Ulrich *et al.* [13] led to similar conclusions. The other genetic polymorphisms, i.e., *GSTM1* and *GSTT1*, seemed not to modify the association between smoking duration and colorectal adenomas.

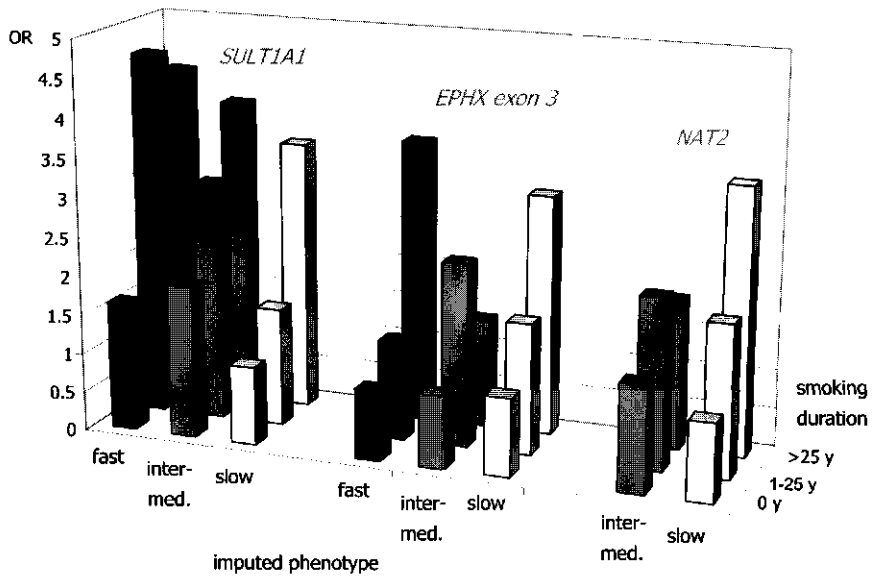

Figure 5.1. *SULT1A1*, *EPHX* and *NAT2* polymorphisms, smoking, and risk of colorectal adenomas.

Discussion

We found that cigarette smoking increased the risk of colorectal adenomas and that duration of cigarette smoking was the main determinant. Giving up smoking reduced the risk after a period of eight years. Smoking characteristics were most strongly associated with risk of small and non-villous adenomas. Smoking was a stronger risk factor for colorectal adenomas in those with *SULT1A1* fast sulfation and possibly, in those with slow *NAT2* acetylation than for those with other inherited variants of these genes.

The study population was enrolled among subjects undergoing endoscopy at the outpatient clinics of eight hospitals. Frequently occurring indications (not related to smoking status) were routine check-up (37%) and anal bleeding (28%) among cases and large bowel complaints (38%) and defecation problems (22%) among controls. This might implicate that our study population is not comparable to the general population. Indeed, the proportion of current smokers is higher in the general Dutch population of the same age (30.6%) than it was in our study population (23.4%), whereas the number

of cigarettes smoked per day was similar †. On the other hand, rates of ever smoking in a sample (n=1935) we randomly selected from the general population inhabiting the same regions as our study population, were in between rates observed for cases and controls (62%, unpublished data). Although the response rate was relatively low in this study, bias by smoking status-specific response [32] did probably not occur, as smoke rates among participants were similar to the rates among invited subjects who decided not to participate in our study (unpublished data). It is unlikely that selection of subjects has introduced bias as smoking status of invited subjects was unknown at the time of recruitment. Moreover, smoking was not related to indication of colonoscopy or sigmoidoscopy in our study. Not surprisingly, therefore, our results did not change after exclusion of those undergoing endoscopy for bowel complaints or defecation problems. We do not think that recall bias occurred. In general, smoking is not a well-known risk factor of colorectal adenomas and it is probably not advised to patients diagnosed with adenomas to give up smoking.

Like almost all other studies [29,30,33-44], our study gives evidence for an increased risk of colorectal adenomas among smokers, especially among those who smoked for a long period. Also in line with other studies, we found that the association with smoking was most pronounced for small (≤ 1cm) and non-villous adenomas [29,30,41].

Strikingly, we observed associations between smoking status and *GSTM1*, *GSTT1*, and *SULT1A1* genotype. To our knowledge, such associations, if reported [45,46], were not present in previous studies. Our results might be due to chance. Certain genetic polymorphisms, however, are known to predispose for risk behavior, as in the case of *ADH2* genotype and alcohol abuse [47].

Only a few studies have been published to date with respect to *SULT1A1* and *EPHX* variants and colorectal adenomas. This implies that more research is needed to verify our finding that *SULT1A1* fast sulfation and *EPHX* slow epoxide hydrolysis (i.e., *HH/HH*) variants may increase adenoma risk irrespective of exposure. Fast sulfation was observed to increase the risk of colorectal adenomas, in contrast to results from another study, in which low *SULT1A1* (STA3) activity in platelets was associated with an elevated risk [48].

To our knowledge, our study is the first study on smoking and colorectal neoplasm in which the potential role of *SULT1A1* was considered. *SULT1A1* allele frequencies were comparable to those reported previously [7,22,49]. Our study indicates that *SULT1A1* polymorphism may modify the association between smoking and colorectal adenomas, implying that fast sulfation might predispose to smoking-associated adenomas. These findings are consistent with those of *in vitro* studies which indicate that *SULT1A1* might activate procarcinogens from cigarette smoke [4-6]. Moreover, results from a study

† see internet site http://www.cbs.nl; Centraal Bureau voor de Statistiek, 2001.

considering the role of *SULT1A1* in the association between well-done meat intake and postmenopausal breast cancer point in the same direction [11].

Although large differences in risk of smoking-associated adenomas were observed over variants of *EPHX*, there was no clear pattern. Possibly, inappropriate classification due to limited knowledge about its functional significance, obscures the effect of *EPHX*. Hassett and colleagues reported a reduction of enzyme activity and/or stability for His113 variants and an increase for Arg139 variants of *EPHX* [50]. The *EPHX HH/HH* variant resulted in the lowest protein half-life, although it was not statistically significantly different from half-lives of other variants [51]. Confusion about how to impute *EPHX* phenotypes from genetic variants has led to the use of different, partly overlapping, classifications. In a previous paper [23], we used a classification that we considered most clear and which was also used by Pastorelli and co-workers [52]. To enable comparison of our results to those of others, we tested all three classifications. The results calculated with the classification we previously used [23] and that of Ulrich *et al.* [13] were more similar to each other than to the results produced with the classification used by Cortessis and colleagues [14]. This was due to the greater similarity between the former two classifications than with the latter classification method. Cortessis and colleagues reported that predicted high *EPHX* stability (presence of three or four stable (fast) alleles) increased risk of adenomas in combination with current smoking [14]. In contrast, Ulrich and co-workers reported that smoking increased risk of adenomas especially in combination with the *EPHX HH/HH* genotype, the *EPHX* exon 3 *HH* (slow) variant being responsible for this finding [13].

Frequencies of the *GSTM1* and *GSTT1* genotypes were in the same range as those reported from other (Western) European studies and were not associated with adenoma risk [15]. There was no evidence for predisposition to smoking-associated adenomas related with genetic variants of *GSTM1* and *GSTT1*, which is in line with other studies [45,46,53,54].

Our finding that *NAT1* slow acetylation (**11* allele present) protects against colorectal adenomas can not be verified with other studies, since these compared *NAT1*10* or rare *NAT1* alleles leading to absence of the NAT1 enzyme or to low enzyme activity, with all other variants. Recently, however, it was found that the more frequently occurring *NAT1*11* allele leads to reduced enzyme activity [27]. However, frequency of this allele was still too low to study the potential interaction of *NAT1* polymorphism with smoking in our population.

Irrespective of smoking status, *NAT2* variants did not predispose to colorectal adenomas, which is in line with almost all of the previous studies [16]. However, we found that risk of colorectal adenomas was especially high among smokers with the imputed slow *NAT2* phenotype. Similar findings were reported by Welfare and co-workers [55]. These findings are in line with metabolic studies. Whereas heterocyclic amines are

mainly activated via *N*-*O*-acetylation, *N*-acetylation executed by NAT2 is a major detoxification route for arylamines present in tobacco smoke, such as 4-aminobiphenyl [17]. It remains puzzling why such associations as reported here were not found in large studies [44,54], of which one considered colorectal adenomas and found smoking to be an important risk factor [44]. Therefore, more studies are needed to verify our results.

In summary, we found that smoking increased the risk of colorectal adenomas, and that this risk was mainly determined by smoking duration. Smoking especially increased risk of small and non-villous adenomas. We found indications for genetic polymorphisms of *SULT1A1* and *EPHX* exon 3 and *NAT2* to influence the association between smoking and colorectal adenomas. The finding that smoking increases risk of adenomas most importantly in combination with *SULT1A1* fast sulfation and *NAT2* slow acetylation is consistent with results from biochemical studies and indicates that *SULT1A1* and *NAT2* are indeed important in the metabolism of arylamines and/or PAHs from tobacco smoke. *GSTM1* and *GSTT1*, however, do not seem to play a role and the potential role of *EPHX* remains to be elucidated.

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6.

Alcohol consumption, alcohol dehydrogenase 3 polymorphism, and colorectal adenomas

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Among cases, main indications for endoscopy were routine check-up for adenoma recurrence (37%), anal bleeding (27%), and large bowel complaints (15%), while controls mainly underwent endoscopy because of large bowel complaints (38%), defecation problems (21%), or anal bleeding (17%).

Medical files were checked for additional information on medical history and information on polyp recurrence, size, localization, histology, and the number of excised polyps.

In retrospect, based on information from questionnaires and medical files, we excluded 170 participants who did not meet the eligibility criteria, mainly because of non-adenomatous or unknown types of polyps (59%). In addition, we used complete information of 64 subjects meeting our criteria, recruited between December 1995 and June 1997, from a preceding study on somatic mutations in colorectal adenomas conducted in one of the eight hospitals [22]. This increased the study population from 861 to 925 subjects.

QUESTIONNAIRES

All invited subjects received a short questionnaire inquiring about important characteristics such as age, gender, alcohol consumption, education level, and smoking. About one third of subjects who did not want to participate in the study completed this short questionnaire. Although they were older, they did not differ from participants with respect to gender, education level, smoking, and alcohol consumption.

Participants further received dietary and lifestyle questionnaires and were requested to complete these according to habits in the year previous to their last endoscopy or complaints.

To assess dietary habits, we used a standardized and validated semi-quantitative food frequency questionnaire described in detail by Ocké and colleagues [23]. By means of this questionnaire, consumption of alcoholic beverages was assessed for beer, white wine, red wine, ports, and liquors separately. Subjects could choose to report average consumption in glasses per day, week, month or year. Reproducibility of alcohol consumption as assessed by this questionnaire was high for both males and females (r= 0.91) as was its relative validity (r=0.74 for males, r=0.87 for females compared with the means of twelve 24-h recalls) [23]. Intakes of total energy and of various nutrients and ethanol were calculated by use of a computerized version of the Dutch food composition table. A Dutch alcoholic consumption contains approximately 10 g of ethanol. Nutrients, except ethanol, were adjusted for total energy intake using the residual regression method [24].

LABORATORY ANALYSES

Blood samples were stored at -20°C. DNA was isolated from 200 µl frozen whole blood, using the QIAamp blood kit (Qiagen Inc., U.S.A.), diluted to a concentration of approximately 20 ng/µl, and stored at 4 °C until analyzed.

We used a PCR-RFLP method for determination of *ADH3* genotype. A 145-basepair (bp) fragment of exon 8 of the *ADH3* gene was amplified using primers described by Groppi and colleagues (5'-GCTTTAAGAGTAAATATTCTGTCCCC-3' and 5'-AATCTACCTCTTTCCAGAGC-3') [25]. To check for DNA cross-contamination, one in eight samples contained no DNA but water instead.

For RFLP analysis, *Ssp*I digested the *ADH3*1* allele into fragments of 67, 63 and 15 bp, and the *ADH3*2* allele into fragments of 130 and 15 bp. DNA fragments were separated on an ethidium bromide stained agarose gel (4%) and visualized under UV light.

To control the specificity of *ADH3* genotyping, a random sample of primary PCR products was digested by *Nla*III, cleaving the closely to *ADH3* related *ADH1* and *ADH2* genes, but leaving *ADH3* intact [25]. Laboratory personnel was blinded to case-control status. DNA was not available of ten participants and it was not possible to genotype another eight samples (< 1%) for *ADH3*.

DATA ANALYSIS

Subjects with incomplete dietary data (n=38) were excluded, as were the 18 subjects of whom *ADH3* genotype was not assessed. The analyses thus included 869 subjects: 433 cases and 436 controls. We studied total alcohol consumption in glasses per week, calculated by summing the separately reported intakes of beer, wines, ports and spirits, as well as total ethanol intake in grams per day from all dietary sources, including small amounts from sauces, puddings, chocolates, and low-alcohol beer. Alcohol consumption was divided in tertiles based on the distribution in the total study population. To additionally evaluate the effect of a combination of *ADH3* polymorphism with high alcohol consumption, we defined high alcohol consumption as the consumption of more than three drinks daily because this amount exceeds the recommended daily maximum for both men and women. *ADH3*1/*1* was considered as the high-risk genotype and compared with the combination of *ADH3*1/*2* and *ADH3*2/*2* genotypes.

The analyses were conducted using Statistical Analysis Software (SAS version 6.12, SAS Institute, Cary, NC). All tests of statistical significance were two-sided. To test for linear trend, we modeled the tertile of alcohol intake as a continuous variable in the logistic regression model, in which each tertile was assigned its median value. Logistic regression models were used to calculate odds ratios (ORs) and 95% confidence intervals (95% CIs). Factors selected as possible confounders were age, gender, body mass index, indication for endoscopy, center, cigarette smoking, physical activity, family history of

colorectal cancer, education level, use of nonsteroidal anti-inflammatory drugs, total energy intake, consumption of vegetables, fruit, total and red meat, and nutrients related to these food groups. Variables related to colorectal adenomatous polyps as well as to exposure at $p < 0.5$ [26] were separately entered as covariates in the regression models. None of these changed the odds ratio for alcohol consumption by more than 10%. However, we included age and indication for endoscopy (complaints-related, screening, and other/unknown) in the multivariate models to control for potential confounding. Analyses on the total population were additionally adjusted for gender. Gender-stratified analyses were conducted because of 1) different male-female ratios between cases and controls, 2) gender-specific patterns of alcohol consumption, and 3) gender-specific differences in alcohol vulnerability [27].

To evaluate the possible interplay between *ADH3* genotype and alcohol consumption, the group with *ADH3*1/*2* or *ADH3*2/*2* genotypes in combination with and low alcohol consumption (lowest tertile) served as the reference category.

As in different stages of carcinogenesis different risk factors may operate, we conducted case-case analyses for adenoma recurrence (primary *vs.* recurrent), size (< 1 cm *vs.* ≥ 1 cm), localization (proximal *vs.* distal) and number (multiple *vs.* single) of polyps.

To check whether former adenomas among cases or undetected right sided polyps in controls could have biased our results, we repeated all analyses after restriction of our study population to cases with first diagnosis of adenomas not longer than one year ago (n=299) and controls with complete visualization of the colon (n=334).

Results

The case group contained more men than the control group (55 *vs.* 37%). Table 6.1 shows characteristics of the study population for cases and controls stratified by gender. Among women and men, cases were significantly older than controls and less often underwent endoscopy because of bowel complaints. Among women, cases had a higher intake of alcohol (especially of spirits and fortified wines), vegetables, fruits, and folic acid, and had less frequently changed their diet because of bowel complaints compared to controls. Among men, cases more frequently (had) smoked and consumed less energy and folic acid than controls. There were no differences with respect to *ADH3* genotype. The distribution of *ADH3* was in Hardy-Weinberg equilibrium.

Alcohol intake from all sources ranged from zero to ten glasses per day. In the control group, the median alcohol consumption among men was nine consumptions per week, while the median consumption of alcohol among women was only one glass per week. Among cases, median alcohol intake was ten drinks per week among men and 2.5 drinks per week among women.

Table 6.1. Characteristics of the study population by gender.

Gender	Women (N=471)		Men (N=398)	
Characteristic	Cases (n=196)	Controls (n=275)	Cases (n=237)	Controls (n=161)
Age (years), mean ± SD	58.6±10.7	49.5±15.0[a]	59.3±10.6	51.9±12.2[a]
Genotype, %				
*ADH3*1/*1*	35.2	36.7	36.3	34.2
*ADH3*1/*2*	48.0	47.6	44.7	46.6
*ADH3*2/*2*	16.8	15.6	19.0	19.3
Alcohol intake, median (25th, 75th percentile)				
Ethanol (g/day)	3.9 (0.3; 14.6)	1.5 (0.1; 8.7)[a]	14.2 (4.3; 30.4)	14.5 (3.5; 26.5)
Alcoholic consumptions/wk, total	2.5 (0.2; 10.0)	1.0 (0; 6.0)[a]	10.0 (2.3; 21.1)	9.3 (2.5; 18.0)
Beer	0 (0; 0)	0 (0; 0.1)	0.5 (0; 7.0)	2.0 (0; 7.0)
Wine (red, white, rosé)	0.5 (0; 4.0)	0.2 (0; 2.7)	0.5 (0; 5.2)	0.7 (0; 4.0)
Spirits (including port, sherry etc)	0.1 (0; 1.1)	0 (0; 0.5)[a]	0.9 (0; 6.5)	0.5 (0; 4.0)
Dietary habits, median (25th, 75th percentile)				
Total energy (KJ/day)	7,684 (6,539; 8,979)	7,440 (6,419; 8,978)	9,293 (7,942; 10,951)	10,239 (8,435; 11,623)[a]
Vegetables (g/d)	122 (98, 154)	114 (90, 144)[a]	110 (86, 140)	112 (83, 137)
Fruit (pieces/d)	1.5 (1.0, 2.3)	1.0 (0.6, 2.0)[a]	1.0 (0.4, 2.0)	1.0 (0.4, 2.0)
Red meat (g/d)	57 (28, 77)	48 (26, 73)	74 (48, 91)	73 (42, 90)
Coffee (cups/day)	4.0 (2.5; 5)	4.0 (2; 6)	4.0 (3; 6)	5.0 (3; 6)
Folic acid (µg/day)	187 (162; 221)	175 (153; 204)[a]	204 (171; 245)	216 (187; 252)[a]
Retinol (µg/day)	554 (392, 785)	499 (346, 744)	736 (524, 1223)	838 (517, 1189)
Medical history, %				
Dietary changes due to bowel complaints	18.9	33.8[a]	17.3	19.3
Family history of colorectal cancer	26.5	19.3	20.3	19.3
Previous diagnosis of adenomas	45.9	n.a.[b]	40.1	n.a.[b]
Complaints-related endoscopy indication	46.9	78.2[a]	55.7	74.5[a]
Other characteristics				
Body Mass Index (kg/m^2), mean ± SD	25.8±4.5	25.1±4.5	26.3±3.3	25.9±3.1
Cigarette smoking, % never smokers	45.9	52.7	32.1	46.6
High physical activity, %	35.7	34.6	20.3	26.7
Low Education level, %	44.4	33.1	30.0	26.1

[a] Significantly different from cases: $p < 0.05$ (Fishers' exact test for categorical variables, t-test for age and BMI, and Wilcoxon's rank test for dietary variables); [b] n.a., not applicable.

Alcohol intake correlated positively with energy intake, and education level. Also, alcohol consumption was related to (history of) cigarette smoking and family history of colorectal cancer (data not shown).

In Table 6.2, risk estimates for the association between the number of drinks per week (in tertiles) and colorectal adenomas are shown. Overall, alcohol intake was weakly but not with statistical significance related to colorectal adenomas. Among women, alcohol consumption significantly increased risk of colorectal adenomas. When consumers of more than 21 alcoholic consumptions per week were compared to consumers of less than one glass weekly, alcohol appeared to be a risk factor also for men (OR 1.8, 95% CI 0.9-3.8). We did not find increased risks for women consuming more than 21 glasses per week, probably because this category only contained ten cases and twelve controls (data not shown).

Table 6.2. Association between alcohol consumption and risk of adenomatous colorectal polyps.

	Alcohol consumption (drinks/week) [a]			
	< 1	1 - 10	≥ 10	p-trend
All				
N (cases/controls)	122/163	139/153	172/120	
Gender and age adjusted OR (95% CI)	1 (REF)	1.22 (0.81-1.83)	1.44 (0.95-2.17)	0.06
Multivariate OR (95% CI) [b]	1 (REF)	0.93 (0.59-1.49)	1.10 (0.69-1.73)	0.17
Women				
N (cases/controls)	76/135	69/99	51/41	
Age adjusted OR (95% CI)	1 (REF)	1.17 (0.75-1.82)	2.19 (1.30-3.68)	0.003
Multivariate OR (95% CI) [b]	1 (REF)	0.99 (0.61-1.60)	1.81 (1.02-3.21)	0.04
Men				
N (cases/controls)	46/28	70/54	121/79	
Age adjusted OR (95% CI)	1 (REF)	0.93 (0.50-1.73)	1.07 (0.60-1.91)	0.63
Multivariate OR (95% CI) [b]	1 (REF)	0.96 (0.50-1.85)	1.12 (0.61-2.05)	0.57

[a] One alcoholic consumption contains approximately 10 g of ethanol; [b] adjusted for (sex), age, and indication for endoscopy (complaints-related, screening, other/unknown).

The analysis of alcohol intake from all sources in grams per day, which was slightly different from the daily alcohol intake in grams imputed from alcoholic beverages only, yielded odds ratios comparable to those presented in Table 6.2. No specific type of beverage was responsible for the increased risk of colorectal adenomas (data not shown). Case-case analyses did not indicate that alcohol consumption was related to specific adenoma characteristics such as location, size, type, and number of adenomas (data not shown).

After exclusion of cases who had previously been diagnosed with adenomas and of controls whose proximal colon was not examined, results remained similar (OR and

95% CI for women in the highest tertile of alcohol consumption 2.2, 1.1-4.2; and for men 1.0, 0.5-2.0). After exclusion of those who underwent endoscopy because of large bowel complaints, gender- and age adjusted odds ratio inflated moderately among women (OR 1.9, 95% CI 1.0-3.4), but not among men (OR 1.1, 95% CI 0.5-2.1).

Table 6.3. Risk [a] of adenomatous colorectal polyps by alcohol consumption and *ADH3* genotype.

		Alcohol consumption (drinks/week) [b]		
		< 1	1-10	≥ 10
All				
*ADH3*1/*2, *2/*2*	N cases/controls	72/96	99/105	107/79
	OR (95% CI)	1 (REF)	0.97 (0.60-1.56)	1.15 (0.70-1.90)
*ADH3*1/*1*	N cases/controls	50/67	40/48	65/41
	OR (95% CI)	0.94 (0.53-1.64)	0.99 (0.54-1.79)	1.76 (1.00-3.11)
Women				
*ADH3*1/*2, *2/*2*	N cases/controls	41/77	51/68	35/29
	OR (95% CI)	1 (REF)	0.94 (0.51-1.74)	1.38 (0.66-2.87)
*ADH3*1/*1*	N cases/controls	35/58	18/31	16/12
	OR (95% CI)	0.90 (0.46-1.73)	0.92 (0.42-2.05)	2.61 (1.05-6.50)
Men				
*ADH3*1/*2, *2/*2*	N cases/controls	31/19	48/37	72/50
	OR (95% CI)	1 (REF)	1.03 (0.47-2.27)	1.05 (0.50-2.20)
*ADH3*1/*1*	N cases/controls	15/9	22/17	49/29
	OR (95% CI)	1.31 (0.41-4.16)	1.06 (0.42-2.69)	1.50 (0.67-3.34)

[a] Adjusted for (sex), age, and indication for endoscopy (complaints-related, screening, other/unknown); [b] one alcoholic consumption contains approximately 10 g of ethanol.

In Table 6.3, we show risk estimates for the combined associations of alcohol consumption and *ADH3* genotype with colorectal adenomas. The association between alcohol and adenomas was not markedly influenced by *ADH3* polymorphism, though the risk of adenomas was highest among subjects who had the *ADH3*1/*1* genotype and were in the upper tertile of alcohol consumption (Table 6.3). When comparing consumers of more than 21 alcoholic drinks weekly to those consuming less than one drink weekly, risk increased most markedly for men with the *ADH3*1/*1* genotype (OR 2.8, 95% CI 1.0-8.3) and less so for men with other genotypes (OR 1.6, 95% 0.7-4.1). However, the interaction term did not reach statistical significance.

Discussion

In this first study on alcohol consumption and *ADH3* genotype in the epidemiology of colorectal adenomas, we observed that alcohol consumption increased risk of colorectal polyps most markedly among women and that this association may be influenced by *ADH3* genotype.

We recruited both cases and controls among those undergoing endoscopy. In the Netherlands, endoscopies are not routinely conducted for screening purposes like in the United States. Consequently, endoscopies were mostly conducted for bowel pain, anal bleeding, or defecation problems (64%) in our study. These complaints may influence dietary patterns. Our study population might also be more health conscious than the general population. This implies that our findings are not easily extrapolated to the general population. However, alcohol consumption in our control group was similar to the habitual alcohol consumption we assessed using the same questionnaire in a random sample (n=1935) from the general population inhabiting the same regions as the controls (unpublished data).

Of those invited, about 54% were willing to participate. Selection bias might have occurred if habitual alcohol consumption influenced the probabilities of being invited or of participating. It is not likely that alcohol consumption influenced the chance of being invited since habitual alcohol consumption was unknown at selection for almost all subjects (>95%). Moreover, participants did not differ in alcohol consumption from those who refused participation but completed the short questionnaire.

The control group consisted of significantly more women, possibly because women were more likely than men to undergo endoscopy for major bowel complaints such as irritable bowel syndrome (IBS) which was found to be more prevalent in Dutch women than in men [28]. Also, cases were older than controls. Gender and age differences between cases and controls were also observed in other case-control studies on colorectal adenomas [29-31]. Cases with history of adenomas might be over-represented in our study population since these had a higher probability of being invited and this might have introduced bias. We therefore included indication for endoscopy in our multivariate model. Exclusion of those with a history of adenomas yielded essentially the same results.

Inclusion of controls with bowel complaints did probably not lead to important overestimation of the true associations between alcohol and adenomas, though bowel complaints occurred more often in the control group and were associated to lower alcohol consumption in women. Odds ratios only marginally inflated after exclusion of those undergoing endoscopy because of bowel complaints. We expect no misclassification by inclusion of controls with incomplete visualization of the colon (22%). In theory, these could have proximal adenomas, leading to bias toward the null. However, exclusion of controls with incomplete colonoscopy did not change our results.

Recall bias might have occurred since most cases and controls were aware of their status at time of completion of the questionnaires. If alcohol would have been known as a risk factor for polyps, cases might have reported lower or higher intake than their true intake of alcohol. However, alcohol consumption is generally believed to increase risk of

several cancers, but probably not of colorectal adenomas. Indeed, none of those previously being diagnosed with adenomas indicated to have changed alcohol consumption because of colorectal adenomas.

We assessed alcohol consumption by use of a food-frequency questionnaire. Although a validation study of our questionnaire showed that habitual alcohol intake might be systematically underestimated, especially by men, subjects were appropriately ranked on alcohol consumption [23]. Ideally, per beverage type, both frequency and the number of drinks per occasion should be inquired [32]. We had no information on drinking patterns or on drinking habits over the years. Recent drinking habits might well reflect those in the past, as in a Dutch cohort, alcohol consumption patterns were found to be relatively stable, especially among men [33]. The same was concluded from a follow-up study among British male doctors [34].

Allele frequencies of *ADH3*1* and *ADH3*2* among controls were 59% and 41%, respectively, which is similar to frequencies reported from other Caucasian populations [10,11,16,19]. We correctly amplified *ADH3* and not *ADH1* or *ADH2* in all samples (as checked by digestion of a random sample of PCR products with the restriction enzyme *Nla*III). Use of an internal control in RFLP analysis proved that all digestions were successful.

Our finding that alcohol consumption increases the risk of adenomatous colorectal polyps corresponds with the results of most previously conducted studies [29-31,35-40]. Among women, risk of adenomas already increased at consumption of ten or more beverages per week, whereas among men, risk was increased only at consumption of more than 21 beverages per week. It is difficult to compare these results with those obtained in other studies, since different cut-off points are used and gender-specific results are not always presented. In general, like in our study population, men consume more alcohol and the range of alcohol consumption is wider in men than in women [33,41]. A possible explanation for our results is that the threshold for an effect of alcohol on adenomas could be higher in men than in women. Women are more vulnerable to alcohol than men mainly because of a lower rate of first-pass ethanol metabolism in the stomach [27,42]. Since blood ethanol levels are higher in women than in men at equal consumption and ethanol reaches the colonic epithelium via the blood circulation, this might imply that at equal intake of alcohol, the colonic epithelium of women is exposed to higher levels of ethanol and acetaldehyde than that of men.

Alcohol did not specifically increase adenoma recurrence and other adenoma characteristics. To our knowledge, only Boutron and colleagues found that alcohol consumption specifically increased risk of large adenomas [43].

We did not find specific types of beverages to be responsible for the observed increase in risk, which is in line with the conclusion of the World Cancer Research Fund expert

committee stating that *'the effect generally seems to be related to total ethanol intake, irrespective of the type of drink'* [44].

The effect of ethanol is probably co-carcinogenic rather than carcinogenic [4,45]. In contrast, its major metabolite acetaldehyde is a probable carcinogen and was found to form adducts, induce DNA cross-links, chromosomal aberrations, and sister chromatid exchanges *in vitro*, and inhibit DNA repair enzymes [5]. Therefore, we hypothesized that polymorphism of the *ADH3* gene, encoding the principal enzyme oxidizing ethanol to acetaldehyde, would play a role in the association between alcohol and adenomas. We found stronger associations between alcohol consumption and colorectal adenomas in carriers of the *ADH3*1/*1* genotype than in those with other *ADH3* genotypes. However, interaction terms for *ADH3* genotype and alcohol consumption were not statistically significant, possibly because modest gene-environment interactions can only be studied in populations with several thousands of subjects [46]. An alternative explanation is that the role of *ADH3* genetic polymorphism might be obscured by production of ADH [47] and/or acetaldehyde production by intestinal microflora [21]. Because of these effects, *ADH3* genotype might be especially important among heavy drinkers. In contradiction to our expectations, the potential role of *ADH3* polymorphism did not become more pronounced with high alcohol consumption, i.e., more than 21 drinks per week. However, our study population only included very few heavy drinkers or alcoholics and this may have influenced our results. Alternatively, ADH-catalyzed oxidation of ethanol might be less important because other enzymatic systems are upregulated in heavy drinkers [48].

ADH3 polymorphism has not been considered in studies on colorectal adenomas so far. Other studies on the role of *ADH3* polymorphism in the association between alcohol and neoplasm were on oropharyngeal, laryngeal, head and neck, and breast cancer [14-20]. Three of these seven studies indicated that drinkers with the *ADH3*1/*1* genotype are at higher risk of neoplasm than those carrying *ADH3*1/*2* and *ADH3*2/*2* genotypes [14-16].

We conclude that alcohol consumption elevates the risk of adenomatous colorectal polyps. *ADH3* genotype may be a modest effect modifier of the association between alcohol consumption and colorectal adenomas. These findings need further confirmation. Our hypothesis that the influence of *ADH3* genotype becomes relevant at high ethanol concentrations should preferably be tested in a large population with higher alcohol consumption. Moreover, exposure of the human colon to ethanol and acetaldehyde and effects of this, and the role and impact of alcohol dehydrogenase synthesis by gastrointestinal bacteria need to be studied in more detail.

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7.

Meat consumption, cigarette smoking, and genetic susceptibility in the etiology of colorectal cancer:

results from a Dutch prospective study

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Cancer Causes and Control, in press

Abstract

We evaluated the effect of meat consumption and cigarette smoking in combination with *N*-acetyltransferases 1 and 2 (*NAT1* and *NAT2*), and glutathione *S*-transferase M1 (*GSTM1*) genotypes on risk of colorectal cancer. From a Dutch prospective study, after 8.5 years of follow-up, data of 102 incident colorectal cancer cases and a random sample of 537 controls frequency-matched for gender and age were analyzed. Baseline information on dietary and smoking habits, as well as blood samples for DNA isolation and genotyping were available. Red meat intake increased colorectal cancer risk among men (OR 2.7, 95% CI 1.1-6.7 highest *vs.* lowest intake), whereas poultry and fish decreased risk among women (OR 0.5, 95% CI 0.2-1.07). Cigarette smoking for at least 16 years increased colorectal cancer risk among former smokers only (OR 2.7, 95% CI 1.0-7.4), compared to those having smoked for 15 years or less. *NAT1* and *NAT2* polymorphisms did not significantly modify these associations. High consumption of poultry and fish was inversely associated with colorectal cancer only in presence of *GSTM1*. In this study, meat consumption and former long-term smoking were associated with colorectal cancer. Associations of colorectal cancer with different types of meat were modified by gender and *GSTM1* genotype.

Introduction

Incidence of colorectal cancer is high and increasing in developed countries. In 1995, the incidence rate of colorectal cancer in the Netherlands was about 95 per 100 000 persons per year (European Standardized Rate) [1]. High consumption of red meat and long-term cigarette smoking are among its potential risk factors [2-3].

Although risk estimates are above one in most studies, in individual studies, they range from 0.8 to 2.5 [2]. A recent meta-analysis of 13 studies showed that meat significantly increased risk of colorectal cancer by 12-17% per 100 g increase in total and red meat consumption. Daily increase in intake of processed meat by 25 g led to an increase in risk of 49% [4]. As was recently reviewed by Giovannucci [5], smoking and specifically long-term cigarette smoking, was associated with a 1.5-2 times increased risk of colorectal cancer in most of the recent studies. These associations were more consistent in US than in European populations [5].

Inconsistencies could of course result from methodological differences (e.g., study design, study size, measurement of exposure), but also from differences between study populations (e.g., sex and age distribution, lifestyle, and genetic susceptibility). Indeed, preferences for meat types, meat preparation methods, and smoking habits differ between countries and populations [6].

Differences in genetic susceptibility to xenobiotics may result from genetic polymorphisms leading to differences in gene expression or to different stability or activity of the encoded metabolic enzymes. Examples of polymorphic genes are *N*-acetyltransferases 1 and 2 (*NAT1* and *NAT2*), involved in metabolism of heterocyclic aromatic amines (HCAs) from heavily cooked meat and tobacco smoke, and glutathione

S-transferases (*GST*s), involved in metabolism of polycyclic aromatic hydrocarbons (PAHs) from tobacco smoke and barbecued meat. To date, 26 alleles for *NAT1* and 29 *NAT2* alleles have been identified †, resulting in fast or slow acetylation. Depending on the substrate, fast or slow acetylation of aromatic amines might result in prolonged exposure to potential carcinogens and increased formation of DNA-adducts [7]. *GSTM1* is important in the detoxification of various xenobiotics. The *GSTM1* null genotype results in absence of the corresponding enzyme and occurs in 39-62% of Caucasians [8].

Overall, results regarding a possible role for genetic susceptibility in the associations of meat consumption and smoking with colorectal cancer are inconsistent [9-14]. *NAT1* and *NAT2* fast acetylators consuming relatively large amounts of meat may be at increased risk of colorectal cancer [9,10]. However, no important roles for *NAT2* and *GSTM1* polymorphisms in the association between meat consumption and colon cancer were found in a large study [11]. Similarly, although one study reported an increased risk of colorectal cancer for *NAT2* slow acetylators having smoked in the past 5 years [12], the role of genetic polymorphisms in smoking-associated colorectal cancer is unclear [13,14].

Although allele frequencies of *NAT1*, *NAT2* and *GSTM1* are thought to be constant over Caucasian populations, exposure to potential carcinogenic substances is more variable. The influence of metabolic genotype on the effect of exposure on disease might be most relevant in those being exposed to relatively high or low levels of potential carcinogens [15]. This variation in exposure and potential variation of its effect warrants analysis of gene-environment interactions in different populations. In this first Dutch study on gene-environment interactions in colorectal cancer, we investigate the possible interplay between meat consumption or tobacco smoking, and genetic susceptibility as represented by *NAT1*, *NAT2* and *GSTM1* genotypes.

Materials and Methods

STUDY POPULATION

We conducted a nested case-control study using data from the prospective Monitoring Project on Cardiovascular Disease Risk Factors which was conducted in three Dutch towns, i.e. Amsterdam, Maastricht and Doetinchem between January 1987 and December 1991. More than 36,000 men and women were enrolled. A detailed description of this project was previously published [16]. In brief, each year, a random sample of men and women, aged 20-59 years, was selected from the municipal registries of the three towns and invited to participate. The overall response rate was 50% for men and 57% for women. The study protocol was approved by the Medical Ethical Committee of the University of Leiden, The Netherlands in 1987.

† See internet site http:\\www.louisville.edu/medschool/pharmacology/NAT.html, last revision

In Doetinchem, some subjects participated more than once and duplicate observations from these participants (n=1,097; first record was used) were excluded. We further excluded subjects who could not be identified in the National Population Database (n=24), whose vital status by 31 December 1997 was unknown (n=343), who disagreed with release of their medical records from the general practitioner and therefore could not be linked to the cancer registry (n=597), who did not provide a blood sample (n=705), who were of presumed non-Caucasian nationality (n=1402), or who had cancer previous to their inclusion into the cohort (except non-melanoma skin cancer and cervix cancer *in situ*, n=542). From the resulting database, we included all incident colorectal cancer cases and a random sample of controls as described below.

Follow-up for incident cancer for the period 1987 to end of 1998 was achieved via computerized record linkage with the Netherlands Cancer Registry (NCR) and with the three regional cancer registries (IKA, IKL, and IKO) serving the areas of Amsterdam, Maastricht and Doetinchem, respectively. NCR is a national registry of all malignant tumors diagnosed from 1989 onwards in people living in the Netherlands. Completeness, data consistency and the possibility of duplicate records are extensively checked [17]. Because data from the NCR were complete only for the period 1989 to the end of 1996, additional information from the regional cancer registries was used. For 1987 and 1988, completeness of data from these registries varied between 60% and 100% depending on registry and year. For 1997, data from all three regional registries were 100% complete and for 1998, data were 100% complete for IKL only. Records from the cohort were linked using a method based on the two-stage process developed by Van den Brandt and colleagues [18].

In total, 108 incident colorectal cancer cases could be identified. A random sample of controls with the same distribution of gender, age (5-year intervals), and center as the cases was drawn. As the success rate of DNA isolation was expected to be low, we sampled six controls for every case to obtain a final case-control ratio of at least five-to-one. After exclusion of one case of whom no exposure data was available, our study population consisted of 107 cases and 600 controls. About 38% of these originated from the Western part (Amsterdam), 23% from the Eastern part (Doetinchem), and 39% from the South-Eastern part of the Netherlands (Maastricht).

Exposure

Meat consumption and smoking habits were estimated by use of a self-administered questionnaire. Dietary habits were estimated using a short semi-quantitative food frequency method, validated by the use of a dietary history method [19]. Spearman rank correlation coefficients for the reproducibility of meat intake as estimated by the questionnaire were $r=0.59$ for men and $r=0.56$ for women; coefficients for relative

December 12, 2000 (*NAT1*) and April 6, 2001 (*NAT2*)

validity were r=0.49 for men and r=0.40 for women [19]. As the questionnaire was designed to estimate exposure to risk factors of cardiovascular diseases, emphasis was on foods supposed to increase this risk (e.g., meat snacks, fats); the questionnaire was designed to rank subjects on their usual intake. Frequency of meat consumption was inquired separately for beef, pork, poultry, and fish. Consumption was assessed in six categories: never, less than once per month, one to three times monthly, once per week, two to four times weekly, and more than four times per week. In addition, frequency of consumption of four typically Dutch meat snacks, among which sausage slices, was asked in categories of never, less than once weekly, once weekly, two to six times a week, and daily. Participants were also asked how many sandwiches with meat filling they commonly consumed daily.

Assuming the median frequency per category (i.e., for the category 'two to four times weekly' we assumed a consumption of three times per week), total meat consumption was calculated by adding up the frequencies of all meat types consumed. Frequency of consumption of fresh red meat was estimated by summation of reported beef and pork intake. Consumption of other foods (e.g., vegetables, fruit) and energy intake were calculated using data from the computerized version of the Dutch food composition table 1993, to estimate portion sizes [20].

Exposure to tobacco smoke was assessed for cigarettes, cigars, and pipe separately. For each type, current smoking status, age at start of smoking, total number of years smoked, and the amounts smoked usually and currently per day were inquired.

GENETIC SUSCEPTIBILITY

All participants provided a blood sample that was separated into plasma, erythrocytes, and buffycoats, and was subsequently stored at -20°C. Mean storage time until DNA isolation was 11.5 years. Of one case and 19 controls respectively, no samples could be retrieved.

DNA was isolated from buffycoats. If DNA isolation failed, the procedure was repeated for cases but not for controls, since controls were over-sampled by 20%. DNA could be isolated for 102 cases and 540 controls (success rate of 96% for cases and 93% for controls). DNA was diluted to a concentration of 20 ng/µl and stored at +4°C in deep-well microtiterplates.

We determined the presence or absence of *GSTM1* with a multiplex PCR procedure, developed by Arand and co-workers [21], which simultaneously determines *GSTM1* and *GSTT1* genes. As a positive PCR control, however, we used primers derived from β–globin [22] instead of albumin. The fragments of *GSTM1, GSTT1*, and β-globin were respectively 215, 480, and 350 bp in size.

Allelic variants of *NAT1* and *NAT2* were determined by an allele specific oligo hybridization assay described by Bunschoten and colleagues [23]. In short, allele specific

oligonucleotide probes were covalently applied to a membrane in lines, followed by hybridization with PCR products comprising the allelic variants of *NAT1* and *NAT2*, perpendicular to the oligonucleotide lines. Using these oligonucleotide probes, we could identify *NAT1* alleles **4, *3, *10* and **11*, and *NAT2* alleles **4, *5, *6, *7, *12* (see [23], for details).

We checked for cross-contamination between samples prior to PCR by inclusion of one sample without DNA after every seven DNA samples. All these controls were negative. Validity and reproducibility of the allele specific oligo hybridization assay were extensively tested and proved to be 100% [23]. To test reproducibility of the *GSTM1* genotype determination, a number of samples (approximately 10%) were genotyped in duplicate; no differences were observed.

Data analysis

All subjects of whom at least one genotype (i.e., *GSTM1* or *NAT1* or *NAT2*) could be determined were included (102 cases and 539 controls). Two controls had missing values on important energy sources resulting in an extremely low calculated energy intake (1890 and 2270 kJ/d) and thus, these were excluded from the analyses, yielding a final data set of 102 cases and 537 controls.

Categorical variables had a separate category for missing information. Data on non-categorical exposure variables on smoking and meat consumption was divided in categories each containing approximately equal numbers of controls, taking the lowest categories of exposure as the reference.

Because there has been debate about the *NAT1* genotype-phenotype correlation [24], we composed the following categories: slow acetylators were those carrying at least one *NAT1*11* allele, fast acetylators had at least one *NAT1*10* allele, and all others were classified as normal acetylators. For *NAT2*, we used the generally accepted imputation [7,24] which classifies carriers of *NAT2*4/*4*, *NAT2*4/*12*, and *NAT2*12/*12* genotypes as fast, carriers of only one *NAT2*4* or *NAT2*12* allele as intermediate, and all others as slow acetylators.

All analyses were conducted using Statistical Analysis Software (SAS version 6.12, SAS Institute, Cary, NC). Statistical significance ($p<0.05$) was tested using two-sided Wilcoxon, Kruskal-Wallis, or Fisher's exact test. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated. Factors considered for confounding were history of gallstones, diabetes and adenomatous polyps, education level, total energy intake, intake of coffee, alcohol, vegetables, and fruit, body mass index, physical activity, use of aspirin, smoking (when modeling meat consumption), and meat consumption (for the smoking model). These were separately included in the model containing the matching factors (i.e., age, sex, and center) and the exposure variables (i.e., meat consumption and smoking). Those factors changing the odds ratios for the exposure variables by more

than 10% without importantly increasing the associated standard errors were included in the model. This resulted in the following multivariate models: the meat consumption models contained total energy intake, alcohol consumption and body height, and the smoking models contained body mass index (BMI), alcohol intake, and coffee consumption.

To study the interplay between genetic susceptibility and meat consumption or smoking, we composed six categories originating from three categories of exposure and two categories of imputed phenotype. The combination of the assumed low-risk imputed phenotype (i.e., *NAT1* slow/normal acetylation (i.e., no *NAT1*10* allele present), *NAT2* slow acetylation, and *GSTM1* present) and the lowest exposure category served as the reference category.

We repeated our analyses after exclusion of those for whom follow-up ended within the first two years after inclusion (13 cases and one control excluded). Similarly, analyses were repeated after exclusion of those with incident colorectal cancer after 31 December 1997 (nine cases), and after exclusion of those below age 50 at the end of follow-up.

Results

Table 7.1 shows the baseline characteristics of the study population. Cases had a higher BMI and more often reported diabetes. Also, cases were taller, more frequently reported a history of gallstones, and were less physically active than controls, but these latter differences were not statistically significant. Cases consumed slightly, but not significantly, more red meat and less poultry and fish than controls. Although there were no apparent differences between the cases and controls with respect to cigarette smoking, relatively more cases than controls had recently given up smoking (Table 7.1). Results on cigar and pipe smoking are not included in this paper because only 12% of cases and 14% of controls reported to have ever smoked cigars and/or pipe regularly, 90% of them in combination with cigarettes (data not shown). Of the 102 cancers, 63 were located in the colon and 39 in the rectum or rectosigmoid.

Table 7.1. General characteristics of the study population at baseline.

Characteristic		Cases N=102	Controls N=537
Demographic			
Female, %		45.1	46.0
Age at baseline, mean (SD)		51.3 (7.8)	51.4 (7.8)
Height, cm, mean (SD)		171.5 (9.4)	169.9 (9.3)
BMI, kg/m^2, mean (SD)		26.9 (3.9)	25.9 (3.4) [a]
Caucasian, %		97.1	97.0
Medical history, %			
Colorectal polyps		2.9	1.7
Gallstones		7.8	3.9
Diabetes		4.9	0.9 [a]
Lifestyle			
Meat, mean intake (SD)			
Total meat, g/day		83.2 (42.9)	80.4 (35.3)
Red meat, g/day		45.5 (21.2)	42.8 (21.3)
White meat, g/day		21.7 (18.8)	25.2 (22.7)
Processed meat, g/day		27.4 (27.7)	26.9 (21.5)
Beef, frequency per month		8.1 (6.0)	7.8 (6.0)
Pork, frequency per month		9.4 (5.9)	8.6 (6.0)
Poultry, frequency per month		3.7 (4.1)	4.0 (4.4)
Fish, frequency per month		2.3 (2.2)	2.9 (3.4)
Meat snacks, frequency per month		5.9 (6.5)	6.0 (6.7)
Other dietary characteristics, mean intake (SD)			
Total energy, kJ/day		6,895 (2,229)	6,773 (1,871)
Total vegetables, g/day		131.0 (61.8)	128.9 (62.1)
Cabbage and Brussels sprouts, g/day		31.4 (22.2)	27.6 (18.7)
Fruit, pieces/day		1.0 (0.7)	1.0 (0.7)
Coffee, cups/day		4.1 (2.6)	4.6 (2.9)
Alcohol, glasses/day		1.5 (1.9)	1.2 (1.6)
Cigarette smoking			
Smoking status,% [b]	never	29.4	29.8
	ex	42.2	33.3
	current	28.4	36.5
Total duration of smoking, years, mean (SD)		18.4 (15.5)	18.0 (15.5)
Number of cigarettes per day, mean (SD)		12.0 (11.2)	11.1 (10.5)
Age at start of smoking, years, mean (SD)		17.9 (4.1)	18.2 (5.2)
Time since quit smoking, years, mean (SD) [c]		11.4 (8.3)	15.0 (9.5) [a]
Other lifestyle characteristics, %			
Regular physical activity in leisure time		63.7	72.4
Occasional use of vitamin supplements		39.2	37.2
Regular use of aspirin		29.7	23.8

[a] $p < 0.05$, estimated by Wilcoxon rank test (continuous variables) or Fishers' exact test (categorical variables); [b] percentages do not count up to 100% as information was missing for two controls; [c] ex-smokers only.

Table 7.2 shows the associations of meat consumption with colorectal cancer. Frequent consumption of fresh red meat increased the risk of colorectal cancer in men only, whereas among women, frequent consumption of poultry and fish tended to decrease this risk. Consumption of sausages or meat as sandwich filling (both important sources of processed meat) were both not associated with colorectal cancer (Table 7.2). After exclusion of those who were under age 50 at the end of follow-up (n=13 cases and 62

Table 7.2. Meat consumption characteristics and risk of colorectal cancer for the total population and by gender: odds ratios and 95% confidence intervals [a].

Meat consumption characteristics	Total population		Stratification by gender			
			Women		Men	
	N ca/co [b]	OR (95% CI)	N ca/co	OR (95% CI)	N ca/co	OR (95% CI)
Total meat (times per week)						
0 - 3.9	30/183	1 (REF)	20/86	1 (REF)	10/97	1 (REF)
4 - 5.9	33/163	0.7 (0.4-1.2)	17/87	0.7 (0.3-1.4)	16/76	0.9 (0.4-2.4)
6+	39/191	1.1 (0.6-1.9)	11/72	0.5 (0.2-1.4)	28/119	1.9 (0.9-4.3)
p-value for trend		0.48		0.50		0.10
Fresh red meat (times per week)						
0 - 3	22/157	1 (REF)	15/72	1 (REF)	7/85	1 (REF)
3.1 - 4.5	35/186	1.3 (0.7-2.3)	18/102	0.8 (0.4-1.8)	17/84	2.7 (1.1-6.9)
5+	45/194	1.6 (0.9-2.9)	15/71	1.2 (0.5-2.8)	30/123	2.7 (1.1-6.7)
p-value for trend		0.10		0.64		0.06
Poultry (times per month)						
0 - < 1	27/116	1 (REF)	15/50	1 (REF)	12/66	1 (REF)
1 - 4	32/166	0.8 (0.5-1.5)	16/70	0.8 (0.3-1.7)	16/96	0.9 (0.4-2.2)
4+	43/255	0.7 (0.4-1.3)	17/125	0.5 (0.2-1.1)	26/130	1.1 (0.5-2.4)
p-value for trend		0.30		0.07		0.68
Fish (times per month)						
0 - < 1	36/177	1 (REF)	21/83	1 (REF)	15/94	1 (REF)
1 - 4	34/150	1.1 (0.7-1.9)	16/60	1.1 (0.5-2.4)	18/90	1.3 (0.6-2.8)
4+	32/210	0.7 (0.4-1.3)	11/102	0.5 (0.2-1.0)	21/108	1.2 (0.6-2.4)
p-value for trend		0.23		0.05		0.29
Sausage as a snack						
No	51/253	1 (REF)	27/121	1 (REF)	24/132	1 (REF)
Yes	51/284	0.9 (0.6-1.3)	21/124	0.8 (0.4-1.4)	30/160	1.0 (0.5-1.9)
p-value for trend		0.50		0.39		1.0
Sandwiches with meat filling (number/day)						
0 - 1	60/274	1 (REF)	38/159	1 (REF)	22/115	1 (REF)
2+	42/263	0.7 (0.4-1.1)	10/86	0.5 (0.2-1.2)	32/177	0.8 (0.4-1.6)
p-value for trend		0.15		0.13		0.58

[a] Adjusted for age, sex, center, total energy intake, alcohol consumption, and body height; [b] N ca/co, number of cases over number of controls.

controls), the association between red meat consumption frequency and colorectal cancer became statistically significant for the total population (OR 2.0, 95% CI 1.1-3.8 highest *vs.* lowest consumption group). Sub-site analyses showed that frequent consumption of fish protected against colon, but not against rectal cancer (highest *vs.*

lowest category OR 0.5, 95% CI 0.3-0.9 for colon, and OR 1.6, 95% CI 0.7-3.6 for rectum tumors). The reduction in risk of colon cancer was largest and statistically significant among women (OR 0.4, 95% CI 0.1-0.9). No other differences were observed.

Table 7.3. Cigarette smoking characteristics and risk of colorectal cancer for the total population and stratified by smoking status: odds ratios and 95% confidence intervals[a].

		Total population[b]		Stratification by smoking status			
Smoking characteristics				Former		Current	
		N ca/co[c]	OR (95% CI)	N ca/co	OR (95% CI)	N ca/co	OR (95% CI)
Cigarette smoking							
Never		30/160	1 (REF)		n.a.[d]		n.a.
Former		43/179	1.4 (0.8-2.5)				
Current		29/196	0.9 (0.5-1.7)				
	p-value for trend		0.27				
Smoking duration (years)							
all	*former/current*						
0	1-15	30/160	1 (REF)	13/77	1 (REF)	3/18	1 (REF)
1-25	16-30	36/178	1.2 (0.7-2.1)	23/71	2.7 (1.03-7.4)	7/60	0.4 (0.1-1.9)
>25	>30	36/197	1.2 (0.7-2.1)	7/31	3.2 (1.04-9.8)	19/118	1.9 (0.5-8.2)
	p-value for trend		0.99		0.04		0.28
Cigarettes per day							
all	*former/current*						
0	1-10	30/160	1 (REF)	12/75	1 (REF)	10/71	1 (REF)
1-14	11-20	26/174	0.9 (0.5-1.7)	21/69	2.1 (0.9-5.0)	14/88	1.1 (0.4-2.8)
>14	>20	46/201	1.5 (0.9-2.6)	10/35	1.7 (0.6-4.6)	5/37	1.2 (0.3-4.0)
	p-value for trend		0.32		0.15		0.75
Time since quit smoking							
all	*former*[e]						
never smoked		30/160	1 (REF)				
> 15 years	> 18 years	15/81	1.1 (0.5-2.3)	18/53	1 (REF)		n.a.
0-15 years	9-18 years	28/97	1.7 (0.9-3.1)	16/65	2.6 (1.0-6.5)		
still smokes	0-8 years	29/196	0.9 (0.5-1.7)	9/60	2.2 (0.8-5.5)		
	p-value for trend		0.24		0.10		

[a] Adjusted for age, sex, center, coffee and alcohol consumption, and body mass index; [b] information on smoking characteristics was missing for two controls; [c] N ca/co, number of cases over number of controls; [d] n.a., not applicable; [e] former smokers only, time since quitting was unknown for one former smoker.

Associations between cigarette smoking and colorectal cancer are shown in Table 7.3. There were no differences between men and women (data not shown). Among former

smokers, smoking for more than 30 years was associated with a three times increased risk of colorectal cancer, but was not associated with a significantly increased colorectal cancer risk among current smokers (Table 7.3). Exclusion of the first two years of follow-up led to an even stronger association between smoking duration and colorectal cancer among former smokers (OR 5.4, 95% CI 1.5-19.6), but remained insignificant in current smokers (data not shown). The increased risk of colorectal cancer among former smokers was strongest among those who had quit smoking 9-18 years before inclusion and was due to an increased risk of rectum cancer (OR 6.4, 95% CI 1.3-32.1). Although the number of cigarettes smoked daily was also related to increased risk of colorectal cancer, this association did not reach statistical significance (Table 7.3).

Table 7.4. Allele frequencies of *NAT1* and *NAT2*, prevalence of imputed phenotypes of *NAT1*, *NAT2*, and *GSTM1* and association of these imputed phenotypes with colorectal cancer.

	Cases		Controls		
Gene	N	Proportion	N	Proportion	OR (95% CI)
NAT1 alleles	204		1074		
*4		0.760		0.713	
*3		0.025		0.034	
*10		0.196		0.227	
*11		0.020		0.026	
NAT1 imputed phenotype [a]	102		536		
Slow		0.04		0.05	1 (REF)
Normal		0.62		0.55	1.4 (0.5-4.2)
Fast		0.34		0.40	1.1 (0.4-3.3)
NAT2 alleles	204		1074		
*4		0.245		0.252	
*5A		0.044		0.031	
*5B/C		0.397		0.371	
*6		0.275		0.307	
*7		0.039		0.035	
*12		0.00		0.005	
*14		0.00		0.00	
NAT2 imputed phenotype [b]	102		536		
Slow		0.57		0.56	1 (REF)
Intermediate		0.36		0.37	0.9 (0.6-1.5)
Fast		0.08		0.07	0.9 (0.4-2.0)
GSTM1	102		537		
Present		0.43		0.47	1 (REF)
Null		0.57		0.53	1.2 (0.8-1.8)

[a] *NAT1* imputed phenotypes; fast: at least one **10* allele, no **11* allele, normal: no **10* or **11* allele, slow: at least one **11* allele. [b] *NAT2* imputed phenotypes; fast: homozygous *NAT2*4* or *NAT2*12*, or *NAT2*4/*12*; intermediate: one **4* or **12* allele present; slow: no **4* or **12* alleles present.

Table 7.5. Odds ratios (95% confidence intervals) [a] for the interplay of meat consumption and smoking with imputed phenotype of *NAT1*, *NAT2*, and *GSTM1* in relation to colorectal cancer.

Characteristic	*NAT1* imputed phenotype		*NAT2* imputed phenotype		*GSTM1* genotype	
	slow and normal	fast [b]	slow	fast / intermediate	present	null
Meat consumption characteristics						
Fresh red meat (times per week)						
0 - 3	1 (REF)	0.7 (0.3-1.9)	1 (REF)	0.7 (0.3-1.9)	1 (REF)	1.7 (0.7-4.4)
3.1 - 4.5	1.2 (0.6-2.4)	0.9 (0.4-2.0)	1.0 (0.5-2.2)	1.1 (0.5-2.4)	1.5 (0.6-3.7)	1.7 (0.7-4.1)
5+	1.4 (0.7-2.9)	1.4 (0.6-3.0)	1.4 (0.7-2.9)	1.4 (0.6-3.1)	2.0 (0.8-5.0)	2.2 (0.9-5.2)
Poultry (times per month)						
0 - 1	1 (REF)	0.7 (0.3-1.6)	1 (REF)	1.4 (0.6-3.4)	1 (REF)	1.0 (0.4-2.4)
1 - 4	0.6 (0.3-1.3)	0.9 (0.4-2.0)	0.8 (0.4-1.8)	1.3 (0.6-2.9)	1.1 (0.5-2.5)	0.6 (0.3-1.5)
4+	0.7 (0.4-1.5)	0.5 (0.2-1.0)	1.1 (0.6-2.4)	0.6 (0.3-1.4)	0.4 (0.2-0.98)	1.1 (0.5-2.2)
Fish (times per month)						
0 - 1	1 (REF)	0.7 (0.3-1.4)	1 (REF)	1.2 (0.6-2.4)	1 (REF)	0.9 (0.4-1.8)
1 - 4	1.0 (0.5-1.9)	0.9 (0.4-1.9)	1.2 (0.6-2.4)	1.2 (0.6-2.6)	0.9 (0.5-2.0)	1.2 (0.6-2.4)
4+	0.6 (0.3-1.2)	0.6 (0.3-1.3)	0.9 (0.5-1.8)	0.7 (0.3-1.4)	0.5 (0.2-1.1)	0.9 (0.4-1.7)
Smoking characteristics						
Smoking duration						
0 years	1 (REF)	0.6 (0.3-1.5)	1 (REF)	1.0 (0.4-2.1)	1 (REF)	1.2 (0.5-2.6)
1 - 25 years	1.1 (0.6-2.2)	0.8 (0.4-1.8)	1.1 (0.5-2.4)	1.2 (0.5-2.6)	1.2 (0.5-2.7)	1.4 (0.6-3.1)
> 25 years	0.9 (0.5-1.9)	1.0 (0.5-2.3)	1.3 (0.6-2.7)	1.0 (0.4-2.2)	1.2 (0.5-2.8)	1.4 (0.6-3.0)
Cigarettes per day						
0	1 (REF)	0.6 (0.3-1.5)	1 (REF)	1.0 (0.4-2.1)	1 (REF)	1.2 (0.5-2.6)
1 - 4	1.0 (0.5-2.1)	0.4 (0.2-1.2)	0.9 (0.4-2.0)	0.9 (0.4-2.1)	0.9 (0.4-2.1)	1.1 (0.5-2.6)
> 14	1.1 (0.6-2.3)	1.5 (0.7-3.1)	1.6 (0.8-3.4)	1.3 (0.6-2.7)	1.6 (0.7-3.7)	1.7 (0.8-3.6)

[a] Analyses on meat consumption were adjusted for age, sex, center, total energy intake, alcohol consumption, and body height; analyses on smoking were adjusted for age, sex, center, coffee and alcohol consumption, and body mass index; [b] *NAT1* fast, at least one **10* allele present, for other imputed phenotypes see text or foot note to Table 7.4.

Since our study is the first to report allele frequencies of *NAT1*, *NAT2*, and *GSTM1* in a Dutch population, these are included in Table 7.4. *NAT1*, *NAT2*, and *GSTM1* genotypes were not associated with colorectal cancer (Table 7.4).

To illustrate the possible interplay of meat consumption and smoking with genotype in colorectal cancer etiology, risk ratios for the combinations of *NAT1*, *NAT2* and *GSTM1* genotypes and different levels of exposure are shown in Table 7.5. We found no indications for important roles of *NAT* polymorphisms in the associations of meat consumption and smoking with colorectal cancer. *GSTM1* appeared to influence the associations of poultry and fish consumption with colorectal cancer. In presence of *GSTM1*, the associations of poultry and fish with colorectal cancer were inverse, whereas no decreased risk was found for the combination of high consumption of poultry and fish and the *GSTM1* null genotype (Table 7.5). The interaction between *GSTM1* genotype and poultry reached statistically significance (p=0.01).

Exclusion of subjects who were diagnosed with incident colorectal cancer (n=13 cases) or died for unknown reason (n=1 control) within the first two years of follow up only marginally changed our β-estimates and did not change our conclusions. Also, exclusion of cases who got incident cancer in 1998 (n=9) did not change the results importantly (data not shown).

Discussion

Red meat consumption was associated with increased risk of colorectal cancer in men, whereas consumption of poultry and fish tended to decrease this risk in women. Frequent consumption of fish decreased colon cancer risk. Former smoking tended to increase the risk of rectal cancer and long-term smoking tended to increase risk of colorectal cancer among former smokers only. Polymorphisms in *NAT1*, *NAT2*, and *GSTM1* genes were not related to colorectal cancer and did in general not influence the associations of meat consumption and smoking with colorectal cancer. Frequent consumption of poultry and fish decreased colorectal cancer risk only in presence of the *GSTM1* gene.

We conducted a nested case-control study with prospective data on exposure. A great advantage of this design is that no information or selection bias is to be expected. Information of exposure was probably not biased by latent disease since exclusion of the first two years of follow-up did not change the results of this study. Since exposure in the past is at least equally (and probably more) relevant to cancer etiology as recent exposure, we do not consider it a major disadvantage of our study that the information on dietary and smoking habits was collected at baseline (almost ten years before most cases occurred).

It is unlikely that one or more controls were misclassified as cases since the linkage method used for identification of cases had a sensitivity of 98% and a specificity of

100% [18]. For the years 1987, 1988, 1997, and 1998, identification of cases had to be based on information of regional cancer registries only. Although this might have led to underestimation of the actual number of cases, this proportion was estimated to be less than 0.3% [17]. Exclusion of cases with incident cancer in 1987 and 1988 or 1998 did not change our results.

Our questionnaire was primarily designed to estimate the cohort members' exposure to risk factors of cardiovascular diseases. As a consequence, no data on family history of cancer was collected. Although only about 5% of cancers are thought to be strictly hereditary [3], family history could have confounded our results since it might both be related to exposure and to the disease.

For estimation of meat consumption, a short semi-quantitative food frequency method developed and validated by Bloemberg and colleagues was used [19]. Since meat consumption was estimated by frequencies of consumption of beef, pork, poultry, and fish, a major source of meat intake in the Dutch population was possibly missed; i.e., minced meat being composed of a mix of pork and beef. Validation by a dietary history method showed, however, that meat consumption was estimated with acceptable reproducibility and validity (see Methods section) [19]. Because only consumption of selected foods was inquired, our questionnaire underestimated energy intake, but accurately ranked energy intake as estimated by twelve 24-hour recalls (r=0.71, Ocké *et al.*, unpublished data).

Smoking habits were assessed by detailed questions and almost no data on smoking were missing. In contrast to Giovannucci and colleagues [25,26], we had no information on smoking habits in the distant past, nor did we record the number of cigarettes smoked daily at different ages. Although the questionnaire included questions on the number of cigarettes smoked daily at present and in the past, the year in which smoking habits were changed was not recorded. Hence, we considered past smoking habits to be most accurate in estimating ones' exposure to cigarette smoke. This might have caused some misclassification and attenuation of the effect of smoking dose among current smokers, since 10% indicated to have increased their daily cigarette consumption.

Red meat consumption was positively associated with colorectal cancer while the association between poultry and fish consumption and colorectal cancer (specifically the association between fish consumption and colon cancer) was negative. These findings correspond with those of two large US prospective studies [27,28], but not with results of European studies [29-31]. These opposite associations are not the result of substitution of red meat by poultry and fish as was suggested earlier [27]: consumption of poultry and fish was not correlated with red meat and those in the highest category of red meat consumption had almost equal probability to be in the lowest or highest of category of poultry and fish consumption. Consequently, adjustment of our analyses on the

association of red meat with colorectal cancer for poultry and fish and *vice versa* did not influence our results.

The observation that red meat intake and colorectal cancer were only related among men could be the result of the higher intake or the greater diversity in beef and pork intake among males (median intake 4 times a week, interquartile range 2 - 6 times weekly) than in females (3.5, 2 - 4, respectively). The inverse association between poultry and fish consumption and colorectal cancer among women was, however, not a result of higher (range of) intake by women. Although red meat was associated with unhealthy behavior (e.g., smoking, high coffee consumption), whereas poultry and fish consumption correlated with healthy habits (e.g., high consumption of vegetables), adjustment in the analyses for these factors did not change our results. However, residual confounding can remain even after adjustment. Our results could also be due to differences in food preparation (leading to differences in HCA concentration), food processing, fat or heme content [3,32]. In contrast to other studies [4,27-30], we found no association between sausage consumption and colorectal cancer, possibly because we had no information about other commonly consumed processed meats, such as smoked ham. Our null findings for consumption of meat on sandwiches could partly be due to the fact that this category comprised processed as well as fresh meats.

We found that duration of cigarette smoking was positively associated with colorectal cancer, which is in accordance with most recently conducted studies [5]. This effect was strongest for rectum cancer and among those who quit smoking 9 - 18 years ago. It is not clear why former smokers had an increased risk of cancer while current smokers had not, as both were of the same age and smoked the same amount of cigarettes daily. Moreover, current smokers had smoked for a longer period than former smokers had. Our findings can not be attributed to a latency effect causing latent cases to quit smoking, because exclusion of the first two years of follow-up strengthened the association between smoking duration and colorectal cancer among former smokers, but did not change the association in current smokers. Finally, our results can not be explained by unbalanced numbers in the different categories of smoking duration, because changing these categories to improve balance did not change our conclusions. We did not observe an increased risk of (colo)rectal cancer for those who quit smoking more than 15 years ago, possibly because the total duration of smoking did, in general, not exceed three or four decades [5].

Despite the large base population and the relatively long follow-up period, we could only include 102 colorectal cancer cases in this study. With a sample of about 100 cases and 500 controls, we estimated the power to be about 90% for a true relative risk of 2.0 for one factor at a time. Thus, the power to study the combined effect of genotype and exposure was low.

DNA isolation was not successful for 7% of samples, possibly because problems with collection of buffycoats at the start of the study. However, DNA isolation success rate was not different between samples collected at the start (1987) and at the end of the study (1991).

The allele frequencies of *NAT1*, *NAT2* and *GSTM1* were similar to frequencies in other studies among European Caucasians [7,8,23]. Consistently with most other studies, *NAT1*, *NAT2* and *GSTM1* genotype did not increase risk of colorectal cancer [8,33]. In contrast to Chen and colleagues [10], we found no indication that *NAT1* genotype modulates the associations of meat consumption and smoking with colorectal cancer. Like these authors [10], we classified those carrying at least one *NAT1*10* allele as fast and all others as normal acetylators. This may be incorrect since recent studies indicate that *NAT1*11*, **14*, and **15* lead to low enzyme activity, whereas activity associated with *NAT1*10* is similar to activity of the wildtype enzyme [24]. Unfortunately, the infrequent presence of these slow alleles did not allow us to study the effect of slow *versus* normal *NAT1* acetylation (see Table 7.4).

There was no indication for interplay between *NAT2* polymorphism and meat consumption or smoking in colorectal cancer, which is in accordance with a large US case-control study [11], but contradicts two other studies [9,10]. However, when we alternatively chose low meat consumption as the reference category in both groups of acetylators, both the positive association of red meat consumption and the inverse association of poultry and fish consumption with colorectal cancer were strongest among those carrying the *NAT2* fast acetylation genotype (as can be concluded from Table 7.5). This effect might be the result of differences in concentrations of specific HCAs between fresh red meat and poultry [34-36], or of differences in metabolism of the different HCAs by NAT2 [37]. However, it is also possible that poultry and fish generally contain less HCAs than red meat in the Netherlands, due to differences in preparation methods. HCAs formed during meat preparation were not considered as a risk factor for colorectal cancer at the time the cohort was enrolled (January 1987 until December 1991). Therefore, we had no information on meat preparation habits and this could have flawed our results on the interactions between genetic susceptibility and meat consumption. Our results on smoking and *NAT2* polymorphism are in accordance with the results of the large US case-control study [13], although another study showed that colorectal cancer risk was confined to current smokers with the slow *NAT2* phenotype [12].

The inverse associations of consumption of fish and poultry with colorectal cancer were strongest in presence of the *GSTM1* gene. The association of poultry with cancer was significantly modified by *GSTM1* genotype, which is not in accordance with the US case-control study [11]. Consistent with other studies [13,14], no indications for a role of *GSTM1* genotype in smoking-associated colorectal cancer was found. The nature of the

observed effect modification is not clear, since the *GSTM1* genotype is thought to be more important in the associations of smoking characteristics with colorectal cancer, as the GST-μ enzyme is probably more involved in detoxification of PAHs than in HCA detoxification [38]. Exposure to PAHs through intake of meat is presumably very low, since this occurs when meat is cooked over an open flame as in barbecuing which is rarely practiced by the Dutch population. Since the GST-μ enzyme itself is highly inducible by a range of substances from food and cigarette smoke, and *GSTM1* genotype influences inducibility of cytochrome P 450 enzymes (CYP1A1 and 1A2) which also play a major role in the metabolism of xenobiotics [38], other mechanisms might be more important. However, adjustment of our analyses on *GSTM1* for possible inducers, such as cruciferous vegetables and coffee, did not change our results importantly. Alternatively, the protective effect of the GST-μ enzyme with high poultry and fish consumption could be associated with the protective effect of the more healthy lifestyle associated with consumption of these meats, such as a relatively high consumption of vegetables and fruit. Apart from GST-μ, other GST enzymes, such as GST-θ, may play a role in the detoxification of carcinogens [39]. Although we had information about *GSTT1* genotype in this study, we did not consider the effect of the polymorphism. The *GSTT1* null genotype occurs in 10 - 20% of Caucasians [8], and in 16.5% of the subjects in our study. Considering the relatively low number of cases, there were too few cases with the null genotype to enable subgroup analyses.

In this relatively young population, red meat consumption and former long-term smoking modestly increased risk, whereas poultry and fish tended to decrease risk of colorectal cancer in subgroups only. *GSTM1* genotype altered the inverse associations of poultry and fish with colorectal cancer. In general, modification by genotype appears to be small and less important than the effect of gender, smoking status, and location of the tumor. However, as mentioned, our study is small and our results need confirmation in other (European) study populations.

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8.

General discussion

In the studies described in this thesis, we investigated the potential influence of genetic susceptibility on the associations of high meat consumption, alcohol intake, and cigarette smoking with colorectal tumor risk. As the strengths and limitations of cohort and case-control studies have been addressed in detail elsewhere and many papers have summarized possible associations between diet and colorectal neoplasm (see e.g., [1-6]), these issues will not be discussed here. Instead, in this Chapter, emphasis will be on issues related to studies investigating the influence of genetic susceptibility (through metabolic polymorphisms) on the association between specific exposures and colorectal tumors. Although specifically addressing colorectal cancer, many of the issues discussed here will also apply to other diseases in which low-penetrance genetic susceptibility plays a role.

First, the main results of the studies described in this thesis will be summarized and compared to results reported by others. Next, the strengths and limitations of epidemiological studies incorporating metabolic polymorphisms and finally, the potential for this type of studies in the future and other possibilities for future research will be discussed.

Main findings

The studies described in this thesis aimed to evaluate the potential interplay between common genetic polymorphisms encoding metabolic enzymes, and the risk of colorectal tumors associated with meat consumption and preparation (Chapters 4 and 7), cigarette smoking (Chapters 5 and 7), and alcohol intake (Chapter 6). Methodological issues related to these studies were addressed in Chapters 2 and 3.

The environmental exposures studied were found to be probable risk factors of colorectal tumors [7,8], possibly through the action of potential carcinogens, which are metabolized by enzymes encoded by polymorphic genes. Genetic polymorphisms may thus influence the risk of neoplasm. The main results of the studies described in Chapters 4 through 7 are depicted in Table 8.1.

As shown in Table 8.1, meat consumption did not increase risk of colorectal adenomas. We also did not find that unfavorable meat preparation methods increased adenoma risk. Frequent red meat consumption was weakly positively associated with increased risk of colorectal cancer. We found no strong indications for genetic polymorphisms to modify the association of meat consumption with colorectal neoplasm (Table 8.1).

Cigarette smoking was a relatively strong risk factor of colorectal adenomas, mainly through smoking duration (Table 8.1). This association was most pronounced in those with inherited variants of *SULT1A1* leading to fast sulfation (OR 4.3, 95% CI 1.6-11.8), and of *NAT2* encoding slow acetylation (OR 3.5, 95% CI 1.9-6.4). Smoking-associated adenoma risk also varied over categories of *EPHX*, but not in a clear pattern. The risk of

colorectal cancer was also increased with smoking duration, although only among former smokers (OR 2.7, 95% CI 1.0-7.4, for those who had smoked for more than 16 years compared to those who had smoked for less than 16 years). We found no indications for any of the studied genotypes to modify this association.

Table 8.1. Summary of main results described in thesis regarding the potential influence of genetic susceptibility on the associations of meat consumption and preparation, cigarette smoking, and alcohol consumption, with colorectal tumor risk.

Environmental exposure		Endpoint	Overall OR (95% CI)	Genetic susceptibility[a]							
				*ADH3*1/*1*	*EPHX* ex 3 *HH*	*GSTM1* null	*GSTT1* null	*NAT1*[b]	*NAT2*[c]	*SULT1A1* fast	Chapter
Total meat intake	7 *vs.* ≤ 5 times per week	adenomas	1.2 (0.8-1.9)	*				*			4
Meat preparation	all *vs.* ≤ 1 meat types dark	adenomas	1.0 (0.6-1.5)	*				*			4
Red meat intake	5+ *vs.* ≤ 3 times per week	cancer	1.6 (0.9-2.9)	*	*		*			*	7
White meat intake[d]	4+ *vs.* < once per month	cancer	0.7 (0.4-1.3)	*	*		*			*	7
Cigarette smoking	> 25 yr. *vs.* never smoking	adenomas	2.4 (1.4-4.1)	*				*			5
Cigarette smoking	> 25 yr. *vs.* never smoking	cancer	1.2 (0.7-2.1)	*	*		*				7
Alcohol intake											
women	10+ drinks *vs.* < 1 drink weekly	adenomas	1.8 (1.0-3.2)		*	*	*	*	*	*	6
men			1.1 (0.6-2.1)		*	*	*	*	*	*	6

[a] Legend to boxes
observed odds ratio: ☐ 1 - 2 ▒ 2 - 3 ▓ 3 - 4 ■ > 4 * not applicable;
[b] *NAT1*10* was considered the high-risk genotype in Chapter 7; [c] *NAT2* fast imputed genotype was considered as the high-risk genotype in all Chapters, but risk was highest for slow *NAT2* acetylators who (had) smoked (Chapter 5); [d] boxes indicate [1/OR] instead of the actual OR observed.

High alcohol consumption was a risk factor for colorectal adenomas especially among women (Table 8.1). Risk of adenomas among men was increased only with the consumption of more than 21 drinks per week (OR 1.8, 95% CI 0.9-3.8). Although the risk of adenomas was higher among alcohol drinkers with the imputed *ADH3* fast than

with the slow phenotype, we found no strong indications that *ADH3* polymorphism modifies the association between alcohol drinking and colorectal adenomas.

Overall, our results show, at best, modest influence of the metabolic polymorphisms studied, with odds ratios for interaction between metabolic polymorphisms and environmental exposures ranging from 1.3 to 1.8 (see Table 2.3, Chapter 2). Our results are in accordance with those of other studies, some of which included large populations (i.e. more than 2,000 cases and controls [9,10]). Like in our studies, in these studies odds ratios for the exposed genetically susceptible group generally ranged from 1.5 to 3.5 and were rarely higher than 5 (see Table 1.1, Chapter 1) [11,12]. There are a number of potential reasons why the studies conducted thus far have produced inconsistent results. These issues will be discussed below.

Studies incorporating genetic susceptibility: strengths and shortcomings

Until recently, in epidemiological studies the association between environmental exposure factors and colorectal neoplasm was analyzed without consideration of genetic susceptibility. Most of these studies found weak and inconsistent associations between exposure and disease [6,13] possibly because the assessment of environmental exposure is prone to errors [1] and genetic susceptibility was not assessed.

Assessment of genetic susceptibility

With the development of methods to detect DNA polymorphisms, the incorporation of genetic susceptibility to common environmental exposures into epidemiological studies evolved. Since the early 1990s, an increasing number of epidemiological studies incorporated data on genetic polymorphisms. This was considered an important improvement, as the incorporation of metabolic polymorphisms may increase our knowledge on which carcinogens potentially increase cancer risk [14]. As genetic polymorphisms, unlike e.g. mutations in tumor suppressor genes, do not directly affect carcinogenesis, their overall effect is expected to be small [15,16]. However, in the presence of exposure to a relevant carcinogen, these polymorphisms might be potent effect modifiers of cancer risk, as susceptible subjects may respond differently to specific carcinogenic substances compared to non-susceptible persons [14]. Moreover, the impact of genetic susceptibility may be small at individual level but may nevertheless be important in terms of population attributable risk, as this type of genetic susceptibility is highly frequent in the general population and determines the effective dose of commonly occurring carcinogens [17,18]. In comparison with environmental exposure assessment, the assessment of genetic susceptibility is considered to be highly sensitive and precise. However, the assessment of genetic susceptibility has several limitations [11], as will be discussed below.

Determination of genetic polymorphisms

Errors in the determination of genotypes or in the subsequent imputation of phenotypes may result in misclassification. Misclassification due to measurement errors is thought to be of minor importance for the assessment of genotypes. In our study, validity and reproducibility of newly developed methods for *NAT1* and *NAT2* were extensively tested (see Chapter 3). Reproducibility of other methods was also tested by determination in duplicate for about 10% of the samples. Reproducibility and validity were close to 100% for all genotypes. Nonetheless, misclassification of imputed phenotypes could have occurred because not all known alleles were included in the analyses [19]. However, this type of misclassification is thought to have only a minor effect for most of the genotypes described in this thesis, since we determined all alleles frequently occurring in Caucasian populations. For example, for *NAT1*, we were not able to distinguish between *NAT1*10* (normal acetylation) and *NAT1*14* and *NAT1*15* (slow, respectively no NAT1 acetylation [20]). However, *NAT1*14* and *NAT1*15* allele frequencies are very low in Caucasian populations [20,21].

In our studies, all genotypes were determined by standardized methods. To control for cross-contamination, we included negative controls in all tests. Where needed, positive controls were also included to test for PCR-performance (as in the case of *GSTM1* and *GSTT1*, where no PCR product is formed in the absence of these genes, i.e., in samples from subjects with homozygous null genotypes). Laboratory personnel was blinded to case-control status. Differential misclassification did probably not occur in our studies, as the genotypes were, in almost all investigated situations, not dependent on other variables under study (see Chapters 4-7).

Genotype-phenotype correlation

For some genes, such as *EPHX* and to a lesser extent *NAT1*, phenotype-genotype correlation is not yet clear [22,23], and it is therefore difficult to impute phenotypes based on the determined genotypes. The use of imputed phenotypes for these genes might introduce phenotype misclassification and might also provide an explanation why we did not observe any effect of these imputed phenotypes on exposure-related neoplasm. Gene expression is influenced by genetic and environmental factors (see under *'Effective dose of carcinogens'*). Genes may contain several polymorphic sites in coding and non-coding regions, which may be present on the same or on the complementary DNA strand. The exact location of these polymorphic sites may determine the nature of the gene product. Often, this location can not be determined by genotyping but haplotype analysis is required instead [24]. Genotype-phenotype correlation may vary over tissues, as expression of many genes is tissue-specific. For example, *NAT2* is mainly expressed in the liver, whereas *NAT1* is expressed in most tissues, including colorectal epithelium [25].

Assumption of independence

One assumption frequently made when studying potential interaction between exposure and genotype is that the occurrence of genotype and exposure are independent of each other [26]. It is questionable whether this assumption always applies, as we found that *GSTM1* and *GSTT1* genotype frequencies differed with smoking status (Chapter 5). However, such dependence has never been published before. Violation is nevertheless possible for ADH3 and alcohol consumption (Chapter 6). The *ADH3*1* allele was found to be in linkage disequilibrium with *ADH2*2*, which decreases the risk of alcoholism, although *ADH3* itself was not associated with drinking behavior within strata of *ADH2* [27-29]. Probably, such dependence does not influence the analyses described in Chapter 6, as we analyzed the combined effect of *ADH3* genotype and alcohol consumption against other combinations of *ADH3* and alcohol intake.

Involvement of multiple polymorphic genes

If one metabolic enzyme is not expressed or has a reduced substrate affinity or a lower stability due to an underlying genetic polymorphism, other metabolic enzymes might take over its function. This was indeed found for the *GST* superfamily, which consists of four closely related enzymes with broad substrate specificity [30]. It may be important to consider all the principal genetically polymorphic enzymes possibly involved in the metabolism of the carcinogen under study. Methods to genotype many polymorphisms at once are rapidly evolving [31], but most epidemiological studies so far do not have enough power to include many polymorphic genes (see under *'Sample size requirements'*). In spite of the availability of these methods, we might not be able to study all genes in a single pathway, simply because for most metabolic routes, not all the genes involved will be known. Moreover, it is not exactly known which are the interactions between the different enzymes involved in the same metabolic route. Some researchers, when including several polymorphisms in one epidemiological study, found indicative gene-gene interactions (e.g., between *GSTs* [32] and between *NAT1* and *NAT2* [33,34]), whereas others did not find evidence for such associations [35]. Whether such interactions are important remains unclear. On the one hand, studies that failed to detect such interactions may have had insufficient power, but on the other hand, the comparison of multiple combinations may have led to the finding and publication of some spurious associations.

ASSESSMENT OF ENVIRONMENTAL EXPOSURE

Obviously, the assessment of exposure to environmental risk factors may be prone to errors and does not directly reflect the amount of the ultimate carcinogen (effective dose) to which the target tissue becomes exposed. The events leading from

environmental exposure, measurable in traditional epidemiological studies, to the ultimate effective dose of the corresponding carcinogen are shown in Figure 8.1.

With respect to assessment of exposure, three issues are important, namely correct assessment of certain sources of potential carcinogens (i.e., diet, smoking), assessment of exposure to specific carcinogens, and assessment of the effective dose of a carcinogen, which is determined by a cascade of events starting with absorption (Figure 8.1). Exposure to environmental (and especially dietary) factors is difficult to assess [1,36-38]. Here, the assessment of exposure to the specific factors studied in this thesis will be discussed briefly. Also, problems will be discussed that may occur with assessment of exposure to potential carcinogens in relation to the effective dose of the ultimate carcinogen, which may initiate DNA mutations and ultimate tumor formation (Figure 8.1).

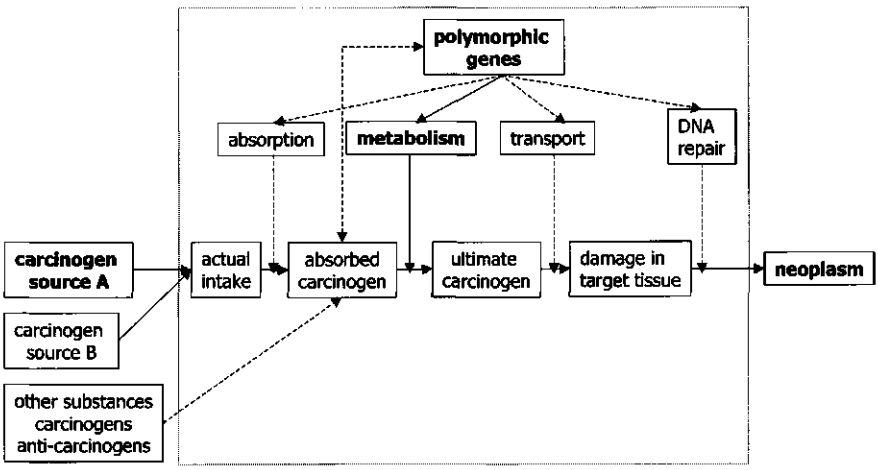

Figure 8.1. Relation of measured exposure (carcinogen of source A) to the ultimate carcinogen and neoplasm risk.

Determination of exposure

The quality of the assessment of environmental exposure in our studies is comparable to that applied in other studies. For the assessment of dietary habits in the adenoma case-control study (Chapters 4-6), we used a validated self-administered semi-quantitative food frequency questionnaire [39], of which reproducibility and validity were judged

sufficiently high (i.e., median correlation coefficients for relative validity 0.6), although the relative validity for some food groups, such as vegetables and fish, was low (i.e., $r<0.4$) [39,40]. Correlation coefficients for the reproducibility and relative validity of alcohol consumption were high ($r>0.8$ and $r>0.7$, respectively) [39]. Meat consumption was assessed by a questionnaire that has not yet been validated but that yielded estimates correlating well with meat consumption assessed from the food frequency questionnaire ($r=0.7$ - 0.9), which was reported to have a reproducibility of about 0.7 and a relative validity of about 0.5 [39]. The individual reproducibility of preparation habits assessed for beef patties was high (Cohen's $\kappa=0.8$, see Chapter 4),

In the prospective nested case-control study on colorectal cancer, dietary habits were estimated by a short semi-quantitative food frequency method validated using a dietary history method (Chapter 7) [41]. Spearman rank correlation coefficients for the reproducibility of meat intake were about 0.6, and between 0.4 and 0.5 for relative validity.

Current smoking rates might have been underreported to a small extent [42]. Underreporting of current smoking in our studies was not considered important, as smoking rates within the adenoma study population did not differ from rates observed in a random sample of the general population and were also similar to rates reported by non-participants (see Chapter 2). The frequency of cigarette smoking among controls of the nested case-control study was equal to that in the general Dutch population by 1989, when the cohort was enrolled [†].

Differential misclassification can result from under- or over-reporting of certain exposures or to differential recall, as has been discussed in detail elsewhere [1]. In this respect, alcohol consumption may be underestimated especially by heavy drinkers. If heavy drinking increases the risk of colorectal adenomas, than this risk might be underestimated if heavy drinkers underestimate the number of drinks they consume.

Thus, misclassification of exposure (especially to dietary factors) may have attenuated the effects of exposure on disease in our studies.

Actual intake of relevant carcinogens

For many carcinogens, it is difficult to estimate the actual intake of the specific carcinogen of interest correctly. One example is the estimation of HCA concentrations in well-done meat. Preparation of meat at high temperatures was consistently found to increase HCA concentrations in laboratory studies [43-46], but HCA concentrations in beef patties prepared as habitual at home by volunteers did not clearly correlate with height of the heat source (used as proxy for cooking temperature, Chapter 4). Thus, estimation of exposure to HCAs by use of databases in which this exposure is imputed from

[†] See http:\\www.cbs.nl; Centraal Bureau voor de Statistiek, 2001.

laboratory assessments [47-50] has its limitation as it may not correctly reflect the true exposure.

Large variations in the intake of HCAs and PAHs from meat can be expected, as these are the result of varying preparation methods [51]. If the concentration of carcinogens is considered to be relatively constant, like in manufactured cigarettes, it may still vary over time and over different brands [52]. Besides, several measurable exposures, such as cigarette smoke, may contain many more carcinogens than just the carcinogen of interest [52]. Also, not all the potential sources of exposure may be known, and not all known sources of a certain exposure might have been assessed. For example, PAH can originate from cigarette smoke and from barbecued meat, but also from many other sources, such as occupational exposure, or through contamination of dietary components (especially grains) [53]. However, in most situations, the main source of exposure will be evaluated. For instance, in our study population, PAH exposure probably mainly originated from cigarette smoke, as occupational exposure will have occurred in only a few subjects, and contamination of dietary components was probably less important (see the Appendix to this thesis).

Absorption of carcinogens

The absorption of carcinogens may depend on the matrix in which they are ingested. For example, when consumed with dietary fiber that may bind carcinogens or decrease bowel transit time, exposure of the colon epithelium to potential carcinogens may be decreased [5]. Carcinogens may also interact with anti-carcinogens leading to inactivation [54] or may be bound by human colon microflora [55]. Furthermore, genetic control of absorption may occur through control of membrane-bound proteins facilitating the absorption of complex substances [56].

Effective dose of carcinogens

To damage DNA of colon epithelial cells, the absorbed carcinogens need metabolic activation and subsequent transportation to the tissue where the DNA damage is induced. Potential carcinogens can be transported directly to the target tissue where metabolic activation takes place, but mainly, they undergo metabolism in the liver, after which carcinogenic metabolites may be transported to the target tissue. Here, further metabolic activation can occur depending on the expression of specific metabolic genes in that tissue, as was proposed for HCAs [57]. During transport, carcinogens may be bound to proteins and this process may also be under genetic control [56].

Metabolism of potential carcinogens occurs via complex pathways involving many enzymes of which a large proportion is encoded by polymorphic genes. These metabolic pathways are under complex control of many genetic and environmental factors [58,59-61]. This especially applies to phase I cytochrome P450 enzymes [61,62], but, more recently, it was discovered that induction and inhibition of several phase II enzymes also occurs [63].

A specific carcinogen may also be metabolized via alternative pathways, as was found for many potential carcinogens such as benzo[a]pyrene [64] and ethanol [65]. The complexity of carcinogen metabolism was clearly illustrated with a figure of the breakdown of arylamines by Grant and colleagues [66] (see Figure 1.2, Chapter 1, for a simplified reproduction of the metabolism).

If one of the metabolic enzymes is not or less functional due to a genetic polymorphism, other enzymes may compensate for this particular enzyme, as discussed before (see under *'Involvement of multiple polymorphic genes'*). Further, metabolic enzymes, such as mEH, have broad substrate specificity and may therefore be involved in the metabolism of many potential carcinogens [67]. Besides, some enzymes (e.g., mEH and NATs) may be involved both in carcinogen detoxification and in activation, the process being dependent on the chemical properties of the intermediary metabolites formed [67].

Thus, the sum of all these processes determines the nature and concentration of the final metabolites of a specific carcinogen and thus, the effective dose. In fact, the final effective dose of the ultimate carcinogen may be too low to result in increased risk of neoplasm [14].

Initiation of tumor formation

If the ultimate carcinogen reaches the target tissue and indeed induces DNA damage, an effect on neoplasm risk is only expected when the damage (such as DNA adducts) is not repaired. It is possible that the ultimate carcinogen mainly forms 'benign' adducts (such as protein adducts), of which the relation to cancer is unknown [68]. The repair of such adducts is under the genetic control of polymorphic genes [69,70]. Multiple genetic alterations are required before cells will convert to malignant cells. Probably, the cells that have accumulated DNA damage will be most sensitive to the effects of further exposure to carcinogenic factors [15]. Once a cell has become malignant, it might undergo multiple cell divisions to form a tumor, or the damaged cell undergoes programmed cell death. Alternatively, mutated cells may be scavenged by cells of the immune system. Recent findings indicate that all these processes are also dependent on genetic polymorphisms [70].

Thus, the formation of tumors depends on multiple processes in which environmental and genetic factors interact at many levels. More knowledge on these processes is needed to investigate the relation of environmental exposure to ultimate tumor formation in more detail.

SAMPLE SIZE REQUIREMENTS TO STUDY GENE-ENVIRONMENT INTERACTIONS

As illustrated above, studies incorporating metabolic polymorphisms might suffer from various biases that may attenuate risk estimates. Especially when evaluating potential interaction between the genetic polymorphism and exposure under study, important

attenuation of risk estimates may occur especially when relatively modest effects are to be expected [71], underlining the need for sufficiently large sample sizes.

For our adenoma case-control study, we calculated sample sizes according to Schlesselman [2]. With 435 cases and an equal number of controls, we were able to detect an odds ratio of about two with a power of 90% for the exposed, genetically susceptible group, provided that the frequency of the high-risk genotype is about 40%, and the exposure is analyzed in tertiles (see Chapter 2). For the nested case-control study on colorectal cancer, with the inclusion of 100 cases and of 500 controls, we calculated that the power would be sufficient (i.e. >80%) to detect odds ratios of two given an exposure prevalence of minimum 20% [2]. Thus, our sample of 102 cases and 537 controls was large enough to detect odds ratios of around 2 for single exposures such as meat consumption, smoking, or genetic susceptibility as predicted from imputed phenotypes [72,73].

With these calculations of required sample sizes, we did not aim to evaluate interactions, but rather we wanted to evaluate the combined effect of exposure and genetic susceptibility, although we had sufficiently large sample sizes to detect interaction odds ratios of 3 to 4 with a power of 80% (one-sided α=0.05, see Table 2.3, Chapter 2), which were expected beforehand. However, we only found indications for weak interactions (i.e., OR<2; see Table 2.3, Chapter 2). To detect relatively weak interactions, large sample sizes are needed [26,74].

As has been illustrated by García-Closas and Rothman and colleagues [71,75,76], misclassification can severely bias odds ratios for relatively weak gene-environment interactions. The bias will tend to weaken multiplicative interaction terms, resulting in a large decrease of study power, but can lead to bias away from the null for additive interaction terms while still decreasing study power [71]. As misclassification of dietary factors [39,41] is considered inevitable whereas minor misclassification may occur in the classification of genetic susceptibility, this implies that, in order to detect truly present but rather weak gene-environment interactions, very large study populations are needed, including several thousands of subjects [71,75].

The application of large sample sizes, however, has consequences for the quality of the data collected and for monitoring of data collection [12]. This, in turn, will decrease study power and increase the minimum sample size required [75] and might be one explanation why no effect of *NAT2* and *GSTM1* was found in some large studies [9,10]. This example illustrates that, although study samples can be expanded to several thousands of subjects, sample sizes that can be enrolled feasibly are still limited and adaptations to designs are thus needed.

Future studies

In the last years, our knowledge about cancer etiology and epidemiology has evolved rapidly. A decade ago much less was known about metabolic polymorphisms. For

example, *NAT1* was thought to be monomorphic, whereas over 20 polymorphic sites have been detected to date [77]. Also, it was relatively unknown that, like phase I enzymes, many phase II enzymes, such as *GSTs*, can be induced by a variety of environmental factors [63]. This has complicated the overall picture of the potential role of metabolic polymorphisms in cancer etiology. Nevertheless, epidemiological studies integrating data on exposure and genetic susceptibility may be useful to indicate candidate genes that may increase individual susceptibility to cancer. It is still possible that these metabolic polymorphisms do modify cancer risk, but that we were not able to prove this because of lack of biological knowledge, of appropriate study populations, and of methodological constraints.

Improve biological knowledge

In this paragraph, several possibilities to improve our biological knowledge on the potential relation between genetic susceptibility to environmental exposure and colorectal cancer will be discussed. Apart from the advantages, potential disadvantages will also be discussed.

Genotype-phenotype correlation

Although importantly determined by the underlying genotype, it is the enzyme activity (phenotype) that determines the potential of formation of ultimate carcinogens. Thus, it is important to understand the correlation between genotypes (which can be easily incorporated in large epidemiological studies) and the resulting phenotypes. This may need a multidisciplinary approach, integrating biochemical studies using recombinant DNA techniques, studies investigating enzyme expression and enzyme extracts, and studies on the level of metabolites formed by different polymorphic variants [16]. The results produced by such studies should still be verified in animal and human experiments, as the situation *in vitro* might be quite different from the *in vivo* situation and might also differ between animals and humans [16].

Inclusion of more metabolic polymorphisms

To study a specific metabolic route of a potential carcinogen in more detail, all the known polymorphisms of important enzymes in this route might be considered at once. This can be achieved using one of the rapidly evolving high-throughput methods, and/or by screening of relevant DNA sequences for new mutations [31]. While offering many new and exciting opportunities, the use of these techniques may also introduce problems. The problem of multiple comparisons is aggravated, and at least some spurious associations will be found if the Bonferroni correction for multiple comparisons is not applied [11]. Further, such data might be difficult to interpret, as the function of newly detected polymorphisms will not yet be clear. As the groups sharing

the same polymorphisms will be relatively small, very large sample sizes are required, and the total costs of such a study will be high [11].

An efficient approach to explore differences between cases and control with respect to known and new potentially interesting polymorphisms is to pool small aliquots of samples from cases and to compare this pooled sample with a pooled sample of all controls. This approach may be applied at the level of gene polymorphisms, gene expression (by investigating expression profiles), and protein concentrations. The subsets that differ most between cases and controls can subsequently be identified, and can than be used to evaluate the presence of interaction with environmental risk factors [11].

Identification of highly susceptible and highly exposed subgroups

It is possible that the effect of genetic susceptibility is greater for certain subgroups, but that this effect is attenuated by the inclusion of many less susceptible groups within the group assumed to be 'genetically susceptible' [12]. Garte [12] proposed the conduct of subgroup analyses comparing the highly susceptible and highly exposed subgroup with all other subjects in the study population. However, sound knowledge about metabolic routes, exposures, and polymorphisms is needed to identify who are susceptible [12], which is not always available. Besides, there should be an identifiable group being highly susceptible, and the formation of an ultimate carcinogen should occur at clearly higher rate in this group compared to the total population. Moreover, if the susceptible group has very specific characteristics and if many factors are known to influence the metabolism, then the number of susceptibles might be too small to detect any potentially present but still relatively small effect. So far, analyses in subgroups defined by multiple genetic susceptibility and exposure factors have indeed resulted in somewhat higher odds ratios [12,78], although this increase could also be the result of the fact that risk estimates based on small groups tend to be higher [1].

Development of biomarkers that can be applied in large populations

Biomarkers may increase our understanding of the many processes that take place between the environmental exposure and ultimate tumor formation [79]. The metabolic polymorphisms described in this thesis may be considered biomarkers of susceptibility [79], and the incorporation of other biomarkers may be useful. To date, few reliable and easily applicable biomarkers are available [80]. The measurement of biomarkers is often complicated and requires large amounts of biological samples, which may lead to decreased participation rates. Most markers reflect recent exposure only and concentrations may be modified in the presence of disease [80]. Moreover, biomarkers do not necessarily reflect the actual exposure or the risk of ultimate disease. Markers of absorption do not include information about metabolism. The concentrations of markers including this information (e.g., urinary or fecal metabolites or mutagenicity,

and protein or DNA adducts in blood) do not necessarily correlate with concentrations in the target tissue. DNA adducts measured in the target tissue may be considered as markers of disease, although these do not necessarily predict risk of ultimate cancer, as most adducts will be repaired [81,82]. Similarly, markers that occur after the induction of DNA damage, such as aberrant crypt foci, probably do not always develop into tumors. Application in epidemiological studies requires biomarkers that can be collected and assessed easily, reliably, and at low cost. These biomarkers need to have a proven connection with either the (long-term) exposure or the disease, and should, after thorough validation, ideally be tested in prospective studies to establish the exact role of the marker in carcinogenesis [79]. This way, valid biomarkers may help to better understand the many processes that take place between the environmental exposure and ultimate tumor formation.

ALTERNATIVE STUDY DESIGNS

Adaptation of epidemiological study designs

Very large numbers of cases and controls are needed to study the interplay between environmental exposure and genetic susceptibility in neoplasm [75]. The enrollment of thousands of study subjects may not be feasible, especially if the disease under study is rare. Moreover, the application of very large samples may result in weak and biologically meaningless associations, which are nevertheless significant. Bayesian statistics, in which background knowledge about the associations under study is incorporated in the analyses, may help to overcome this problem [83].

Several alternative study designs by which the potential effects of genetic susceptibility to environmental carcinogens can be studied more efficiently have been proposed. Assuming that the genetic polymorphism and the exposure under study occur independently, Piegorsch and colleagues proposed the case-only design [84]. Case-only studies include the same cases as would be enrolled for normal case-control studies, but no controls. The non-exposed cases are then considered as the pseudo-control group, whereas the exposed cases form the pseudo-case group. Odds ratios calculated for the effect of genetic susceptibility represent the gene-environment interaction effect [84,85]. Case-only studies offer better precision than traditional case-control studies, as the variability of the control group is excluded, and the power to detect gene-environment interaction is comparable to the power calculated for assessment of a single main effect in a case-control study [86]. However, this design has several disadvantages. First, it is questionable whether the assumption of independence between genotype and exposure is correct [87]. Second, main effects of the exposure and of the genotype cannot be considered. Third, these studies would miss gene-environment effects that do not depart from multiplicativity, but do nevertheless depart from additivity [86]. To be able to study main effects in case-only studies, adaptations of this design have been proposed.

One of these is the 'incomplete-data' case-control design, in which both genotype and exposure are assessed among cases, but only one of these among the controls, so that main effects can be studied as well, whereas required sample sizes are half of those needed for traditional case-control studies [88]. However, this design also requires independence between genotype and exposure. To test whether this assumption is valid, genotype and exposure data should be collected in a random sample of controls [88].

Genetic polymorphisms that occur at lower frequencies than those studied in this thesis (e.g., lower than 10%) may be studied more efficiently using study designs in which susceptible subjects are over-sampled, such as two-stage case-control studies and case-family studies [86].

The advantages and disadvantages of these alternative designs should be studied in more detail [86]. It has to be emphasized that case-control studies are generally conducted with multiple purposes, some of which are not attainable using the designs described above.

Intervention studies

To study the potential influence of genetic susceptibility toward a specific environmental exposure factor in more detail, one might conduct a controlled intervention study in which intermediate endpoints are used, such as adenoma recurrence [89,90], DNA-adduct formation [91-94], or colon epithelial proliferation [95,96]. To more efficiently study the effect of genetic susceptibility, susceptible subjects can be over-sampled [97]. The great advantage of such a design is that a generally occurring environmental exposure can be carefully controlled and that the inclusion of several biomarkers of exposure or of early effect may increase our knowledge on different processes occurring between exposure and cancer. However, intervention studies require specific and relatively simple hypotheses [59], as only one exposure and a limited number of susceptibility markers can be studied. Moreover, the choice of a suitable endpoint is difficult. The association with ultimate cancer is only weak for early endpoints such as DNA adducts (and these might even be considered as late markers of exposure), and the inclusion of late endpoints, such as recurrence of colorectal adenomas [89,90], might not be feasible as it requires long intervention periods.

Shifting hypotheses: who did *not* get the disease?

Alternatively, instead of investigating what factors increase colorectal cancer risk, one might also investigate which factors protect subjects who are highly susceptible to colorectal cancer (such as persons harboring a rare mutation that increases risk of a certain cancer dramatically) [98]. For example, about 30% of the women carrying *BRCA1* or *BRCA2* mutations reaches the age of 70 without diagnosis of breast cancer, and it is not known what protects these women. Studies among carriers of highly penetrant mutations in mismatch repair genes (leading to hereditary nonpolyposis colorectal cancer) or the *APC* gene (causing familial adenomatous polyposis coli) might increase

evidence on protective colorectal cancer factors. The discovery that at least some of the mutations found in hereditary and sporadic colorectal tumors are identical suggests similar mechanisms of carcinogenesis [99]. The disadvantage of such studies is that it may be difficult to disentangle genetic and exposure effects, as these hereditary disorders are, by definition, clustered within families. Moreover, recruitment of study populations may be difficult, as the diseases studied are rare, although participation rates may be higher.

Concluding remarks

Thus far, epidemiological studies have only found weak indications that metabolic polymorphisms modify colorectal cancer risk. In the future however, with increasing knowledge of the underlying biological processes, we might be able to elucidate the influence of metabolic polymorphisms in cancer etiology [100]. At present, the application of alternative study designs may help uncovering the role of specific genetic polymorphisms. In this respect, the application of intervention studies over-sampling genetically susceptible subjects may be promising. Also, epidemiological studies including individuals with inherited genetic defects that greatly increase colorectal cancer risk may be used. Both these designs are applied in projects currently conducted at our division. Certainly, the investigation of the potential influence of metabolic polymorphisms on cancer susceptibility needs an integrative approach in which many types of studies should be conducted.

If, in the future, genetic susceptibility to xenobiotics from environmental sources will be found to increase colorectal neoplasm risk, this will strengthen the hitherto weak evidence that environmental risk factors for colorectal cancer may increase cancer risk through their potentially carcinogenic constituents. The frequencies of metabolic polymorphisms are high in the general population (generally, between 20 and 60%) and the effect of these is expected to be small on an individual level but, because of their high frequency, high in terms of population attributable risks [17,18]. Future screening of the total population for such highly frequent low-penetrance polymorphisms is not expected [18]. The general population will only be served with genetic screening if the risk of developing the disease if susceptible (positive predictive value) is relatively high (greater than 50%), which is not to be expected for most metabolic polymorphisms [18]. In the light of ethical constraints, the relevance of genetic screening should thus be thoroughly considered, even if certain subgroups will be found to be highly susceptible toward several carcinogens. More gain for cancer prevention is to be expected from reduction of potential exposure to carcinogens, e.g. by the application of low-risk meat cooking methods, quitting smoking, or reduction of alcohol intake.

As explained in this Chapter, molecular epidemiological studies alone will not solve the issue of genetic susceptibility to carcinogens. The many studies conducted during the

last decade have shown that genetic susceptibility is complex and may be important at all stages between environmental exposure and the formation of a malignant tumor. Cancer, and surely colorectal cancer, is a complex disease evolving via many different routes and involving many factors. The exact cascade of processes leading to colorectal cancer will be different for each and every individual. For researchers, it remains however important to detect common patterns in this complex variety of processes. Thus, to study just one or a few metabolic polymorphisms in this cascade is probably too simplistic and integrated approaches are needed, requiring new study designs and methods for analysis, and involving biochemical and molecular studies, animal experiments, and controlled intervention trials, together with studies like the ones described in this thesis.

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Summary

Colorectal cancer is the second most common cancer in the Western world, whereas it rarely occurs in non-Western, developing countries. Colorectal cancer is thought to arise almost uniquely from colorectal adenomas and has been estimated to be attributable to environmental exposure (mainly diet) for about 90%. The factors that probably increase colorectal cancer risk are (red) meat and alcohol intake, and (long-term) smoking, possibly through their potentially (co-)carcinogenic constituents. However, their effects were found to be relatively small. Certain subgroups may be more susceptible to specific carcinogens than the general population on average, and the risk of colorectal cancer resulting from exposure to these substances may be higher in these subgroups. This increased susceptibility is thought to arise via polymorphisms in genes that code for enzymes metabolizing potential carcinogens.

Genetic susceptibility to environmental carcinogens

Most carcinogens need metabolic activation in the human body before they can cause DNA damage, and thus, possibly increase cancer risk. This implicates that the metabolism of these potential carcinogens is crucial with respect to cancer risk. The extent to which potential carcinogens become activated or detoxified depends on the (genetically determined) properties of metabolic enzymes and determines the individuals' genetic susceptibility to environmentally induced cancer. These properties are importantly determined by genetic polymorphisms resulting in differences in enzyme activity by alteration of gene expression or differences in enzyme activity, stability or substrate affinity. Polymorphisms are associated with a low individual cancer risk, but because they occur at high frequency (generally, between 20% and 60%) in populations, they could importantly influence population attributable risk.

Aim of studies

In this thesis, we studied the potential influence of genetic susceptibility to carcinogens determined by genetic polymorphisms, on associations of meat consumption, (long-term) cigarette smoking, and alcohol intake with colorectal tumor risk.

Methods and population

For this purpose, efficient and reliable techniques for the detection of genetic polymorphisms are needed. In Chapter 3, we described the development of such a method, the reverse line blot method. The method was tested for *N*-acetyltransferases (*NAT*) 1 and 2. We demonstrated that our method was reliable (sensitivity and specificity, as compared to commonly used methods for *NAT1* and *NAT2* genotype determination, were both 100%), quick, and relatively cheap. Moreover, expansion of the method with other allelic variants is achieved relatively easy. Our method is thus

useful for the analyses of multiple polymorphisms in relatively large epidemiological studies.

The impact of genetic susceptibility was studied in two populations. The first was a case-control population recruited among those undergoing endoscopy at the outpatient clinics of eight hospitals in the central region of the Netherlands, between June 1997 and June 2000. The recruitment procedures and the main characteristics of the study population were described in Chapter 2. In this chapter, methodological strengths and weaknesses of our population were also discussed. After three years of recruitment, 887 subjects were included, 440 cases and 447 controls. We concluded that the results produced from our study population were internally valid, although they should not be extrapolated inconsiderately to the general population. The second population originated from the prospective cohort recruited within the Monitoring Project on Cardiovascular Disease Risk factors, including more than 36,000 men and women recruited in three Dutch towns between January 1987 and December 1991. After about 8.5 years of follow-up, we analyzed data of all cases of colorectal cancer (n=102) that had arisen in the cohort and of a random sample of 537 controls frequency-matched with cases on age, sex and town.

Meat

The association between meat consumption (and preparation) and colorectal neoplasm was described in Chapters 4 and 7. At present, it is not known how high meat consumption increases the risk of colorectal neoplasm. Possibly, heterocyclic aromatic amines (HCAs) which are formed in meat cooked at high temperature may be responsible for this increase in risk. We therefore first investigated if HCA exposure occurs in the general Dutch population and found that HCAs were indeed present in beef patties habitually prepared at home by 63 apparently healthy volunteers. Next, we investigated if commonly occurring polymorphisms in genes that encode enzymes involved in HCA metabolism modified the associations of meat consumption and preparation with colorectal neoplasm. In our adenoma case-control study, we collected detailed data on meat consumption and meat preparation. We also determined the genotypes of the polymorphic *NAT1*, *NAT2*, sulfotransferase (*SULT*) *1A1*, and glutathione *S*-transferase (*GST*) *M1* and *T1* genes (see Chapter 4). From the nested case-control study on colorectal cancer, we used data on meat consumption and we determined genetic polymorphisms of *NAT1*, *NAT2* and *GSTM1* (Chapter 7). Meat consumption did not increase risk of colorectal adenomas (OR 1.2, 95% CI 0.8-1.9), nor did meat preparation methods assumed to be associated with HCA formation increase risk, possibly because the preparation methods inquired do not clearly reflect HCA concentrations (Chapter 4). Frequent red meat consumption was weakly positively associated with increased risk of colorectal cancer (OR 1.6, 95% CI 0.9-2.9, highest *vs.* lowest intake, Chapter 7). We found no strong indications for genetic polymorphisms to

modify the association of meat consumption with colorectal neoplasm (Chapters 4 and 7).

Cigarette smoking

To investigate the potential association between (long-term) cigarette smoking and colorectal neoplasm in more detail, we incorporated data on genetic polymorphisms encoding enzymes that metabolize cigarette smoke carcinogens, such as arylamines and polycyclic aromatic hydrocarbons. Results for colorectal adenomas were presented in Chapter 5, whereas results for colorectal cancer were described in Chapter 7. We found that cigarette smoking was a relatively strong risk factor of colorectal adenomas and that this risk was mainly determined by smoking duration. Smoking for more than 25 years more than doubled adenoma risk (OR 2.4, 95% CI 1.4-4.1). Cigarette smoking was most strongly associated with adenomas less than 1 cm in size and of tubular histology. The association between long-term cigarette smoking and adenomas was most pronounced in those with inherited variants of *SULT1A1* leading to fast sulfation (OR 4.3, 95% CI 1.6-11.8), and of *NAT2* encoding slow acetylation (OR 3.5, 95% CI 1.9-6.4), although we found no indications for statistically significant interactions (i.e., more than multiplicative). We found no indications of effect modification by genetic polymorphisms of epoxide hydrolase (*EPHX*) at exons 3 and 4, *GSTM1* and *GSTT1*. The frequency of the imputed *NAT1* slow phenotype was too low to allow evaluation of potential effects (Chapter 5). Similar to colorectal adenomas, risk of colorectal cancer was also increased with smoking duration, although this association was found among former smokers only (OR 2.7, 95% CI 1.0-7.4, for 16-30 years of past smoking and OR 3.2, 95% CI 1.0-9.8, for more than 30 years of past smoking, compared to former smokers having smoked for less than 16 years). In this nested case-control study, we found no indications for any of the studied genotypes to modify this association (Chapter 7).

Alcohol

Finally, we studied modification of the association between alcohol consumption and colorectal adenomas by the alcohol dehydrogenase (*ADH*) 3 genetic polymorphism. The results are described in Chapter 6. Alcohol consumption in itself was a risk factor for colorectal adenomas, especially among women drinking ten or more beverages weekly (OR 1.8, 95% CI 1.0-3.2) in comparison with women drinking less than one consumption per week. Risk of adenomas among men was increased only with consumption of more than 21 drinks per week (OR 1.8, 95% CI 0.9-3.8). Although the risk of adenomas was highest among those with the *ADH3* fast imputed phenotype, we found no strong indications that the *ADH3* polymorphism indeed modifies the association between alcohol drinking and colorectal adenomas.

Discussion and concluding remarks

In summary, the results of our studies do not point toward strong modifying effects of genetic polymorphisms of enzymes involved in carcinogen metabolism, although weak indications were found that some genetic polymorphisms might indeed cause subjects to be more susceptible toward environmental carcinogens. These findings are in accordance with the growing amount of epidemiological studies incorporating data on metabolic polymorphisms (see Chapters 1 and 8). The modifying effects of genetic polymorphisms may be diluted in several ways, of which one is carcinogen metabolism. Most carcinogens are metabolized via various complex metabolic routes, involving numerous genetically polymorphic metabolic enzymes, which are under the complex control of many other genes and environmental substances. The sum of all these processes determines the nature and concentration of the final metabolites and thus, the effective dose of the carcinogen, thereby influencing ultimate tumor risk. It is also possible that the statistical power to detect relatively weak interactions between environmental exposures and genetic susceptibility was insufficient because it rapidly decreases in the presence of misclassification. Misclassification of environmental and especially dietary exposure assessed via self-administered questionnaires is considered inevitable, but misclassification may also occur in the determination of genetic polymorphisms and subsequent imputation of phenotypes, as phenotype-genotype correlations are not always clear.

Apart from the fact that more knowledge is needed on the diverse factors involved in carcinogenesis, to study the impact of genetic susceptibility on colorectal tumor risk, alternative methods are needed using an integrated approach. Intervention studies among genetically susceptible subjects, in which the environmental exposure factor of interest is carefully monitored and several markers of exposure and disease are incorporated, might be most promising. Further, epidemiological studies may include individuals with inherited genetic defects that greatly increase colorectal cancer risk to investigate why some subjects are not affected with cancer. Both these designs are applied in projects currently conducted at our division.

Samenvatting

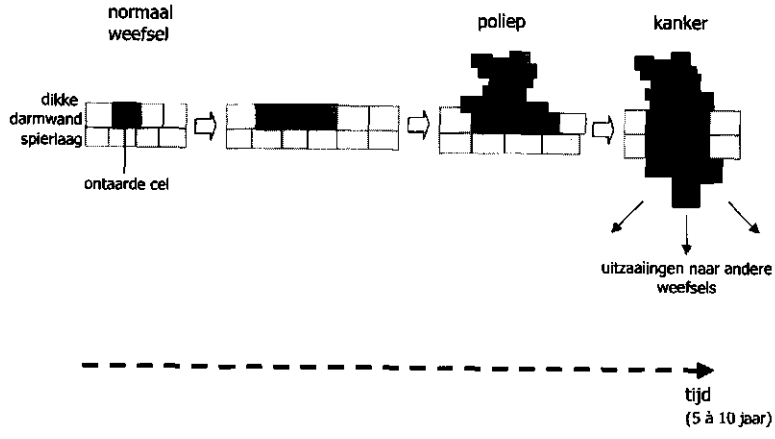
- voor iedereen die meer over het onderzoek wil weten-

De titel van het proefschrift in het Nederlands luidt:

Vlees, roken, alcohol en dikke darm tumoren:
de rol van aangeboren gevoeligheid

Darmkanker: vóórkomen en ontstaan

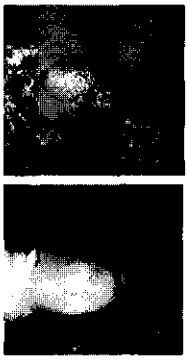
In landen met een hoge levensstandaard is dikke darmkanker één van de belangrijkste vormen van kanker. In Nederland worden er elk jaar ongeveer 6000 nieuwe gevallen van dikke darmkanker geconstateerd. Darmkanker komt even vaak voor bij mannen als bij vrouwen. Bij mannen komt het na longkanker en prostaatkanker het meeste voor, terwijl het bij vrouwen na borstkanker de meest voorkomende kankersoort is. In Figuur 1 is geïllustreerd hoe darmkanker waarschijnlijk ontstaat.



Figuur 1. Van normaal dikke darmweefsel naar kanker.

Dikke darmkanker ontstaat voornamelijk uit zogenaamde adenomateuze dikke darmpoliepen (Figuur 2), goedaardige gezwellen in de dikke darm. Lang niet alle poliepen ontaarden uiteindelijk in kanker. Uit voorzorg worden echter alle poliepen die worden gevonden tijdens een kijkonderzoek van de dikke darm (endoscopie) verwijderd. Zowel poliepen als kankergezwellen noemen we tumoren, hoewel de eerste (nog) goedaardig zijn.

Risicofactoren en aangeboren gevoeligheid



Figuur 2. Voorbeelden van dikke darmpoliepen.

Het is nog lang niet duidelijk hoe dikke darmkanker precies ontstaat. Wel lijken het eten van veel vlees, het bereiden van vlees bij hoge temperaturen, het drinken van alcohol en het langdurig roken de kans op dikke darmkanker te verhogen. Hoe dat precies gebeurt, is niet duidelijk. Wel weten we dat vlees, alcohol en sigaretten kankerverwekkende stoffen kunnen bevatten. De resultaten van onderzoek naar de risicofactoren voor dikke darmkanker en -poliepen zijn echter niet eenduidig. Soms, maar lang niet altijd, blijkt uit onderzoek dat deze factoren de kans op kanker licht kunnen verhogen. Een mogelijke verklaring hiervoor is dat vlees, alcohol en sigarettenrook niet voor iedereen even schadelijk zijn, omdat de gevoeligheid van mensen voor kankerverwekkende stoffen gedeeltelijk is aangeboren. Op deze manier kan erfelijkheid dus een rol spelen bij de manier waarop het lichaam met schadelijke stoffen uit onze omgeving en uit onze voeding omgaat.

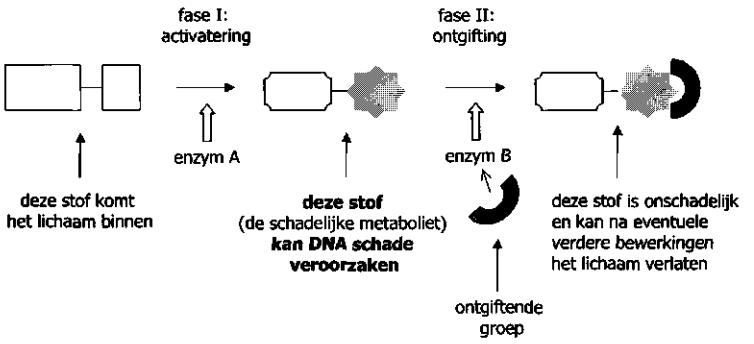
Een voorbeeld

Een goed voorbeeld van aangeboren gevoeligheid is de relatie tussen de kleur van de huid (erfelijk) en blootstelling aan zonlicht. Mensen met een lichte huid verbranden sneller dan mensen met een donkere huid en hebben bovendien een hogere kans op het krijgen van huidkanker door zonlicht. We kunnen in dit geval niet spreken van een erfelijke ziekte, omdat lang niet alle mensen met een lichte huid huidkanker krijgen – zelfs niet als ze veel en lang in de zon zitten. Bovendien gaat het om een veel voorkomende variatie in het DNA: er zijn immers heel veel mensen die een lichte huidkleur hebben. Wel zeggen we dat mensen met een lichte huid een aangeboren gevoeligheid hebben voor het krijgen van huidkanker door zonlicht. Zo is het ook met de vormen van darmkanker waarnaar wij onderzoek hebben gedaan. Wij onderzochten bepaalde veel voorkomende variaties in het erfelijke materiaal die ervoor kunnen zorgen dat iemand gevoeliger is voor bepaalde mogelijk kankerverwekkende stoffen. Benadrukt dient te worden dat we géén zeldzame erfelijke afwijkingen onderzochten die in bijna alle mensen met die afwijking tot dikke darmkanker leiden.

Activering van mogelijk kankerverwekkende stoffen

Kankerverwekkende stoffen zijn meestal pas echt schadelijk na activering in het lichaam. Na opname door de darmen worden deze stoffen naar de lever getransporteerd, waar ze worden omgezet in andere stoffen. Voordat ze onschadelijk kunnen worden gemaakt, moeten deze stoffen eerst worden geactiveerd. De geactiveerde stoffen zijn het meest

schadelijk. Aan deze geactiveerde stof (ook wel de schadelijke metaboliet genoemd) kan een zogenaamde ontgiftende groep worden gekoppeld, waardoor de stof onschadelijk wordt (Figuur 3). Enzymen kunnen deze omzettingen vergemakkelijken. Door variaties in deze enzymen varieert de snelheid van de omzettingen.



Figuur 3. Schema van de omzetting (het metabolisme) van mogelijk kankerverwekkende stoffen in het menselijk lichaam.

Uit Figuur 3 kunnen we afleiden dat de eerste fase (activatering door enzym A) snel gevolgd wordt door de tweede fase (ontgifting door enzym B). Als op de één of andere manier het proces na de eerste fase stagneert (bijvoorbeeld doordat enzym A zeer snel werkt, of doordat enzym B niet goed werkt), kan er stapeling van de geactiveerde stof (de schadelijke metaboliet) ontstaan. Deze schadelijk metaboliet kan binden aan het DNA en dit kan tot gevolg hebben dat de cel niet meer normaal zal functioneren en een tumorcel wordt. Nu komt de aangeboren gevoeligheid die wij hebben bestudeerd in beeld. In veel gevallen is het namelijk erfelijk bepaald hoe goed enzym A en B functioneren. Daarom is het mogelijk dat de ene mens bij een zelfde dosis van een mogelijk schadelijke stof (bijvoorbeeld door dezelfde hoeveelheid sigaretten per dag te roken), toch veel meer aan de schadelijke metabolieten van deze stof is blootgesteld dan de andere mens.

Doel van ons onderzoek

Toen we met het onderzoek dat in dit proefschrift is beschreven begonnen, wisten we nog maar weinig over deze vorm van aangeboren gevoeligheid voor dikke darmkanker. Het enige dat we wisten was dat vlees, alcohol en sigarettenrook de kans op darmkanker kunnen verhogen, maar hoe precies wisten we niet. We dachten dat dat wel eens aan de

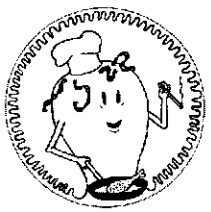
eventuele schadelijke stoffen in deze producten zou kunnen liggen. Als deze schadelijke stoffen hiervoor inderdaad verantwoordelijk zijn, dan zouden mensen die gevoelig zijn voor die stof (die dus veel van de schadelijke metaboliet aanmaken), een hogere kans op darmkanker moeten hebben dan mensen die hiervoor niet of minder gevoelig zijn.

We vroegen ons het volgende af:

- Verhoogt het eten van vlees, het bereiden van vlees bij hoge temperaturen, het drinken van alcohol en het roken van sigaretten de kans op dikke darmtumoren?
- Speelt aangeboren gevoeligheid voor kankerverwekkende stoffen uit deze producten hierbij een rol?

Dit werd apart onderzocht voor darmpoliepen (zie Hoofdstuk 4 tot en met 6) en voor dikke darmkanker (zie Hoofdstuk 7).

Opzet van het onderzoek



Figuur 4. Logo van de *POLIEP*-studie.

Het onderzoek naar dikke darmpoliepen, de *POLIEP*-studie (Figuur 4), werd uitgevoerd in acht ziekenhuizen in Nederland tussen juni 1997 en juni 2000. Voor dit onderzoek vroegen we alle mensen bij wie tijdens een kijkonderzoek in de dikke darm adenomateuze poliepen waren gevonden om deel te nemen (*patiënten*). Ook vroegen we mensen bij wie juist geen poliepen waren gevonden om mee te doen (*controles*). Al deze mensen kregen een aantal vragenlijsten waarmee we hun voedingspatroon, medicijngebruik, en overige leefgewoonten probeerden te achterhalen. Ook stonden alle mensen wat bloed af waaruit wij DNA isoleerden om er erfelijke variaties in te bepalen. Er deden uiteindelijk 440 mensen met poliepen en 447 mensen zonder poliepen mee aan het onderzoek. Details over deze onderzoekspopulatie zijn beschreven in Hoofdstuk 2.

De invloed van erfelijke gevoeligheid bij dikke darmkanker werd onderzocht in een groot onderzoek, uitgevoerd door het Rijksinstituut voor Volksgezondheid en Milieu (RIVM). Voor dit onderzoek werden in Amsterdam, Doetinchem en Maastricht gedurende vijf jaar (januari 1987 tot en met december 1991) alle personen tussen de 20 en de 59 jaar oud benaderd. Deze mensen werd gevraagd of ze vragenlijsten wilden invullen en een beetje bloed wilden afstaan. In totaal deden er meer dan 36.000 mensen aan dit onderzoek mee. Ruim acht jaar na aanvang van het onderzoek bleek dat in deze groep 102 mensen dikke darmkanker hadden gekregen. Deze groep van 102 mensen (de *patiënten*) werd vergeleken met een groep van 537 mensen zonder kanker (de *controles*) die aan hetzelfde onderzoek meededen. Uit het bloed dat bij deze mensen was verzameld werd DNA geïsoleerd, zodat we de aangeboren gevoeligheid voor mogelijk kankerverwekkende stoffen uit vlees en sigarettenrook konden bepalen.

In beide onderzoeken vergeleken we de eet- en rookgewoonten van de mensen met darmpoliepen of darmkanker (de patiënten) met die van de controlegroep. Zo konden we zien of mensen met darmpoliepen bijvoorbeeld over het algemeen meer vlees aten of meer rookten dan mensen zonder darmpoliepen. Uit deze vergelijkingen konden we vervolgens de kans op darmpoliepen of darmkanker afleiden, onder andere voor het eten van veel ten opzichte van weinig vlees.

Resultaten: Vlees

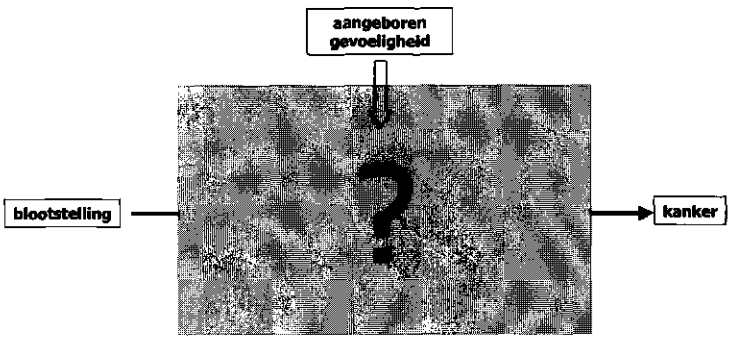
Als vlees bereid wordt bij zeer hoge temperaturen kunnen er kankerverwekkende stoffen in ontstaan. Deze stoffen worden omgezet door enzymen die erfelijke variaties vertonen. We konden deze schadelijke stoffen aantonen in vlees bereid door Nederlanders, maar mensen die vaak vlees aten hadden niet meer kans op poliepen dan mensen die weinig vlees aten. Wel leek het erop dat mensen die elke dag varkens-, rund-, of ander 'rood' vlees aten ongeveer anderhalf keer zoveel kans hadden op het krijgen van dikke darmkanker dan mensen die dit minder dan vijf keer per week aten. We konden echter niet vaststellen dat dit verschil in risico niet op toeval berustte. We vonden geen aanwijzingen dat aangeboren gevoeligheid voor kankerverwekkende stoffen hierbij een rol speelde.

Resultaten: Roken

Het is algemeen bekend dat roken schadelijk is voor de gezondheid. Roken wordt vooral in verband gebracht met hart- en vaatziekten en longkanker. Het wordt steeds duidelijker dat het ook de kans op andere vormen van kanker, zoals dikke darmkanker, kan verhogen. We konden aantonen dat mensen die gedurende lange tijd (meer dan 25 jaar) sigaretten rookten of hadden gerookt, bijna tweeëneenhalf keer zoveel kans hadden op het krijgen van dikke darmpoliepen als mensen die nooit gerookt hebben. Ditzelfde gold ook voor darmkanker: mensen die langer dan 16 jaar hadden gerookt, hadden ongeveer drie keer zoveel kans darmkanker te krijgen dan mensen die nooit hadden gerookt. De schadelijke stoffen in sigarettenrook kunnen door verschillende enzymen worden geactiveerd, waarvan sommige in verschillende varianten voorkomen. Er waren inderdaad verschillen tussen mensen met snelwerkende en langzame varianten van enkele van deze enzymen, maar die verschillen waren erg klein. Zo bleek dat de kans op dikke darmpoliepen groter is bij rokers die de 'snelle' variant van het enzym sulfotransferase (vergelijkbaar met enzym A in Figuur 3) hadden, dan onder rokers die een 'langzame' variant hadden. Verder verhoogden 'langzame' varianten van *N*-acetyltransferase (vergelijkbaar met enzym B in Figuur 3) de kans op dikke darmpoliepen onder rokers.

Resultaten: Alcohol

Wij vonden in ons onderzoek een verband tussen het drinken van alcohol en het vóórkomen van dikke darmpoliepen. Dit verband was sterker voor vrouwen dan voor mannen. Vrouwen die meer dan tien glazen alcohol per week dronken hadden een bijna twee keer zo hoge kans op poliepen dan vrouwen die minder dan één glas per week dronken. Een zelfde risicoverhoging werd bij mannen pas gevonden als ze meer dan 21 glazen per week dronken. Uit onderzoek is bekend dat een afbraakproduct van alcohol, aceetaldehyde, veel schadelijker is dan alcohol zelf. Het enzym alcoholdehydrogenase zet alcohol om in aceetaldehyde. Dit enzym heeft enkele erfelijk bepaalde varianten. De 'snelle' variant verhoogt mogelijk de kans op dikke darmpoliepen omdat er stapeling van het schadelijke aceetaldehyde kan plaatsvinden. In ons onderzoek vonden we echter geen sterke aanwijzingen hiervoor.

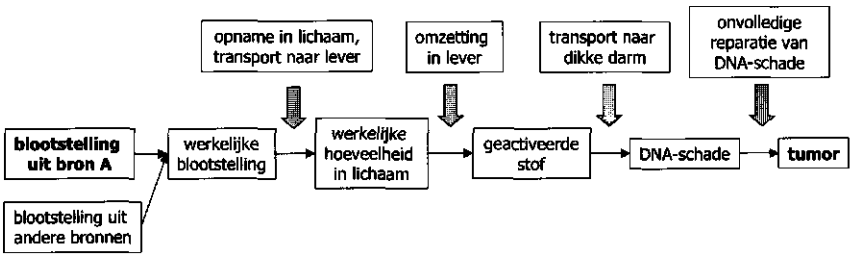


Figuur 5. Dikke darmkanker: een zwarte doos?

Discussie en conclusie

Ons onderzoek bevestigde dat het eten van vlees, het drinken van alcohol en het langdurig roken van sigaretten de kans op dikke darmtumoren kunnen verhogen. We vonden echter geen aanwijzingen voor een belangrijke rol van aangeboren gevoeligheid voor mogelijk kankerverwekkende stoffen afkomstig uit deze producten. Onze resultaten kwamen goed overeen met die van andere onderzoekers. Dit wil echter niet zeggen dat aangeboren gevoeligheid niet belangrijk is. Het is goed mogelijk dat aangeboren gevoeligheid wel een rol speelt, maar dat we deze rol niet zichtbaar konden maken. Kanker kan worden gezien als een 'zwarte doos'. We weten immers maar weinig over het ontstaan ervan en vaak hebben we alleen informatie over de blootstelling aan

een bepaalde risicofactor (bijvoorbeeld roken) en de uiteindelijke 'ziekte' (darmkanker of darmpoliepen). Door het bestuderen van aangeboren gevoeligheid hoopten we meer informatie te krijgen over het belang van blootstelling aan kankerverwekkende stoffen bij het ontstaan van darmkanker. Echter, we tasten nog steeds in het duister over wat er nu precies gebeurt. De 'zwarte doos' van kanker is eigenlijk niet veel kleiner geworden (Figuur 5). Dat komt misschien omdat het ontstaan van tumoren een complex en langdurig proces is, waarbij veel verschillende factoren van binnen en buiten het lichaam een rol spelen. Vaak is het al moeilijk om de blootstelling aan een bepaalde kankerverwekkende stof te meten, bijvoorbeeld omdat deze afkomstig kan zijn van verschillende bronnen en ook nog varieert in de tijd (sigaretten zijn in de loop van de jaren bijvoorbeeld minder giftige stoffen gaan bevatten). Daarnaast is het afhankelijk van bijvoorbeeld de voeding hoeveel het lichaam van de stof opneemt. Bij activering van de stof in de lever speelt vervolgens aangeboren gevoeligheid een rol. Na activering wordt een deel van de stof naar de dikke darm getransporteerd en kan daar DNA-schade veroorzaken. Gelukkig kan DNA-schade in veel gevallen worden gerepareerd. Pas als er ernstige schade aan het DNA is ontstaan die niet wordt gerepareerd, dan kan de cel ontaarden en kwaardaardig worden. Sommige cellen zijn echter zo ernstig beschadigd, dat ze dood gaan en niet kwaardaardig worden. Uit het bovenstaande blijkt dat kanker een proces is waar vele stappen aan vooraf gaan (Figuur 6). Pas als het bij al die stappen 'fout' gaat, zal een tumor ontstaan.



Figuur 6. Kanker ontstaat na vele stappen. Deze Figuur geeft een versimpelde weergave van dit complexe proces. NB: tussen DNA-schade en de vorming van een tumor zitten ook nog vele stappen die hier niet zijn weergegeven.

Om dikke darmkanker beter te kunnen bestrijden is meer inzicht over het ontstaan ervan nodig. Meer inzicht kunnen we waarschijnlijk alleen krijgen door op veel vlakken

meer onderzoek te doen. Zo moeten we bijvoorbeeld te weten komen welke enzymen nog meer belangrijk zijn bij de omzetting van mogelijk kankerverwekkende stoffen, welke aangeboren varianten er van deze enzymen bestaan en wat het effect is van deze varianten op de omzetting van de kankerverwekkende stoffen. Daarnaast moeten we ook meer weten over de blootstelling aan mogelijk kankerverwekkende stoffen en over de gevolgen van deze blootstelling. Onderzoek naar het effect van aangeboren gevoeligheid op het ontstaan van dikke darmkanker vereist dus onderzoek vanuit verschillende wetenschapsgebieden. Hierbij zal epidemiologisch onderzoek zoals beschreven in dit proefschrift, zeker een belangrijke rol blijven spelen.

Dankwoord

Vele handen maken licht werk. De hulp van velen zorgde ervoor dat dit proefschrift er kwam! Zonder iedereen bij name te noemen, wil ik hier daarom allen die, op welke manier dan ook, aan dit proefschrift hebben bijgedragen heel hartelijk danken.

Voor epidemiologisch onderzoek zijn vaak veel vrijwilligers nodig, en dat geldt zeker ook voor het onderzoek beschreven in dit proefschrift. Daarom ben ik de deelnemers aan de *POLIEP*-studie erg dankbaar voor hun medewerking. Ook dank ik de deelnemers aan het Peilstationsproject Hart- en Vaatziekten. Verder veel dank aan alle vrijwilligers van de populatie-steekproef en aan hen die daarna tartaartjes bakten. Zonder al deze deelnemers was dit proefschrift er zeker niet gekomen!

Ik ben mijn co-promotor Ellen Kampman heel dankbaar voor haar vertrouwen in mijn kunnen. Toen ik aan het AIO-schap begon wist ik niets van kanker en heel weinig van moleculaire technieken. Je hebt me aardig bijgespijkerd Ellen! Bedankt voor je persoonlijke, betrokken en nauwgezette manier van begeleiden. Ik heb het je al vaker gezegd: ik heb geboft met zo'n begeleider! Dank ook aan mijn beide promotores, Frans Kok en Daan Kromhout. Ik heb goede herinneringen aan onze vergaderingen (ik zal jullie sterke verhalen niet vergeten!). Jullie leerden mij dat mijn teksten korter en scherper kunnen. Dank voor jullie waardevolle inbreng!

Veel lof voor ons *POLIEP*-studie team! Annelies Bunschoten heeft vrijwel alle moleculaire bepalingen opgezet en uitgevoerd en mij geduldig ingewijd op het lab. Annelies, heerlijk dat ik zelf mocht 'aanrommelen' zonder dat je me echt liet zwemmen. Dank ook voor begeleiden van enkele van mijn studenten. En niet in de laatste plaats een pluim voor jouw teamspirit! Leuk dat je bij de afsluiting van ons project weer naast me staat. Elly Monster en Maria van Vugt hebben het leeuwendeel van de werving uitgevoerd. Hen bedank ik voor het prikken van die vele bloedjes in alle uithoeken van het land. Elly, jij hebt menig deelnemer voor de studie gewonnen door je prettige omgang met de deelnemers en je vasthoudendheid. Dank ook voor de gezellige ritjes naar de ziekenhuizen, waarbij ik dikwijls in jouw autootje mocht rijden. Maria, jou wil ik ook met name bedanken voor het invoeren van die duizenden vragenlijsten. Niemand die dat zo snel en goed kan als jij! Ik zal nooit dat moment vergeten dat we er achter kwamen dat er 600 vragenlijsten op een inmiddels gewiste harde schijf stonden... We hebben met z'n allen een week moeten typen om de schade weer in te halen.

Voor het werven van de deelnemers ben ik zeer veel dank verschuldigd aan de gastro-enterologen, endoscopie-verpleegkundigen en secretaresses van Ziekenhuis Gelderse Vallei te Ede, het Rivierenland Ziekenhuis te Tiel, het Universitair Medisch Centrum Sint Radboud in Nijmegen, het Slingeland Ziekenhuis te Doetinchem, het Sint Antonius Ziekenhuis te Nieuwegein, het Eemland Ziekenhuis in Amersfoort, het Bosch MediCentrum in Den Bosch, en het Slotervaart Ziekenhuis te Amsterdam. Verder ook dank aan de diverse artsen van ziekenhuizen waarin de *POLIEP*-studie pas in een later

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About the author

Edine (Everdina Wubbina) Tiemersma was born on 27 February 1972 in Haarlem, the Netherlands. After graduation from secondary school (*VWO* at the *Christelijke Scholengemeenschap Zuid-Oost Drenthe*, Emmen) in 1990, she started with a M.Sc. Biology at Wageningen University in the same year. She conducted four research projects focused on the biology and epidemiology of tropical infectious diseases. After obtaining her M.Sc. degree in September 1996, she was appointed as a scientific advisor at TNO Prevention and Health in Leiden for a period of five months. In January 1997, she started with a Ph.D. project entitled *'Gene-environment interactions in colorectal adenomas and colorectal cancer: influence of diet, smoking and genetic susceptibility'* at the Division of Human Nutrition and Epidemiology at Wageningen University, of which the main results are described in this thesis. In 1997, she attended the Annual New England Epidemiology Summer Program of the New England Epidemiology Institute in Boston, U.S.A., and in 1999 the Molecular Epidemiology course of the International Agency for Research on Cancer in Turin, Italy. She participated in the European Nutrition Leadership Program of 2001 in Luxembourg. She was a member of the Ph.D. study tour committee organizing a two-week study tour for Ph.D. students to South Africa in 1999, including two symposia. From 1997 to 1999, she was the secretary of the daily board of the committee for temporary scientific staff members within the Division. Since January 2002, she is working on the epidemiology of antimicrobial resistance in the Netherlands and Europe at the Department for Infectious Diseases Epidemiology at the National Institute for Public Health and the Environment in Bilthoven, the Netherlands.

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Appendix

Role of EPHX in the associations of smoking and diet with colorectal adenomas

E.W. Tiemersma, J. Kloosterman, A. Bunschoten, F.J. Kok, and E. Kampman

Abstract; *IARC Scientific Publications*, in press

Introduction

Humans can be exposed to polycyclic aromatic hydrocarbons (PAH) via cigarette smoke and possibly, via intake of foods containing PAH residues formed during production, packaging, or preparation of food. PAH are metabolized by the microsomal epoxide hydrolase enzyme, encoded by the polymorphic *EPHX* gene. Polymorphisms occur in the third and fourth exon. The exon 3 polymorphism leads to a tyrosine (*Y*) 113 → histidine (*H*) substitution in 30-35% of Caucasians, resulting in a markedly lower enzyme activity in *HH* homozygotes. A histidine (*H*) 139 → arginine (*R*) substitution in exon 4 leads to a higher enzyme activity and is found in 15-20% of Caucasians. As *EPHX* is involved in both activation and detoxification of PAH, genetic variation in the underlying gene may influence the rate of PAH metabolism, and through that, the effect of smoking and intake of foods potentially containing PAH residues on the risk of colorectal adenomas.

Methods

Cases (n=385) and polyp-free controls (n=396) were recruited from an ongoing study between 1997 and 2000 among those undergoing endoscopy at the outpatient clinics of eight Dutch hospitals. Eligible subjects were Dutch speaking, of European origin, aged 18 to 75 years at time of endoscopy, had no hereditary colorectal cancer syndromes, chronic inflammatory bowel disease, history of colorectal cancer, or previous bowel resection. We also excluded subjects with only hyperplastic or unknown types of polyps.

Smoking and habitual consumption of foods known to be possibly containing PAH residues (e.g., (barbecued) meat, green leafy vegetables, and fat and oil) were assessed through self-administered questionnaires, one of which was a validated semi-quantitative food frequency questionnaire described in detail elsewhere [1]. Information on histology of excised polyps was obtained through medical files. Blood samples were drawn from all participants for DNA extraction. *EPHX* polymorphisms in exon 3 and 4 were determined as we described previously [2].

Exposure variables describing smoking habits and consumption of relevant foods were divided in quartiles based on the distribution in the control group. We considered the highest exposure category and the slow genotypes of *EPHX*, i.e. *YH* and *HH* for exon 3 and *HH* for exon 4, as high-risk categories. We calculated odds ratios adjusted for age, gender, and constipation history, and several other potential confounders, depending of the variable under study.

Results

Selected characteristics of the study population are summarized in Table A.1. Exposure to cigarette smoke was higher among cases than among controls. Also, cases consumed more fat and oil and green leafy vegetables than controls. The distributions of *EPHX* exon 3 and 4 polymorphisms did not differ between cases and controls.

Table A.2 shows risk estimates for colorectal adenomas for the total population and for *EPHX*-defined subgroups. Smoking increased risk of colorectal adenomas. For exon 3, this risk was confined to those with the fast (*YY*, *YH*) genotype. There was no difference in risk between carriers of fast or slow variants of exon 4. Intake of fat and oil was also positively associated with adenomas, but there were no differences between the *EPHX* variants. Total meat intake, green leafy vegetables consumption, and barbecue frequency were not associated with adenomas, and *EPHX* genotype did not influence these associations (data not shown).

Table A.1. General characteristics of the study population.

	Cases n=385	Controls n=396
Sex, % male	54.0 [a]	37.6
Age, years	59.5 ± 10.5 [a]	51.2 ± 13.7
BMI, kg/m^2	26.1 ±3.8 [a]	25.5 ± 4.2
History of constipation in last three years, %	26.2 [a]	42.9
Ever smoked cigarettes, %	62.4 [a]	53.3
Cigarette smoking, pack-years [b]	25.0 ± 20.7 [a]	18.8 ± 18.9
Total energy, kJ/day	8725 ± 2594	8652 ± 2629
Fat and oils, g/day	27.5 ± 14.7 [a]	23.8 ± 15.3
Vegetables, g/day	127.9 ± 52.3	124.2 ± 45.5
Green leafy vegetables, g/day	23.8 ± 17.8 [a]	21.1 ± 15.7
Total meat, g/day	109.4 ± 54.2	104.6 ± 54.7
Barbecue, frequency/year	3.1 ± 8.0 [a]	3.0 ± 5.7
EPHX exon 3 genotype, % fast (*YY*)	49.4	48.2
EPHX exon 4 genotype, % fast (*HR* or *RR*)	34.8	37.2

[a] Significantly different from controls ($p<0.05$); [b] smokers only.

Discussion

In this study, we found indications for interplay between *EPHX* genotype and smoking in the etiology of colorectal adenomas. Of two recently published studies, one provided results similar to those of our study [3], but the other presented opposite findings [4]. These conflicting results can partly be due to differences between the three studies with respect to classification of *EPHX* polymorphisms. Data on genotype-phenotype correlation *in vitro* and *in vivo* are limited, especially for the different combinations of exon 3 and 4 polymorphisms, and we therefore did not combine both. Cortessis and Ulrich both used different classifications of *EPHX* imputed phenotypes (from fast to very slow) based on the combinations of exon 3 and 4 polymorphisms. Whereas the role of epoxide hydrolase in PAH metabolism is well-established [5], more research

on the enzyme activity of combinations of exon 3 and 4 variants and on its effect on PAH-associated neoplasm is needed.

From our study, we conclude that *EPHX* genotype at exon 3 possibly modulates the association between smoking and colorectal adenomas, the fast variant being related to highest risk.

Table A.2. Smoking, intake of fat and oil, *EPHX* imputed phenotypes, and risk of colorectal adenomas: odds ratios (95% confidence intervals).

		EPHX			
		Exon 3		Exon 4	
	Total population	Fast (*YY*)	Slow (*YH* or *HH*)	Fast (*HR* or *RR*)	Slow (*HH*)
Smoking status [a]					
Never	1.0 (REF)	1.0 (REF)	0.9 (0.6-1.5)	1.0 (REF)	1.6 (0.98-2.8)
Former	1.2 (0.8-1.7)	1.1 (0.6-1.8)	1.2 (0.7-2.1)	1.7 (0.9-3.2)	1.6 (0.9-2.7)
Current	1.7 (1.1-2.5)	2.0 (1.1-3.5)	1.3 (0.7-2.2)	2.6 (1.3-5.0)	2.1 (1.2-3.7)
Pack-years [a]					
None	1.0 (REF)	1.0 (REF)	0.9 (0.6-1.5)	1.0 (REF)	1.7 (1.0-2.8)
≤ 10	1.1 (0.7-1.7)	0.8 (0.4-1.6)	1.3 (0.7-2.3)	2.5 (1.2-5.1)	1.1 (0.6-2.1)
10 - 20	1.2 (0.8-2.0)	1.5 (0.8-2.8)	0.9 (0.4-1.9)	1.5 (0.7-3.4)	1.8 (0.9-3.6)
> 20	1.9 (1.2-2.9)	2.5 (1.3-4.6)	1.4 (0.8-2.6)	2.3 (1.1-4.7)	2.8 (1.5-5.2)
Intake of fat and oil (g/day) [b]					
< 15	1.0 (REF)	1.0 (REF)	1.8 (0.9-3.4)	1.0 (REF)	1.6 (0.8-3.3)
15 - 25	1.5 (0.96-2.4)	2.7 (1.4-5.2)	1.6 (0.9-3.1)	2.1 (0.96-4.6)	2.0 (0.99-4.2)
25 - 35	1.5 (0.9-2.4)	2.5 (1.2-5.0)	1.5 (0.7-3.1)	1.6 (0.7-3.7)	2.3 (1.1-5.0)
≥ 35	1.8 (1.0-3.1)	2.2 (1.1-4.7)	2.5 (1.2-5.2)	2.2 (0.95-5.3)	2.5 (1.1-5.4)

[a] Adjusted for age, gender, constipation history, and alcohol consumption; [b] adjusted for age, gender, constipation history, total energy intake, intake of cereals, and duration of smoking.

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