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Dietary acrylamide intake and the risk of colorectal cancer with specific mutations in KRAS and APC

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Acrylamide, a probable human carcinogen, is present in heat-treated carbohydrate-rich foods. Epidemiological studies have not shown a clear association between acrylamide intake and colorectal cancer (CRC) risk. This may be due to the molecular heterogeneity in colorectal tumors, which was not taken into consideration before. Since the acrylamide metabolite glycidamide induces specific DNA mutations in rodents, we investigated whether acrylamide is associated with CRC risk characterized by mutations in Kirsten-ras (*KRAS*) and adenomatous polyposis coli (*APC*); key genes in colorectal carcinogenesis. This case–cohort analysis, within the Netherlands Cohort Study on diet and cancer, was based on 7.3 years of follow-up. Acrylamide intake was assessed with a food frequency questionnaire. Mutation analysis of codons 1286–1520 in exon 15 in *APC* and codons 12 and 13 in exon 1 in *KRAS* was performed on tumor tissue of 733 cases. Hazard ratios (HR) were calculated using Cox proportional hazards analysis. Among men, acrylamide intake was statistically significantly associated with an increased risk of particularly tumors with an activating *KRAS* mutation {HR fourth versus first quartile: 2.12 [95% confidence interval (CI): 1.16–3.87], *P* trend: 0.01}. Among women, acrylamide intake was statistically significantly associated with a decreased risk of particularly tumors with a truncating *APC* mutation (fourth versus first quartile: 0.47 (95% CI: 0.23–0.94), *P* trend: 0.02), but only in the highest quartile of intake. This is the first study to show that acrylamide might be associated with CRC with specific somatic mutations, differentially in men and women. More research is needed to corroborate or refute these findings.

Introduction

In Europe, in 2008, colorectal cancer (CRC) was the most common cancer (436 000 cases; 13.6% of all cancer cases) and the second leading cause of cancer death (212 000 deaths; 12.3%) (1). Throughout the world, CRC incidence rates vary widely, which is likely to be the result of environmental factors, especially specific components in the diet.

Acrylamide is used in industrial chemistry as a precursor in the production of polyacrylamides, which are used for clarifying drinking water and other industrial applications. Acrylamide is a neurotoxin in humans and has shown to be carcinogenic in rodents (2). In 1994, it was classified by the International Agency for Research on Cancer as a probable human carcinogen (group 2A) (2). In 2002, the Swedish National Food Administration reported high levels of acrylamide in commonly consumed heat-processed foods (3), such as French fries, potato chips and cookies.

In rodents, acrylamide given in drinking water led to several tumors, especially in hormone-sensitive organs such as the testis and the mammary gland (4,5). *In vivo*, acrylamide is oxidized to the epoxide glycidamide, catalyzed by the enzyme cytochrome P4502E1 (CYP2E1) (6). Contrary to acrylamide itself, glycidamide forms adducts with DNA bases and is mutagenic (7). In rodents, glycidamide shows a characteristic DNA mutation pattern. The most frequently observed mutations induced by acrylamide administration were A>G transitions and G>C transversions. Direct administration of glycidamide appeared to induce more mutations than acrylamide at any given dose and additionally rendered G>T transversions (7–9).

A hormonal pathway for acrylamide-induced carcinogenesis has been hypothesized (10). In rats, acrylamide exposure has been shown to influence hormone levels (11–13), and in mice (14,15) and human breast and colorectal cells, acrylamide increased the expression of genes involved in the generation of sex hormones (16).

In epidemiological studies, no clear association was found between acrylamide exposure and CRC risk. One case–control study observed a statistically significant inverse association, hypothesized to be due to residual confounding (17). Three prospective cohort studies [among which was the Netherlands Cohort Study on diet and cancer (NLCS) study using 13.3 years of follow-up] and a case–control study did not show an association between acrylamide intake and CRC risk (18–21). This may either indicate that acrylamide has no role in causing CRC or the studies may have missed a true association. Associations may become more apparent when the molecular heterogeneity of colorectal tumors is taken into account.

The inactivation of the tumor suppressor gene adenomatous polyposis coli (*APC*) and the activation of the proto-oncogene Kirsten-ras (*KRAS*) are thought to be key events in CRC initiation and progression (22). *APC* has been proposed to function as a 'gatekeeper' gene, and inactivation of the *APC* gene seems to trigger the cascade of events that leads to malignant transformation of epithelial cells into adenocarcinoma (23). Somatic mutations in the *APC* gene are found in the majority of sporadic colorectal tumors (24) and most of these mutations occur within the codons 1286–1520 of exon 15, the so-called mutation cluster region (25). Missense or frameshift mutations in the *APC* gene lead to truncated, and therefore inactive APC proteins (22). Mutations in *KRAS* are thought to lead to increased and unregulated cellular proliferation and malignant transformation from an intermediate adenoma to a late adenoma or carcinoma. About 30–60% of the colorectal adenocarcinomas have a *KRAS* mutation (26). The most frequently affected codons are codons 12 and 13 in exon 1 and to a lesser extent codon 61 in exon 2.

We hypothesized that acrylamide intake is associated with the risk of CRC with a specific molecular signature characterized by activating mutations in the *KRAS* gene and truncating mutations in the *APC* gene and we focused on the specific point mutations induced by acrylamide and its metabolite glycidamide in rodents: G>C transversions, G>T transversions and A>G transitions. The latter mutation can, however, not be investigated in the current study, because from a previous analysis, it was known that A>G mutations do not lead to truncating *APC* mutations in this study population (27) and activating *KRAS* mutations are only G mutations.

Materials and methods

Study design

This study was embedded in the NLCS. The NLCS was initiated in September 1986 and included 58 279 men and 62 573 women aged 55–69 years, who

Abbreviations: AA, acrylamide-associated; APC, adenomatous polyposis coli; CI, confidence interval; CRC, colorectal cancer; FFQ, food frequency questionnaire; HR, hazard ratio; KRAS, Kirsten-ras; NLCS, Netherlands Cohort Study on diet and cancer.

were identified through 204 municipal computerized population registries throughout the Netherlands (28). The study design for the present analyses was a case–cohort study: cases were accumulated from the entire NLCS cohort and a subcohort (n = 5000) was randomly selected from the entire cohort at baseline. The number of person–years at risk for the entire cohort was estimated from the subcohort, for efficiency reasons.

Identification of the incident histologically confirmed CRC cases was done by record linkage to the Netherlands Cancer Registry and the Dutch Pathology Registry (in Dutch: PALGA), providing a near 100% coverage of the municipalities included in the NLCS (29). Cases and subcohort members were excluded from the analyses if they had been diagnosed with another cancer (except skin cancer) at baseline and if their dietary data were incomplete or inconsistent. Due to incomplete coverage by PALGA in the earlier years, the first 2.3 years of follow-up were excluded from the analyses.

Tissue samples

As described previously, tumor material of CRC cases was collected after approval by the ethical review boards of Maastricht University, the Netherlands Cancer Registry and PALGA (30). All relevant pathology laboratories in the Netherlands agreed to make tissue samples available upon request from PALGA. From the 815 eligible tissue samples distributed among 54 pathology laboratories throughout the Netherlands, 771 samples could be retrieved. After excluding samples that contained only healthy colorectal mucosa, that were revised as a benign adenoma instead of a carcinoma by a pathologist or that did not yield sufficient DNA, tumor tissue from 733 CRC patients was available for mutation analysis.

Mutation analysis

The methods of DNA isolation, PCR and sequencing are extensively described elsewhere (27,30). Tumor material was analyzed for mutations in codons 1286–1520 (mutation cluster region) of exon 15 of the *APC* gene and in codons 12 and 13 of exon 1 of the *KRAS* gene using nested PCR, followed by direct sequencing of purified segments. Mutation analysis of the *APC* gene was successful and complete for 662 samples, and of the *KRAS* gene for all 733 samples.

Acrylamide intake assessment

At the start of the NLCS in 1986, the cohort members completed a semiquantitative, self-administered questionnaire on diet, other environmental risk factors, medical history and family history of cancer. The dietary part was a 150 item food frequency questionnaire (FFQ), which assessed habitual consumption (frequency and portion size) of foods and beverages during the year preceding the start of the study. Acrylamide intake was assessed with this FFQ, as described previously (31). In short, data from the Dutch Food and Consumer Product Safety Authority were used to assign a mean acrylamide concentration to each food from the NLCS FFQ. An estimation of the total dietary acrylamide intake was made using the mean acrylamide level in the foods, and the consumption frequency and portion size of the foods, with a subsequent summation across all of the foods.

Data analysis

Data analysis was conducted for men and women separately, because of the potential sex hormonal effect of acrylamide (10). Tumor DNA contains thousands of mutations, but most mutations do not alter protein function and therefore are unlikely to have played a role in cancer causation. Because of that, we only examined mutations leading to the activation of the *KRAS* gene and to introduction of a stop codon (truncating mutation) in the *APC* gene, which are mutations that are likely to have played an important role in the carcinogenic process. The molecular endpoints that we analyzed were: an activating *KRAS* and/or truncating *APC* mutation; a G>C or G>T activating *KRAS* and/or truncating *APC* mutation; an activating *KRAS* mutation; a G>C or G>T activating *KRAS* mutation; a truncating *APC* mutation and a truncating G>C or G>T *APC* mutation. In addition, we zoomed in on the singular mutations leading to activating *KRAS* and truncating *APC* mutations. From now on, for brevity, we will talk about *KRAS* mutations when we mean activating *KRAS* mutations and about *APC* mutations when we mean truncating *APC* mutations. For readability, we will from now on use the wording AA (acrylamide-associated) mutations to signify G>C or G>T mutations.

Hazard ratios (HR) and corresponding 95% confidence intervals (CI) were determined using Cox proportional hazards models with person–years at risk as the time metric. In subgroup analyses, we restricted to never smokers because of the high concentration of acrylamide in tobacco smoke that may blur the estimation of dietary acrylamide intake. However, for men, the number of never smokers was too small, and therefore, we combined never smokers with ex-smokers that quit >10 years before baseline. In order to avoid analyses in very small groups, acrylamide intake was included in the models as a continuous variable, as tertiles or as quartiles with a minimum of 20, 60 and 80 cases, respectively. When there were <60 cases, acrylamide intake was only modeled continuously. HRs were adjusted for age and variables known as CRC risk factors in this population (*a priori* chosen covariables), namely smoking (status, quantity and duration), body mass index, family history of CRC and total energy intake. Variables considered as potential confounders were included in the multivariable-adjusted model if they changed the age-adjusted HR of acrylamide by >10%. The following variables, selected from the literature, were tested: physical activity, education level, intake of fat, fiber,

Table I. Characteristics of male CRC cases with specific molecular endpoints and male subcohort members[a]

	Subcohort	Case groups						
		Total CRC	Activating *KRAS* and/or truncating *APC* mutation	G>C or G>T activating *KRAS* and/or truncating *APC* mutation	Activating *KRAS* mutation	G>C or G>T activating *KRAS* mutation	Truncating *APC* mutation	G>C or G>T truncating *APC* mutation
n[b]	1904	341	183	72	114	48	117	28
Acrylamide intake, µg/day	22	23	24	25	24	25	23	26
Acrylamide intake, µg × kg·BW^{-1} × day^{-1}	0.29	0.29	0.31	0.32	0.31	0.32	0.30	0.34
Main food sources of acrylamide								
Coffee, g/day	573	560	591	598	586	581	603	621
Dutch spiced cake, g/day	4	5	5	6	5	6	4	8
Cookies, g/day	14	14	14	16	15	18	13	14
Potato crisps, g/day	0.43	0.55	0.53	0.48	0.60	0.37	0.42	0.69
French fries, g/day	7	7	8	6	8	6	8	6
A priori chosen covariables								
Age, years	61	63	63	63	63	63	63	61
BMI, kg/m^2	25	25	26	25	26	25	25	25
Family history of CRC, n (% yes)	6	12	11	14	11	13	12	14
Total energy intake, kJ/day	2173	2139	2162	2193	2139	2146	2166	2258
Smoking status, %								
Never	14	11	10	10	11	13	10	4
Ex-smoker	52	64	64	64	64	62	62	64
Current smoker	34	25	26	26	25	25	28	32
Duration of cigarette smoking, years	29	30	30	32	30	31	31	34
Frequency of cigarette smoking, n/day	15	17	17	15	18	14	16	16

BMI, body mass index; BW, body weight.
[a]Data represent means or percentages.
[b]n without missing values for *a priori* chosen covariables.

Table II. HR and 95% CI for CRC risk according to categories of dietary acrylamide intake among men

Endpoint		Continuous acrylamide intake (per 10 μg/day)	Quartile 1/ tertile 1	Quartile 2/ tertile 2	Quartile 3/ tertile 3	Quartile 4	*P* trend
Total CRC							
Smokers and non-smokers	*n* cases/*n* person–years	341/9115	86/2246	85/2281	76/2335	94/2253	
	HR (95% CI)[a]	1.03 (0.94–1.14)	1.00 (ref)	1.10 (0.78–1.55)	1.00 (0.70–1.43)	1.17 (0.82–1.66)	0.44
Non-smokers[b]	*n* cases/*n* person–years	255/7285	60/1761	69/1836	55/1853	71/1835	
	HR (95% CI)[a]	1.02 (0.92–1.14)	1.00 (ref)	1.31 (0.88–1.96)	1.10 (0.73–1.66)	1.32 (0.88–1.99)	0.32
KRAS and/or *APC* mutation							
Smokers and non-smokers	*n* cases/*n* person–years	183/9115	39/2246	39/2281	47/2335	58/2253	
	HR (95% CI)[a]	1.10 (0.98–1.23)	1.00 (ref)	1.11 (0.69–1.79)	1.36 (0.85–2.18)	1.58 (1.00–2.51)	0.04
Non-smokers[b]	*n* cases/*n* person–years	140/7285	27/1761	34/1836	35/1853	44/1835	
	HR (95% CI)[a]	1.07 (0.94–1.21)	1.00 (ref)	1.37 (0.79–2.37)	1.49 (0.86–2.57)	1.72 (1.00–2.95)	0.07
AA *KRAS* and/or *APC* mutation							
Smokers and non-smokers	*n* cases/*n* person–years	72/9115	19/3038	23/3088	30/2989		
	HR (95% CI)[a]	1.13 (0.96–1.34)	1.00 (ref)	1.29 (0.70–2.41)	1.66 (0.88–3.11)		0.12
Non-smokers[b]	*n* cases/*n* person–years	55/7285	15/2390	17/2433	23/2462		
	HR (95% CI)[a]	1.09 (0.91–1.31)	c	c	c	c	c
KRAS mutation							
Smokers and non-smokers	*n* cases/*n* person–years	114/9115	21/2246	24/2281	30/2335	39/2253	
	HR (95% CI)[a]	1.15 (1.00–1.31)	1.00 (ref)	1.34 (0.72–2.49)	1.76 (0.96–3.22)	2.12 (1.16–3.87)	0.01
Non-smokers[b]	*n* cases/*n* person–years	87/7285	13/1761	21/1836	21/1853	32/1835	
	HR (95% CI)[a]	1.16 (1.00–1.33)	1.00 (ref)	1.84 (0.89–3.80)	1.99 (0.96–4.13)	2.78 (1.37–5.67)	0.007
AA *KRAS* mutation							
Smokers and non-smokers	*n* cases/*n* person–years	48/9115	11/3038	16/3088	21/2989		
	HR (95% CI)[a]	1.17 (0.96–1.42)	c	c	c	c	c
Non-smokers[b]	*n* cases/*n* person–years	37/7285	9/2390	12/2433	16/2462		
	HR (95% CI)[a]	1.16 (0.94–1.43)	c	c	c	c	c
APC mutation							
Smokers and non-smokers	*n* cases/*n* person–years	117/9115	28/2246	25/2281	32/2335	32/2253	
	HR (95% CI)[a]	1.04 (0.91–1.20)	1.00 (ref)	0.94 (0.53–1.66)	1.22 (0.71–2.11)	1.16 (0.67–2.02)	0.49
Non-smokers[b]	*n* cases/*n* person–years	91/7285	20/1761	22/1836	27/1853	22/1835	
	HR (95% CI)[a]	0.99 (0.84–1.15)	1.00 (ref)	1.13 (0.59–2.17)	1.47 (0.79–2.71)	1.09 (0.56–2.11)	0.84
AA *APC* mutation							
Smokers and non-smokers	*n* cases/*n* person–years	28/9115	8/3038	7/3088	13/2989		
	HR (95% CI)[a]	1.15 (0.91–1.46)	c	c	c	c	c
Non-smokers[b]	*n* cases/*n* person–years	20/7285	6/2390	5/2433	10/2462		
	HR (95% CI)[a]	1.01 (0.77–1.32)	c	c	c	c	c

Median acrylamide intake in the quartiles of acrylamide intake in men (subcohort): 11.7, 17.0, 23.0 and 35.8 μg/day.
[a]Adjusted for age (years), smoking [status (current/non-current), quantity (*n* cigarettes/day) and duration (years)], body mass index (kg/m^2), family history of CRC (yes/no) and total energy intake (kcal/day).
[b]Non-smokers: never smokers and ex-smokers that quit smoking >10 years before baseline.
[c]Insufficient number of cases.

Table III. Endpoints for which the scaled Schoenfeld residuals indicated violation of the proportional hazards assumption: analysis stratified by follow-up time

Endpoint	2.3–4.8 years of follow-up		4.8–7.3 years of follow-up	
	n cases/*n* person–years	HR (95% CI)	*n* cases/*n* person–years	HR (95% CI)
KRAS and/or *APC* mutation, men[a]	76/4667	1.23 (1.06–1.42)	107/4448	1.00 (0.86–1.16)
AA *KRAS* and/or *APC* mutation, men	33/4667	1.33 (1.08–1.62)	39/4448	0.95 (0.73–1.24)
KRAS mutation, men[b]	45/4667	1.30 (1.07–1.57)	69/4448	1.06 (0.89–1.25)
APC mutation, women	46/5162	0.69 (0.47–1.01)	40/5033	1.10 (0.87–1.38)
APC mutation, never smokers, women	31/3087	0.66 (0.39–1.11)	28/3024	1.13 (0.82–1.56)

HRs adjusted for age (years), smoking [status (current/non-current), quantity (*n* cigarettes/day) and duration (years)], body mass index (kg/m^2), family history of CRC (yes/no) and total energy intake (kcal/day).
[a]*KRAS* and/or *APC* mutation, men: 2.3–4.8 years of follow-up: tertile 2: 1.61 (0.85–3.03), tertile 3: 2.35 (1.26–4.37), $P = 0.01$; 4.8–7.3 years of follow-up: tertile 2: 1.17 (0.71–1.95), tertile 3: 1.07 (0.64–1.81), $P = 0.88$.
[b]*KRAS* mutation, men: 4.8–7.3 years of follow-up: tertile 2: 1.38 (0.73–2.63), tertile 3: 1.37 (0.71–2.64), $P = 0.43$.

heme iron, dietary vitamin B6, vegetables, fruits, dairy, meat, alcohol and tea. In addition, carbohydrate and *trans* unsaturated fatty acid intake were checked because of their correlation with acrylamide intake. Dose–response trends were tested by fitting the median acrylamide intake per quantile as a continuous variable and evaluated using the Wald χ^2 test.

The proportional hazards assumption was checked using scaled Schoenfeld residuals and by visually inspecting the −ln[−ln(survival)] curves. Standard errors were estimated using the robust Huber–White sandwich estimator to account for additional variance introduced by sampling from the cohort.

All statistical analyses were performed using STATA version 12 and the tests were performed two sided with a *P* value <0.05 considered as statistically significant.

Results

For the Cox proportional hazards analyses, only the *a priori* chosen covariables: smoking (status, quantity and duration), body mass index,

family history of CRC and total energy intake were included in the multivariable-adjusted models, since adding the potential confounders did not change the HRs by >10%. Multivariable-adjusted results did not differ importantly from age-adjusted results and did not lead to different conclusions. Therefore, only the multivariable-adjusted results are shown and discussed; first for men and then for women.

Men

At baseline, male cases were older than male subcohort members (except cases with a tumor with an AA *APC* mutation) and more often showed a family history of CRC (Table I).

Among men, acrylamide intake was not associated with CRC risk overall (Table II). There was a statistically significant positive, dose-dependent association between acrylamide intake and tumors with a *KRAS* and/or *APC* mutation (HR for the highest versus the lowest quartile = 1.58 (95% CI: 1.00–2.51), *P* trend: 0.04), and the HR was 1.10 (95% CI: 0.98–1.23) per 10 μg/day increment. Among non-smoking men, the corresponding HRs were 1.72 (95% CI: 1.00–2.95; *P* trend: 0.07) and 1.07 (95% CI: 0.94–1.21), respectively. When looking at tumors with an AA *KRAS* and/or *APC* mutation, weaker associations were seen when looking at the tertiles of acrylamide intake: HR for the highest versus the lowest tertile = 1.66 (95% CI: 0.88–3.11; *P* trend: 0.12), but the HR per 10 μg/day increment was 1.13 (95% CI: 0.96–1.34). Among non-smoking men, the HR per 10 μg/day increment was 1.09 (95% CI: 0.91–1.31). The number of non-smokers was too small for an analysis based on categories of acrylamide intake for this endpoint. It appeared that the abovementioned associations were driven by the association with the risk of tumors with a *KRAS* mutation, because the HR for tumors with a *KRAS* mutation were stronger: the HR for the highest versus the lowest quartile was 2.12 (95% CI: 1.16–3.87; *P* trend: 0.01) and a HR of 1.15 (95% CI: 1.00–1.31) per 10 μg/day increment. Among non-smokers, the corresponding HRs were 2.78 (95% CI: 1.37–5.67; *P* trend: 0.007) and 1.16 (95% CI: 1.00–1.33). There were not enough cases to look at the dose–response relationship, but the HR per 10 μg/day increment for a tumor with an AA *KRAS* mutation was slightly higher than for a tumor with any kind of activating *KRAS* mutation: 1.17 (95% CI: 0.96–1.42) for all men and 1.16 (95% CI: 0.94–1.43) for non-smokers. Acrylamide intake was not statistically significantly associated with tumors with an *APC* mutation among men, but the continuous acrylamide variable was associated with the risk of tumors with an AA *APC* mutation with approximately the same strength as with the risk of a tumor with an AA *KRAS* mutation. The small number of cases precluded looking at the dose–response relationship for this endpoint. Among non-smoking men, this association was considerably reduced.

The proportional hazards assumption was violated according to the scaled Schoenfeld residuals for the continuous acrylamide intake and the highest category of acrylamide intake in the analyses of tumors with a *KRAS* and/or *APC* mutation, tumors with an AA *KRAS* and/or *APC* mutation and tumors with a *KRAS* mutation. However, only for tumors with a *KRAS* and/or *APC* mutation there was a borderline statistically significant interaction with follow-up time. Inspection of the −ln[−ln(survival)] curves showed crossing of survival curves for the different acrylamide intake categories. The results of splitting of the follow-up period halfway are presented in Table III. The positive associations appeared to be confined to the follow-up period 2.3–4.8 years for tumors with a *KRAS* and/or *APC* mutation, tumors with an AA *KRAS* and/or *APC* mutation and tumors with a *KRAS* mutation.

Table IV shows the associations between acrylamide intake and the risk of CRC stratified by the singular point mutations. Among men, a borderline statistically significant positive association was observed between acrylamide intake and the risk of tumors with an G:C>T:A activating *KRAS* mutation [HR per 10 μg/day: 1.22 (95% CI: 0.99–1.50)]. For G:C>A:T activating *KRAS* mutations, there was a statistically non-significant positive association with acrylamide intake, whereas the number of G:C>C:G activating *KRAS* mutations was too small for a meaningful analysis. Sample sizes were too small for analyses of the singular mutations among non-smoking men.

Table IV. HR and 95% CI for CRC risk specified by specific mutations in the *KRAS* and *APC* gene according to categories of dietary acrylamide intake

	Mutation induced by glycidamide in rodents					Other functional mutation			
	G>T activating *KRAS* mutation	G>C activating *KRAS* mutation	G>T truncating *APC* mutation	G>C truncating *APC* mutation	A>G truncating *APC* mutation	G>A activating *KRAS* mutation[a]	G>A truncating *APC* mutation[b]	A>T truncating *APC* mutation	A>C truncating *APC* mutation
Men									
n cases/person–years	40/13 490	9/13 490	23/13 490	6/13 490	0/13 490	67/13 490	71/13 490	4/13 490	1/13 490
HR (per 10 μg/day increment) (95% CI)	1.22 (0.99–1.50)	[c]	0.98 (0.73–1.33)	[c]	[c]	1.15 (0.97–1.35)	1.11 (0.93–1.31)	[c]	[c]
Women									
n cases/person–years	36/14 982	9/14 982	13/14 982	3/14 982	0/14 982	51/14 982	52/14 982	6/14 982	3/14 982
HR (per 10 μg/day increment) (95% CI)	0.79 (0.55–1.14)	[c]	1.19 (0.85–1.68)	[c]	[c]	0.95 (0.72–1.26)	0.82 (0.60–1.11)	[c]	[c]

HRs adjusted for age (years), smoking [status (current/non-current), quantity (*n* cigarettes/day) and duration (years)], body mass index (kg/m^2), family history of CRC (yes/no) and total energy intake (kcal/day).
[a]G:C>A:T activating *KRAS* mutation, men: tertile 2: 1.67 (0.84–3.30), tertile 3: 1.89 (0.98–3.64), $P = 0.08$.
[b]G:C>A:T truncating *APC* mutation, men: tertile 2: 1.05 (0.56–1.99), tertile 3: 1.45 (0.80–2.62), $P = 0.19$.
[c]Insufficient number of cases.

Table V. Characteristics of female CRC cases with specific molecular endpoints and female subcohort members[a]

	Subcohort	Case groups						
		Total CRC	*KRAS* and/or *APC* mutation	AA *KRAS* and/ or *APC* mutation	*KRAS* mutation	AA *KRAS* mutation	*APC* mutation	AA *APC* mutation
n[b]	2084	282	136	60	94	45	86	16
Acrylamide intake, µg/day	21	20	20	20	20	19	20	23
Acrylamide intake, ug × kg BW^{-1} × day^{-1}	0.32	0.29	0.29	0.27	0.28	0.26	0.29	0.33
Main food sources of acrylamide								
Coffee, g/day	497	518	497	463	490	454	492	503
Dutch spiced cake, g/day	6	5	5	5	5	4	4	7
Cookies, g/day	14	14	14	15	14	15	14	16
Potato crisps, g/day	0.40	0.33	0.38	0.22	0.17	0.24	0.49	0.17
French fries, g/day	4	4	5	4	3	3	6	4
A priori chosen covariables								
Age, years	61	63	63	63	63	63	63	63
BMI, kg/m^2	25	26	26	26	26	26	26	26
Family history of CRC, *n* (% yes)	6	10	7	5	6	4	8	6
Total energy intake, kJ/day	1684	1668	1723	1737	1707	1728	1737	1733
Smoking status, %								
Never	60	63	65	65	67	67	69	62
Ex-smoker	20	22	20	22	21	20	14	25
Current smoker	20	15	15	13	12	13	17	13
Duration of cigarette smoking, years	11	10	10	10	10	10	9	8
Frequency of cigarette smoking, *n*/day	5	4	4	3	4	4	3	2

BMI, body mass index; BW, body weight.
[a]Data represent means (SD) or percentages.
[b]*n* without missing values for *a priori* chosen covariables.

Women

Female cases were older at baseline than subcohort members and had a higher body mass index (Table V). In addition, female cases were generally more often never smokers, less often current smokers, and smoked fewer cigarettes per day and had smoked for a shorter duration than subcohort members.

Among women, there was a modest tendency toward an inverse association for all of the endpoints, but this was mostly restricted to the highest acrylamide intake category (Table VI). The most prominently decreased risks were observed for tumors with a *KRAS* and/or *APC* mutation [HR for the highest versus the lowest quartile = 0.59 (95% CI: 0.34–1.02); *P* trend: 0.04], and tumors with an *APC* mutation [HR for the highest versus the lowest quartile = 0.45 (95% CI: 0.23–0.91); *P* trend: 0.02]. Among never-smoking women, the corresponding HRs were 0.42 (95% CI: 0.20–0.88; *P* trend: 0.01) and 0.48 (95% CI: 0.24–0.98; *P* trend: 0.03). Despite the statistically significant *P* for trend in these analyses, the HRs did not decrease consistently over the categories of intake, and the HR per 10 µg/day increment was never statistically significantly <1.

Scaled Schoenfeld residuals indicated a violation of the proportional hazards assumption for the continuous acrylamide variable and the highest category of acrylamide intake for tumors with an *APC* mutation, both among all women and among never-smoking women. Only for the highest quartile of intake for all women was there a statistically significant interaction with follow-up time. Inspection of the −ln[−ln(survival)] indicated crossing of the survival curves for the different acrylamide categories. In Table III, it is shown that the inverse association between acrylamide intake and tumors with an *APC* mutation was confined to the follow-up period 2.3–4.8 years.

Only two singular mutations leading to truncation of the *APC* gene could be studied, namely G:C>T:A and G:C>A:T truncating *APC* mutations, and only the former showed a statistically non-significantly increased HR (Table IV). For the other mutations, case numbers were deemed too small. Subgroup analyses among never-smoking women with respect to specific single types of truncating *APC* mutations could not be performed due to the limited case numbers.

Discussion

In this study, we investigated associations between dietary acrylamide intake and the CRC risk characterized by mutations in the *KRAS* and *APC* gene. Since, to the best of our knowledge, this is the first study on acrylamide intake in relation to specific mutations in these genes and we performed many analyses within small subgroups, caution must be exercised in interpreting the results.

Among men, we found a statistically significant positive multivariable-adjusted association between acrylamide intake and the risk of colorectal tumors harboring activating *KRAS* mutations. When we zoomed in to see which specific mutations were behind this, we observed a statistically significant positive association for the only mutation that we were able to study out of the two relevant mutations caused by the acrylamide metabolite glycidamide in rodents, namely G>T mutations.

Among women, a decreased risk in the highest category of acrylamide intake was observed for all molecular endpoints; the most prominent and statistically significant multivariable-adjusted inverse association was found for tumors with a truncating *APC* mutation. There were no indications for any specific singular mutations, but in the case of a putative protective association, this would not be expected. We have no explanation for why acrylamide intake is positively associated with the risk of CRC with activating *KRAS* mutations among men and not among women.

The associations for which a violation of the proportional hazards assumption was shown appeared to be confined to the follow-up period 2.3–4.8 years. We do not have a clear-cut explanation for this, but it calls for a cautious interpretation of the observed associations, especially because we are not able to investigate what the association between acrylamide intake and CRC risk is in the first 2.3 years of follow-up because the first 2.3 years were excluded due to the fact that tumor material of cases was only collected for the subsequent 5 years of follow-up. Protopathic bias, however, is therefore unlikely to have taken place.

The relationship between acrylamide intake and overall CRC risk for men and women separately was only investigated in four other epidemiological studies. A case–control study (20), a cohort study

Table VI. HR and 95% CI for CRC risk according to categories of dietary acrylamide intake among women

Endpoint		Continuous acrylamide intake (per 10 μg/day)	Quartile 1/ tertile 1	Quartile 2/ tertile 2	Quartile 3/ tertile 3	Quartile 4	*P* trend
Total CRC							
Smokers and non-smokers	*n* cases/*n* person–years	282/10 195	76/2515	74/2568	75/2527	57/2585	
	HR (95% CI)[a]	0.95 (0.85–1.07)	1.00 (ref)	1.00 (0.70–1.43)	1.09 (0.76–1.55)	0.76 (0.52–1.11)	0.13
Never smokers	*n* cases/*n* person–years	179/6111	59/1628	51/1472	47/1467	32/1544	
	HR (95% CI)[a]	0.95 (0.82–1.11)	1.00 (ref)	1.13 (0.73–1.75)	1.10 (0.70–1.74)	0.66 (0.41–1.08)	0.04
KRAS and/or *APC* mutation							
Smokers and non-smokers	*n* cases/*n* person–years	136/10 195	35/2515	37/2568	41/2527	23/2585	
	HR (95% CI)[a]	0.93 (0.78–1.10)	1.00 (ref)	1.04 (0.64–1.69)	1.17 (0.72–1.91)	0.60 (0.34–1.05)	0.04
Never smokers	*n* cases/*n* person–years	88/6111	24/1628	26/1472	27/1467	11/1544	
	HR (95% CI)[a]	0.87 (0.69–1.11)	1.00 (ref)	1.12 (0.62–2.04)	1.18 (0.64–2.18)	0.42 (0.20–0.88)	0.006
AA *KRAS* and/or *APC* mutation							
Smokers and non-smokers	*n* cases/*n* person–years	60/10 195	24/3413	19/3353	17/3429		
	HR (95% CI)[a]	0.86 (0.67–1.11)	1.00 (ref)	0.78 (0.42–1.47)	0.65 (0.33–1.27)		0.24
Never smokers	*n* cases/*n* person–years	39/6111	17/2151	12/1922	10/2038		
	HR (95% CI)[a]	0.82 (0.55–1.20)	[b]	[b]	[b]	[b]	[b]
KRAS mutation							
Smokers and non-smokers	*n* cases/*n* person–years	94/10 195	24/2515	26/2568	29/2527	15/2585	
	HR (95% CI)[a]	0.90 (0.73–1.10)	1.00 (ref)	1.16 (0.65–2.07)	1.33 (0.75–2.39)	0.61 (0.30–1.20)	0.08
Never smokers	*n* cases/*n* person–years	63/6111	25/2151	23/1922	15/2038		
	HR (95% CI)[a]	0.82 (0.62–1.08)	1.00 (ref)	1.09 (0.58–2.02)	0.63 (0.32–1.28)		0.15
AA *KRAS* mutation							
Smokers and non-smokers	*n* cases/*n* person–years	45/10 195	18/3413	15/3353	12/3429		
	HR (95% CI)[a]	0.79 (0.58–1.07)	[b]	[b]	[b]	[b]	[b]
Never smokers	*n* cases/*n* person–years	30/6111	13/2151	9/1922	8/2038		
	HR (95% CI)[a]	0.77 (0.50–1.19)	[b]	[b]	[b]	[b]	[b]
APC mutation							
Smokers and non-smokers	*n* cases/*n* person–years	86/10 195	24/2515	23/2568	26/2527	13/2585	
	HR (95% CI)[a]	0.90 (0.72–1.12)	1.00 (ref)	0.90 (0.49–1.64)	1.01 (0.56–1.83)	0.47 (0.23–0.94)	0.02
Never smokers	*n* cases/*n* person–years	59/6111	23/2151	22/1922	14/2038		
	HR (95% CI)[a]	0.90 (0.67–1.20)	[b]	[b]	[b]		[b]
AA *APC* mutation							
Smokers and non-smokers	*n* cases/*n* person–years	16/10 195	6/3413	4/3353	6/3429		
	HR (95% CI)[a]	[b]	[b]	[b]	[b]	[b]	[b]
Never smokers	*n* cases/*n* person–years	11/6111	5/2151	3/1922	3/2038		
	HR (95% CI)[a]	[b]	[b]	[b]	[b]	[b]	[b]

Median acrylamide intake in the quartiles of acrylamide intake among women (subcohort): 10.2, 15.2, 21.2 and 35.0 μg/day; tertiles: 11.4, 17.9 and 32.0 μg/day.
[a]Adjusted for age (years), smoking [status (current/non-current), quantity (*n* cigarettes/day) and duration (years)], body mass index (kg/m^2), family history of CRC (yes/no) and total energy intake (kcal/day).
[b]Insufficient number of cases.

among men (18), a cohort study among women (19) and the NLCS study with a follow-up of 13.3 years (21) did not observe a statistically significant association. Mucci *et al.* (17) reported an inverse association among non-smokers (men and women combined), a subgroup that probably contains a substantial amount of women.

Our study has some limitations. Most importantly, chance findings due to multiple subgroup analyses cannot be excluded, although the subgroups were *a priori* chosen. Another limitation is the potential non-differential misclassification of acrylamide exposure assessed with a FFQ. However, a validation study showed that using mean acrylamide levels for individual foods does not preclude reliable ranking of consumers in terms of their acrylamide intake (32). Together with the high validity and reproducibility of the NLCS FFQ (33,34), this strengthens our confidence that the acrylamide intake assessment in our study was of reasonable quality.

Concurrently with acrylamide, people consuming heat-generated foods are exposed to a number of other carcinogenic substances in those foods, e.g. polycyclic aromatic hydrocarbons, furans and furan derivatives, such as 5-hydroxymethylfurfural, and these other compounds may thus be confounders in the relationship between acrylamide intake and cancer risk. However, the reaction in which acrylamide is generated heavily relies on specific conditions, such as the presence of asparagine, whereas for other heat-generated toxicants other specific conditions are needed. Thus, across the span of a whole diet, the correlation between acrylamide and other heat-generated compounds is probably limited and there seems little potential for confounding. For 5-hydroxymethylfurfural this has been clearly shown, as no clear correlation between the estimated dietary intake of 5-hydroxymethylfurfural and acrylamide was observed in a correlation study (35).

The strengths of this study are the prospective design, the selection of an older cohort, in which dietary habits are relatively stable and the large size of this cohort. The combination of available molecular data and detailed information on several potential confounding factors provided by a validated and reproducible FFQ is unique.

We would like to stress that our findings need to be validated by results from other prospective studies, preferably with several studies pooled in order to obtain larger case numbers, stratified by sex. We can only speculate about the possible mechanisms underlying the observed associations. The positive association between acrylamide intake and the risk of colorectal tumors with an activating *KRAS* mutation, and within this endpoint the strongest association with G>T (an acrylamide-induced mutation in rodents), is compatible with glycidamide being a mutagen.

It is hypothesized that acrylamide may influence sex hormonal systems (10,36). The conspicuously lower incidence rate of CRC among women compared with men (37) is suggestive of sex hormonal involvement in its development. Decreased risks of CRC associated with hormone replacement therapy were reported in women (38), In addition, several prospective studies observed an inverse association

between oral contraceptive use and CRC risk (39). Little is known about the interaction between sex hormones and the *KRAS* and *APC* genes with respect to CRC development. There are some indications that estrogen receptors α and β protect against *APC*-dependent colorectal carcinogenesis (40), which would fit with the observed inverse association between acrylamide intake and a reduced risk of a tumor with a truncating *APC* mutation among women. In normal colonic mucosa, estrogen receptor β levels are higher in women than in men, whereas in tumors the level of this receptor is much more reduced compared with normal tissue in women than in men. This may indicate that this receptor, and thus the role of estrogenic substances, is more important in the etiology of CRC in women than in men (41).

Conclusions

In conclusion, this study showed a statistically significant positive association between acrylamide intake and the risk of colorectal tumors with, in particular, an activating mutation in the *KRAS* gene among men, and a statistically significant inverse association with the risk of tumors with a truncating mutation in the *APC* gene among women. Thus, acrylamide intake may influence the risk of CRC with activating *KRAS* mutations or truncating *APC* mutations in men and women differentially. However, a cautious interpretation of these results is necessary, since they are the first results considering tumor heterogeneity, because we performed many subgroup analyses, and because of the important differences in associations over the rather short follow-up period. We encourage replication of these associations in other prospective cohort studies with high-quality estimation of the acrylamide intake, e.g. studies using acrylamide and glycidamide to hemoglobin adducts as biomarkers of internal acrylamide exposure.

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