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Citation for published version (APA):

Schouten, L. J., van Dijk, B. A. C., Oosterwijk, E., van Engeland, M., Hulsbergen van Kaa, C. A., Kiemeneij, L. A., Goldbohm, R. A., Kester, A. D. M., de Voge, S., Schalken, J. A., & van den Brandt, P. A. (2008). Alcohol consumption and mutations or promoter hypermethylation of the von Hippel-Lindau gene in renal cell carcinoma. *Cancer Epidemiology Biomarkers & Prevention*, 17(12), 3543-3550. <https://doi.org/10.1158/1055-9965.EPI-08-0321>

Document status and date:

Published: 01/01/2008

DOI:

[10.1158/1055-9965.EPI-08-0321](https://doi.org/10.1158/1055-9965.EPI-08-0321)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

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Alcohol Consumption and Mutations or Promoter Hypermethylation of the *von Hippel–Lindau* Gene in Renal Cell Carcinoma

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Abstract

Alcohol consumption has been associated with a decreased risk for renal cell cancer in several studies. We investigated whether alcohol is associated with (epi)genetic changes of the *von Hippel–Lindau* (*VHL*) gene in renal cell cancer. The Netherlands Cohort Study (NLCS) on Diet and Cancer started in 1986 ($n = 120,852$) and uses the case-cohort method. After 11.3 years of follow-up, 314 renal cell cancer cases and 4,511 subcohort members were available for analysis. DNA was isolated from paraffin-embedded tumor tissue from 235 cases. *VHL* mutations were analyzed by sequencing, whereas *VHL* promoter methylation was analyzed using methylation-specific PCR. In multivariate analysis, hazard ratios of renal cell cancer for cohort members who consumed up to 5, 15, 30, and ≥ 30 g of alcohol per day were 0.72, 0.64, 0.81, and 0.69, respectively, compared with nondrinkers [95% confidence interval (95% CI) for the ≥ 30 category, 0.44–1.07;

P for trend, 0.17]. Alcohol intake from beer, wine, and liquor was associated with decreased risks for renal cell cancer, although not statistically significant. Hazard ratios were not different for clear-cell renal cell cancer with and without *VHL* mutations, except for alcohol from beer, which was associated with an increased risk for clear-cell renal cell cancer without *VHL* mutations (hazard ratio for ≥ 5 g of alcohol from beer compared with nondrinkers, 2.74; 95% CI, 1.35–5.57). Alcohol was associated with a decreased risk for clear-cell renal cell cancer without *VHL* gene promoter methylation (hazard ratio for >15 g compared with nondrinkers, 0.58; 95% CI, 0.34–0.99). In this study, a not statistically significant inverse association was observed between alcohol and renal cell cancer. There was no statistical significant heterogeneity by *VHL* mutation or methylation status. (Cancer Epidemiol Biomarkers Prev 2008;17(12):3543–50)

Introduction

In a recent pooled analysis of 12 prospective cohort studies, alcohol consumption was associated with a decreased risk for renal cell cancer (1). Persons who drank >15 g of alcohol per day had a decreased risk by 28% compared with nondrinkers. However, this pooled analysis could not investigate whether the association was different for the different histologic subtypes of renal cell cancer because histology was not specified for most cases (1).

Renal cell cancer is classified in different subtypes. Most renal cell cancers are of the clear-cell type ($\sim 80\%$); other subtypes are papillary renal cell cancer (10%), chromophobe renal cell cancer (5%), collecting-duct

carcinoma (1%), and unclassified renal cell cancer (3–5%; ref. 2). *von Hippel–Lindau* (*VHL*) disease is a rare inherited disorder associated with (among others) an increased risk for clear-cell renal cell cancer (3). After the identification of the *VHL* gene on chromosome 3p25, it became evident that this gene is also involved in the development of sporadic clear-cell renal cell cancer. It is estimated that $\sim 75\%$ of all sporadic clear-cell renal cell cancer harbor biallelic *VHL* defects (4). Besides mutations, the *VHL* gene can be inactivated by other mechanisms like hypermethylation of the *VHL* gene promoter region (5). It has been suggested that risk factors might be associated with mutations in the *VHL* gene: occupational exposure to trichloroethylene and fruit consumption were associated with mutations in the *VHL* gene in renal cell cancer (6, 7).

Whether alcohol consumption is associated with clear-cell renal cell cancer as such or more specifically with (epi)genetic alterations of the *VHL* gene has not been investigated before. Alcohol degrades methyl donors and has been found to be associated with hypomethylation of the genome in cancer (8). However, paradoxically, in cancer the promoter region of tumor suppressor genes

Received 4/8/08; revised 8/28/08; accepted 9/16/08.

Grant support: The Dutch Kidney Foundation (grant C99.1863) and the Netherlands Cancer Society.

Presented in part at the Annual Meeting of the AACR, April 2006, Washington.

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doi:10.1158/1055-9965.EPI-08-0321

are often hypermethylated. It has been hypothesized that the hypermethylation of promoter regions of genes has been induced by a compensatory up-regulation of DNA methyltransferase activity (9). In that case, however, alcohol consumption is not expected to be inversely associated with hypermethylation of the *VHL* gene in renal cell cancer.

We studied whether alcohol consumption is associated with risk for renal cell cancer and more specifically with mutational status or promoter hypermethylation of the *VHL* gene in clear-cell renal cell cancer within a large prospective cohort study.

Materials and Methods

Subjects. The NLCS on Diet and Cancer is a prospective cohort study, which started in September 1986. The study design has been reported in detail elsewhere (10). Briefly, the cohort included 120,852 men and women, aged 55 to 69 y, at the beginning of the study. The study was designed as a case-cohort study, using all cases and a random sample of 5,000 persons from the cohort (subcohort), who have been followed to estimate the accumulated person-years in the entire cohort (11). The subcohort was sampled randomly from the cohort after the baseline exposure measurement. Follow-up for incident cancer has been established by computerized record linkage with the Netherlands Cancer Registry and Pathologisch-Anatomisch Landelijk Geautomatiseerd Archief (PALGA), a national database of pathology reports. The method of record linkage to obtain information on cancer incidence has been described previously (12). The completeness of cancer follow-up was estimated to be >96% (13). From 1986 to 1997 (11.3 y follow-up), 355 kidney cancer cases (International Classification of Diseases for Oncology 3, C64.9) were identified. Urothelial cell carcinomas were excluded and only histologically confirmed epithelial cancers were included (International Classification of Diseases for Oncology: M8010-8119, 8140-8570), leaving 337 cases. The PALGA database was used to identify the location of tumor tissue storage in the Dutch pathologic laboratories. For 273 cases, a PALGA record including information on the location of paraffin material could be identified within the PALGA database at the start of the collection of paraffin blocks. For 251 (92%) of 273 cases, paraffin blocks were collected. Failure to retrieve material was the result of the refusal of the pathology laboratory to cooperate (3 laboratories with material for 10 cases), the unavailability of suitable material (that is, only material from a biopsy, cytology, or a metastasis was present; 8 cases), not being able to locate the paraffin block at the laboratory (3 cases), and for 1 case, the reason was not recorded.

Material of 16 cases was discarded after revision. The collected material was unsuitable for analysis because it concerned a biopsy ($n = 2$) or a metastasis ($n = 2$), no tumor tissue was present ($n = 4$), or material contained <10% malignant cells ($n = 7$; tumor samples had to contain at least 10% malignant cells to decrease the possibility of missing mutations). Material from one case was reclassified as urothelial cell carcinoma. Thus, tumor DNA from 235 cases was available for further analysis (235 of 337 or 70% of all kidney cancer cases and 235 of

273 or 86% of the renal cell cancer cases with information on the location of the paraffin block).

All subcohort members who reported prevalent cancer (excluding skin cancer) at baseline were excluded from analyses (leaving 4,774 subcohort members).

Cases and subcohort members with incomplete or missing information on alcohol consumption were excluded from analysis, leaving 314 cases and 4,511 subcohort members available for analysis (14 cases were also part of the subcohort). Hazard ratios for renal cell cancer were calculated for intake of alcohol.

Case groups were defined as follows: total renal cell cancer, all histologically confirmed cases of renal cell cancer detected by linkage to cancer and pathology registries ($n = 314$); clear-cell renal cell cancer, tumor block collected and classified as clear-cell renal cell cancer after pathologic revision ($n = 176$); mutated clear-cell renal cell cancer, clear-cell renal cell cancer with a mutation in the *VHL* gene ($n = 106$), wildtype clear-cell renal cell cancer, clear-cell renal cell cancer without a mutation in the *VHL* gene ($n = 70$); methylated clear-cell renal cell cancer, clear-cell renal cell cancer with a methylated *VHL* promoter ($n = 14$); and unmethylated clear-cell renal cell cancer, clear-cell renal cell cancer without a methylated *VHL* promoter ($n = 124$).

Questionnaire. At baseline, cohort members completed a self-administered questionnaire on risk factors for cancer. The food-frequency section concentrated on habitual consumption during the preceding year. Consumption of alcoholic beverages was addressed by questions on beer, red wine, white wine, sherry, other fortified wines, liqueur, and liquor. The questionnaire data of all cases and subcohort members were key-entered twice and processed in a manner blinded with respect to case or subcohort status to minimize observer bias in the coding and interpretation of data. The questionnaire has been validated against a 9-d diet record (14). The Pearson r between the mean daily ethanol intake assessed by the questionnaire and that estimated by the 9-d record was 0.86 for all subjects and 0.78 for users of alcoholic beverages (14). Respondents who took alcoholic beverages less than once a month were considered nondrinkers. Four items from the questionnaire (that is, red wine, white wine, sherry, and liqueur) were combined into one wine variable because these items were substantially correlated. Mean daily alcohol consumption was calculated using the Dutch food composition table (15). On the basis of pilot study data, standard glass sizes were defined as 200 mL for beer, 105 mL for wine, 80 mL for sherry, and 45 mL for both liqueur and liquor, corresponding to 8, 10, 11, 7, and 13 g of alcohol, respectively.

***VHL* Analysis.** Paraffin blocks of tumors were collected from 51 pathology laboratories; the procedures have been described in detail elsewhere (16). We were able to collect material for 251 cases. One experienced pathologist (C.A. Hulsbergen-van de Kaa) revised all HE-stained slides according to the WHO Classification of Tumours of 2002 (17). The protocols for DNA isolation and mutation analyses have been described previously (16). Briefly, paraffin was removed with xylene, and tumor DNA was extracted by salt precipitation. The entire gene was amplified using six primer sets as described before (16). Samples were first subjected to PCR-single-strand conformational polymorphism

Table 1. Description of exposure variables and potential confounders in subcohort members, renal cell cancer cases, and renal cell cancer cases with tissue blocks collected according to sex; NLCS, 1986 to 1997

	Males [mean (SD)]			Females [mean (SD)]		
	Subcohort members (n = 2,273)	RCC cases (n = 211)	RCC cases with collected tumor material (n = 145)	Subcohort members (n = 2,238)	RCC cases (n = 103)	RCC cases with collected tumor material (n = 74)
Alcohol intake						
Total alcohol intake						
Drinkers of alcohol*	1,944 (85.5%)	178 (84.4%)	123 (84.8%)	1,507 (67.3%)	56 (54.4%)	42 (56.8%)
Alcohol intake (g/d) †	17.5 (16.9)	16.9 (15.5)	16.0 (15.3)	8.6 (10.4)	10.9 (12.6)	8.7 (10.2)
Alcohol intake from beer						
Drinkers of beer*	1,296 (57.0%)	114 (54.0%)	84 (57.9%)	222 (9.9%)	11 (10.7%)	9 (12.2%)
Intake of alcohol from beer (g/d) †	6.4 (9.9)	5.8 (10.1)	6.0 (10.8)	2.7 (4.5)	2.4 (3.0)	2.2 (3.1)
Alcohol intake from wine						
Drinkers of wine*	1,187 (52.2%)	109 (51.7%)	73 (50.3%)	1,423 (63.6%)	53 (51.5%)	40 (54.1%)
Alcohol intake from wine (g/d) †	7.6 (9.7)	6.4 (8.1)	6.2 (8.8)	7.0 (8.6)	8.0 (9.3)	6.5 (7.9)
Alcohol intake from liquor						
Drinkers of liquor*	1,324 (58.3%)	121 (57.4%)	87 (60.0%)	296 (13.2%)	11 (10.7%)	8 (10.8%)
Alcohol intake from liquor (g/d) †	12.6 (13.4)	13.6 (13.5)	11.6 (12.0)	8.1 (9.9)	14.7 (16.3)	10.7 (13.1)
Potential confounders						
Age at baseline	61.4 (4.2)	62.0 (3.8)	62.2 (3.8)	61.4 (4.3)	61.5 (3.9)	61.4 (3.9)
BMI	25.0 (2.6)	25.4 (2.7)	25.4 (2.6)	25.1 (3.5)	25.8 (3.3)	25.8 (3.2)
Current smokers of cigarettes*	829 (36.5%)	89 (42.2%)	57 (39.3%)	483 (21.6%)	25 (24.3%)	17 (23.0%)
Cigarettes/d †, ‡	17.1 (10.6)	19.3 (12.8)	19.7 (12.8)	11.5 (8.3)	12.7 (8.1)	12.5 (8.3)
Years of smoking †	33.8 (11.9)	35.7 (11.7)	35.4 (11.4)	28.0 (12.4)	28.8 (12.3)	27.3 (12.8)
Diagnosis of hypertension: yes*	525 (23.1%)	55 (26.1%)	36 (24.8%)	649 (29.0%)	39 (37.9%)	29 (39.2%)
Diagnosis of diabetes mellitus: yes*	77 (3.4%)	7 (3.3%)	6 (4.1%)	84 (3.8%)	4 (3.9%)	3 (4.1%)
Family history of RCC: yes*	14 (0.6%)	3 (1.4%)	2 (1.4%)	32 (1.4%)	1 (1.0%)	1 (1.4%)
Energy intake (including alcohol)	2,168 (511)	2,122 (483)	2,114 (490)	1,692 (397)	1,654 (395)	1,645 (394)

Abbreviation: RCC, renal cell cancer.

*Number (%).

†Among drinkers only.

‡Only for ever-smokers.

analysis, which was followed by direct sequencing in case of aberrant or unclear results. Separate PCRs were set up for single-strand conformational polymorphism and sequencing to reduce the risk for false-positive results. Mutations were identified by visual inspection of sequences provided by the ABI basecaller. After revision and *VHL* gene mutation analyses, data were available for 235 cases (16).

DNA methylation in the CpG island of the *VHL* gene promoter was determined by chemical modification of genomic DNA with sodium bisulfite and subsequent methylation-specific PCR analysis as described in detail elsewhere (18). In brief, 500 ng of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega), again treated with NaOH, precipitated with ethanol, and resuspended in H₂O. To enable methylation-specific PCR analysis on formalin-fixed, paraffin-embedded tissue, *VHL* methylation-specific PCR primers as described in ref. 18 and designed in a region of the promoter CpG island, of which methylation has been associated with inhibition of *VHL* gene expression, were adapted for nested methylation-specific PCR analysis. Primers are available upon request. *VHL* methylation-specific PCR reproducibility was 93% (37 of 40 duplicate methylation-specific PCRs).

Data Analysis. Confounders considered for multivariable analysis were age at baseline (years), sex, cigarette smoking (never, former, or current), body mass index (BMI; kg/m²), energy intake (kcal/d), kidney cancer in

first degree family (yes or no), a diagnosis of diabetes mellitus (yes or no), a history of hypertension (yes or no), educational level (four categories: primary school, junior high school, senior high school, higher vocational school, or university), and nonoccupational physical activity (four categories). Those variables that were associated with alcohol consumption, that were an independent risk factor of renal cell cancer, and that changed the risk estimates for the association of alcohol consumption and renal cell cancer by >10% were included as confounders in multivariable analyses. Using these criteria, confounders entered in the analyses were age, sex, and cigarette smoking.

Hazard ratios and corresponding 95% confidence intervals (95% CI) for renal cell cancer were estimated using Cox proportional hazard models processed with the STATA statistical software package (release 9.1, 2005, STATA Corporation) after testing the proportional hazards assumption using scaled Schoenfeld residuals (19). SEs were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling person-time from the cohort (20). To obtain *P* values for dose-response trends, ordinal exposure variables were fitted as continuous terms.

Tests for heterogeneity were done to evaluate differences between subtypes of tumors (e.g., *VHL* mutated versus *VHL* wildtype) using the competing risks procedure in STATA. However, the SE for the difference of the log-hazard ratios from this procedure assumes independence of both estimated hazard ratios, which would

Table 2. Multivariable adjusted hazard ratios and 95% CIs of renal cell cancer for alcohol consumption; NLCS on Diet and Cancer, 1986 to 1997

Variable	Alcohol consumption at baseline					Alcohol consumption at baseline			Alcohol consumption among stable users*		
	No. of cases/ person-years subcohort	Age and sex adjusted		Multivariable adjusted		No. of cases/ person-years subcohort	Multivariable adjusted		No. of cases/ person-years subcohort	Multivariable adjusted	
		HR	95% CI	HR [†]	95% CI		HR [‡]	95% CI		HR [†]	95% CI
Total alcohol intake (g/d)											
No alcohol intake	80/11,035	1	Ref	1	Ref	75/10,150	1	Ref	61/8,462	1	Ref
0.1-4.9	74/13,706	0.72	0.52-1.00	0.72	0.51-0.99	69/12,643	0.71	0.50-1.00	47/9,147	0.69	0.46-1.02
5-14.9	64/10,823	0.66	0.47-0.94	0.64	0.46-0.91	61/9,986	0.66	0.46-0.94	40/6,702	0.66	0.43-0.99
15-29.9	63/7,437	0.87	0.61-1.24	0.81	0.57-1.16	59/6,976	0.79	0.54-1.15	38/4,003	0.94	0.60-1.47
≥30	33/4,299	0.74	0.48-1.15	0.69	0.44-1.07	27/3,972	0.61	0.38-0.98	17/2,333	0.69	0.39-1.23
<i>P</i> for linear trend		0.34		0.17			0.09			0.40	
Alcohol increment per 10 g	314/47,299	0.98	0.90-1.06	0.96	0.89-1.05	291/43,728	0.94	0.86-1.02	203/30,647	0.98	0.88-1.09
Alcohol from beer (g/d)											
No alcohol from beer	189/31,533	1	Ref	1	Ref	174/29,157	1	Ref	126/21,406	1	Ref
0.1-4.9	81/10,208	0.95	0.70-1.30	0.95	0.69-1.29	77/9,448	0.98	0.71-1.35	50/6,009	1.03	0.70-1.52
5-14.9	36/4,215	0.98	0.65-1.46	0.96	0.64-1.43	32/3,912	0.93	0.61-1.43	23/2,433	1.10	0.66-1.83
≥15	8/1,344	0.67	0.32-1.41	0.63	0.30-1.33	8/1,209	0.69	0.32-1.45	4/799	0.55	0.19-1.56
<i>P</i> for linear trend		0.46		0.36			0.41			0.69	
Alcohol increment per 10 g	314/47,299	0.92	0.74-1.15	0.91	0.72-1.13	291/43,728	0.92	0.73-1.16	203/30,647	0.83	0.62-1.11
Alcohol from wine (g/d)											
No alcohol from wine	152/19,503	1	Ref	1	Ref	140/17,770	1	Ref	101/13,457	1	Ref
0.1-4.9	97/16,418	0.83	0.63-1.09	0.85	0.64-1.12	90/15,325	0.85	0.64-1.14	64/10,711	0.87	0.62-1.22
5-14.9	46/7,311	0.85	0.60-1.21	0.85	0.60-1.21	44/6,811	0.91	0.64-1.30	27/4,377	0.84	0.53-1.32
≥15	19/4,067	0.65	0.40-1.05	0.63	0.38-1.03	17/3,821	0.64	0.38-1.08	11/2,103	0.72	0.38-1.37
<i>P</i> for linear trend		0.06		0.06			0.12			0.23	
Alcohol increment per 10 g	314/47,299	0.90	0.76-1.07	0.89	0.75-1.06	291/43,728	0.87	0.73-1.03	203/30,647	0.88	0.69-1.12
Alcohol from liquor (g/d)											
No alcohol from liquor	182/30,543	1	Ref	1	Ref	170/28,057	1	Ref	131/20,708	1	Ref
0.1-4.9	51/7,473	0.84	0.60-1.18	0.84	0.60-1.17	48/7,002	0.80	0.57-1.13	24/4,666	0.60	0.38-0.94
5-14.9	32/4,602	0.80	0.54-1.20	0.78	0.52-1.16	30/4,350	0.73	0.48-1.10	20/2,699	0.79	0.48-1.30
≥15	49/4,681	1.17	0.82-1.67	1.10	0.77-1.57	43/4,318	0.98	0.68-1.43	28/2,574	1.11	0.70-1.76
<i>P</i> for linear trend		0.76		0.99			0.55			0.94	
Alcohol increment per 10 g	314/47,299	1.05	0.94-1.17	1.03	0.92-1.15	291/43,728	0.98	0.87-1.11	203/30,647	1.08	0.92-1.26

Abbreviations: HR, hazard ratio; Ref, reference category.

*Participants who reported that they had the same drinking habits at baseline and 5 y before baseline.

†Adjusted for sex, age, and cigarette smoking (never, ex, current). Hazard ratios for alcohol intake from beer, wine, and liquor are mutually adjusted for intake of alcohol from the other drinks.

‡Adjusted for sex, age, and cigarette smoking (never, ex, current), BMI (continuous), and energy intake (continuous, not including energy from alcohol). Hazard ratios for alcohol intake from beer, wine, and liquor are mutually adjusted for intake of alcohol from the other drinks. Because of missings for BMI and inconsistent or incomplete dietary information, only 291 cases and 4168 subcohort were available for analysis.

underestimate that SE and thus overestimate the *P* values for their difference. Therefore, these *P* values and the associated confidence intervals were estimated based on a bootstrapping method that was developed for the case-cohort design (21). For each bootstrap sample, *X* subcohort members were randomly drawn from the subcohort of *X* subjects and *Y* cases from the total of *Y* cases outside the subcohort, both with replacement, out of the data set of *X* + *Y* observations. The log-hazard ratios were obtained from this sample using STATA's competing risks procedure and recalculated for each bootstrap replication. The confidence interval and *P* value of the differences in hazard ratio of the subtypes were then calculated from the replicated statistics using the accelerated bias corrected method in STATA. Each bootstrap analysis was based on 1,000 replications.

Results

Among men, the proportion of alcohol drinkers was approximately equal in the subcohort to that in the renal cell cancer cases, whereas in women, the proportion of alcohol drinkers was considerably higher in the subcohort than in the renal cell cancer cases. Average alcohol intake among consumers was slightly higher in male subcohort members than in male renal cell cancer cases, whereas in females, the average alcohol intake was lower in the subcohort (Table 1). Renal cell cancer cases had a higher BMI at baseline, were more often current smokers, and were more often diagnosed with hypertension than subcohort members. In males, energy intake (including energy from alcohol) was higher in subcohort members than in cases.

Multivariable hazard ratios for alcohol intake adjusted for sex, age, and cigarette smoking were slightly decreased, although not statistically significant (Table 2). Cohort members with an alcohol intake of >30 g/d had a multivariable adjusted hazard ratio of 0.69 (95% CI, 0.44-1.07) compared with cohort members who did not drink alcohol (P for trend, 0.17). Similar hazard ratios were observed when BMI and energy intake were added as covariates to the multivariable model. Relative risks for men who consumed up to 5, 15, 30, and >30 g/d were 0.96, 0.84, 0.98, and 0.75, respectively, compared with nondrinkers (95% CI for the top category, 0.44-1.28). Relative risks for women who consumed up to 5, 15, 30, and >30 g/d were 0.52, 0.40, 0.68, and 1.08, respectively, compared with nondrinkers (95% CI for the top category, 0.44-2.64). The interaction, however, was not statistically significant (P for interaction, 0.14).

Alcohol intake from beer and wine (each adjusted for alcohol intake from other sources) were also associated with nonsignificantly decreased risks, whereas the highest alcohol intake group from liquor was not inversely associated with renal cell cancer risk. The hazard ratios did not change substantially when analyses were restricted to subjects who were abstainers or who reported stable use during 5 years preceding baseline, although the confidence intervals were wider because of decreased power (Table 2).

We did not show a difference about hazard ratios for clear-cell renal cell cancer with and without *VHL* mutations (Table 3: P for heterogeneity between subtypes, 0.53). Some heterogeneity seemed to be present for

alcohol intake from beer, although this was not statistically significant with a P for heterogeneity of 0.30. We observed an increased risk for clear-cell renal cell cancer without *VHL* mutations, with the hazard ratios for cohort members who consumed up to 5 and ≥ 5 g of alcohol from beer being 1.71 (95% CI, 0.82-3.56) and 2.74 (95% CI, 1.35-5.57), respectively, compared with nondrinkers of beer (P for trend, 0.005). Alcohol originating from wine and liquor was not associated with renal cell cancer risk, with or without *VHL* mutations. The prevalence of *VHL* promoter hypermethylation was the highest in cases that reported an alcohol intake of ≥ 15 g/d: 20%, compared with 11.1% and 4.5% in cases that reported no or 0.1 to 14.9 g alcohol per day, respectively. In multivariate analysis (Table 4), alcohol intake was associated with a decreased risk for clear-cell renal cell cancer without hypermethylation of the *VHL* gene, the hazard ratios for cohort members who consumed up to 5 and ≥ 5 g of alcohol from beer were 0.81 (95% CI, 0.52-1.25) and 0.58 (95% CI, 0.34-0.99), respectively, compared with nondrinkers (P for trend, 0.04). Hazard ratios for clear-cell renal cell cancer with a hypermethylated promoter region of the *VHL* gene were mostly greater than one, although the number of cases was small and the P for heterogeneity was far from statistically significant.

Discussion

In this study, we observed that alcohol intake was associated with a decreased risk for renal cell cancer, although not statistically significant. We did not show a

Table 3. Multivariable adjusted hazard ratios and 95% CIs of renal cell cancer according for alcohol consumption according to *VHL* mutational status; NLCS on Diet and Cancer, 1986 to 1997

Variable and level	Person-years subcohort	Clear-cell carcinoma			Clear-cell carcinoma, <i>VHL</i> mutated			Clear-cell carcinoma, <i>VHL</i> wildtype			P for heterogeneity
		No. of cases	HR*	95% CI	No. of cases	HR*	95% CI	No. of cases	HR*	95% CI	
Total alcohol intake (g/d)											
No alcohol intake	11,035	46	1	Ref	28	1	Ref	18	1	Ref	
0.1-4.9	13,706	46	0.76	0.50-1.16	27	0.73	0.42-1.25	19	0.82	0.43-1.58	
5-14.9	10,823	33	0.59	0.37-0.93	15	0.42	0.22-0.81	18	0.86	0.45-1.64	
≥ 15	11,735	51	0.74	0.48-1.14	36	0.83	0.48-1.43	15	0.59	0.29-1.19	0.53
P for linear trend			0.16			0.44			0.16		
Alcohol increment per 10 g	47,299	176	0.94	0.83-1.05	106	0.99	0.86-1.14	70	0.83	0.68-1.01	0.049
Alcohol from beer (g/d)											
No alcohol from beer	31,533	105	1	Ref	68	1	Ref	37	1	Ref	
0.1-4.9	10,208	42	1.00	0.65-1.56	25	0.75	0.44-1.28	17	1.71	0.82-3.56	
≥ 5	5,559	29	1.19	0.74-1.91	13	0.67	0.35-1.29	16	2.74	1.35-5.57	0.30
P for linear trend			0.54			0.19			0.005		
Alcohol increment per 10 g	47,299	176	1.00	0.77-1.29	106	0.92	0.56-1.49	70	1.09	0.88-1.35	0.69
Alcohol from wine (g/d)											
No alcohol from wine	19,503	86	1	Ref	54	1	Ref	32	1	Ref	
0.1-4.9	16,418	57	0.85	0.59-1.21	31	0.73	0.46-1.17	26	1.04	0.60-1.79	
≥ 5	11,379	33	0.66	0.43-1.00	21	0.67	0.40-1.12	12	0.63	0.31-1.30	0.68
P for linear trend			0.049			0.10			0.25		
Alcohol increment per 10 g	47,299	176	0.80	0.60-1.06	106	0.87	0.63-1.20	70	0.67	0.38-1.17	0.49
Alcohol from liquor (g/d)											
No alcohol from liquor	30,543	104	1	Ref	62	1	Ref	42	1	Ref	
0.1-4.9	7,473	30	0.94	0.61-1.45	14	0.67	0.36-1.24	16	1.43	0.79-2.57	
≥ 5	9,283	42	0.96	0.64-1.42	30	1.06	0.65-1.72	12	0.77	0.39-1.53	0.10
P for linear trend			0.81			0.93			0.59		
Alcohol increment per 10 g	47,299	176	0.99	0.85-1.16	106	1.09	0.92-1.29	70	0.79	0.57-1.09	0.04

NOTE: Relative risks for alcohol intake from beer, wine, and liquor are mutually adjusted for intake of alcohol from the other drinks.

*Adjusted for sex, age, and cigarette smoking (never, ex, current).

Table 4. Multivariable adjusted hazard ratios and 95% CIs of renal cell cancer according for alcohol consumption according to methylation status of the promoter region of the *VHL* gene; NLCS on Diet and Cancer, 1986 to 1997

Variable and level	Person-years subcohort	Clear-cell carcinoma, <i>VHL</i> methylated			Clear-cell carcinoma, <i>VHL</i> not methylated			<i>P</i> for heterogeneity
		No. of cases	HR*	95% CI	No. of cases	HR*	95% CI	
Alcohol intake								
No.	11,035	4	1	Ref	32	1	Ref	
0.1-14.9	24,529	3	0.28	0.06-1.35	64	0.81	0.52-1.25	
≥15	11,735	7	1.43	0.31-6.62	28	0.58	0.34-0.99	0.29
<i>P</i> for linear trend			0.53			0.04		
Alcohol increment per 10 g	47,299	14	1.08	0.80-1.45	124	0.82	0.70-0.97	0.17
Alcohol intake from beer								
Alcohol increment per 10 g	47,299	14	1.12	0.59-2.12	124	0.84	0.62-1.12	0.34
Alcohol intake from wine								
Alcohol increment per 10 g	47,299	14	1.30	0.74-2.29	124	0.68	0.45-1.03	0.32
Alcohol intake from liquor								
Alcohol increment per 10 g	47,299	14	0.84	0.41-1.72	124	0.90	0.72-1.12	0.09

NOTE: Relative risks for alcohol intake from beer, wine, and liquor are mutually adjusted for intake of alcohol from the other drinks.

*Adjusted for sex, age, and cigarette smoking (never, ex, current).

statistical significant difference in hazard ratios for renal cell cancer with or without mutations in the *VHL* gene, although hazard ratios of alcohol intake from beer were increased in clear-cell renal cell cancer without mutations in the *VHL* gene. Hazard ratios were significantly decreased in clear-cell renal cell cancer without methylation of the promoter region of the *VHL* gene. To our knowledge, this is the first study investigating the association between alcohol intake and mutational status and promoter-region hypermethylation of the *VHL* gene in renal cell cancer.

The overall results of this analysis are in agreement with a recent publication of the Pooling Project of Prospective Studies on Diet and Cancer, including 12 prospective cohort studies (1). The NLCS was included in that analysis, as well as most prospective studies that have published results on this association thus far (22-27). Three prospective cohort studies were not included in the analysis of the pooling project (27-29), two of which (28, 29) did not observe an association between alcohol intake and renal cell cancer risk, but the numbers of cases in these studies were rather small (19 and 44 cases, respectively). The third prospective cohort study did observe an inverse association (27). Results among case-control studies are more heterogeneous. Most case-control studies observed no association or a positive association (30-45), whereas some published a negative association between alcohol intake and renal cell cancer risk (46-50). The International Renal Cancer Case-Control study published a statistically significant negative association in women and no association in men (51). The discrepancies between case-control and prospective studies may be explained by the fact that case-control studies are more liable to recall and selection bias.

In our analysis, we observed that the association did not change when the analysis was restricted to the cohort members who reported a stable use. This does suggest that the effect is not limited to the latest phases of renal cell cancer development. It would have been interesting to study the subgroups who reported a lower or higher consumption at baseline than 5 years before, but the numbers in these subgroups were too small for a meaningful analysis.

In the current study, it was possible to study the association with specific subtypes of renal cell cancer according to histology and (epi)genetic aberrations of the *VHL* gene. The results in clear-cell renal cell cancer were not significantly different from renal cell cancer overall. Likewise, we did not observe a statistically significant difference in the association of alcohol intake and clear-cell carcinoma, with or without mutations in the *VHL* gene. Unexpectedly, alcohol consumption from beer was associated with an increased risk for renal cell cancer without a *VHL* mutation. This could indicate an effect of specific constituents in beer. For example, nitrosamines have been associated with increased risk for cancer (52) and was present in large quantities in Dutch beer in the past (53). Although some studies reported a negative association between beer intake and renal cell cancer risk that was weaker than the association between total alcohol intake and renal cell cancer risk (1, 46), other studies did not observe a difference (39, 50). In these studies, only the association with renal cell cancer was investigated, disregarding histology and *VHL* mutation status. It cannot be excluded, however, that this result is a chance finding because the number of exposed cases with this specific endpoint is small.

The negative association between alcohol intake and renal cell cancer risk was strongest and also statistically significant in the subgroup of clear-cell renal cell cancer without promoter hypermethylation of the *VHL* gene. In the subgroup with promoter hypermethylation, risks were mostly higher than one, but the number of cases was very small, hampering more definitive conclusions. The observed heterogeneity between the subgroups according to methylation status are, however, in agreement with the theory that alcohol is associated with an increased risk of hypermethylation of gene promoters such as the *VHL* gene (8). In addition, other genes that may play a role in renal carcinogenesis can be inactivated by promoter hypermethylation as well, for example, *APAF1*, *JUP*, *RASSF1*, *COL1A1*, and *TIMP3* (54). Alcohol consumption may also be associated with hypermethylation of the promoter regions of these and other tumor suppressor genes and thus promote cancer development in a specific subgroup of renal cell cancer (those with

hypermethylation of tumor suppressor genes), although it may be overall associated with a decreased risk for renal cell cancer.

There are different explanations for the negative association between alcohol intake and renal cell cancer risk. Moderate alcohol intake is associated with a lower risk for diabetes type II and may be associated with increased insulin sensitivity (55). Hyperinsulinemia is possibly associated with risk for kidney cancer (56) and might therefore explain the observed inverse association between alcohol and risk for cancer. Another possible mechanism is the diuretic effect of alcohol, which may decrease the exposure of renal cells to carcinogens because of dilution and a shorter duration of exposure. In a pooled analysis of two US prospective studies, no association was observed between water intake and risk for renal cell cancer (22), making this mechanism less likely.

Although tumors with a *VHL* mutation in our population were slightly larger than tumors without a *VHL* mutation (16), *VHL* mutational status was not associated with nuclear grade, tumor-node-metastasis, stage, or survival (16, 57). In previous analyses, the association of cigarette smoking, hypertension and use of antihypertensive medication, and the intake of carotenoids and vitamins with *VHL* mutational status in renal cell cancer has been investigated. Cigarette smoking was associated with renal cell cancer risk for men but not specifically with *VHL* gene mutations, irrespective of sex, suggesting that smoking may cause renal cell cancer independent of *VHL* gene mutations (58). With respect to hypertension and use of antihypertensive medications, the association of hypertension was stronger in renal cell cancer cases with *VHL* gene mutations, whereas use of diuretics was associated with renal cell cancer without *VHL* gene mutations (59). Results were suggestive of higher relative risks for wildtype *VHL* tumors with α -carotene, β -cryptoxanthin, folate, and supplemental vitamin C and multivitamin intake (60).

These results from the NLCS are most likely not affected by selection or information bias. Selection bias is unlikely, given the high level of follow-up in terms of cases and subcohort person-years (13). In theory, selection bias may have occurred in the collection of tissue samples. For 235 (70%) of the 337 cases, tumor material could be collected. There was no indication for bias in the selection of cases with tumor material according to the risk factors and potential confounders studied. Information bias is unlikely in our study because the information with respect to the risk factors was collected before the diagnosis of renal cell cancer. Alcohol consumption and information about potential confounders were self-reported, however, and misclassification of exposure is a potential source of bias. The questionnaire has been validated against a 9-day diet record (14), and the correlation with respect to alcohol consumption was high.

VHL mutations were determined with sequencing using single-strand conformational polymorphism as a screening tool. This was considered sensible because, in a pilot study with 20 cases, we never detected a mutation by direct sequencing after a negative result on the single-strand conformational polymorphism (0% false negatives; ref. 16).

In this prospective study, we observed that alcohol consumption is inversely associated with the risk for

renal carcinoma. There was overall no heterogeneity with respect to mutation status of the *VHL* gene, but alcohol consumption from beer was positively associated with renal cell cancer with wildtype *VHL* gene. Alcohol consumption was negatively associated with renal cell cancer without hypermethylation of the *VHL* gene. Replication of our results in larger data sets is required. It should also be investigated whether alcohol consumption is associated with hypermethylation of other tumor suppressor genes that are involved in renal carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank the participants of this study; the cancer registries (Integraal Kankercentrum Amsterdam, Integraal Kankercentrum Limburg, Integraal Kankercentrum Midden Nederland, Integraal Kankercentrum Noord-Nederland, Integraal Kankercentrum Oost, Integraal Kankercentrum Rotterdam, Integraal Kankercentrum Stedendriehoek Twente, Integraal Kankercentrum West, Integraal Kankercentrum Zuid, and Vereniging van Integrale Kankercentra); the Netherlands nationwide registry of pathology (PALGA) for providing cancer follow-up data; the pathology laboratories for providing the tissue samples (for a complete list see ref. 16); Dr. E. Dorant, C.A. de Brouwer, Prof. Dr. A. Geurts van Kessel, and Prof. Dr. D.J. Ruiters for their preparatory work for this study; K.P. van Houwelingen, H. Gorissen, and K. Wouters for the laboratory analysis; Dr. A. Volovics for statistical advice; S. van de Crommert, H. Brants, J. Nelissen, C. de Zwart, M. Moll, and A. Pisters for assistance; and H. van Montfort, L. van den Bosch, and J. Berben for programming assistance.

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