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Cancer and Diet

Promoter CpG island methylation in ion transport mechanisms and associated dietary intakes jointly influence the risk of clear-cell renal cell cancer

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Abstract

Background: Sodium intake, but not potassium or fluid intake, has been associated with higher renal cell cancer (RCC) risk. However, risk factors may differ by molecular subtypes of the tumour. In renal physiology, electrolyte and water homeostasis is facilitated by ion transport mechanisms (ITM). Aberrant regulation of ITM genes, for example by promoter CpG island methylation, may modify associations between sodium, potassium and fluid intake and RCC risk.

Methods: We identified *ARHGDIG*, *ATP1A1*, *SCNN1B* and *SLC8A3* as ITM genes exhibiting RCC-specific promoter methylation and down-regulation. Methylation-specific polymerase chain reaction (PCR) was used to analyse promoter CpG island methylation in tumour DNA of 453 RCC cases from the Netherlands Cohort Study ($n = 120\,852$) after 20.3 years of follow-up. Diet was measured at baseline using food-frequency questionnaires. Cox regression analyses were restricted to clear-cell (cc)RCC ($n = 306$) and stratified by tumours with no, low (1 gene) and high (≥ 2 genes) methylation.

Results: Sodium intake (high vs low) increased ccRCC risk particularly in tumours with a high methylation index: hazard ratio (HR) [95% confidence interval (CI)]: 2.04 (1.16–3.58), whereas heterogeneity across the methylation index was not significant (*P*-heterogeneity $= 0.26$). Potassium intake was differentially associated with ccRCC risk (*P*-heterogeneity $= 0.008$); the risk for high (vs low) potassium intake was low for unmethylated tumours [HR (95% CI): 0.60 (0.36–1.01)], but high for tumours with a high methylation index

[HR (95% CI): 1.60 (0.96–2.65)]. Risks similarly differed for fluid intake, though not significantly (*P*-heterogeneity = 0.54).

Conclusions: Our findings suggest for the first time that dietary intakes are differentially associated with ccRCC risk according to molecular subtypes defined by ITM gene-specific promoter methylation.

Key words: Promoter CpG island methylation, clear-cell renal cell cancer risk, ion transport mechanisms, dietary intakes, prospective cohort

Key messages

- Potassium intake was differentially associated with ccRCC risk when the methylation status of the investigated ITM genes was considered.
- Specific dietary intakes and gene-specific promoter methylation may have a joint influence in relation to ccRCC risk.
- This study provides the first indications of a possible biological rationale for the role of ITM in the development of ccRCC.

Introduction

The regulation of electrolyte and water homeostasis is a key aspect of renal physiology and is, in the kidney, achieved by tubular reabsorption and secretion of water and solutes, such as sodium and potassium.[1] Transcellular reabsorption and secretion of solutes is facilitated by protein carriers or ion-specific channels.[1] Ion- transport mechanisms (ITM), including ion channels, transporters, exchangers, pumps and associated enzymes, have recently been put forward as novel mechanisms underlying carcinogenesis.[2] Given their role in renal physiology, ITM may be involved in the pathophysiology of renal cell cancer (RCC).

Previously, we reported that sodium intake, but not potassium or fluid intake, was associated with RCC risk in the Netherlands Cohort Study on diet and cancer (NLCS).[3] However, consensus has emerged that to better understand the aetiological mechanisms of human cancers, the molecular characterization of the tumour should be considered.[4] Therefore, it is worthwhile to further investigate these risk factors based on such characterization.

Promoter CpG island methylation is a frequently observed mechanism in gene silencing and occurs more often than genetic inactivation in many tumour types.[5–7] Few studies have focused on promoter CpG island methylation of genes involved in ITM in relation to RCC.[8,9] Promoter CpG island methylation of genes involved in ITM may be particularly useful for the molecular characterization of RCC when studying risk factors related to ITM, as aberrant regulation of ITM gene expression by promoter CpG island methylation may sensitize individuals to the effects of dietary intakes of sodium, potassium and fluid.

In the present study, we use data from the NLCS to investigate if associations between dietary intakes of sodium, potassium and fluid and the RCC risk differ among tumours according to a promoter CpG island methylation index of genes regulating ion transport or homeostasis.

Methods

Study design and study population

The NLCS is a prospective cohort study that was initiated in 1986 and included 120 852 participants aged 55–69 years at baseline.[10] The NLCS was designed as case-cohort study for efficiency in questionnaire processing and follow-up. Cases were derived from the entire cohort, whereas a subcohort of 5000 subjects was randomly sampled at baseline to estimate person-years at risk for the entire cohort.[11] Subcohort members were regularly followed up for vital status information, whereas all cohort members were followed up for cancer occurrence using record linkage with the Netherlands cancer registry and with the Dutch pathology registry (PALGA).[12] The coverage of cohort members by the Netherlands cancer registry and PALGA to establish cancer follow-up is estimated to be over 96%.[13] Cases and subcohort members with prevalent cancer (excluding skin cancer) at baseline were excluded. A

unique population-based collection of DNA material of RCC cases is nested within the NLCS. Initially, this collection of DNA material included only cases from the first 11.3 years of follow-up,[14] yet recently efforts were made to expand the collection up to 20.3 years of follow-up.

Tissue collection and DNA isolation

A total of 608 RCC cases were identified within the NLCS between 1986 and 2006. Only histologically confirmed epithelial RCC cases ($n = 568$) were eligible for the collection of formalin-fixed paraffin-embedded (FFPE) tumour tissues from ~ 50 pathology laboratories throughout The Netherlands. Overall, 79.8% of the FFPE tumour tissues were retrieved ($n = 453$), including 80.6% clear-cell (cc)RCC, 13.3% papillary (p)RCC, 3.3% chromophobe (chr)RCC and 2.8% other or undefined RCC. Adjacent normal FFPE tissues were also collected for 76.4% of the cases with FFPE tumour tissues. A detailed description of the tissue collection is available as Supplementary data at *IJE* online.

Methods used for DNA isolation of FFPE tissues from RCC cases identified during the first 11.3 years of follow-up included in the initial collection have been described previously.[14] For recently added RCC cases, vital tissue areas were dissected before DNA isolation. DNA was isolated using the QIAamp DNA Mini Kit (Qiagen), according to manufacturer's instructions.

Dietary assessment

All NLCS participants returned a mailed, self-admini stered, baseline questionnaire, including a 150-item, semi-quantitative food-frequency questionnaire (FFQ), which was used to assess intakes of sodium, potassium and fluid before diagnosis. Participants with incomplete dietary questionnaires were excluded, leaving 4439 subcohort members and 434 cases eligible for analyses.[15] The FFQ ranked individuals adequately according to dietary intakes when compared with 9-day dietary records,[15] and reflected nutrient intakes for at least 5 years.[16] Average daily nutrient intakes were calculated using the Dutch food composition table 1986–87[17] and defined as intakes through foods and beverages per day, including amounts naturally present in foods and beverages plus amounts added during food processing by food manufacturers. Salt (sodium chloride) added during home preparation and before consumption (i.e. discretionary salt intake) was assessed separately using specific questions.[3,18]

Gene selection

Potential candidate genes involved in common ITM mechanisms were selected from the KEGG pathway database.[19] Four of these genes were selected based on a literature search showing evidence for promoter DNA hypermethylation: *Rho GDP dissociation inhibitor gamma* (*ARHGDIG*), which is involved in vasopressin-related water reabsorption; *ATPase Na+/K+ transporting alpha 1 polypeptide* (*ATP1A1*) and *sodium channel non-voltage-gated 1 beta subunit* (*SCNN1B*), which are involved in aldosterone-regulated sodium reabsorption; and *solute carrier family 8 member 3* (*SLC8A3*), which is a sodium to calcium exchanger in the calcium signalling pathway. For *ATP1A1*, *SCNN1B* and *SLC8A3*, a significant, negative correlation was observed between promoter CpG island methylation and gene expression in the ccRCC samples from The Cancer Genome Atlas (TCGA), indicating a potential functional epigenetic control for these genes.[20]. This significant negative correlation between expression and DNA hypermethylation was not present at the promoter regions of *ARHGDIG*; however, only limited data were available for these key loci.

Promoter methylation

Promoter CpG island hypermethylation, in short promoter methylation, was analysed using nested methylation-specific polymerase chain reaction (PCR; MSP), as previously described elsewhere.[21–23] MSP primer design was based on the MBD-affinity massive parallel sequencing data. Primer sequences and MSP conditions are shown in Supplementary Table S1, available as Supplementary data at *IJE* online. MSP analyses of *ARHGDIG*, *ATP1A1*, *SCNN1B* and *SLC8A3* were performed successfully for 98.5%, 97.8%, 92.7% and 96.7% of the 453 RCC cases, respectively. Reproducibility was 99.7% (*ATP1A1*) to 100% (other genes) in ~ 40 samples. A methylation index was calculated combining all four individual genes into three subgroups representing tumours with no (no gene methylated), low (one gene methylated) and high (≥ two genes methylated) methylation. Additionally, we successfully performed MSP analyses of all genes in adjacent normal FFPE tissues of 42 ccRCC cases selected to represent the full spectrum of the methylation index, to evaluate if the observed promoter methylation was cancer specific.

Statistical analysis

All analyses were conducted in Stata version 12 (Stata Corp., College Station, TX). Associations between sodium, potassium and fluid intake and RCC risk were tested for total RCC and by histological subtype. Analyses including the

methylation index were restricted to ccRCC cases, as for other histological subtypes the statistical power was too low. Hazard ratios (HR) and 95% confidence intervals (CI) were estimated using Cox proportional hazards analyses adjusted for the case-cohort design.[24] Analyses were performed for an age- and sex-adjusted model and a multivariable-adjusted model, including a priori selected potential confounders: age, sex, total energy intake, body mass index (BMI), smoking (status, intensity and duration), self-reported doctor's diagnosis of hypertension and/or use of antihypertensive medication, and alcohol consumption. Analyses regarding sodium intake were additionally adjusted for discretionary salt intake in sensitivity analyses. The proportional hazards assumption was tested using the scaled Schoenfeld residuals. A violation was apparent for alcohol consumption, which was therefore analysed as time-dependent covariate. Tests for heterogeneity were performed to evaluate differences across tumours with different promoter methylation profiles, using an adapted version of the competing risks procedure in Stata developed for the case-cohort design, as described previously.[25,26] All tests were two-sided.

Results

The prevalences of promoter methylation of *ARHGDIG*, *ATP1A1*, *SCNN1B* and *SLC8A3* in ccRCC tumours were 23.1%, 15.9%, 46.0% and 18.2%, respectively (Table 1). To verify these proportions, probes matching the MSP regions were identified in the TCGA ccRCC database. For *SLC8A3*, a probe in the same region as the MSP amplicon indicated a prevalence of 16.6–22.5%. Neighbouring probes were identified for *ARHGDIG* and *SCNN1B*, with estimated prevalences of 12.8–22.1% and 22.0–27.8%, respectively. For *ATP1A1*, no suitable probes could be identified. We observed substantial differences in proportions of promoter methylation among the histological subtypes of RCC, most clearly for *ARHGDIG* and *SLC8A3* ($P = 0.005$ and 0.004, respectively). The methylation index did not differ across the histological subtypes ($P = 0.109$).

At baseline, ccRCC cases and subcohort members were relatively similar, except that ccRCC cases were more frequently male (Table 2). Among ccRCC cases, those with a high methylation index had a higher baseline potassium intake, a higher BMI and less frequently hypertension, compared with those without promoter methylation.

The overall associations between sodium, potassium and fluid intake and ccRCC risk showed a higher HR for high (vs low) sodium intake [HR (95% CI): 1.42 (1.06-1.89)] and a significantly increasing trend in HRs over the successive tertiles (P-trend = 0.02; Table 3). There was no association between potassium and fluid intake and ccRCC risk. Associations regarding pRCC and

Table 1. Proportions of promoter methylation of *ARHGDIG*, *ATP1A1*, *SCNN1B* and *SLC8A3* in renal cell tumours and in histological subtypes of renal cell tumours

		Histological subtypes			
Gene	Overall RCC, *n* (%)	ccRCC, *n* (%)	pRCC, *n* (%)	chrRCC, *n* (%)	***P*-value**[a]
Total *N*	453 (100.0)	365 (80.6)	60 (13.3)	15 (3.3)	–
ARHGDIG					
no	351 (78.7)	277 (76.9)	54 (91.5)	9 (60.0)	
yes	95 (21.3)	83 (23.1)	5 (8.5)	6 (40.0)	0.005
ATP1A1					
no	373 (84.2)	301 (84.1)	48 (82.8)	14 (93.3)	
yes	70 (15.8)	57 (15.9)	10 (17.2)	1 (6.7)	0.68
SCNN1B					
no	237 (56.4)	182 (54.0)	39 (67.2)	10 (71.4)	
yes	183 (43.6)	155 (46.0)	19 (32.8)	4 (28.6)	0.09
SLC8A3					
no	369 (84.3)	288 (81.8)	57 (96.6)	14 (93.3)	
yes	69 (15.8)	64 (18.2)	2 (3.4)	1 (6.7)	0.004
Methylation index[b]					
0	178 (44.1)	134 (41.2)	32 (58.2)	7 (50.0)	
1	107 (26.5)	88 (27.1)	14 (25.5)	4 (28.6)	
2-4	119 (29.5)	103 (31.7)	9 (16.4)	3 (21.4)	*0.109*

RCC, renal cell cancer; ccRCC, clear-cell RCC; pRCC, papillary RCC; chrRCC, chromophobe RCC; *ARHGDIG*, Rho GDP dissociation inhibitor gamma; *ATP1A1, ATPase Na+/K+ transporting alpha 1 polypeptide; SCNN1B, sodium channel non-voltage-gated 1 beta subunit; SLC8A3, solute carrier family 8 member 3.*

[a]*P*-value for difference among histological subtypes calculated using Fisher's exact test.

[b]Numbers do not add up to total due to missing values.

Table 2. Baseline characteristics of subcohort members, total clear-cell renal cell cancer cases and clear-cell renal cell cancer cases according to methylation index in the Netherlands Cohort Study, 1986-2006

Baseline characteristics, mean (SD)	Subcohort members	Total ccRCC	Methylation index[a,b]			*P*-value[c]
			No methylation	Low methylation	High methylation	
Total (*N*)	3980	306	110	74	90	
Age (years)	61.3 (4.2)	60.7 (3.9)	60.7 (3.7)	61.4 (4.0)	60.4 (3.9)	0.26
Male sex (%)	49.1	63.7	62.7	62.2	66.7	0.79
Dietary intakes						
Sodium intake (g/d)[d]	2.3 (0.6)	2.5 (0.6)	2.4 (0.7)	2.5 (0.6)	2.5 (0.6)	0.22
Discretionary salt intake (g/d)[e]	2.8 (2.7)	2.7 (2.9)	2.6 (2.6)	2.7 (3.6)	2.5 (2.6)	0.79
Fluid intake (ml/d)	2087 (487)	2103 (471)	2077 (525)	2102 (361)	2139 (455)	0.50
Potassium intake (mg/d)[d]	3529 (602)	3607 (651)	3490 (633)	3614 (674)	3742 (636)	0.02
Alcohol consumption (g ethanol/d)[f]	13.5 (15.1)	15.1 (15.0)	16.5 (17.2)	14.8 (15.2)	14.0 (12.1)	0.91
Total energy intake (kcal/d)	1926 (515)	1987 (507)	2024 (581)	1946 (446)	1978 (473)	0.68
Lifestyle factors						
BMI (kg/m^2)	25.0 (3.1)	25.5 (3.0)	24.8 (2.8)	25.9 (3.5)	25.8 (2.8)	0.04
Cigarette smoking						
Status: current (%)	27.3	29.4	28.2	33.8	30.0	0.72
Duration in current smokers (years)	38.7 (9.7)	39.7 (7.7)	40.3 (7.8)	41.0 (7.9)	37.7 (8.4)	0.16
Intensity in current smokers (cigarettes/d)	15.2 (8.8)	15.9 (7.8)	17.3 (8.1)	16.3 (8.5)	13.6 (6.5)	0.24
Hypertension: yes (%)	32.1	35.6	44.6	35.1	25.6	0.02
Prescribed low-salt diet: yes (%)	7.6	9.5	10.0	8.1	8.9	0.91

d, day; ccRCC, clear-cell renal cell cancer; sd, standard deviation; BMI, body mass index; *ARHGDIG, Rho GDP dissociation inhibitor gamma; ATP1A1, ATPase Na+/K+ transporting alpha 1 polypeptide; SCNN1B, sodium channel non-voltage-gated 1 beta subunit; SLC8A3, solute carrier family 8 member 3*.

[a]The methylation index of a tumour is based on frequencies of methylation in *ARHGDIG*, *ATP1A1*, *SCNN1B* and *SCL8A3*; no, low and high methylation corresponds to methylation in 0, 1 and ≥ 2 genes, respectively.

[b]*N* does not correspond with the overall *N*, due to missing values.

[c]Differences tested using Kruskal-Wallis test (continuous) and χ^2-test (categorical).

[d]Intakes are energy-adjusted by using the residual mean method.

[e]Salt intake refers to sodium-chloride intake.

[f]In consumers only (*n* subcohort = 3033).

chrRCC are shown in Supplementary Table S2, available as Supplementary data at *IJE* online.

Heterogeneous associations between dietary intakes and ccRCC risks for tumours with a different methylation index were particularly observed for potassium intake (*P*-heterogeneity = 0.008; Table 3). In tumours without promoter methylation, high (vs low) potassium intake was associated with a lower ccRCC risk [HR (95% CI): 0.60 (0.36–1.01), *P*-trend = 0.05], whereas in tumours with a high methylation index, high (vs low) potassium intake was associated with a higher ccRCC risk [HR (95% CI): 1.60 (0.96–2.65), *P*-trend = 0.06]. For fluid intake, ccRCC risks were not statistically different by tumours with a different methylation index (*P*-heterogeneity = 0.54), though HRs for high (vs low) fluid intake varied between tumours without promoter methylation and tumours with a high methylation index in the same direction as for potassium intake [HR (95% CI): 0.66 (0.39–1.12) and 1.53 (0.79–3.01), *P*-trend = 0.15 and 0.22; respectively]. High (vs low) sodium intake was associated with a higher ccRCC risk particularly in tumours with a high methylation index [HR (95% CI): 2.04 (1.16–3.58), *P*-trend = 0.01]; however heterogeneity across the methylation index was not significant (*P*-heterogeneity = 0.26). High (vs low) sodium intake was also associated with a higher ccRCC risk for tumours without promoter methylation and for tumours with a low methylation index [HR (95% CI): 1.29 (0.81– 2.05) and 1.55 (0.90–2.65), *P*-trend = 0.29 and 0.10; respectively], though risk estimates were much lower compared with tumours with a high methylation index.

The contribution of each of the four individual genes to the methylation index is evaluated in single gene analyses (Tables 4 and 5). High (vs low) sodium intake increased ccRCC risk, regardless of promoter methylation of any of the genes, but in tumours with *ATP1A1* promoter methylation, the ccRCC risk for high (vs low) sodium intake was markedly high [HR (95% CI): 3.19 (1.43–7.12)]. However, the number of cases with *ATP1A1* promoter methylation in this analysis was low and the test for heterogeneity not significant. Furthermore, the observed heterogeneity in the association between high (vs low) potassium intake and ccRCC risk by methylation index was largely determined by

Table 3. Hazard ratios and 95% confidence intervals of renal cell cancer risk, clear-cell renal cell cancer risk and clear-cell renal cell cancer risk according to a methylation index, in the Netherlands Cohort Study, 1986-2006

Dietary intakes	Person-years[a]	RCC cases			ccRCC cases			Methylation index[a]									*P*-value[c]
								No methylation			Low methylation			High methylation			
		n	HR[b]	95% CI	*n*	HR[b]	95% CI	*n*	HR[b]	95% CI	*n*	HR[b]	95% CI	*n*	HR[b]	95% CI	
Sodium intake[d,e]																	
T1	22 584	147	1.00	(ref)	88	1.00	(ref)	34	1.00	(ref)	22	1.00	(ref)	19	1.00	(ref)	
T2	22 548	158	1.06	(0.83–1.35)	93	1.02	(0.75–1.40)	35	1.08	(0.65–1.78)	17	0.73	(0.39–1.37)	31	1.52	(0.84–2.76)	
T3	22 115	193	1.33	(1.05–1.67)	125	1.42	(1.06–1.89)	41	1.29	(0.81–2.05)	35	1.55	(0.90–2.65)	40	2.04	(1.16–3.58)	0.255
P for trend				0.016			0.016			0.287			0.099			0.011	
Increment 1 g/day			1.19	(1.02–1.39)		1.18	(0.98–1.42)		1.19	(0.87–1.64)		1.18	(0.83–1.69)		1.41	(1.05–1.90)	0.410
Potassium intake[d,e]																	
T1	21 775	156	1.00	(ref)	98	1.00	(ref)	39	1.00	(ref)	26	1.00	(ref)	24	1.00	(ref)	
T2	22 941	168	1.00	(0.79–1.27)	103	0.97	(0.73–1.30)	46	1.13	(0.73–1.75)	20	0.71	(0.39–1.28)	24	0.92	(0.52–1.63)	
T3	22 531	174	1.03	(0.82–1.30)	105	0.98	(0.73–1.31)	25	0.60	(0.36–1.01)	28	1.00	(0.58–1.74)	42	1.60	(0.96–2.65)	0.008
P for trend				0.799			0.909			0.050			0.972			0.063	
Increment 1 g/day			1.04	(0.88–1.23)		1.02	(0.82–1.27)		0.72	(0.49–1.06)		1.08	(0.68–1.69)		1.45	(1.04–2.02)	0.012
Fluid intake[f]																	
Low	15 841	111	1.00	(ref)	70	1.00	(ref)	30	1.00	(ref)	14	1.00	(ref)	17	1.00	(ref)	
Moderate	29 740	217	0.99	(0.77–1.28)	135	0.98	(0.72–1.34)	46	0.73	(0.45–1.18)	38	1.47	(0.76–2.86)	39	1.28	(0.71–2.32)	
High	21 667	170	0.98	(0.73–1.30)	101	0.94	(0.66–1.35)	35	0.66	(0.39–1.12)	22	1.17	(0.53–2.58)	34	1.53	(0.78–3.01)	0.541
P for trend				0.881			0.730			0.146			0.793			0.218	
Increment 1l/day			0.91	(0.73–1.14)		0.97	(0.73–1.29)		0.74	(0.45–1.19)		1.09	(0.68–1.76)		1.25	(0.77–2.04)	0.644

RCC, renal cell cancer; ccRCC, clear-cell RCC; HR, hazard ratio; 95% CI, 95% confidence interval; T1-3, tertile 1-3; *ARHGDIG, Rho GDP dissociation inhibitor gamma; ATP1A1, ATPase Na+/K+ transporting alpha 1 polypeptide; SCNN1B, sodium channel non-voltage-gated 1 beta subunit; SLC8A3, solute carrier family 8 member 3*.

[a]The methylation index of a tumour is based on frequencies of methylation in *ARHGDIG*, *ATP1A1*, *SCNN1B* and *SCL8A3*; no, low and high methylation correspond to methylation in 0, 1 and ≥2 genes, respectively.

[b]HRs are adjusted for age (years), sex (male/female), BMI (kg/m^2), smoking (status (non-current/current), duration (years), intensity (cigarettes/day)), hypertension status (yes/no), alcohol intake (g ethanol/day) and total energy intake (kcal/day).

[c]*P*-value for heterogeneity tested using an adapted competing risk procedure for case-cohort design.

[d]Tertile boundaries are sex-specific and based on subcohort members.

[e]Intake is energy-adjusted by using the residual mean method.

[f]Categories low, moderate and high fluid intake correspond to, respectively, ≤ 1.75; 1.75-=-2.25; and >2.25 l/day.

Table 4. Hazard ratios and 95% confidence intervals of clear-cell renal cell cancer risk according to promoter methylation of *ARHGDIG* and *ATP1A1* for intakes of sodium, potassium and fluid in the Netherlands Cohort Study, 1986-2006

Dietary intakes	*ARHGDIG*						***P-value***[b]	*ATP1A1*						***P-value***[b]
	Unmethylated			Methylated				Unmethylated			Methylated			
	n	**HR**[a]	95% CI	*n*	**HR**[a]	95% CI		*n*	**HR**[a]	95% CI	*n*	**HR**[a]	95% CI	
Sodium intake[c,d]														
T1	68	1.00	(ref)	18	1.00	(ref)		79	1.00	(ref)	8	1.00	(ref)	
T2	68	0.98	(0.69–1.40)	23	1.19	(0.63–2.27)		94	0.91	(0.65–1.27)	16	1.95	(0.83–4.58)	
T3	94	1.40	(1.01–1.94)	31	1.65	(0.90–3.04)	0.723	97	1.23	(0.90–1.69)	26	3.19	(1.43–7.12)	0.159
P for trend			0.038			0.100				0.179			0.003	
Increment 1 g/d		1.17	(0.95–1.45)		1.30	(0.91–1.86)	0.644		1.16	(0.94–1.43)		1.36	(0.96–1.93)	0.583
Potassium intake[c,d]														
T1	76	1.00	(ref)	22	1.00	(ref)		83	1.00	(ref)	15	1.00	(ref)	
T2	80	0.98	(0.71–1.36)	20	0.84	(0.45–1.55)		89	0.99	(0.72–1.35)	10	0.62	(0.28–1.39)	
T3	74	0.89	(0.64–1.25)	30	1.27	(0.73–2.22)	0.335	78	0.86	(0.63–1.19)	25	1.51	(0.78–2.92)	0.019
P for trend			0.504			0.384				0.372			0.204	
Increment 1 g/d		0.95	(0.73–1.22)		1.29	(0.85–1.95)	0.346		0.96	(0.74–1.23)		1.30	(0.84–2.02)	0.199
Fluid intake[e]														
Low	54	1.00	(ref)	15	1.00	(ref)		60	1.00	(ref)	8	1.00	(ref)	
Moderate	99	0.90	(0.64–1.29)	33	1.23	(0.64–2.36)		109	0.91	(0.65–1.27)	24	1.61	(0.70–3.71)	
High	77	0.87	(0.58–1.30)	24	1.29	(0.60–2.75)	0.964	81	0.86	(0.58–1.26)	18	1.57	(0.58–4.26)	0.464
P for trend			0.502			0.523				0.443			0.421	
Increment 1l/d		0.91	(0.66–1.26)		1.18	(0.68–2.06)	0.843		0.92	(0.67–1.26)		1.23	(0.66–2.28)	0.256

ARHGDIG, *Rho GDP dissociation inhibitor gamma*; *ATP1A1*, *ATPase Na+/K+ transporting alpha 1 polypeptide*; HR, hazard ratio; 95% CI, 95% confidence interval; T1-3, tertile 1-3; d, day.

[a]HRs are adjusted for age (years), sex male/female), BMI (kg/m^2), smoking status (non-current/current), duration (years), intensity (cigarettes/day), hypertension status (yes/no), alcohol intake (g ethanol/day) and total energy intake (kcal/day).

[b]*P*-value for heterogeneity tested using an adapted competing risk procedure for case-cohort design.

[c]Tertile boundaries are sex-specific and based on subcohort members.

[d]Intake is energy-adjusted by using the residual mean method.

[e]Categories low, moderate and high fluid intake correspond to, respectively, ≤ 1.75; 1.75-2.25 and > 2.25 l/day.

the promoter methylation of *SCNN1B* (*P*-heterogeneity < 0.001). In tumours without *SCNN1B* methylation, high (vs low) potassium intake decreased the ccRCC risk [HR (95% CI): 0.59 (0.38–0.91), *P*-trend = 0.02], whereas in tumours with *SCNN1B* methylation high (vs low) potassium intake increased the ccRCC risk [HR (95% CI): 1.68 (1.10–2.56), *P*-trend = 0.01]. Similarly, differential ccRCC risks for high (vs low) potassium intake were observed by promoter methylation of the three other genes, yet only for *ATP1A1* was heterogeneity significant (*P*-heterogeneity = 0.02). For high (vs low) fluid intake, differential ccRCC risks were only, though not significantly, observed by promoter methylation of *SCNN1B*, showing ccRCC risks of 0.65 (95% CI: 0.40–1.05, *P*-trend = 0.08) and 1.58 (95% CI: 0.90–2.78, *P*-trend = 0.12) in tumours without and with *SCNN1B* methylation, respectively.

Discussion

To our knowledge, this is the first study to report on dietary sodium, potassium and fluid intake and a methylation index of four ITM genes in relation to ccRCC risk. We previously reported the overall associations between these dietary intakes and RCC risk in the same study population, then using 17.3 years of follow-up and including all RCC cases regardless of histological and molecular subtype.[3] The present study suggests that high sodium intake may increase ccRCC risk, regardless of the methylation index, whereas high potassium and fluid intake may be differentially associated with ccRCC risk across the methylation index.

Here, we used a hypothesis-driven, integrated approach including a pathway analysis and an exhaustive search of publicly available data to identify genes of interest with ccRCC-specific properties. Thereby, we sought to select the most relevant set of candidate genes to study the modifying effect of gene-specific promoter methylation in the association between dietary intakes and ccRCC risk, i.e. *ARHGDIG*, *ATP1A1*, *SCNN1B* and *SLC8A3*. In 42 selected ccRCC tumours and their adjacent normal tissues, promoter methylation was present in the tumour but not in the normal tissues, indicating that methylation of these genes may indeed be cancer-specific (data not shown).

Table 5. Hazard ratios and 95% confidence intervals of clear-cell renal cell cancer risk characterized by promoter methylation of *SCNN1B* and *SLC8A3* for intakes of sodium, potassium and fluid in the Netherlands Cohort Study, 1986-2006

Dietary intakes	*SCNN1B*						***P-value***[b]	*SLC8A3*						**P-value**[b]
	Unmethylated			Methylated				Unmethylated			Methylated			
	n	**HR**[a]	95% CI	*n*	**HR**[a]	95% CI		*n*	**HR**[a]	95% CI	*n*	**HR**[a]	95% CI	
Sodium intake[c,d]														
T1	47	1.00	(ref)	33	1.00	(ref)		73	1.00	(ref)	13	1.00	(ref)	
T2	42	0.89	(0.58–1.38)	44	1.27	(0.79–2.03)		71	0.95	(0.67–1.35)	19	1.36	(0.64–2.86)	
T3	62	1.34	(0.91–1.98)	56	1.67	(1.07–2.61)	0.437	101	1.43	(1.04–1.96)	23	1.70	(0.84–3.46)	0.624
P for trend			0.133			0.022				0.025			0.135	
Increment 1 g/d		1.16	(0.88–1.52)		1.29	(1.00–1.67)	0.237		1.19	(0.96–1.47)		1.30	(0.91–1.84)	0.479
Potassium intake[c,d]														
T1	56	1.00	(ref)	35	1.00	(ref)		83	1.00	(ref)	13	1.00	(ref)	
T2	59	0.98	(0.67–1.43)	35	0.92	(0.57–1.49)		82	0.90	(0.65–1.24)	18	1.26	(0.61–2.61)	
T3	36	0.59	(0.38–0.91)	63	1.68	(1.10–2.56)	<0.001	80	0.89	(0.64–1.23)	24	1.69	(0.85–3.36)	0.271
P for trend			0.015			0.013				0.483			0.126	
Increment 1 g/d		0.70	(0.51–0.98)		1.52	(1.14–2.02)	<0.001		0.97	(0.75–1.25)		1.39	(0.90–2.14)	0.134
Fluid intake[e]														
Low	39	1.00	(ref)	24	1.00	(ref)		59	1.00	(ref)	9	1.00	(ref)	
Moderate	68	0.82	(0.54–1.25)	59	1.36	(0.83–2.24)		107	0.94	(0.67–1.34)	25	1.59	(0.74–3.44)	
High	44	0.65	(0.40–1.05)	50	1.58	(0.90–2.78)	0.232	79	0.88	(0.58–1.31)	21	1.92	(0.84–4.34)	0.644
P for trend			0.075			0.117				0.511			0.119	
Increment 1l/d		0.74	(0.49–1.12)		1.32	(0.90–1.93)	0.141		0.93	(0.67–1.29)		1.33	(0.81–2.20)	0.535

SCNN1B, *sodium channel non-voltage-gated 1 beta subunit*; *SLC8A3*, *solute carrier family 8 member 3*; T1-3, tertile1-3; d, day.

[a]HRs are adjusted for age (years), sex (male/female), BMI (kg/m^2), smoking status (non-current/current), duration (years), intensity (cigarettes/day), hypertension status (yes/no), alcohol intake (g ethanol/day) and total energy intake (kcal/day).

[b]*P*-value for heterogeneity tested using an adapted competing risk procedure for case-cohort design.

[c]Tertile boundaries are sex-specific and based on subcohort members.

[d]Intake is energy-adjusted by using the residual mean method.

[e]Categories low, moderate and high fluid intake correspond to, respectively, ≤ 1.75; 1.75-2.25 and > 2.25 l/day.

Although potassium intake was not associated with the overall ccRCC risk, we show differential associations between potassium intake and ccRCC risk by the methylation index. This highlights the crucial role of molecular characterization of the tumour to better understand aetiology.[27] Heterogeneity for this association was particularly present for *SCNN1B* and to lesser extent for *ATP1A1*. There are many observations that have lent theoretical plausibility for a link between potassium and these ion transporters and their role in the development of ccRCC. *SCNN1B* encodes for the epithelial Na+ channel beta subunit (ENaC-β), which is located on the cell surface in many organs including the kidney and, together with other ENaC subunits, ENaC-β mediates the first step of active sodium reabsorption and controls electrolyte and water homeostasis.[28] Furthermore, mutations in *SCNN1B* have been associated with plasma potassium levels[29] and hyper- and hypokalaemia.[30,31] *ATP1A1* encodes for Na^+-K^+-ATPase, which is responsible for maintaining the electrochemical gradients of sodium and potassium ions across the plasma membrane. This transmembrane potential is influenced by potassium concentrations in intracellular and extracellular fluid, which are determined by dietary intakes and urinary excretion.[32] Changes in potassium concentrations and the transmembrane potential may disturb or may be disturbed by any condition that enhances cell breakdown or cell production, such as cancer, because the transmembrane potential influences the progression through the cell cycle.[32,33] However, concentration changes are related to the ability of the kidney to augment potassium excretion.[32] Under normal conditions, urinary potassium excretion increases after potassium load. Aberrant regulation of ion channel expression and function may diminish the kidneys' ability to regulate potassium excretion and may be a functional element in carcinogenesis.[33]

However, for biological plausibility, especially within this type of aetiological research, it is warranted to first establish the link between promoter methylation on the one hand and gene expression on the other. Since no expression data were available on the tissue samples of the NLCS, we could only rely on the samples in the TCGA to evaluate the correlation between DNA methylation and expression of the investigated genes. Methylation of *ATP1A1*, *SCNN1B*

and *SLC8A3* showed only a weak, inverse correlation with gene expression in ccRCC tumour samples of the TCGA. It should, however, be noted that these correlations may be underestimated due to the single CpG site measurement of the probe-based Infinium platform used by TCGA compared with our MSP integrating multiple CpG sites in one single reaction.

Given the sodium-potassium exchange mechanisms in the kidney, there is a theoretical link between the intake of potassium and sodium in relation to ccRCC. Although for sodium intake the ccRCC risk increased particularly in tumours with a high methylation index, we did not observe a significant heterogeneity across the methylation index, as was found for potassium intake. Several methodological reasons might explain the lack of heterogeneity in ccRCC risk for sodium intake. Our FFQ-based measurement of sodium intake may be less accurate than that of potassium intake, as total sodium intake consists for approximately 30% of the intake of sodium from discretionary salt (sodium chloride) and no such discretionary intake is common for potassium.[3] As a result, the FFQ may partially misclassify sodium intake of some participants, leading to compromised power in the analyses. This is particularly relevant when using the Wald test as a test for heterogeneity, because it is relatively conservative when it comes to rejecting the null hypothesis. Nevertheless, sodium intake performed not markedly worse in validation against 9-day dietary records than did potassium intake ($r = 0.64$ and 0.67, respectively) and additional analyses provided no evidence for residual confounding by discretionary salt intake.

Using the present study design, it is not possible to investigate the temporal relationship between the dietary intakes and the methylation index of the tumour. It is plausible that gene-specific promoter methylation, as an early event in carcinogenesis, sensitizes the renal environment to the dietary intakes under study, so promoting the development of ccRCC. However, we cannot exclude that the observed promoter methylation of the carefully selected genes may represent a general CpG island methylator phenotype (CIMP), which has been widely studied for other cancer types.[34] Neither can we exclude the possibility that dietary intakes may have caused gene-specific promoter methylation in the tumour, although there is no evidence for any methylation-promoting effects of sodium, potassium or fluid intake.

The prospective study design and the completeness of cancer follow-up make selection bias and information bias unlikely. Furthermore, we were able to retrieve tumour material of an exceptional amount of RCC cases (i.e. 80%) and found no indication for selection bias due to the unsuccessful retrieval of the remaining cases. Although the proportion of retrieved tumour material was lower in cases with distant metastases, risk factors and potential confounders did not differ between cases with and without tumour material. This study is among the largest prospective studies on RCC and, so far, the only prospective study including FFPE tumour tissues; yet the number of cases for some RCC subtypes is still too low to conduct analyses with adequate power. Moreover, risk estimates may be attenuated due random misclassification of dietary intakes, introduced by the long follow-up and single baseline measurement, even though intakes were rather stable during the first 5 years of follow-up.[16]

In conclusion, potassium intake was differentially associated with ccRCC risk only when the methylation index of the investigated genes was considered. Using this approach of molecular pathological epidemiology, this study provides the first indications of a biological rationale for the possible role of ITM in the development of ccRCC. However, for biological plausibility, it is warranted to first establish the effect of promoter methylation on the expression of these genes.

Supplementary Data

Supplementary data are available at *IJE* online.

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