Renal cell cancer : a molecular-epidemiological approach to unravel new pathways underlying disease etiology and prognosis

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Renal Cell Cancer

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*Aan mijn oma
voor al haar wijsheid*

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CHAPTER 1

General introduction

Cancer of the kidney is the 7th most common cancer in the more developed regions of the world [1]. Globally, kidney cancer accounted for 337.860 new cases in 2012, which was 2-3% of the total human cancer burden [1]. Renal cell cancer (RCC) is the most common type of kidney cancer (90%) [2,3]. In the Netherlands, the age-standardized incidence (per 100,000 per year) of RCC has been increasing from 8.85 in 1989 to 10.76 in 2007, but stabilized over the last five years (10.33 in 2012) [4]. The incidence of RCC has been reported to be twice as high among men as women and increases with advancing age [5].

In addition to sex and age, several risk factors have been established to increase the risk of RCC, including hypertension, cigarette smoking, obesity and familial cancer syndromes [6]. For several other factors, including diabetes, end-stage renal disease, low parity in women, low physical activity, trichloroethylene exposure and genetic predisposition, positive associations with RCC risk are suggested, but conflicting results have been observed among different studies [6]. In addition, epidemiological evidence suggests that moderate alcohol consumption and diets rich in fruits and vegetables might be associated with a lower RCC risk [6]. However, the effects that these factors have on RCC risk are rather modest and, in general, the etiology of RCC is still poorly understood.

Remarkably, despite that dietary intakes affect the homeostasis of sodium, potassium and water in the human body, and that the regulation of this homeostatic balance is one of the main aspects in renal physiology, the number of epidemiological studies investigating dietary intakes of sodium, potassium [7] and fluid [8-10] in relation to RCC risk is limited. In order to advance in the field of RCC research and gain insight into its etiology and prognosis, it is important to search beyond current perspectives and generate new hypotheses for future research. Therefore, this thesis describes the endeavor to unravel the associations between dietary sodium, potassium and fluid intake as potential novel risk factors for RCC and to discover novel disease pathways that may link renal (patho)physiology to RCC etiology and prognosis using molecular epidemiology.

RENAL PHYSIOLOGY: THE ESSENTIALS

In humans, the kidneys are located in the abdominal cavity behind the peritoneum, one on each side of the spine. The renal parenchyma is the functional tissue of the kidney and is divided into two major structures: the outer renal cortex and the inner renal medulla (Figure 1.1). A healthy parenchyma contains more than 1 million nephrons. The nephron is the main working unit of the kidney and is involved in the production of urine. A nephron consists of a glomerulus, a tuft of capillaries in between the afferent and the efferent arterioles, and a series of renal tubules, which

are divided into four histo-physiological segments: the proximal tubule, the loop of Henle, the distal tubule and the collecting tubule.

Figure 1.1 The kidney. The renal parenchyma includes an outer cortex and an inner medulla. The nephron consists of the glomerulus (1) and a series of renal tubules, which are divided into four histo-physiological segments: proximal tubule (2), loop of Henle (3), distal tubule (4) and collecting tubule (5). Adapted from: Baldewijns *et al.*, 2008. [11]

Each part of the nephron has different permeability and transport characteristics and, therefore, performs a distinct function. The glomerulus forms the pre-urinary filtrate from blood plasma. The proximal tubule and loop of Henle reabsorb large amounts of water and important nutrients into the systemic circulation to prevent essential losses. The remaining part of the renal tubular system is involved in the final composition of the urine. Many of the kidney's functions are accomplished by the processes of filtration, reabsorption, secretion and excretion of water, electrolytes and waste products, which all take place in the nephron. The kidney is involved in a wide scala of physiologic processes, including the removal of waste products of metabolism, acid-base balance, electrolyte balance, osmoregulation, volume regulation and blood pressure regulation. Volume regulation and osmoregulation are two functions of the kidney that are of particular interest when investigating dietary intakes of sodium, potassium and water.

The process of *volume regulation* keeps the effective circulating volume (ECV), which is the volume in the arterial system effectively perfusing human tissues, at adequate levels [12]. Volume regulation is a vital and acute process in the human body. There are several types of conditions that may cause an inappropriate decrease the ECV. In order to restore the ECV, sodium is reabsorbed from the renal tubules into the blood vessels. Via the principle of osmosis, water will naturally follow. Sodium is thus the main determinant of volume regulation.

If the aforementioned ECV is maintained at adequate levels, day-to-day fluctuations in dietary sodium and water intakes are handled by *osmoregulation* [12]. The adult human body contains 65% water [13]. This water is distributed over two major body fluid compartments (the intracellular fluid volume and the extracellular fluid volume, including the interstitial fluid volume and the plasma volume). Water and the bodily solute content within these compartments is maintained within narrow limits, which is essential for adequate cell functioning. Sodium is the primary determinant of the extracellular fluid volume, whereas potassium is the primary determinant of the intracellular fluid volume. Changes in water content across these compartments are facilitated by changes in sodium. In order to maintain an electroneutral balance in the cells after a shift of sodium across the compartments, sodium is exchanged for potassium. This exchange is primarily governed by the sodium-potassium-ATPase pump in the cell membrane, which is therefore an important mechanism in ion transport.

THE ROLE OF DIETARY INTAKES IN VOLUME- AND OSMOREGULATION

The kidney is programmed to reabsorb large amounts of sodium from the diet [14,15], because sodium is evolutionary important to our body, given its vital role in volume regulation. However, the sodium content in our diet is nowadays much higher than what we would physically need. This chronic excess of sodium intake in populations to date results in a chronic volume overload [13]. Therefore, excess of sodium has been associated with multiple diseases, such as a high blood pressure and the development of hypertension across many populations [15].

The role of dietary sodium intake in osmoregulation may best be explained using the example of consuming a high sodium product such as chips, an isotonic drink (in which the sodium concentration is equal to that of the extracellular volume) and plain water. In healthy humans, consuming chips will raise the plasma sodium concentration. This will result in a movement of water out of the cells into the extracellular space. The net effect is an increase of osmolality of both the intra- and extracellular volume. Since there is no change in total body volume, urinary sodium excretion is low. However, the decrease in intracellular volume influences the hormonal mechanism that induces thirst and the secretion of antidiuretic hormone (ADH) in the hypothalamus. As a result, also the water excretion by the kidney is low. When consuming an isotonic drink, the plasma sodium concentration does not change, while the consumed volume will result in an increase of extracellular volume and a subsequent raise in blood pressure. Even though consuming an isotonic drink will not change the plasma sodium concentration, the raise in blood pressure will result in excretion of excess sodium and water. Finally, the consumption of plain water decreases the plasma sodium

concentration and will subsequently result in a movement of water out of the extracellular space into the cells. The net effect is a decrease of osmolality of both the intra- and extracellular volume, with an overall volume expansion, which will increase the urinary sodium excretion. ADH is inhibited due to the increase in intracellular volume. Without ADH, water excretion by the kidney is high.

Dietary intakes may also affect potassium homeostasis (i.e. the distribution of potassium between intracellular and extracellular fluids) [12]. When drinking a potassium-rich drink, such as orange juice, plasma potassium concentration increases and, because such increases could be lethal, potassium immediately enters the cell. Excess of potassium is excreted within 6 to 8 hours. In addition to variations in dietary intakes, the distribution of potassium between intracellular and extracellular fluids is also influenced, among many others, by any condition that changes the rates of cell breakdown and cell production, such as cancer [12,16]. Cell breakdown results in release of potassium. The subsequent change in plasma potassium concentration depends on the degree of cellular potassium uptake by other cells and the ability of the kidney to excrete potassium.

Taken together, sodium is a key determinant in two major aspects of renal physiology, including volume regulation and osmoregulation. In these processes, water and sodium balance is directly related to each other, yet independently regulated, and sodium and potassium are exchanged. Therefore, we hypothesized that dietary sodium, potassium and fluid intake may be novel risk factors for RCC and that their potential effects may be influenced by each other.

HYPERTENSION AND OTHER POTENTIAL BIOLOGIC MECHANISMS IN RENAL CELL CANCER

In order to better understand the role of dietary intakes in RCC etiology, it is important to investigate these associations in more detail. This thesis particularly focuses on the role of hypertension as intermediate, because high sodium intake is an important determinant of hypertension, while hypertension is an established risk factor for RCC [6]. Therefore, we hypothesized that dietary sodium intake may influence RCC risk, through hypertension. Hypertension is a major chronic disease, which affected approximately 40% of the world's population in 2008 [17]. Uncontrolled hypertension can lead to chronic kidney disease, worsening renal function, and enhancing progression to end-stage renal disease, conditions that may predispose to the development of RCC [6].

Furthermore, two potentially cancer-causing, biologic mechanisms in renal physiology, i.e. ion transport mechanisms (ITMs) and the renin-angiotensin-aldosterone-system

(RAAS), will be investigated in this thesis to further explore the association between sodium, potassium and fluid intake and RCC risk.

Ion transport mechanisms

In the nephron, ion channels, transporters, exchanger pumps and associated enzymes, collectively referred to as ion transport mechanisms (ITMs), facilitate tubular reabsorption and secretion, because the lipid layers of the cell membranes hinder free diffusion of ions [12]. Tubular reabsorption consists of two steps; from the tubular lumen, through the luminal membrane, into the cell and from the cell across the basolateral membrane, into the interstitium, which is connected to the systemic circulation (Figure 1.2).

Figure 1.2 Schematic presentation of renal reabsorption and secretion of sodium and potassium. Sodium (Na^+) is reabsorbed actively, in exchange for potassium (K^+), from the tubular cell into the circulation and passively from to luminal pre-urinary filtrate into the tubular cell. The excess of potassium is secreted out of the tubular cell into the luminal pre-urinary filtrate, which will eventually be excreted as urine.

Firstly, sodium is, in exchange for potassium, actively transported from the tubular cell into the circulation using the aforementioned sodium-potassium-ATPase pump. Sodium-potassium-ATPase is an abundantly expressed protein in all epithelial cells, not only in the kidney, and maintains trans-membrane sodium and potassium electrochemical gradients, which are essential for other ITMs to function properly [18]. After the cellular sodium concentration is decreased, sodium is reabsorbed

passively from the luminal pre-urinary filtrate into the tubular cell via ITMs. Since different functions are performed throughout the tubular system of the nephron, ITMs are site-specific. In the proximal tubule, the sodium/hydrogen exchanger type 3 (NHE3 also SLC9A3) is for example the principle mediator of the proximal reabsorption of 70-80% of the sodium filtered at the glomerulus [19,20]. In the distal nephron, epithelial sodium channels (ENaC) facilitate the process of sodium transport from the lumen into the cell in order to maintain electrolyte and water homeostasis [21]. ITMs have recently emerged as novel mechanisms that drive carcinogenesis throughout the multi-stage process [16].

Renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system (RAAS) is a peptide-based system that has been recognized for decades as a principal determinant of arterial blood pressure, but is also involved in fluid and electrolyte balance [22]. Basically, the RAAS focuses on the processing of the precursor angiotensinogen to the active peptide angiotensin II and the interactions with its receptors [22]. Renin is activated in response to low stretch of the afferent arteriole resulting from hypotension or volume depletion. Subsequently, renin induces the generation of angiotensin, which will be converted into angiotensin II by the angiotensin II converting enzyme (ACE). To restore blood pressure, angiotensin II induces sodium reabsorption and thereby extracellular volume expansion, through increasing the secretion of aldosterone and, simultaneously, higher systemic blood pressure and cardiac output by arterial vasoconstriction. The kidney influences the RAAS and blood pressure by secreting the hormones such as renin and angiotensin II [12]. It has been established that the RAAS does not only play a role in normal renal physiology, but also has important implications in the pathogenesis and treatment of disease. Mostly, the RAAS has been investigated in relation to cardiovascular diseases. For example, up-regulated RAAS is known to be involved in the development of hypertension and several forms of antihypertensive therapy intervene on various aspects of the RAAS [22,23]. Recent developments indicate that aberrant regulation of the RAAS may also contribute to carcinogenesis through stimulation of angiogenesis, invasion, cell survival and proliferation [22].

Taken together, ITMs and the RAAS are two potentially cancer-causing, biologic mechanisms involved in both renal (patho)physiology and carcinogenesis. Therefore, we hypothesize that these mechanisms may be underlying disease pathways in the association between sodium, potassium and fluid intake and RCC risk.

MOLECULAR (PATHOLOGICAL) EPIDEMIOLOGY

In an attempt to investigate the role of ITMs and the RAAS in the associations under study, the present thesis uses a molecular-epidemiological approach. Molecular epidemiology evaluates the relationship between a biomarker on the one hand and exposures, susceptibility and disease on the other hand, in order to understand the mechanisms through which environmental exposures (e.g. nutrients) interact with host susceptibility factors (e.g. genetic variation) and influence disease risk (e.g. RCC risk) by combining laboratory and population research [24]. In this thesis, we use molecular data, in addition to dietary exposure data, to investigate gene-environment interactions and tumor heterogeneity.

Gene-environment interactions

A gene-environment interaction may be defined as: “a joint effect of one or more genes with one or more environmental factors that cannot readily be explained by their separate marginal effects” [25]. Although gene-environment interactions are worth studying for many reasons [25], in this thesis we used them mainly to investigate RAAS and ITMs as underlying, potentially cancer-causing biological pathways that may link dietary intakes of sodium, potassium and fluid to RCC risk. However, in complex diseases, such as RCC, it has emerged that part of the inter-individual differences in disease risk may be explained by gene-environment interactions [25]. To address this issue, we will additionally try to identify and specify subpopulations that are particularly at risk for RCC.

Approximately 1% of the DNA differs between individuals. The most common type of genetic variation is the single nucleotide polymorphism (SNP). SNPs in RAAS and ITM genes have mainly been investigated in relation to cardiovascular disease risk [26-30]. Few SNPs were, in this context, associated with response to sodium, for example SNPs in *adducin1* (*ADD1*) and *angiotensinogen* (*AGT*) [31]. Only one study previously investigated SNPs in RAAS genes in relation to RCC risk [32]. This study showed that several SNPs in *AGT* increased RCC risk, particularly among the subpopulations with hypertension or obesity [32]. In this thesis, we seek to replicate this finding and additionally investigate SNPs in other candidate genes encoding for major RAAS factors that cover the essential part of the system. SNPs in ITM genes have not yet been investigated in relation to RCC risk.

Tumor heterogeneity

It is increasingly recognized that RCC is not a single disease. RCC is usually classified based upon extensive evaluation of both histological and molecular features of the tumor [2]. The World Health Organization (WHO) distinguishes three major subtypes

of malignant renal tumors. The most common subtype is clear-cell RCC (80-90%), which is characterized by cells containing clear cytoplasm and which shows often a specific deletion of chromosome 3p and mutations in the Von Hippel-Lindau (*VHL*) gene [2,33]. Two other common subtypes are papillary RCC (10-15%) and chromophobe RCC (4-5%) [2]. Besides heterogeneity with respect to histological features, resulting in the traditional histological subtypes of RCC, it has been acknowledged that substantial, molecular heterogeneity exists within subtype-specific tumors. Molecular heterogeneity may reflect the unique carcinogenic route along which a tumor has been developing and may progress. Therefore, molecular subgroups may be associated with different risk factors and a different clinical behavior. The emerging field of molecular pathological epidemiology (MPE) integrates molecular subgroups into traditional epidemiological research [34]. MPE aims to increase our knowledge on the underlying biologic pathways that are responsible for these differences and to explain observed differences in risk factors across populations and studies [34]. MPE is, however, not (yet) commonly used in relation to RCC [35].

In addition to gene-environment interactions with germline genetic variations (i.e. SNPs), we focus in this thesis also on the molecular characterization of clear-cell RCC in terms of acquired epigenetic abnormalities. Epigenetic mechanisms regulate gene expression on multiple levels without changing the sequence of the genome [11]. DNA methylation is one such process, in which a methyl group (CH_3) binds to a cytosine at a CpG dinucleotide where a cytosine precedes a guanine [36]. CpG-rich areas, in for example the promoter region of a gene, are so-called 'CpG islands'. Most promoter CpG islands are normally unmethylated. Promoter CpG island hypermethylation has been associated with gene silencing [37]. Gene silencing and subsequent inappropriate loss of function of tumor suppressor genes (TSGs) has been associated with carcinogenesis [38]. The degree to which promoter methylation is present can be used to classify tumors [39]. Promoter hypermethylation of TSGs is thought to occur early and frequently in carcinogenesis and, therefore, may contribute to both cancer development and progression [38,40-42]. Therefore, we hypothesized that promoter methylation status may be used to classify the RCC tumor in terms of molecular heterogeneity and that promoter methylation may play a role in the etiology and prognosis of RCC. In this thesis, we use three approaches to study promoter methylation. Firstly, we will study the interplay between promoter methylation of ITM genes and dietary intakes related to ITMs (i.e. sodium, potassium and fluid intakes) in relation to RCC risk. Secondly, we will investigate whether promoter methylation in RCC is associated with dietary alcohol and folate intakes. Finally, we will use promoter methylation to distinguish RCC subtypes with a different survival.

Promoter CpG island hypermethylation and ITMs

In addition to the aforementioned gene-environment interactions with SNPs, the role of ITMs in the associations between sodium, potassium and fluid intake and RCC risk is also investigated using promoter CpG island hypermethylation. This multidimensional approach highlights two distinct mechanisms by which the genes involved in ITMs may be aberrantly regulated or silenced, including genetics (SNPs) and epigenetics (promoter methylation) and, therefore, this multidimensional approach provides a broader view of the role of ITMs in RCC carcinogenesis.

Promoter CpG island hypermethylation and dietary intakes

Several factors have been hypothesized to mediate the general process of methylation, i.e. methyl metabolism, including dietary intakes. An adequate folate metabolism is essential to properly regulate the methylation of DNA. Folate, as 5-methyltetrahydrofolate, has a central role in methyl metabolism, because it supplies a methyl group to convert homocysteine to methionine, which is then converted to S-adenosylmethionine, the universal methyl donor [43]. Folate deficiency is reported to be associated with global *hypomethylation* [44] and might therefore also affect *hypermethylation*. However, the relation between hypo- and hyper-methylation remains inconclusive [45,46]. The methyl metabolism may indirectly also be influenced by the intake of alcohol [47]. High alcohol intake decreases the absorption of folate from the intestinal lumen [48,49]. Moreover, folate is degraded by acetaldehyde, which is a metabolite of alcohol.

Promoter CpG island hypermethylation and prognosis of clear-cell renal cell cancer

Currently, the prognosis of patient outcome in a clinical setting is predicted using several prognostic parameters, including cancer stage, for which the tumor-node-metastasis (TNM) staging system is commonly used [50], and Fuhrman nuclear grading, in which nuclear grades between 1 and 4 were defined by means of increasing nuclear size, irregularity and nucleolar prominence [51]. However, in this era of molecular diagnostics, tumor behavior in response to molecular therapies can no longer accurately be predicted without considering the molecular heterogeneity of the tumor. In addition, molecular heterogeneity for example with respect to promoter methylation may also be used to classify the tumor to improve early detection and to determine prognosis [52]. Promoter methylation of cysteine dioxygenase type 1 (*CDO1*) has been identified as marker of poor breast cancer survival. *CDO1* may be related to general carcinogenic processes, such as oxidative stress responses [42,53,54], but is also indirectly involved in renal physiology through the taurine pathway [55,56]. Therefore, we investigated promoter methylation of *CDO1* in relation to RCC survival.

AIM OF THE THESIS

The overall aim of this thesis is to unravel novel disease pathways implicated in renal (patho)physiology in the etiology and prognosis of RCC. In the first part of this thesis, we evaluated dietary intakes of sodium, potassium and fluid as potential novel risk factors for RCC. In the second part of this thesis, we studied gene-environment interactions with genetic variation in the RAAS and ITMs to elucidate these renal (patho)physiological pathways as novel disease pathways that may link dietary intakes of sodium, potassium and fluid intake to RCC risk. In the third part of the thesis, we use promoter methylation status to classify the RCC tumor in terms of molecular heterogeneity and aim to clarify its role in disease pathways in the etiology and prognosis of RCC.

STUDY DESIGN AND STUDY POPULATION

All studies in this thesis were conducted within the framework of the Netherlands Cohort Study on diet and cancer (NLCS). The NLCS is a prospective cohort study that was initiated in 1986 and included 120,852 participants aged 55-69 years at baseline [57]. All participants returned a mailed, self-administered, baseline questionnaire on diet and other potential risk factors for cancer, such as smoking habits and hypertension. This baseline questionnaire included a 150-item, semi-quantitative food frequency questionnaire (FFQ), which was used for the assessment of dietary habits. In addition to the baseline questionnaire, approximately 90,000 participants provided toenail clippings, which showed to be a valid source of DNA for the genotyping of a limited set of SNPs when using the Agena Bioscience (formerly Sequenom Bioscience) MassARRAY® platform using the iPLEX™ assay [58,59]. 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 5,000 subjects was randomly sampled at baseline to estimate person years at risk for the entire cohort [60]. Subcohort members were regularly followed up for vital status information. All cohort members were followed up for cancer occurrence using record linkage with the Netherlands cancer registry and with the Dutch pathology registry (PALGA) [61].

A large and unique population-based collection of tumor DNA of incident RCC cases is nested within the NLCS. Formalin-fixed paraffin-embedded (FFPE) tumor tissues from ~50 pathology laboratories throughout the Netherlands were collected. Clinical information and tumor characteristics, such as age at diagnosis and tumor size, were available from the pathological reports and Netherlands cancer registry. Information on the survival time was accomplished by record linkage to the municipal population registries and the causes of death registry from Statistics Netherlands.

In this thesis, the overall associations between dietary exposure data and RCC risk were studied using follow-up data between 1986 and 2003. An overview of the study designs including two types of molecular data is shown in Figure 1.3. All analyses with molecular data included cases from the maximum follow-up period until 2006. During this period of 20.3 years, 608 RCC cases were identified.

1. the NLCS cohort study design for gene-environment interactions

2. the NLCS cohort study design for molecular pathological epidemiology

Figure 1.3 Overview of study designs for molecular analyses in the Netherlands Cohort Study (NLCS). Molecular analyses regarding gene-environment interactions (design 1) are used to determine the interactions between the exposures of interest (e.g. sodium intake) established at baseline and a particular genotype based on SNP genotyping of toenail DNA in relation to RCC risk. Molecular (pathological) analyses (design 2) are used to compare the effect of the exposure of interest (e.g. sodium intake) established at baseline on RCC risk for different subtypes of the tumor (methylated vs. unmethylated) determined using tumor DNA. Abbreviations: RCC, renal cell cancer; AA, homozygote common genotype; AB, heterozygote genotype; BB, homozygote rare genotype; meth (+), tumors with promoter CpG island hypermethylation; meth (-), tumors without promoter CpG island hypermethylation.

OUTLINE OF THE THESIS

Part one (chapter 2) of this thesis describes the overall associations between dietary intakes of sodium, potassium and fluid and, the risk of RCC from the traditional epidemiological perspective. In **part two**, gene-environment interactions are used to highlight the interplay between SNPs in candidate genes involved in the RAAS (**chapter 3**) and candidate genes involved in ITMs (**chapter 4**) and the dietary intakes of sodium, potassium and fluid in relation to RCC risk. Finally, in **part three**, the clear-cell RCC tumor is classified according to promoter methylation status of a subset of genes to study molecular subtype-specific etiology (**chapters 5 and 6**) or prognosis (**chapter 7**) using the approach of MPE. Finally, in **chapter 8** we summarize, discuss and interpret our main findings and conclude with some final remarks on our vision on future directions in RCC research.

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PART I

Dietary intakes

CHAPTER 2

Long-term dietary sodium, potassium and fluid intake; exploring potential novel risk factors for renal cell cancer in the Netherlands Cohort Study on diet and cancer

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ABSTRACT

Introduction

As sodium, potassium and fluid intake are related to hypertension, an established risk factor for renal cell cancer (RCC), they may be independent risk factors for RCC.

Methods

The NLCS with case-cohort design includes 120,852 participants aged 55-69 years. At baseline, diet and lifestyle were assessed with questionnaires. After 17.3 years follow-up, 485 RCC cases and 4,438 subcohort members were available for analyses.

Results

Sodium intake increased RCC risk (P -trend=0.03), whereas fluid and potassium intake did not. For high sodium and low fluid intake RCC risk additionally increased (P -interaction=0.02).

Conclusion

Sodium intake is a potential risk factor for RCC, particularly if fluid consumption is low.

INTRODUCTION

The suggested risk factor pattern for renal cell cancer (RCC), including an increased risk with hypertension, smoking and obesity and a decreased risk with moderate alcohol consumption [1] also determines cardiovascular disease risk. In addition, evidence clearly suggests that high sodium intake may cause a rise in blood pressure, the development of hypertension [2] and affects renal function [3-5]. Furthermore, prolonged sodium excess may contribute directly to kidney damage [6]. Therefore, high sodium intake may be a risk factor for RCC, potentially through hypertension.

The effect of sodium on blood pressure may, however, be modified by potassium intake or fluid intake, given their influence on blood pressure regulation and sodium homeostasis, which are both key functions of the kidney [7,8]. That considered, potassium intake and fluid intake may individually influence RCC risk, or modify potential effects of sodium intake on RCC risk. Until now, only few studies investigated the potential association between RCC risk and sodium intake, potassium intake [9] or fluid intake [10-12]. In these studies, no attention has been given to their joint effects on renal function or on the potential influence of hypertension. In this study, we investigate whether sodium, potassium and fluid intake and their potential interactions are associated with RCC risk in a prospective study.

METHODS

Study design, study population and follow-up

The Netherlands Cohort Study is a prospective cohort study initiated to investigate the association between diet and the development of cancer [13-15]. The cohort included 58,279 men and 62,573 women between the ages of 55-69 years at baseline (1986). The case-cohort design [16] was used for data processing and analysis. Cases were derived from the entire cohort, whereas a subcohort of 5,000 subjects was randomly sampled at baseline to estimate person years at risk for the entire cohort. Participants with prevalent cancer (excluding skin cancer) at baseline were excluded. All cohort members were followed-up for cancer occurrence through computerized record linkage with the Netherlands cancer registry and the Dutch pathology registry [17]. The completeness of cancer follow-up is over 96% [18]. After 17.3 years of follow-up, 514 histological confirmed, epithelial RCC cases were identified [International Classification of Diseases for Oncology 3 (ICD-O-3): C64].

Assessment of dietary intakes

At baseline, all participants returned a mailed, self-administered questionnaire on diet and other risk factors for cancer, including smoking habits and hypertension. Participants with incomplete or inconsistent questionnaires were excluded [13]. A 150-item, semi-quantitative food frequency questionnaire (FFQ) represented the dietary part of the questionnaire, which particularly focused on habitual consumption of food and beverages during the preceding year [13,14]. The daily intakes were defined as the intakes through both foods and beverages, including amounts naturally present in foods and beverages plus amounts added during food processing by food manufacturers. Intakes were calculated using the computerized Dutch food composition table [19]. Sodium, potassium and fluid intake were validated against nine-day dietary records ($r=0.64$, 0.67 and 0.74 , respectively) and tested for reproducibility [13,14,20].

The daily sodium intake did not include sodium from salt added during home-preparation, yet the baseline questionnaire included specific questions on salt added during home-preparation or before consumption [20]. Together, the questions on cooking salt intake (no/yes, number of teaspoons per person) and table salt intake (ranging from “never” to “very often”) represent discretionary salt intake. We considered the actual intake of discretionary salt through consumption of a cooked meal to be two third of the amount of salt added to the meal during cooking [21], as the rest may be discarded in cooking fluid or left in pans or on the plate. The use of table salt was converted into frequencies and quantified by multiplying this frequency by a standard serving size of 0.88 g [22]. As salt preference may be related to (sodium) salt intake, participants were asked to indicate the saltiness of restaurant food and soups from package or can (ranging from “not salty enough” to “much too salty”).

Both sodium and potassium intake were adjusted for total energy intake by using the residual method [23] and modelled as sex-specific quintile distribution and as continuous variable (i.e. per increment of 1 g/d). Similarly, fluid intake was modelled as sex-specific quintile distribution and per increment of 1 l/d, whereas discretionary salt intake was modelled using a categorical variable (i.e. non-consumers plus quartiles of consumers) and per increment of 1 g/d. For discretionary salt intake and perceived saltiness, the second category was used as reference, as this category seemed most normal in our population.

Statistical analyses

In total, 485 RCC cases and 4,438 subcohort members with complete dietary information were included in Cox proportional hazards analyses for the case-cohort design [24], which was used to obtain hazard ratios (HR) and 95% confidence intervals (CI). Associations between sodium, potassium and fluid intake and RCC risk were

analysed in the total population, by hypertension status and when participants with a low-salt diet were excluded, as these subpopulations may differ in dietary behaviours, so influencing the associations under study. Confounders were selected *a priori*. Analyses were adjusted for sex (men/women), age at baseline (y), smoking status (never or ex/current), smoking duration (y), smoking intensity (cigarettes/d), body mass index (BMI, kg/m²), alcohol intake (g ethanol/d), total energy intake (kcal/d), a diagnosis of hypertension (no/yes) and use of diuretic medication (no/yes). Age at baseline, smoking intensity and use of diuretic medication were modelled as time-dependent variables, due to violation of the proportional hazards assumption, tested using scaled Schoenfeld residuals. Robustness of data was investigated by excluding the first two years of follow-up and by reanalysing data separately for the first and second half of follow-up. Interactions with sodium and discretionary salt intake were investigated for potassium and fluid intake and for suggested RCC risk factors using the Wald χ^2 test. A *P*-value<0.05 was considered significant. Data were analysed with STATA (version 12).

RESULTS

Energy-adjusted sodium intake was lower in subcohort members than in RCC cases (Table 2.1). Discretionary salt intake, perceived saltiness, potassium intake and fluid intake were similar among RCC cases, subcohort members and quintiles of sodium intake. Sodium intake was associated with an increased RCC risk (Table 2.2). In multivariable adjusted analyses, we observed a significantly increasing trend across quintiles (*P*-trend=0.03) to a maximum in the highest quintile [HR(95%CI): 1.40(0.99-1.97)] and a significant increase in RCC risk per increment of 1 g/d [HR(95%CI): 1.07(1.00-1.15)]. For discretionary salt intake, the RCC risk was u-shaped, with a significantly increased HR for non-users and a non-significantly increased HR for the fourth quartile of users compared to first quartile of users [HR(95%CI): 2.36(1.56-3.56) and 1.16(0.85-1.58), respectively]. Only after excluding non-users, there was a significant increase in RCC risk per increment of 1 g/d [HR(95%CI): 1.04(1.00-1.08)]. Furthermore, participants who indicated soups and restaurant food as 'much too salty' (*versus* 'good') had the highest RCC risk [HR(95%CI): 1.70(1.24-2.33)]. Fluid and potassium intake were not associated with RCC risk. We observed no difference between multivariable adjusted models with and without hypertension as confounder, indicating that mediation was not present (data not shown). In addition, no significant interactions between hypertension and any of the exposures under study regarding RCC risk were observed (Suppl Table S2.1).

Table 2.1 Characteristics of renal cell cancer cases, subcohort members, and per quintile of sodium intake in the subcohort, NLCS 1986-2003.

Baseline characteristics (mean (SD))	Cases	Subcohort	Quintiles of sodium intake ^{a,b,c}					P-value ^d
			1 (low)	2	3	4	5 (high)	
Total (n)	485	4,438	889	887	888	887	887	
Exposures (in g/d)								
Sodium intake ^c	2.5 (0.7)	2.3 (0.6)	1.6 (0.3)	2.0 (0.2)	2.3 (0.3)	2.6 (0.3)	3.2 (0.5)	-
Discretionary salt intake ^{e,f}	2.9 (3.1)	2.8 (2.7)	3.0 (2.9)	2.7 (2.7)	2.8 (2.6)	2.8 (2.8)	2.7 (2.7)	0.27
Perceived saltiness (%) ^g								
Not salty enough	1.3	1.9	2.7	1.4	1.7	1.8	2.1	
Good	38.4	41.9	41.5	40.8	41.3	43.3	42.7	
A little too salty	42.5	41.2	42.0	42.2	42.3	40.0	39.8	
Much too salty	17.9	14.9	13.9	15.7	14.8	15.0	15.4	0.86
Fluid intake (ml/d)	2,081 (463)	2,081 (486)	2,055 (467)	2,025 (439)	2,050 (512)	2,068 (459)	2,206 (528)	<0.001
Potassium intake (mg/d) ^c	3,519 (569)	3,524 (572)	3,406 (551)	3,494 (516)	3,529 (561)	3,580 (565)	3,611 (637)	<0.001
Potential confounders								
Age at baseline(y)	61.4 (3.9)	61.4 (4.2)	61.6 (4.4)	61.6 (4.3)	61.3 (4.3)	61.2 (4.1)	61.2 (4.1)	0.10
Male sex (%)	64.5	49.4						^b
Total energy intake (kcal/d)	1,971 (490)	1,923 (516)						^c
Alcohol consumption (g ethanol/d) ^h	14.8 (14.8)	13.6 (15.1)	18.9 (19.2)	13.1 (13.8)	12.5 (14.7)	11.3 (11.6)	11.9 (13.5)	<0.001
BMI (kg/m ²) ⁱ	25.5 (3.0)	25.0 (3.1)	24.6 (3.1)	24.7 (3.0)	25.2 (3.1)	25.2 (3.1)	25.4 (3.3)	<0.001
Current smokers (%) ^f	32.4	28.3	33.6	28.1	27.0	24.9	27.7	0.001
Smoking duration (y) ^j	39.8 (9.1)	38.6 (9.9)	39.0 (10.4)	38.6 (9.4)	38.7 (9.7)	38.8 (9.4)	38.0 (10.2)	0.68
Smoking intensity (cig/d) ^j	16.0 (8.5)	15.0 (8.7)	16.5 (9.6)	14.1 (8.7)	14.8 (8.6)	15.2 (8.3)	14.4 (7.9)	0.02
Prescribed low-salt diet (%)	8.0	7.5	7.2	8.7	7.8	5.5	8.1	0.11
Hypertension (%)	36.1	32.0	26.3	36.5	31.4	33.7	31.8	<0.001

^a Quintile boundaries are based on subcohort members; ^b Quintile boundaries are specific for sex; ^c Intakes are energy-adjusted; ^d P-values are tested with Kruskal-Wallis test (continuous) or Chi-square test (categorical); ^e Salt intake refers to sodium-chloride intake; ^f n does not correspond with the overall n, due to missing values; ^g Saltiness of soups and restaurant food; ^h In consumers only; ⁱ In current smokers. BMI = body mass index, cig = cigarettes, NLCS = Netherlands Cohort Study, SD = standard deviation.

Table 2.2 Renal cell cancer risk according to indicators of sodium intake, fluid intake and potassium intake, NLCS 1986-2003.

Exposures in g/d (median in subcohort (men/women))	Cases	Person years	Age- and sex-adjusted model ^a		Multivariable adjusted model ^b	
			HR	95% CI	HR	95% CI
Sodium intake						
Quintile 1 (1.9/1.5)	70	11842	1.00	(ref)	1.00	(ref)
Quintile 2 (2.2/1.8)	76	11,803	1.10	(0.78-1.55)	1.07	(0.75-1.52)
Quintile 3 (2.5/2.0)	81	11,833	1.17	(0.83-1.63)	1.11	(0.78-1.57)
Quintile 4 (2.8/2.3)	89	11,553	1.35	(0.97-1.88)	1.28	(0.91-1.79)
Quintile 5 (3.4/2.8)	93	11,370	1.41	(1.01-1.95)	1.40	(0.99-1.97)
<i>P</i> -trend				0.02		0.03
Increment per 1 g/d			1.07	(1.00-1.14)	1.07	(1.00-1.15)
Discretionary salt intake ^c						
No salt	45	3,033	2.51	(1.69-3.74)	2.36	(1.56-3.56)
Quartile 1 (0.7/0.7)	91	14,392	1.00	(ref)	1.00	(ref)
Quartile 2 (2.0/1.7)	79	13,981	0.89	(0.65-1.23)	0.92	(0.66-1.26)
Quartile 3 (3.4/2.9)	100	13,985	1.12	(0.83-1.51)	1.14	(0.84-1.55)
Quartile 4 (5.5/5.1)	94	13,009	1.16	(0.85-1.57)	1.16	(0.85-1.58)
<i>P</i> -trend				0.27		0.39
Increment per 1 g/d			1.01	(0.97-1.06)	1.02	(0.98-1.06)
Perceived saltiness ^d						
Not salty enough	4	1,084	0.52	(0.19-1.45)	0.48	(0.17-1.35)
Good	148	22,589	1.00	(ref)	1.00	(ref)
A little too salty	167	23,599	1.14	(0.90-1.44)	1.17	(0.92-1.48)
Much too salty	77	8,101	1.62	(1.20-2.18)	1.70	(1.24-2.33)
<i>P</i> -trend				0.001		0.001
Fluid intake (l/d)						
Quintile 1 (1.6/1.5)	83	11,084	1.00	(ref)	1.00	(ref)
Quintile 2 (1.9/1.8)	76	11,599	0.88	(0.63-1.22)	0.88	(0.62-1.24)
Quintile 3 (2.1/2.0)	90	12,066	1.00	(0.73-1.37)	1.01	(0.72-1.42)
Quintile 4 (2.4/2.2)	89	11,686	1.02	(0.75-1.41)	1.01	(0.69-1.47)
Quintile 5 (2.8/2.6)	71	11,966	0.81	(0.58-1.14)	0.78	(0.49-1.23)
<i>P</i> -trend				0.49		0.62
Increment per 1 l/d			0.89	(0.72-1.10)	0.83	(0.60-1.16)
Potassium intake						
Quintile 1 (2.8/2.9)	71	11,228	1.00	(ref)	1.00	(ref)
Quintile 2 (3.1/3.2)	83	11,582	1.15	(0.82-1.60)	1.16	(0.83-1.62)
Quintile 3 (3.4/3.6)	91	12,195	1.20	(0.87-1.67)	1.21	(0.85-1.71)
Quintile 4 (3.7/3.8)	74	11,680	1.01	(0.72-1.42)	1.04	(0.72-1.50)
Quintile 5 (4.2/4.3)	90	11,716	1.24	(0.89-1.72)	1.28	(0.87-1.90)
<i>P</i> -trend				0.42		0.41
Increment per 1 g/d			1.06	(0.89-1.27)	1.10	(0.87-1.39)

^a HR adjusted for age at baseline (y) and sex (men/women); ^b HR adjusted for age at baseline (y), sex (men/women), total energy intake (kcal/d), alcohol consumption (g ethanol/d), BMI (kg/m²), smoking status (never or ex/current), smoking duration (y), smoking intensity (cig/d) and if applicable for sodium intake (g/d), discretionary salt intake (g/d), fluid intake (l/d), potassium intake (g/d) and hypertension (no/yes); ^c Salt intake refers to sodium-chloride intake; ^d Saltiness of soups and restaurant food. BMI = body mass index, cig = cigarettes, NLCS = Netherlands Cohort Study.

In participants without hypertension, the RCC risk for discretionary salt intake ('no' *versus* quartile 1) was no longer significant [HR(95%CI): 1.74(0.84-3.63)]. Excluding the first two years follow-up or participants with low-salt diet did not change results (data not shown). However, the association between sodium intake and RCC risk clearly attenuated over time. We observed a higher HR for quintile 5 (*versus* 1) of sodium intake during the first nine years follow-up and a lower HR during the second nine years follow-up [HR(95%CI): 1.71(1.08-2.71) and 1.12(0.69-1.82), respectively]. There was a significant interaction between sodium and fluid intake but not between sodium and potassium intake regarding RCC risk (P -interaction=0.02 and 0.73, respectively; Table 2.3). Participants with high sodium and low fluid intake compared to low sodium and low fluid intake had a high RCC risk [HR(95%CI): 1.91(1.10-3.32)]. Other interactions between sodium or discretionary salt intake and RCC risk factors were not significant (data not shown).

DISCUSSION

To our knowledge, this is the first prospective study to investigate sodium, potassium and fluid intake and their interactions as potential risk factors for RCC. For sodium intake, the association with RCC risk has been reported in a case-control study, showing a decreased risk for higher sodium intake in men, but not in women [9]. In the present study, we observed an increased RCC risk for higher sodium intake and no interaction between sex and sodium intake for RCC risk. In addition, we found no indications for mediation or effect-modification by hypertension, suggesting that sodium intake may be associated with RCC risk independently of hypertension. Fluid intake was not associated with RCC risk, which is in line with previous prospective cohort studies [10,12], but not with a case-control study showing an increased RCC risk for higher fluid intake [11]. All studies, however, ignored potential confounding by or effect-modification through sodium intake. Yet, we observed an interaction between sodium and fluid intake for RCC risk, which supports our hypothesis that the balance between sodium and fluid intake is important in RCC etiology. Potassium intake was not associated with RCC risk, which is similar to previous research [9].

We recognise that salt (sodium-chloride) intake is a difficult concept to measure accurately, particularly with questionnaires. However, our baseline questionnaire was specifically designed to study salt intake as it additionally included specific questions on discretionary salt intake. Although the FFQ was able to rank individuals adequately according to sodium intake when compared to nine-day dietary records [13,20], information on the validity of discretionary salt intake was not available.

Table 2.3 Renal cell cancer risk for sodium intake by fluid intake and potassium intake, NLCS 1986-2003.

Exposures	Sodium intake ^{a,b}						P-value ^d			
	Tertile 1 (low)			Tertile 2				Tertile 3 (high)		
	cases/py	HR ^c	95% CI	cases/py	HR ^c	95% CI		cases/py	HR ^d	95% CI
Fluid intake										
Low ^e	22/4,902	0.78	(0.43-1.43)	33/5,106	1.03	(0.60-1.76)	42/3,653	1.91	(1.10-3.32)	
Moderate ^e	57/8,994	1.00	(0.63-1.56)	56/9,042	0.96	(0.60-1.54)	64/7,872	1.31	(0.83-2.06)	
High ^e	39/5,803	1.00	(ref)	48/5,463	1.28	(0.81-2.02)	48/7,567	0.93	(0.59-1.46)	0.02
Potassium intake ^b										
Tertile 1 (low)	42/7,421	1.03	(0.62-1.70)	43/6,232	1.23	(0.74-2.03)	38/5,164	1.35	(0.81-2.24)	
Tertile 2	47/6,659	1.32	(0.81-2.15)	47/6,718	1.27	(0.78-2.07)	52/6,621	1.47	(0.92-2.37)	
Tertile 3 (high)	29/5,619	1.00	(ref)	47/6,661	1.32	(0.80-2.17)	64/7,306	1.72	(1.07-2.77)	0.73

^a Tertile boundaries are based on subcohort members and are specific for sex; ^b Intakes are energy-adjusted; ^c HR adjusted for age at baseline (y), sex (men/women), total energy intake (kcal/d), discretionary salt intake (g/d), alcohol consumption (g ethanol/d), BMI (kg/m²), smoking status (never or ex/current), smoking duration (y), smoking intensity (cig/d), hypertension (no/yes) and if applicable for fluid intake (l/d) and potassium intake (g/d); ^d P-value for interaction tested with Wald χ^2 test; ^e Categories low, moderate and high fluid intake correspond to respectively <1.75, 1.75-2.25 and >2.25 l/d. BMI = body mass index, cig = cigarettes, NLCS = Netherlands Cohort Study, py = person years.

Nevertheless, the mean salt intake (sodium from ready-to-eat foods plus discretionary salt) in our population (8.7 g/d) is comparable to that of the Dutch population in 1986 [25]. Exposures were only assessed at baseline. Although nutrient intakes were rather stable during the first five years follow-up [14], they may have changed during later follow-up, possibly explaining the attenuation in risk estimate over time.

Compared to sodium intake, which is calculated using standardized measurement units of the FFQ, the inter-individual range of discretionary salt intake is very large (up to factor 10). To capture all variability, it might be desirable to combine both indicators of sodium intake into one overall intake variable. However, discretionary salt intake is highly influenced by habits, attitudes and beliefs [26]. In fact, those who wish to reduce salt intake may particularly reduce discretionary salt intake. The observed u-shape in RCC risk for discretionary salt intake may perhaps reflect one's ability to reduce discretionary salt intake to prevent or control hypertension. Indeed, participants with hypertension were overrepresented in the lowest category of discretionary salt intake (data not shown). Therefore, an overall intake variable was less informative than both individual indicators of sodium intake separately, as HRs for this overall variable fully reflected the arbitrary combination of a linear and u-shaped trend (data not shown).

We used high perceived saltiness as indicator of low salt preference. Salt preference may indicate actual sodium intake, although no consensus has been reached [27]. Indeed, we observed an inverse relation with perceived saltiness for discretionary salt intake (P -value<0.001), but not for sodium intake (P -value=0.86). The association between high perceived saltiness and RCC risk was, however, in opposite direction as expected, as previously observed for stomach cancer [20]. Perhaps, this association may be explained by the hedonic shift, in which the taste of reduced-sodium products becomes more acceptable over time [27].

The mechanism through which sodium intake may influence RCC risk is unknown. Perhaps, high renal sodium load constitutes to RCC carcinogenesis through inflammation, as sodium is suggested to have inflammatory properties, which can have tumor-promoting consequences [28,29].

In conclusion, sodium intake is a potential risk factor for RCC, particularly if fluid consumption is low, but potassium and fluid intake are not associated with RCC risk. However, these findings await confirmation in other prospective studies and for specific RCC subtypes. Special attention should be given to the role of hypertension and other related diseases, such as end stage renal disease, in the association between sodium intake and RCC risk.

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Suppl Table S2.1 Renal cell cancer risk according to indicators of sodium intake, fluid intake and potassium intake by strata of hypertension, NLCS 1986-2003.

Exposures in g/d (median in subcohort (men/women))	Hypertension						<i>P</i> -value ^b
	No			Yes			
	Cases/py	HR ^a	95% CI	Cases/py	HR ^a	95% CI	
Sodium intake							
Quintile 1 (1.9/1.5)	48/8,765	1.00	(ref)	22/3,076	1.00	(ref)	1.00
Quintile 2 (2.2/1.8)	44/7,589	1.08	(0.70-1.68)	32/4,214	1.05	(0.58-1.89)	
Quintile 3 (2.5/2.0)	50/8,203	1.11	(0.72-1.72)	31/3,630	1.09	(0.60-1.99)	
Quintile 4 (2.8/2.3)	53/7,616	1.27	(0.84-1.93)	36/3,937	1.27	(0.71-2.28)	
Quintile 5 (3.4/2.8)	62/8,008	1.50	(0.99-2.28)	31/3,362	1.28	(0.70-2.36)	
<i>P</i> -trend			0.04			0.30	
Increment per 1 g/d		1.08	(0.99-1.18)		1.07	(0.95-1.21)	
Discretionary salt intake ^c							
No salt	11/1,034	1.74	(0.84-3.63)	34/1,999	2.70	(1.57-4.65)	0.87
Quartile 1 (0.7/0.7)	51/8,974	1.00	(ref)	40/5,418	1.00	(ref)	
Quartile 2 (2.0/1.7)	56/10,223	0.94	(0.63-1.41)	23/3,758	0.86	(0.50-1.48)	
Quartile 3 (3.4/2.9)	71/10,088	1.20	(0.81-1.77)	29/3,897	1.04	(0.62-1.75)	
Quartile 4 (5.5/5.1)	68/9,861	1.19	(0.80-1.76)	26/3,148	1.19	(0.65-1.92)	
<i>P</i> -trend			0.52			0.06	
Increment per 1 g/d		1.04	(0.99-1.08)		0.97	(0.88-1.06)	
Perceived saltiness ^d							
Not salty enough	3/846	0.46	(0.14-1.53)	1/239	0.59	(0.07-4.70)	0.39
Good	112/17,186	1.00	(ref)	36/5,403	1.00	(ref)	
A little too salty	93/15,334	1.03	(0.77-1.39)	74/8,265	1.45	(0.95-2.23)	
Much too salty	41/4,508	1.75	(1.17-2.61)	36/3,593	1.65	(0.97-2.82)	
<i>P</i> -trend			0.01			0.40	
Fluid intake (l/d)							
Quintile 1 (1.6/1.5)	53/7,593	1.00	(ref)	30/3,491	1.00	(ref)	0.90
Quintile 2 (1.9/1.8)	45/7,944	0.81	(0.52-1.26)	31/3,655	0.99	(0.56-1.73)	
Quintile 3 (2.1/2.0)	59/8,259	1.04	(0.67-1.60)	31/3,807	0.94	(0.54-1.66)	
Quintile 4 (2.4/2.2)	53/7,892	0.95	(0.59-1.55)	36/3,794	1.10	(0.59-2.02)	
Quintile 5 (2.8/2.6)	47/8,493	0.78	(0.44-1.38)	24/3,473	0.76	(0.25-1.67)	
<i>P</i> -trend			0.71			0.70	
Increment per 1 l/d		0.85	(0.57-1.26)		0.79	(0.43-1.44)	
Potassium intake							
Quintile 1 (2.8/2.9)	44/8,253	1.00	(ref)	27/2,975	1.00	(ref)	0.94
Quintile 2 (3.1/3.2)	55/8,176	1.31	(0.86-2.00)	28/3,406	0.91	(0.51-1.64)	
Quintile 3 (3.4/3.6)	58/8,383	1.33	(0.86-2.06)	33/3,812	0.97	(0.54-1.71)	
Quintile 4 (3.7/3.8)	46/7,749	1.19	(0.75-1.90)	28/3,931	0.81	(0.44-1.48)	
Quintile 5 (4.2/4.3)	54/7,620	1.47	(0.90-2.40)	36/4,096	0.97	(0.50-1.88)	
<i>P</i> -trend			0.25			0.89	
Increment per 1 g/d		1.12	(0.84-1.50)		1.04	(0.69-1.58)	

^a HR adjusted for age at baseline (y), sex (men/women), total energy intake (kcal/d), alcohol consumption (g ethanol/d), BMI (kg/m²), smoking status (never or ex/current), smoking duration (y), smoking intensity (cig/d) and if applicable for sodium intake (g/d), discretionary salt intake (g/d), fluid intake (l/d) and potassium intake (g/d); ^b P-value for interaction tested with Wald χ^2 test; ^c Salt intake refers to sodium-chloride intake; ^d Saltiness of soups and restaurant food. BMI = body mass index, cig = cigarettes, NLCS = Netherlands Cohort Study, py = person years.

PART II

Gene-environment interactions

CHAPTER 3

Polymorphisms in genes of the renin-angiotensin-aldosterone system and renal cell cancer risk: interplay with hypertension and dietary intakes of sodium, potassium and fluid

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ABSTRACT

Introduction

Hypertension is an established risk factor for renal cell cancer (RCC). The renin-angiotensin-aldosterone system (RAAS) regulates blood pressure and is closely linked to hypertension. RAAS additionally influences homeostasis of electrolytes (e.g. sodium and potassium) and fluid. We investigated single nucleotide polymorphisms (SNPs) in RAAS and their interactions with hypertension and intakes of sodium, potassium and fluid regarding RCC risk.

Methods

The Netherlands Cohort Study (NLCS) was initiated in 1986 and included 120,852 participants aged 55-69 years. Diet and lifestyle were assessed by questionnaires and toenail clippings were collected. Genotyping of toenail DNA was performed using the SEQUENOM® MassARRAY® platform for a literature-based selection of 13 candidate SNPs in seven key RAAS genes. After 20.3 years of follow-up, Cox regression analyses were conducted using a case-cohort approach including 3583 subcohort members and 503 RCC cases.

Results

Two SNPs in *AGTR1* were associated with RCC risk. *AGTR1*_rs1492078 (AA *versus* GG) decreased RCC risk [hazard ratio (HR) (95% confidence interval (CI)): 0.70(0.49-1.00)], whereas *AGTR1*_rs5186 (CC *versus* AA) increased RCC risk [HR(95%CI): 1.49(1.08-2.05)]. Associations were stronger in participants with hypertension. The RCC risk for *AGT*_rs3889728 (AG+AA *versus* GG) was modified by hypertension (*P*-interaction=0.039). SNP-diet interactions were not significant, although HRs suggested interaction between SNPs in *ACE* and sodium intake.

Conclusion

SNPs in *AGTR1* and *AGT* influenced RCC susceptibility, and their effects were modified by hypertension. Sodium intake was differentially associated with RCC risk across genotypes of several SNPs, yet some analyses had probably inadequate power to show significant interaction. Results suggest that RAAS may be a candidate pathway in RCC etiology.

INTRODUCTION

Hypertension is an established risk factor for renal cell cancer (RCC) [1], yet the underlying biological mechanism is poorly understood. The renin-angiotensin-aldosterone system (RAAS), which is a hormonal mechanism that regulates blood pressure, is closely linked to hypertension. Up-regulated RAAS is known to influence the development of hypertension, while inhibition of the RAAS with angiotensin-converting-enzyme (ACE) inhibitors or angiotensin-receptor blockers (ARBs) is commonly used as antihypertensive therapy. Yet, recent developments indicate that deregulated RAAS may also contribute to carcinogenesis, as it may enhance malignant transformation through stimulation of angiogenesis, invasion, cell survival and proliferation [2]. Therefore, it is biologically plausible to consider RAAS as a potential candidate pathway in RCC carcinogenesis. In fact, single nucleotide polymorphisms (SNPs) in genes of the RAAS have been associated with RCC [3].

Recently, we showed that high sodium intake, which is recognized to cause a rise in blood pressure and to influence the development of hypertension [4], might also be a potential risk factor for RCC [5]. Renal sodium homeostasis is regulated by local RAAS processes, for example in the proximal tubule of the kidney, where the concentration of angiotensin II, one of the key factors in RAAS, may be approximately 1000 times higher than that in the circulation to stimulate local sodium reabsorption [6]. Conversely, local RAAS may also be enhanced by a high-salt diet [7]. This suggests that, when considering RAAS in RCC etiology, attention should be given to the interplay between both genetic and environmental factors. In contrast to sodium intake, potassium and fluid intake, which are also related to blood pressure regulation and electrolyte balance [6,8], did not appear to be independent risk factors for RCC [5], yet they may still interact with RAAS genes to influence RCC susceptibility regardless of main effects.

We use a candidate SNP approach to investigate the associations between genetic variation in genes encoding for key factors in RAAS and the risk of RCC in a large population-based cohort study after 20.3 years of follow-up. In addition, we specifically aimed to study gene-environment interactions between these SNPs and factors that may influence RAAS, such as hypertension or the intake of sodium, potassium and fluid in relation to RCC risk.

METHODS

Study design, study population and follow-up

The Netherlands Cohort Study on diet and cancer (NLCS) is a prospective cohort study initiated in 1986 to investigate the association between diet and the development of

cancer [9-11]. The cohort includes 58,279 men and 62,573 women between the ages of 55-69 years at baseline. The case-cohort design was used for efficiency in questionnaire processing and follow-up [12]. Cases were derived from the entire cohort, whereas a subcohort of 5,000 subjects was randomly sampled at baseline to estimate person years at risk for the entire cohort and regularly followed-up for vital status information. All cohort members were followed-up for cancer occurrence through computerized record linkage with the Netherlands cancer registry and the Dutch pathology registry [13]. The completeness of cancer follow-up is over 96% [14]. Cases and subcohort members with prevalent cancer (excluding skin cancer) at baseline were excluded. After 20.3 years of follow-up, 608 microscopically confirmed RCC cases were identified [International Classification of Diseases for Oncology 3 (ICD-O-3): C64].

Dietary assessment

All participants returned a mailed, self-administered, baseline questionnaire on diet and other risk factors for cancer such as smoking status and hypertension. A 150-item, semi-quantitative food-frequency questionnaire (FFQ) was used for the dietary assessment. For each food item, participants indicated how often they consumed a standardized unit [9]. We calculated the mean daily item intake by multiplying the consumption frequency and the standardized item unit [9]. Subsequently, we converted mean daily item intakes into nutrient intakes using the Dutch food composition table 1986–87 [15]. Participants with incomplete or inconsistent dietary questionnaires were excluded [9]. The FFQ ranked individuals adequately according to dietary intake when compared to a 9-day dietary record [9], and reflected nutrient intakes for at least 5 years [10]. The daily sodium, potassium and fluid intakes were defined as grams or litres of intake through both foods and beverages per day, including amounts naturally present in foods and beverages plus amounts added during food processing by food manufacturers. Sodium intake did not include sodium from added salt (sodium-chloride), yet the baseline questionnaire included specific questions on salt added during home-preparation and before consumption [16]. Together these variables indicate discretionary salt intake [5]. Sodium and potassium intake were adjusted for total energy intake by using the residual-mean method¹⁷ and modelled as sex-specific tertile distribution. Fluid intake was modelled as categorical variable using the categories low (≤ 1.75 l/d), medium (1.75-2.25 l/d) and high (> 2.25 l/d).

Gene and SNP selection

We chose to select genes and SNPs on the basis of currently available literature. We selected seven genes from the RAAS because they are known to encode for key factors that cover the essential part of RAAS, namely angiotensin I converting enzyme (ACE), angiotensinogen (AGT), angiotensin II receptor, type 1 (AGTR1), angiotensin II

receptor, type 2 (*AGTR2*), cytochrome P450, family 11, subfamily B, polypeptide 2 (*CYP11B2*), nuclear receptor subfamily 3, group C, member 2 (*NR3C2*) and renin (*REN*) (Figure 3.1).

SNPs were carefully selected through extensive literature search considering the number of previously reported associations with i) the risk of RCC, ii) the risk of hyper- or hypotension iii) systolic or diastolic blood pressure, iv) enzymes or proteins in RAAS and v) salt handling or salt sensitivity and ranked accordingly. We gave priority to SNPs with a minor allele frequency (MAF) $\geq 20\%$ in Caucasians and SNPs that were not in high linkage disequilibrium (LD) with each other ($r^2 < 0.8$). Together, these criteria gave over 100 potential SNPs of interest classified into high and low priority SNPs.

Figure 3.1 Selected key factors in the renin-angiotensin-aldosterone system and the corresponding genes. In response to a low blood pressure the angiotensinogen precursor (AGT), which is expressed in the liver, is cleaved by the enzyme renin (REN). The resulting product, angiotensin I, is then converted into the physiologically active enzyme angiotensin II by the angiotensin converting enzyme (ACE). Angiotensin II is a vasopressor hormone that acts through two membrane receptors, receptor type 1 and type 2 (AGTR1/AGTR2), to induce aldosterone synthesis. Aldosterone synthase (i.e. cytochrome P450 family 11, subfamily B, polypeptide 2; CYP11B2) is a monooxygenase located on the mitochondrial inner membrane. Actions of aldosterone, which are mediated by actions of the nuclear receptor subfamily 3, group C, member 2 proteins (NR3C2), enhance water and sodium reabsorption and potassium excretion in the kidney, eventually increasing blood pressure.

DNA isolation

Approximately 90,000 participants provided toenail clippings at baseline, which have been shown to be a valid source of DNA for the genotyping of germline genetic variants [18]. DNA was isolated using an optimized protocol for DNA isolation based on the method of Cline *et al.* [18,19]. To increase our case sample, for 67 RCC cases without toenail clippings, DNA was isolated from formalin-fixed paraffin-embedded (FFPE) healthy tissues, as described by van Houwelingen *et al.* [20] and added to the sample collection of toenail DNA. The use of DNA was approved by the review board of Maastricht University (Maastricht, the Netherlands).

SNP genotyping and quality control

SNP genotyping was performed on the SEQUENOM® MassARRAY® platform using the iPLEX™ assay (Sequenom Inc., Hamburg, Germany), which showed to be suitable for our toenail DNA [21]. This platform allows high-throughput genotyping of up to 40 SNPs at once. Extension primers were designed using MassARRAY® software, version 4.0. Polymerase chain reaction (PCR) amplification and extension reactions were performed according to the manufacturer's instructions. Extension product sizes were determined by mass spectrometry with the use of the iPLEX Gold system [22].

Selected SNPs with high priority were first designated to the assay. Nevertheless, due to potential sequence incompatibilities among primers, not all high priority SNPs could be included in the design. For example, we included *CYB11B2*_rs7831617 (G>T) as a proxy for *CYB11B2*_1799998 (T>C, $r^2=1.0$ and $D'=1.0$), which was listed as high priority SNP, but could not be combined with other SNPs on the multiplex. Subsequently, SNPs with less priority were used to fill up the multiplex. We were able to genotype 13 SNPs in seven key RAAS genes (Table 3.1). All SNPs had a call rate of at least 97%.

Genotyping was performed using 100 ng of DNA, which was pipetted onto 384-well plates. In addition, 302 water controls and one duplicate sample for a random selection of ~5% of the samples ($n=210$) were scattered across the plates for quality control. The average concordance of the duplicates was 99.6%. One duplicate pair showed highly irreproducible results and was therefore excluded from further analysis. Initially, genotyping data were available for 4,238 samples (excluding duplicates), including 78% of the subcohort and 88% of the RCC cases. We excluded 171 samples representing 4% of the data for further analyses, as those samples called less than 90% of the SNPs. Because of the case-cohort design, the remaining 4,067 samples correspond to 3583 subcohort members and 503 RCC cases. There were no substantial differences in quality parameters between toenail samples and FFPE healthy tissues samples.

Table 3.1 Characteristics of SNPs in RAAS genes in subcohort members in the NLCS, 1986-2006.

SNPs in RAAS genes	Chr. location	Reference alleles	Minor allele	MAF	Genotypes					
					11 ^a		12 ^a		22 ^a	
					n	%	n	%	n	%
<i>ACE</i> _rs4295	17q23.3	C/G	G	0.375	1,409	39.39	1,650	46.13	518	14.48
<i>ACE</i> _rs4343	17q23.3	A/G	A	0.489	929	26.00	1,797	50.29	847	23.71
<i>AGT</i> _rs2493137	1q42.2	C/T	C	0.287	1,824	50.91	1,458	40.69	301	8.40
<i>AGT</i> _rs3889728	1q42.2	A/G	A	0.240	2,070	57.79	1,307	36.49	205	5.72
<i>AGT</i> _rs699	1q42.2	C/T	C	0.385	1,359	38.60	1,615	45.87	547	15.54
<i>AGTR1</i> _rs1492078	3q24	A/G	A	0.369	1,386	39.69	1,632	46.74	474	13.57
<i>AGTR1</i> _rs5186	3q24	A/C	C	0.300	1,753	48.95	1,508	42.11	320	8.94
<i>AGTR2</i> _rs5193	Xq22-q23	G/T	T	0.247	2,362	65.94	674	18.82	546	15.24
<i>CYP11B2</i> _rs7831617	8q21-q22	G/T	T	0.423	1,206	33.68	1,721	48.06	654	18.26
<i>NR3C2</i> _rs11099681	4q31.1	C/T	C	0.283	1,830	51.12	1,473	41.15	277	7.74
<i>NR3C2</i> _rs1879829	4q31.1	C/T	C	0.477	999	27.88	1,747	48.76	837	23.36
<i>REN</i> _rs2887284	1q32	A/C	A	0.205	2,258	63.02	1,180	32.93	145	4.05
<i>REN</i> _rs5705	1q32	A/C	C	0.126	2,742	76.55	778	21.72	62	1.73
										0.45

^a Genotypes 11, 12 and 22 refer to the homozygote common, heterozygote and homozygote rare genotype, respectively; ^b *p*-value for HWE; ^c Tested in women only. *ACE* = angiotensin-converting enzyme, *AGT* = angiotensinogen, *AGTR1* = Type 1 angiotensin II receptor, *AGTR2* = Type 2 angiotensin II receptor, Chr. = chromosomal, *CYP11B2* = aldosterone-synthesizing enzyme, HWE = Hardy-Weinberg equilibrium, MAF = minor allele frequency, NLCS = Netherlands Cohort Study, *NR3C2* = mineralocorticoid receptor, RAAS = renin-angiotensin-aldosterone system, *REN* = renin-binding protein, SNPs = single nucleotide polymorphisms.

Statistical analyses

Genotypes were analysed under a co-dominant mode of inheritance, which showed the best model fit according to the Akaike information criterion (AIC), and in an allelic model. SNPs with a MAF<0.25 were analysed in a dominant instead of a co-dominant model to maintain optimal power. Hazard ratios (HR) and 95% confidence intervals (CI) for the independent main effects of each SNP on RCC risk were evaluated in a crude model using Cox proportional hazards analyses adjusted for the case-cohort design [23]. Analyses were conducted in the total population and separately for participants with and without hypertension. Hypertension was defined as a self-reported doctors' diagnosis and/or self-reported use of anti-hypertensive medication. Gene-environment interactions were tested using the Wald χ^2 test by including the interaction term into the model. Interaction analyses including dietary exposures were performed for an age- and sex-adjusted model and a multivariable-adjusted model, including *a priori* selected potential confounders: age at baseline (y), sex (men/women), total energy intake (kcal/d), body mass index (BMI; kg/m²), smoking status (never or ex/current), smoking duration (y), smoking intensity (cigarettes/d), hypertension status (no/yes) and alcohol consumption (g ethanol/d). We recognise that the FFQ-based sodium intake did not include discretionary salt intake. Earlier we indicated that combining both variables into an overall intake variable was not informative, because both variables represent different aspects of total sodium intake [5]. Instead, in sensitivity analyses, we additionally adjusted each SNP-sodium interaction for discretionary salt intake (g/d). Cox proportional hazard analyses were conducted using Stata version 12 (Stata Corp., College Station, TX). For all the models including covariates, the proportional hazards assumption was tested using the scaled Schoenfeld residuals. A violation was apparent for age, which was therefore modelled as time dependent covariate. A *P*-value<0.05 was considered statistically significant.

Additional analyses were conducted to investigate potential higher-order gene-environment interactions among all candidate SNPs in RAAS and all environmental exposures of interest (i.e. sodium, discretionary salt, potassium and fluid intake and hypertension status) and, in particular, to investigate the relevance of the candidate SNPs in a previously observed significant interaction between sodium and fluid intake in relation to RCC risk [5]. We used multifactor dimensionality reduction (MDR; version 3.0.2) as analyses technique, as it is a non-parametric and genetic model-free data mining method that is able to detect higher-order interactions in absence of main effects [24,25]. We investigated all 2- to 4-way interactions, but only selected models with a testing balanced accuracy>0.55 to limit the chance of 'overfitting'. The statistical significance of these models was estimated using permutation testing with 1000 permuted datasets. In addition, an explicit test for interaction has been used to obtain *P*-values for interaction, independent of main effects. MDR analyses did not include the X-chromosomal SNP *AGTR2*_rs5193, to avoid the strong influence of sex on the results.

RESULTS

Genotype and allele frequencies of the RAAS SNPs in subcohort members of the NLCS are given in Table 3.1. All SNPs adhered to the Hardy Weinberg Equilibrium (HWE), as tested with Pearson χ^2 test ($P > 0.05$). HWE of the X-chromosomal SNP *AGTR2_rs5193* was tested in women only. All SNPs showed no or weak LD with each other ($r^2 \leq 0.50$), except for *AGT_rs2493137* which was in moderate LD with *AGT_rs3889728* and *AGT_rs699* ($r^2 = 0.78$ and 0.62 , respectively; Haploview, version 4.2).

Table 3.2 Baseline characteristics of subcohort members and RCC cases in the NLCS, 1986 -2006.

Baseline characteristics (mean (SD))	Subcohort members	RCC Cases
Total (n)	3379	480
Dietary intakes (in g/d)		
Sodium ^a	2.3 (0.6)	2.5 (0.7)
Discretionary salt ^{b,c}	2.8 (2.7)	2.8 (3.0)
Potassium ^a	3.5 (0.6)	3.6 (0.6)
Fluid (l/d)	2.1 (0.5)	2.1 (0.5)
Potential confounders		
Age at baseline (y)	61.3 (4.2)	61.0 (4.0)
Male sex (%)	49.8	65.2
Total energy intake (kcal/d)	1925 (506.2)	1994 (515.9)
Alcohol consumption (g ethanol/d) ^d	13.7 (15.0)	15.7 (16.1)
BMI (kg/m ²) ^c	25.0 (3.1)	25.5 (3.0)
Current smokers (%)	27.4	31.9
Smoking duration (y) ^e	38.7 (8.7)	39.6 (8.5)
Smoking intensity (cig/d) ^e	14.7 (8.7)	15.9 (8.7)
Hypertension (%)	31.8	35.4

^a Intakes are energy-adjusted; ^b Salt intake refers to sodium-chloride intake; ^c n does not correspond with the overall n, due to missing values; ^d In consumers only; ^e In current smokers. BMI = body mass index, cig = cigarettes, RCC = renal cell cancer, SD = standard deviation.

Baseline characteristics of subcohort members and cases are described in Table 3.2. Compared to subcohort members, RCC cases had a similar mean age at baseline, but were more frequently male. Consequently, RCC cases had a slightly higher BMI, were more frequently smokers and consumed more alcohol. Baseline dietary intakes were comparable between subcohort members and RCC cases, except for sodium intake, which was slightly higher in RCC cases.

Main effects of candidate RAAS SNPs

Of the 13 candidate RAAS SNPs, two SNPs both in *AGTR1* (i.e. *AGTR1_rs1492078* and *AGTR1_rs5186*) were associated with RCC risk (Table 3.3). In the total population, the AA (*versus* GG) genotype of *AGTR1_rs1492078* showed a significantly lower RCC risk [HR(95%CI): 0.70(0.49-1.00)], whereas the CC (*versus* AA) genotype of *AGTR1_rs5186* showed a significantly higher RCC risk [HR(95%CI): 1.49(1.08-2.05)]. Associations for both SNPs were stronger in participants with hypertension compared to the total

population and lost significance in participants without hypertension. The remaining candidate RAAS SNPs were neither associated with RCC risk in the total population nor in participants with or without hypertension. However, for *AGT_rs3889728*, the RCC risk for the AG+AA (*versus* GG) genotype was higher in participants without hypertension and lower in participants with hypertension. Indeed, the corresponding SNP-hypertension multiplicative interaction was statistically significant (P -interaction=0.039).

Gene-environment interactions

None of the investigated multiplicative SNP-sodium interactions significantly influenced RCC risk (P -interaction=0.114-0.988; Table 3.4). Nevertheless, we observed a suggestive interaction with sodium intake for some candidate RAAS SNPs, particularly those in *ACE*, since HRs markedly differed across genotypes and tertiles of sodium intake. For *ACE_rs4295*, on the one hand, analyses stratified by genotype showed a clear positive association between sodium intake and RCC risk for the CC genotype (P -trend=0.005) and no association for the GG genotype (P -trend=0.949). On the other hand, analyses stratified by tertiles of sodium intake showed a suggestive positive association between the G allele and RCC risk in participants with a low sodium intake [HR(95%CI): 1.20(0.91-1.57)] and not in those with moderate or high sodium intake. Similarly, the association between sodium intake and RCC risk differed across genotypes of *ACE_rs4343* and the A allele of *ACE_rs4343* was inversely associated with RCC risk [HR(95%CI): 0.77(0.59-1.00)] only in participants with a low sodium intake. Despite these differences, the SNP-sodium interactions for *ACE_rs4295* and *ACE_rs4343* were not significant (P -interaction=0.385 and 0.114, respectively). Different associations between sodium intake and RCC risk were also observed across genotypes of *AGT_rs2493137*, *AGTR1_rs1492078* and *AGTR2_rs5193* (women). None of the associations including sodium intake changed after additional adjustment for discretionary salt intake (data not shown).

SNPs in RAAS genes	No Hypertension						Hypertension					
	Person years ^a	RCC Cases	HR	95% CI	P- trend		Person years ^a	RCC Cases	HR	95% CI	P- trend	
ACE_rs4295												
CC	20,737	161	1.00	(ref)			14,484	98	1.00	(ref)		
CG	23,940	204	1.10	(0.88-1.39)			16,521	134	1.20	(0.91-1.58)		
GG	7,248	54	0.96	(0.69-1.33)			5,108	37	1.07	(0.72-1.60)		
G ^c	51,925	419	1.01	(0.87-1.17)	0.894		36,113	269	1.07	(0.90-1.28)	0.450	0.511
ACE_rs4343												
GG	13,117	104	1.00	(ref)			9,200	70	1.00	(ref)		
AG	26,492	220	1.05	(0.81-1.34)			18,687	147	1.03	(0.76-1.40)		
AA	12,272	92	0.95	(0.70-1.27)			8,196	51	0.82	(0.56-1.20)		
A ^c	51,881	416	0.97	(0.84-1.13)	0.726		36,083	268	0.91	(0.76-1.09)	0.326	0.463
AGT_rs2493137												
TT	26,236	216	1.00	(ref)			18,717	135	1.00	(ref)		
CT	21,357	165	0.94	(0.76-1.15)			14,500	109	1.04	(0.79-1.36)		
CC	4,453	38	1.04	(0.71-1.50)			2,957	25	1.17	(0.74-1.85)		
C ^c	52,046	419	0.98	(0.84-1.16)	0.849		36,174	269	1.06	(0.87-1.30)	0.538	0.328
AGT_rs3889728												
GG	29,781	239	1.00	(ref)			21,082	146	1.00	(ref)		
AG + AA	22,246	180	1.01	(0.82-1.24)			15,071	123	1.18	(0.91-1.52)		
A ^c	52,027	419	1.03	(0.86-1.22)	0.945		36,153	269	1.16	(0.94-1.42)	0.216	0.039
AGT_rs699												
TT	19,625	162	1.00	(ref)			13,891	101	1.00	(ref)		
MT	23,646	180	0.92	(0.73-1.16)			16,299	115	0.97	(0.73-1.29)		
MM	7,960	65	0.99	(0.73-1.35)			5,404	45	1.14	(0.78-1.67)		
M ^c	51,231	407	0.98	(0.84-1.14)	0.775		35,594	261	1.05	(0.87-1.27)	0.621	0.423
AGTR1_rs1492078												
GG	20,054	171	1.00	(ref)			13,997	111	1.00	(ref)		
AG	23,718	191	0.94	(0.76-1.18)			16,447	118	0.91	(0.69-1.19)		
AA	6,907	41	0.70	(0.49-1.00)			4,604	30	0.82	(0.54-1.26)		
A ^c	50,679	403	0.87	(0.75-1.01)	0.075		35,048	259	0.91	(0.75-1.10)	0.322	0.277
AGTR1_rs5186												
AA	25,621	210	1.00	(ref)			17,864	138	1.00	(ref)		
AC	21,725	152	0.85	(0.68-1.07)			15,166	99	0.85	(0.64-1.11)		
CC	4,681	57	1.49	(1.08-2.05)			3,123	32	1.33	(0.87-2.01)		
C ^c	52,027	419	1.09	(0.92-1.29)	0.300		36,153	269	1.03	(0.84-1.27)	0.130	0.728

Table 3.3 (continued).

SNPs in RAAS genes	Person years ^a	RCC Cases	No Hypertension				Hypertension				P- Interaction ^b
			HR	95% CI	P- trend	Person years ^a	RCC Cases	HR	95% CI	P- trend	
AGTR2_rs5193 (men)											
G	18,384	210	1.00	(ref)		12,914	148	1.00	(ref)		
T	5,815	66	0.99	(0.73-1.34)		4,280	47	0.96	(0.67-1.37)		
T ^c	24,199	276	1.00	(0.86-1.16)	0.964	17,194	195	0.98	(0.82-1.17)	0.803	0.686
AGTR2_rs5193 (women)											
GG	16,091	80	1.00	(ref)		11,018	45	1.00	(ref)		
GT + TT	11,757	61	1.04	(0.74-1.48)		7,962	27	0.83	(0.51-1.36)		
T ^c	68,117	498	1.02	(0.77-1.34)	0.809	47,171	313	0.89	(0.59-1.33)	0.463	0.216
CYP11B2_rs7831617											
GG	17,276	135	1.00	(ref)		11,907	83	1.00	(ref)		
GT	25,089	199	1.01	(0.80-1.28)		17,503	131	1.07	(0.80-1.43)		
TT	9,661	84	1.11	(0.83-1.49)		6,743	54	1.15	(0.80-1.65)		0.841
T ^c	52,026	418	1.05	(0.91-1.21)	0.518	36,153	268	1.07	(0.89-1.28)	0.458	0.894
NR3C2_rs11099681											
TT	26,499	214	1.00	(ref)		18,358	134	1.00	(ref)		
CT	21,345	171	0.99	(0.80-1.23)		14,789	111	1.03	(0.79-1.34)		
CC	4,141	34	1.02	(0.70-1.50)		2,986	24	1.10	(0.69-1.75)		
C ^c	51,985	419	1.00	(0.85-1.18)	0.989	36,133	269	1.04	(0.85-1.27)	0.698	0.835
NR3C2_rs1879829											
TT	14,289	115	1.00	(ref)		9,819	81	1.00	(ref)		
CT	25,286	214	1.05	(0.82-1.34)		17,716	132	0.90	(0.67-1.22)		
CC	12,471	90	0.90	(0.67-1.20)		8,639	56	0.79	(0.55-1.13)		
C ^c	52,046	419	0.95	(0.83-1.10)	0.493	36,174	269	0.89	(0.74-1.06)	0.194	0.502
REN_rs2887284											
CC	32,952	269	1.00	(ref)		22,701	169	1.00	(ref)		
AC + AA	19,095	150	0.96	(0.78-1.19)		13,474	100	1.00	(0.77-1.30)		
A ^c	52,047	419	0.97	(0.80-1.16)	0.726	36,175	269	0.99	(0.79-1.23)	0.985	0.698
REN_rs5705											
AA	39,865	329	1.00	(ref)		27,475	216	1.00	(ref)		
AC + CC	12,162	90	0.90	(0.70-1.15)		8,679	53	0.78	(0.57-1.07)		
C ^c	52,027	419	0.89	(0.71-1.12)	0.386	36,154	269	0.80	(0.60-1.07)	0.118	0.122

^a In the subcohort; ^b Using Wald χ^2 test for interaction; ^c Trend for genotype is based on the allelic model. ACE = angiotensin-converting enzyme, AGT = angiotensinogen, AGTR1 = Type 1 angiotensin II receptor, AGTR2 = Type 2 angiotensin II receptor, CYP11B2 = aldosterone-synthesizing enzyme, NLCS = Netherlands Cohort Study, NR3C2 = mineralocorticoid receptor, RAAS = renin-angiotensin-aldosterone system, RCC = renal cell cancer, REN = renin-binding protein, SNPs = single nucleotide polymorphisms.

Table 3.4 Hazard ratios and 95% confidence intervals for the association between renal cell cancer and SNPs in RAAS genes by low, medium and high sodium intake in the NICS, 1986-2006.

SNPs in RAAS genes	Teriles of sodium intake (median) ^{a,b}														
	Low (1.7 g/d)					Medium (2.2 g/d)					High (2.9 g/d)				
	Person			Person			Person			Person			Person		
	years ^c	RCC	Cases	HR ^d	95% CI	years ^c	RCC	Cases	HR ^d	95% CI	years ^c	RCC	Cases	HR ^d	95% CI
ACE_rs4295															
CC	7,042	40	100	(ref)		7,104	53	133	1.33	(0.85-2.07)	6,591	68	191	1.91	(1.25-2.92)
CG	8,103	64	1.48	(0.97-2.27)		8,058	57	1.25	(0.80-1.94)		7,778	83	190	(1.26-2.85)	0.005
GG	2,301	16	1.29	(0.69-2.41)		2,333	18	1.40	(0.77-2.57)		2,614	20	1.37	(0.77-2.44)	0.205
G ^h	17,446	120	1.20	(0.91-1.57)		17,495	128	1.02	(0.77-1.35)		16,983	171	0.89	(0.70-1.13)	0.949
ACE_rs4343															
GG	4,206	33	1.00	(ref)		4,234	34	0.97	(0.58-1.63)		4,676	37	1.01	(0.60-1.68)	0.972
AG	8,888	67	0.95	(0.60-1.49)		9,122	59	0.78	(0.50-1.26)		8,481	94	1.35	(0.88-2.08)	0.064
AA	4,307	19	0.54	(0.30-0.99)		4,110	34	0.99	(0.59-1.66)		3,855	39	1.25	(0.75-2.08)	0.009
A ^h	17,401	119	0.77	(0.59-1.00)		17,466	127	1.00	(0.75-1.32)		17,012	170	1.11	(0.88-1.40)	0.114
AGT_rs2493137															
TT	8,790	59	1.00	(ref)		9,509	66	0.98	(0.67-1.44)		7,938	91	1.66	(1.16-2.37)	0.005
CT	7,187	48	0.93	(0.62-1.40)		6,646	51	1.08	(0.72-1.63)		7,524	66	1.25	(0.85-1.84)	0.146
CC	1,531	13	1.29	(0.67-2.48)		1,360	11	1.13	(0.56-2.27)		1,562	14	1.28	(0.68-2.42)	0.805
C ^h	17,508	120	1.05	(0.77-1.44)		17,515	128	1.10	(0.82-1.46)		17,024	171	0.80	(0.60-1.05)	
AGT_rs3889728															
GG	9,831	68	1.00	(ref)		10,639	71	0.93	(0.65-1.33)		9,311	100	1.53	(1.09-2.13)	0.015
AG + AA	7,656	52	0.95	(0.64-1.40)		6,876	57	1.14	(0.78-1.68)		7,713	71	1.29	(0.89-1.86)	0.084
A ^h	17,487	120	0.97	(0.70-1.35)		17,515	128	0.16	(0.87-1.56)		17,024	171	0.90	(0.67-1.20)	0.333
AGT_rs699															
TT	6,535	44	1.00	(ref)		6,848	50	1.04	(0.67-1.61)		6,242	68	1.61	(1.07-2.43)	0.024
MT	7,786	49	0.91	(0.59-1.41)		8,019	61	1.09	(0.72-1.67)		7,841	70	1.29	(0.86-1.94)	0.068
MM	2,899	22	1.17	(0.67-2.04)		2,403	14	0.84	(0.44-1.60)		2,659	29	1.59	(0.95-2.67)	0.354
M ^h	17,220	115	1.05	(0.79-1.41)		17,270	125	0.96	(0.73-1.26)		16,742	167	0.93	(0.72-1.20)	0.673
AGTR1_rs1492078															
GG	7,322	51	1.00	(ref)		6,693	53	1.07	(0.70-1.63)		6,039	67	1.51	(1.01-2.25)	0.043
AG	7,851	55	0.96	(0.64-1.45)		7,950	53	0.91	(0.60-1.38)		7,917	83	1.44	(0.99-2.11)	0.037
AA	1,917	11	0.74	(0.37-1.50)		2,385	15	0.86	(0.46-1.59)		2,605	15	0.79	(0.42-1.45)	0.999
A ^h	17,220	117	0.89	(0.66-1.21)		17,028	121	0.87	(0.65-1.16)		16,561	165	0.80	(0.63-1.02)	0.782

Table 3.4 (continued).

SNPs in RAAS genes	Tertiles of sodium intake (median) ^{a,b}											
	Low (1.7 g/d)				Medium (2.2 g/d)				High (2.9 g/d)			
	Person		RCC		Person		RCC		Person		RCC	
	years ^c	HR ^d	95% CI	cases	years ^c	HR ^d	95% CI	cases	years ^c	HR ^d	95% CI	cases
AGTR1_rs5186												
AA	8,405	57	1.00 (ref)	8,900	77	1.22 (0.84-1.78)	8,316	76	8,316	1.32 (0.91-1.92)	0.142	76
AC	7,517	44	0.86 (0.56-1.32)	6,800	37	0.79 (0.79-1.23)	7,408	71	7,408	1.41 (0.96-2.05)	0.019	71
CC	1,586	19	1.66 (0.93-2.96)	1,795	14	1.16 (0.62-2.19)	1,300	24	1,300	2.78 (1.60-4.83)	0.187	24
C ^h	17,508	120	1.16 (0.85-1.59)	17,495	128	0.83 (0.61-1.13)	17,024	171	17,024	1.34 (1.02-1.75)		171
AGTR2_rs5193 (men)												
G	6,476	60	1.00 (ref)	6,025	64	1.07 (0.72-1.60)	5,883	86	5,883	1.56 (1.08-2.26)	0.014	86
T	1,901	19	1.10 (0.62-1.95)	2,120	22	1.16 (0.68-1.98)	1,794	25	1,794	1.46 (0.87-2.46)	0.394	25
T ^h	8,377	79	1.03 (0.77-1.40)	8,145	86	1.01 (0.76-1.33)	7,677	111	7,677	0.95 (0.74-1.24)		111
AGTR2_rs5193 (women)												
GG	5,299	19	1.00 (ref)	5,249	26	1.40 (0.75-2.60)	5,543	35	5,543	1.66 (0.91-3.03)	0.077	35
GT + TT	3,831	22	1.59 (0.83-3.02)	4,121	15	0.99 (0.49-2.01)	3,805	24	3,805	1.71 (0.90-3.22)	0.336	24
T ^h	9,130	41	1.38 (0.87-2.19)	9,370	41	0.80 (0.44-1.46)	9,348	59	9,348	0.96 (0.62-1.49)		59
CYP11B2_rs7831617												
GG	5,966	38	1.00 (ref)	5,840	40	1.04 (0.65-1.68)	5,470	57	5,470	1.59 (1.02-2.49)	0.032	57
GT	8,550	60	1.09 (0.70-1.68)	8,209	59	1.11 (0.71-1.71)	8,330	80	8,330	1.49 (0.98-2.26)	0.079	80
TT	2,992	22	1.15 (0.65-2.03)	3,466	28	1.20 (0.71-2.04)	3,204	34	3,204	1.69 (1.02-2.80)	0.189	34
T ^h	17,508	120	1.08 (0.81-1.43)	17,515	127	1.08 (0.83-1.41)	17,004	171	17,004	1.03 (0.81-1.32)		171
NR3C2_rs11099681												
TT	9,025	65	1.00 (ref)	8,470	68	1.03 (0.71-1.50)	9,004	81	9,004	1.21 (0.85-1.72)	0.355	81
CT	7,068	42	0.77 (0.51-1.16)	7,509	55	0.98 (0.66-1.45)	6,768	74	6,768	1.44 (1.00-2.08)	0.001	74
CC	1,394	13	1.27 (0.66-2.45)	1,495	5	0.44 (0.17-1.13)	1,253	16	1,253	1.70 (0.92-3.14)	0.614	16
C ^h	17,487	120	0.97 (0.70-1.35)	17,474	128	0.80 (0.60-1.07)	17,025	171	17,025	1.22 (0.94-1.58)		171
NR3C2_rs1879829												
TT	4,774	35	1.00 (ref)	4,832	29	0.84 (0.49-1.42)	4,684	51	4,684	1.48 (0.93-2.37)	0.092	51
CT	8,452	56	0.93 (0.59-1.47)	8,368	74	1.19 (0.76-1.84)	8,467	84	8,467	1.39 (0.90-2.13)	0.022	84
CC	4,282	29	0.97 (0.57-1.64)	4,316	25	0.80 (0.46-1.38)	3,873	36	3,873	1.30 (0.79-2.16)	0.320	36
C ^h	17,507	120	0.99 (0.76-1.30)	17,515	128	0.98 (0.77-1.25)	17,024	171	17,024	0.95 (0.75-1.21)		171

Table 3.4 (continued)

SNPs in RAAS genes	Tertiles of sodium intake (median) ^{a,b}											
	Low (1.7 g/d)				Medium (2.2 g/d)				High (2.9 g/d)			
	Person years ^c	RCC Cases	HR ^d	95% CI	Person years ^c	RCC Cases	HR ^d	95% CI	Person years ^c	RCC Cases	HR ^d	95% CI
<i>REN_rs2887284</i>												
CC	11,025	75	1.00	(ref)	10,997	84	1.07	(0.76-1.51)	10,929	110	1.46	(1.06-2.00)
AC + AA	6,482	45	1.01	(0.68-1.51)	6,518	44	0.99	(0.66-1.48)	6,095	61	1.47	(1.01-2.13)
A ^h	17,507	120	0.93	(0.66-1.30)	17,515	128	0.96	(0.68-1.34)	17,024	171	1.03	(0.76-1.38)
<i>REN_rs5705</i>												
AA	13,587	96	1.00	(ref)	13,434	100	1.01	(0.75-1.37)	12,843	133	1.44	(1.08-1.91)
AC + CC	3,920	24	0.86	(0.53-1.38)	4,060	28	0.99	(0.63-1.56)	4,181	38	1.32	(0.88-1.99)
C ^h	17,507	120	0.82	(0.53-1.27)	17,494	128	0.91	(0.61-1.60)	17,024	171	0.94	(0.65-1.35)

^a Tertile distribution is sex-specific and based on subcohort members; ^b Sodium intake is energy-adjusted by using the residual mean method; ^c In the subcohort; ^d Hazard ratio adjusted for age at baseline (y), sex (men/women), BMI (kg/m²), hypertension (no/yes), smoking status (never or ex/current), smoking duration (y), smoking intensity (cig/d), alcohol consumption (g ethanol/d) and total energy intake (kcal/d); ^e Dose-response relationships of tertiles of sodium intake are tested for each genotype separately; ^f Using Wald χ^2 test for interaction; ^h Trend for genotype is based on the allelic model and tested for each tertile of sodium intake separately. ACE = angiotensin-converting enzyme, AGT = angiotensinogen, AGTR1 = Type 1 angiotensin II receptor, AGTR2 = Type 2 angiotensin II receptor, CYP11B2 = aldosterone-synthesizing enzyme, NLCS = Netherlands Cohort Study, NR3C2 = mineralocorticoid receptor, RAAS = renin-angiotensin-aldosterone system, RCC = renal cell cancer, REN = renin-binding protein, SNPs = single nucleotide polymorphisms.

For potassium and fluid intake, none of the multiplicative interactions with candidate RAAS SNPs were statistically significant (P -interaction=0.065-0.976; Suppl Tables S3.1 and S3.2). In general, associations were mainly inconsistent. A borderline significant multiplicative SNP-potassium interaction in relation to RCC risk was observed for *AGT*_rs669 (P -interaction=0.080). RCC risk was particularly low for low potassium consumers with the MM (*versus* TT) genotype [HR(95%CI): 0.59(0.31-1.10)]. The interaction between this SNP and fluid intake did not influence RCC risk (P -interaction=0.976). In addition, a borderline significant multiplicative SNP-fluid interaction in relation to RCC risk was observed for *AGTR1*_rs5186 (P -interaction=0.065). RCC risk was highest for high fluid consumers with the CC (*versus* AA) genotype [HR(95%CI): 1.89(1.06-3.38)]. The interaction between this SNP and potassium intake was not statistically significant (P -interaction=0.586), yet a similarly high RCC risk was observed for high potassium consumers with the CC (*versus* AA) genotype [HR(95%CI): 1.86(1.08-3.21)].

Additional analyses

In additional analyses, MDR identified no 2-way interaction models that met the selection criterion of having a testing balanced accuracy $\geq$ 0.55 (Table 3.5). None of the selected 3-way interaction models with a testing balanced accuracy $\geq$ 0.55 included both sodium and fluid intake. Among the selected 4-way interaction models, five models did include both sodium and fluid intake, of which the model including sodium intake, fluid intake, *AGT*_rs2493137 and *AGTR1*_rs5186 had the highest testing balanced accuracy. High (*versus* low) risk combinations of this 4-way interaction showed a [HR(95%CI): 2.59(2.07-3.24)] in multivariable adjusted Cox proportional hazards analysis.

Table 3.5 All 2- to 4-way interaction models with testing balanced accuracy of 0.55 or higher using multifactor dimensionality reduction (MDR).

Interaction components				Training Balanced accuracy		Testing Balanced accuracy		P-value ^a	P-value ^b
2-way									
-									
3-way									
AGT_rs2493137	AGTR1_rs1492078	REN_rs5705							
AGTR1_rs5186	fluid	hypertension		0.574	0.563	0.010	0.017		
AGTR1_rs5186	NR3C2_rs11099681	sodium		0.561	0.557	0.018	0.030		
AGT_rs2493137	AGTR1_rs1492078	sodium		0.574	0.556	0.021	0.034		
				0.572	0.551	0.043	0.057		
4-way									
AGT_rs2493137	AGT_rs699	AGTR1_rs1492078	REN_rs5705	0.585	0.570	0.003	0.007		
AGT_rs2493137	AGTR1_rs5186	Fluid	sodium	0.612	0.565	0.008	0.014		
ACE_rs4343	AGT_rs2493137	AGTR1_rs1492078	REN_rs5705	0.602	0.563	0.010	0.016		
AGT_rs699	AGTR1_rs1492078	NR3C2_rs1879829	potassium	0.614	0.563	0.010	0.016		
AGT_rs2493137	AGT_rs699	AGTR1_rs1492078	NR3C2_rs1879829	0.590	0.561	0.011	0.021		
ACE_rs4343	AGT_rs3889728	Fluid	hypertension	0.591	0.561	0.012	0.021		
AGT_rs2493137	AGTR1_rs1492078	NR3C2_rs1879829	potassium	0.601	0.560	0.013	0.022		
AGT_rs2493137	AGTR1_rs5186	NR3C2_rs11099681	NR3C2_rs2887284	0.593	0.560	0.013	0.022		
AGT_rs2493137	AGTR1_rs1492078	NR3C2_rs2887284	sodium	0.599	0.559	0.013	0.023		
AGT_rs2493137	AGTR1_rs1492078	Fluid	sodium	0.616	0.558	0.015	0.026		
ACE_rs4343	AGT_rs2493137	NR3C2_rs2887284	fluid	0.601	0.558	0.017	0.030		
AGTR1_rs5186	NR3C2_rs11099681	NR3C2_rs2887284	sodium	0.603	0.557	0.018	0.031		
ACE_rs4295	AGTR1_rs1492078	Fluid	sodium	0.612	0.555	0.023	0.036		
AGTR1_rs1492078	NR3C2_rs1879829	hypertension	sodium	0.586	0.555	0.023	0.036		
AGT_rs699	AGTR1_rs5186	NR3C2_rs1879829	potassium	0.608	0.555	0.024	0.039		
AGT_rs3889728	NR3C2_rs11099681	Fluid	sodium	0.599	0.554	0.025	0.040		
AGT_rs699	AGTR1_rs5186	NR3C2_rs11099681	NR3C2_rs2887284	0.599	0.554	0.029	0.042		
AGT_rs699	NR3C2_rs11099681	NR3C2_rs1879829	potassium	0.603	0.553	0.034	0.048		
AGT_rs2493137	AGT_rs699	NR3C2_rs1879829	fluid	0.589	0.552	0.037	0.051		
AGT_rs2493137	AGTR1_rs1492078	NR3C2_rs11099681	sodium	0.597	0.552	0.038	0.052		
AGT_rs2493137	NR3C2_rs11099681	Fluid	sodium	0.603	0.552	0.039	0.053		
AGT_rs3889728	AGTR1_rs5186	NR3C2_rs11099681- hypertension	NR3C2_rs2887284	0.588	0.552	0.039	0.053		
ACE_rs4295	AGT_rs2493137	potassium		0.587	0.550	0.043	0.059		

^a P-value for 1000 permuted datasets without explicit test for interaction; ^b P-value for 1000 permuted datasets with explicit test for interaction. ACE = angiotensin-converting enzyme, AGT = angiotensinogen, AGTR1 = Type 1 angiotensin II receptor, MDR = multifactor dimensionality reduction, NR3C2 = mineralocorticoid receptor, REN = renin-binding protein.

DISCUSSION

In the present population-based cohort study, we investigated the influence of 13 candidate SNPs in seven key RAAS genes on RCC susceptibility and the potential interplay between these SNPs, hypertension and intakes of dietary sodium, potassium and fluid. We showed for the first time that SNPs in *AGTR1* (*AGTR1*_rs1492078 and *AGTR1*_rs5186) were associated with RCC risk in the total population and, more strongly, in participants with hypertension. In addition, we found a significant multiplicative interaction between *AGT*_rs3889728 and hypertension in relation to RCC risk. Together these findings indicate that RAAS may indeed be an underlying candidate pathway explaining previously observed associations between hypertension and RCC risk. Candidate SNPs in *ACE*, appeared to have no main effect of the RCC risk, yet we observed suggestive interactions between *ACE*_rs4295 and *ACE*_rs4343 and sodium intake. Additionally, differential trends in RCC risk for successive tertiles of sodium intake were present across the genotypes of several RAAS genes, suggesting that the association between sodium intake and RCC risk, which has previously been reported in the NLCS [5], may be influenced by the RAAS.

Actions of angiotensin II, a major component of the RAAS and important regulator of blood pressure, salt homeostasis and hormone secretion, are mediated by the angiotensin II receptors (type 1 and 2). Both angiotensin II receptors are membrane receptors on the epithelial cells of the proximal tubule, the major site of renal sodium reabsorption and the site where the majority of RCC cancers is thought to arise from [26]. Associations between SNPs in the gene encoding for angiotensin II receptor, type 1 (*AGTR1*) have been reported in relation to RCC susceptibility [3]. For *AGTR1*_rs12639531 (T>A), the RCC risk was significantly increased under the additive genotypic model (P -trend=0.04) [3]. Two other SNPs in *AGTR1* were investigated in the present study; *AGTR1*_rs5186 (A>C) showing a higher RCC risk and *AGTR1*_rs1492078 (G>A) showing a lower RCC risk. Associations were more strongly present in participants with hypertension, suggesting that effects of *AGTR1* candidate SNPs may be mediated by hypertension. *AGTR1*_rs5186 (A>C) has previously been associated with breast cancer, where it showed to have protective effects [27]. This inconsistency may be explained by the fact that the impact of SNPs in RAAS may be site-specific, because local RAAS influences local processes [6]. Given that incidence rates of RCC are twice as high in men compared to women, the other gene encoding for an angiotensin II receptor, the *AGTR2* gene, was of particular interest as candidate gene for modifying RCC risk, as it is located on the X-chromosome. However, our results indicate no association between *AGTR2*_rs5193 and RCC susceptibility in men and women.

Our findings on *AGT*, encoding for the angiotensin precursor, suggest that there is interplay between *AGT*_rs3889728 and hypertension in relation to RCC risk, while no

main effects of any of the candidate SNPs in *AGT* were present. The RCC risk for the AG+AA (*versus* GG) genotype of *AGT*_rs3889728 was higher in participants without hypertension and lower in participants with hypertension. This association is reversed compared to the association previously reported by Andreotti *et al.* [3], who showed a higher RCC risk for AG+AA (*versus* GG) genotype of *AGT*_rs3889728 particularly in individuals with either hypertension or obesity. It should be noted, however, that the previously reported association was reported in a hospital-based case-control study, in which by study design the likelihood of selection and information bias is higher than in the present prospective cohort study. In addition, both studies only focused on hypertension as a categorical self-reported trait. Although this may not explain such inconsistent associations, it may have masked potential other SNP-hypertension interactions, as it runs the risk of losing information. Another of our candidate SNPs in the *AGT* gene, *AGT*_rs699 (T>M), has not previously been investigated in relation to RCC susceptibility, but was associated with the risk of hypertension, blood pressure and plasma angiotensin levels in Caucasians [28-31]. In the present study, we found no association between *AGT*_rs699 and RCC risk. Recently, Lin *et al.* concluded in their meta-analysis that *AGT*_rs699 was not associated with cancer risk in general [32].

In general, our results indicate that the association between sodium intake and RCC risk, which has previously been reported in the NLCS [5], differs across genotypes of several of the investigated candidate RAAS SNPs. Despite this, tests for multiplicative SNP-diet interaction were never significant. Perhaps, a lack of adequate power may have caused insignificant tests; we examined SNP-diet interactions using the Wald test, which is a relative conservative test when it comes to rejecting the null-hypothesis. Alternatively, risk differences across genotypes may represent biologic interaction, which may best be defined as participation of two component causes in the same sufficient cause [33], rather than as statistical interaction ('effect-modification' or 'heterogeneity of effect'). Biological interaction is not tested as such, yet may still be present when the test for multiplicative interaction is not statistically significant. In particular, SNPs in *ACE* showed a suggestive interaction with sodium intake in relation to RCC risk. For *ACE*_rs4343, we observed that a trend in RCC risk over successive tertiles of sodium intake was only present among participants with the AA genotype and that an inverse association between the A allele and RCC risk was only present among participants with a low sodium intake, while a main effect of the A allele was absent in the total population. Similarly, a meta-analysis on the risk of hypertension also failed to show an overall significant effect of *ACE*_rs4343 (G>A), potentially because of this SNP-sodium interaction [34]. For potassium and fluid intake, which were not associated with RCC risk in previous research in the total population [5], multiplicative interactions with candidate RAAS SNPs were not significant and associations across genotypes not consistent, indicating that neither statistical nor biologic interaction may be present.

RCC risks were investigated regardless of histological subtype. It is plausible that risk estimates and associations may have differed across the different histological subtypes, but it is unlikely that our results would have changed dramatically when restricting current analyses to the main histological RCC subtype (i.e. clear-cell subtype), as it accounts for approximately 80% of the RCC cases.

As RAAS is a complex mechanism, in which proteins, enzymes and receptors work together, it is plausible that higher-order gene-gene or gene-environment interactions may contribute to the risk of RCC, which is a multi-factorial disease. In particular, we sought to investigate the role of genetic variation in RAAS genes in our previously found interaction between sodium and fluid intake [5]. There is no consensus on the best strategy for detecting higher-order gene-gene and gene-environment interactions in humans. We took an endeavor in investigating such interactions among the candidate SNPs and several environmental factors using MDR. In the present study, we identified several potentially relevant 3- and 4-way interaction models, yet none of the 2-way interaction models met the selection criterion. This suggests that, when investigating the RAAS in relation to RCC etiology, interactions of higher-order may indeed be of particular interest. Out of the selected 4-way interaction models, five models represented a potentially relevant sodium-fluid-SNP-SNP interaction, of which the model including sodium and fluid intake and the SNPs *AGT*_rs2493137 and *AGTR1*_rs5186 represented the best model. In Cox proportional hazard analyses, risk estimates of this specific binary MDR model (high *versus* low risk) were highly significant. Interpretation of the individual risks based on such MDR model is, however, challenging [24], and becomes exponentially more difficult with each increasing order of interaction. Moreover, the significance of the models should be interpreted with caution, since results from MDR analyses in general have hardly been validated in external settings.

In the present study, we use a candidate SNP approach to study gene-environment interactions, in which we selected SNPs in a common pathway (i.e. RAAS) based on prior biological knowledge from current literature. None of the selected candidate SNPs have previously been identified as SNPs of interest in genome-wide association studies (GWAS), indicating the complementary nature of both approaches. Using the hypothesis-driven selection makes interaction models potentially more plausible and better interpretable compared to a genome-wide association study (GWAS) approach [35]. In addition, it limits the number of tests performed and can therefore be used as an approach to limit the chance of type 1 errors. Although we selected the SNPs carefully, using a candidate SNP approach runs the risk of investigating SNPs that may be a marker of disease linked to another disease-causing SNP, rather than the disease-causing SNP itself. In addition, considering the complexity of the genetic and environmental network in which the investigated SNPs are included, true effects might still be diluted or masked by effects of those not included in the present study.

In conclusion, we showed that candidate SNPs in several RAAS genes (i.e. *AGT*, *AGTR1* and *ACE*), potentially modified by hypertension or sodium intake, were associated with RCC risk, indicating that RAAS may be a candidate pathway in RCC etiology. Nevertheless, confirmation of these results in other, large prospective studies is warranted.

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SNPs in RAAS genes	Tertiles of potassium intake (median) ^{a,b}												P- interaction ^f
	Low (3.0 g/d)				Medium (3.5 g/d)				High (4.1 g/d)				
	Person years ^c	RCC Cases	HR ^d	95% CI	Person years ^c	RCC Cases	HR ^d	95% CI	Person years ^c	RCC Cases	HR ^d	95% CI	
ACE_rs4295													
CC	6,849	43	1.00	(ref)	7,107	54	1.21	(0.79-1.87)	6,781	64	1.49	(0.98-2.26)	0.115
CG	7,879	71	1.49	(0.98-2.24)	8,023	69	1.36	(0.90-2.05)	8,038	64	1.25	(0.82-1.89)	0.363
GG	2,202	12	0.90	(0.46-1.77)	2,584	20	1.27	(0.72-2.26)	2,463	22	1.38	(0.79-2.41)	0.273
G ^h	16,93	126	1.08	(0.83-1.40)	17,714	143	1.04	(0.81-1.33)	17,282	150	0.91	(0.69-1.18)	0.300
ACE_rs4343													
GG	4,314	25	1.00	(ref)	4,511	40	1.48	(0.86-2.52)	4,292	39	1.52	(0.88-2.60)	0.127
AG	8,798	74	1.44	(0.88-2.34)	9,053	71	1.30	(0.80-2.12)	8,640	75	1.43	(0.88-2.32)	0.922
AA	3,826	25	1.09	(0.60-1.97)	4,147	31	1.26	(0.72-2.22)	4,298	36	1.34	(0.77-2.32)	0.676
A ^h	16,938	124	1.05	(0.81-1.37)	17,711	142	0.92	(0.71-1.19)	17,23	150	0.96	(0.75-1.24)	0.630
AGT_rs2493137													
TT	8,498	71	1.00	(ref)	9,072	61	0.82	(0.57-1.18)	8,666	84	1.15	(0.81-1.62)	0.427
CT	6,919	47	0.83	(0.56-1.23)	7,216	64	1.03	(0.71-1.48)	7,222	54	0.84	(0.57-1.25)	0.751
CC	1,552	8	0.64	(0.29-1.38)	1,507	18	1.38	(0.77-2.47)	1,394	12	1.04	(0.53-2.02)	0.223
C ^h	16,969	126	0.81	(0.60-1.10)	17,795	143	1.29	(0.98-1.71)	17,282	150	0.86	(0.64-1.17)	0.141
AGT_rs3889728													
GG	9,794	78	1.00	(ref)	10,243	71	0.88	(0.62-1.25)	9,743	90	1.14	(0.82-1.58)	0.506
AG + AA	7,155	48	0.86	(0.59-1.27)	7,551	72	1.15	(0.81-1.63)	7,539	60	0.96	(0.66-1.39)	0.474
A ^h	16,949	126	0.87	(0.59-1.29)	17,794	143	1.31	(0.91-1.89)	17,282	150	0.87	(0.60-1.26)	0.177
AGT_rs699													
TT	6,248	51	1.00	(ref)	6,752	47	0.85	(0.55-1.30)	6,625	64	1.15	(0.77-1.72)	0.472
MT	7,519	60	0.98	(0.66-1.47)	8,124	64	0.94	(0.63-1.40)	8,003	56	0.81	(0.54-1.23)	0.390
MM	2,933	14	0.59	(0.31-1.10)	2,649	28	1.28	(0.77-2.14)	2,378	23	1.22	(0.71-2.09)	0.014
M ^h	16,700	125	0.83	(0.64-1.08)	17,525	139	1.22	(0.93-1.60)	17,006	143	0.94	(0.70-1.26)	0.080

Suppl Table S3.1 (continued).

SNPs in RAAS genes	Teriles of potassium intake (median) ^{a,b}											
	Low (3.0 g/d)				Medium (3.5 g/d)				High (4.1 g/d)			
	Person years ^c	RCC Cases	HR ^d	95% CI	Person years ^c	RCC Cases	HR ^d	95% CI	Person years ^c	RCC Cases	HR ^d	95% CI
AGTR1_rs1492078												
GG	6,78	48	1.00	(ref)	6,755	59	1.23	(0.81-1.86)	6,519	64	1.32	(0.88-1.99)
AG	7,568	55	1.02	(0.67-1.55)	8,295	71	1.15	(0.77-1.72)	7,855	65	1.13	(0.75-1.70)
AA	2,2	19	1.18	(0.66-2.10)	2,259	10	0.60	(0.29-1.24)	2,448	12	0.64	(0.33-1.26)
A ^h	16,548	122	1.07	(0.79-1.44)	17,309	140	0.80	(0.61-1.03)	16,822	141	0.75	(0.58-0.97)
AGTR1_rs5186												
AA	8,406	65	1.00	(ref)	8,926	78	1.08	(0.75-1.55)	8,290	67	0.97	(0.67-1.41)
AC	6,920	45	0.83	(0.55-1.25)	7,374	46	0.79	(0.52-1.18)	7,430	61	1.02	(0.70-1.49)
CC	1,623	16	1.16	(0.63-2.11)	1,495	19	1.63	(0.92-2.91)	1,562	22	1.86	(1.08-3.21)
C ^h	16,949	126	0.98	(0.73-1.33)	17,795	143	1.03	(0.76-1.40)	17,282	150	1.29	(0.96-1.72)
AGTR2_rs5193 (men)												
G	6,159	67	1.00	(ref)	6,165	70	1.01	(0.70-1.64)	6,059	73	1.07	(0.75-1.55)
T	1,819	19	0.97	(0.56-1.69)	2,055	21	0.95	(0.55-1.63)	1,941	26	1.25	(0.75-2.07)
T ^h	7,978	86	1.00	(0.75-1.33)	8,220	91	0.96	(0.73-1.26)	8,000	99	1.10	(0.85-1.43)
AGTR2_rs5193 (women)												
GG	5,372	24	1.00	(ref)	5,443	28	0.99	(0.55-1.79)	5,276	28	1.06	(0.58-1.91)
GT + TT	3,620	16	0.99	(0.51-1.92)	4,131	24	1.18	(0.65-2.16)	4,006	21	0.99	(0.53-1.87)
T ^h			1.10	(0.54-2.24)			1.16	(0.65-2.09)			0.96	(0.52-1.76)
CYP11B2_rs7831617												
GG	5,791	39	1.00	(ref)	5,785	46	1.17	(0.74-1.85)	5,700	50	1.28	(0.82-2.02)
GT	8,157	64	1.18	(0.77-1.82)	8,858	66	1.10	(0.72-1.69)	8,074	69	1.24	(0.81-1.90)
TT	3,021	23	1.18	(0.68-2.05)	3,132	31	1.47	(0.88-2.47)	3,508	30	1.21	(0.73-2.03)
T ^h			1.09	(0.83-1.43)			1.11	(0.85-1.46)			0.96	(0.75-1.23)
NR3C2_rs11099681												
TT	8,559	66	1.00	(ref)	8,941	64	0.87	(0.60-1.26)	8,999	84	1.13	(0.80-1.62)
CT	7,202	50	0.84	(0.57-1.26)	7,550	68	1.10	(0.76-1.59)	6,593	53	0.97	(0.66-1.43)
CC	1,168	10	1.03	(0.49-2.13)	1,304	11	1.16	(0.58-2.33)	1,669	13	0.92	(0.48-1.75)
C ^h			0.94	(0.68-1.30)			1.20	(0.91-1.59)			0.87	(0.65-1.16)

Suppl Table S3.2 (continued).

SNPs in RAAS genes	Categories of fluid intake											
	Low (<1.75 l/d)				Medium (≥1.75, <2.25 l/d)				High (≥2.25 l/d)			
	Person years ^a	RCC Cases	HR ^b	95% CI	Person years ^a	RCC Cases	HR ^b	95% CI	Person years ^a	RCC Cases	HR ^b	95% CI
<i>NR3C2_rs1879829</i>												
TT	3,129	30	1.00	(ref)	6,254	44	0.68	(0.41-1.13)	4,905	41	0.73	(0.43-1.25)
CT	5,877	44	0.74	(0.45-1.24)	11,263	93	0.80	(0.50-1.26)	8,146	77	0.86	(0.53-1.40)
CC	2,821	17	0.62	(0.33-1.19)	5,681	48	0.83	(0.50-1.39)	3,969	25	0.57	(0.32-1.04)
C ^c			0.78	(0.56-1.08)			1.11	(0.90-1.38)			0.91	(0.71-1.15)
<i>REN_rs2887284</i>												
CC	7,315	63	1.00	(ref)	15,073	118	0.87	(0.62-1.22)	10,564	88	0.88	(0.61-1.27)
AC + AA	4,512	28	0.75	(0.47-1.21)	8,126	67	0.95	(0.65-1.38)	6,457	55	0.88	(0.58-1.33)
A ^e			0.77	(0.48-1.25)			1.08	(0.78-1.49)			0.98	(0.67-1.43)
<i>REN_rs5705</i>												
AA	9,021	77	1.00	(ref)	17,917	140	0.89	(0.65-1.21)	12,927	112	0.92	(0.66-1.29)
AC + CC	2,807	14	0.65	(0.36-1.19)	5,282	45	0.99	(0.67-1.48)	4,073	31	0.80	(0.50-1.28)
C ^c			0.63	(0.34-1.16)			1.11	(0.77-1.61)			0.87	(0.56-1.35)

^a in the subcohort; ^b hazard ratio adjusted for age at baseline (y), sex (men/women), BMI (kg/m²), hypertension (no/yes), smoking status (never or ex/current), smoking duration (y), smoking intensity (cig/d), alcohol consumption (g ethanol/d) and total energy intake (kcal/d); ^c dose-response relationships of categories of fluid intake are tested for each genotype separately; ^d using Wald χ^2 test for interaction; ^e trend for genotype based on the allelic model and tested for each category of fluid intake separately. ACE = angiotensin-converting enzyme, AGT = angiotensinogen, AGTR1 = Type 1 angiotensin II receptor, AGTR2 = Type 2 angiotensin II receptor, CYP11B2 = aldosterone-synthesizing enzyme, NLCS = Netherlands Cohort Study, NR3C2 = mineralocorticoid receptor, RAAS = renin-angiotensin-aldosterone system, RCC = renal cell cancer, REN = renin-binding protein, SNPs = single nucleotide polymorphisms.

Suppl Table S3.2 Hazard ratios and 95% confidence intervals for the association between renal cell cancer and SNPs in RAAS genes by low, medium and high fluid intake in the NLCS, 1986-2006.

SNPs in RAAS genes	Categories of fluid intake												
	Low (<1.75 l/d)				Medium (≥1.75, <2.25 l/d)				High (≥2.25 l/d)				
	Person years ^a	RCC Cases	HR ^b	95% CI	Person years ^a	RCC Cases	HR ^b	95% CI	Person years ^a	RCC Cases	HR ^b	95% CI	
ACE_rs4295													
CC	4,368	34	1.00	(ref)	9,331	70	0.93	(0.60-1.47)	7,038	57	0.94	(0.58-1.52)	0.651
CG	5,695	45	1.07	(0.66-1.74)	10,637	94	1.10	(0.72-1.69)	7,607	65	0.97	(0.61-1.54)	0.385
GG	1,724	12	0.89	(0.44-1.80)	3,190	21	0.83	(0.46-1.49)	2,335	21	1.08	(0.59-1.96)	0.722
G ^e			0.98	(0.70-1.35)			1.00	(0.80-1.25)			1.04	(0.80-1.36)	0.875
ACE_rs4343													
GG	2,982	18	1.00	(ref)	6,125	57	1.57	(0.88-2.80)	4,009	29	1.19	(0.63-2.25)	0.572
AG	6,224	52	1.53	(0.86-2.72)	11,777	94	1.34	(0.78-2.32)	8,491	74	1.32	(0.75-2.34)	0.490
AA	2,598	19	1.26	(0.63-2.52)	5,245	34	1.06	(0.58-1.97)	4,428	39	1.39	(0.74-2.58)	0.334
A ^e			1.11	(0.82-1.50)			0.83	(0.66-1.03)			1.08	(0.84-1.40)	0.336
AGT_rs2493137													
TT	6,058	41	1.00	(ref)	11,359	103	1.28	(0.86-1.89)	8,820	72	1.05	(0.69-1.62)	0.524
CT	4,604	42	1.31	(0.82-2.10)	9,789	64	0.89	(0.58-1.35)	6,964	59	1.08	(0.70-1.67)	0.245
CC	1,166	8	0.98	(0.43-2.21)	2,051	18	1.23	(0.67-2.24)	1,236	12	1.28	(0.63-2.61)	0.493
C ^e			1.05	(0.76-1.45)			0.85	(0.65-1.11)			1.10	(0.82-1.47)	0.229
AGT_rs3889728													
GG	6,885	46	1.00	(ref)	12,941	111	1.23	(0.85-1.78)	9,954	82	1.09	(0.73-1.63)	0.442
AG + AA	4,942	45	1.34	(0.86-2.10)	10,257	74	1.01	(0.68-1.50)	7,046	61	1.13	(0.74-1.72)	0.201
A ^e			1.29	(0.82-2.05)			0.82	(0.60-1.13)			1.07	(0.74-1.54)	
AGT_rs699													
TT	4,301	33	1.00	(ref)	8,616	71	1.03	(0.66-1.62)	6,708	58	1.00	(0.62-1.59)	0.565
MT	5,339	41	1.02	(0.62-1.68)	10,536	77	0.90	(0.58-1.40)	7,771	62	0.88	(0.56-1.40)	0.282
MM	2,041	15	0.95	(0.49-1.83)	3,703	31	1.05	(0.62-1.78)	2,216	19	1.03	(0.56-1.90)	0.738
M ^e			0.96	(0.70-1.33)			0.98	(0.78-1.24)			1.01	(0.76-1.34)	0.976

Suppl Table S3.2
(continued).

SNPs in RAAS genes	Categories of fluid intake													
	Low (<1.75 l/d)				Medium (≥1.75, <2.25 l/d)				High (≥2.25 l/d)					
	Person years ^a	RCC Cases	HR ^b	95% CI	Person years ^a	RCC Cases	HR ^b	95% CI	Person years ^a	RCC Cases	HR ^b	95% CI	P _{trend} ^c	P _{interaction} ^d
AGTR1_rs1492078														
GG	4,720	34	1.00	(ref)	8,992	80	1.13	(0.73-1.75)	6,342	57	1.03	(0.64-1.66)	0.632	
AG	5,460	42	1.03	(0.63-1.68)	10,242	81	0.99	(0.64-1.53)	8,017	68	0.99	(0.63-1.56)	0.511	
AA	1,365	11	1.03	(0.49-2.16)	3,320	19	0.72	(0.39-1.30)	2,222	11	0.56	(0.27-1.17)	0.191	0.761
A ^e			0.98	(0.68-1.40)			0.82	(0.65-1.03)			0.83	(0.63-1.08)		
AGTR1_rs5186														
AA	5,925	46	1.00	(ref)	11,670	109	1.13	(0.77-1.65)	8,026	55	0.77	(0.50-1.19)	0.637	
AC	4,905	33	0.87	(0.53-1.41)	9,246	53	0.72	(0.47-1.10)	7,574	66	0.99	(0.64-1.52)	0.887	
CC	998	12	1.48	(0.73-3.00)	2,282	23	1.21	(0.69-2.12)	1,401	22	1.89	(1.06-3.38)	0.911	0.065
C ^e			1.07	(0.74-1.54)			0.87	(0.67-1.13)			1.51	(1.12-2.03)		
AGTR2_rs5193 (men)														
G	3,481	44	1.00	(ref)	7,849	85	0.90	(0.60-1.37)	7,054	81	0.98	(0.64-1.52)	0.928	
T	991	12	1.00	(0.49-2.02)	2,582	23	0.77	(0.44-1.35)	2,242	31	1.21	(0.70-2.09)	0.394	0.573
T ^e			1.01	(0.70-1.45)			0.91	(0.71-1.18)			1.11	(0.88-1.40)		
AGTR2_rs5193 (women)														
GG	4,062	20	1.00	(ref)	7,584	42	1.01	(0.55-1.85)	4,445	18	0.72	(0.35-1.48)	0.222	
GT + TT	3,292	15	0.91	(0.45-1.85)	5,185	33	1.17	(0.62-2.19)	3,279	13	0.70	(0.31-1.54)	0.865	0.835
T ^e			0.83	(0.39-1.76)			1.14	(0.69-1.87)			0.93	(0.45-1.96)		
CYP11B2_rs7831617														
GG	3,841	31	1.00	(ref)	7,965	53	0.77	(0.47-1.25)	5,469	51	1.01	(0.61-1.66)	0.680	
GT	5,546	43	0.94	(0.57-1.56)	11,113	92	0.97	(0.62-1.52)	8,430	64	0.84	(0.51-1.37)	0.775	
TT	2,440	17	0.86	(0.45-1.63)	4,100	39	1.14	(0.68-1.92)	3,121	28	0.95	(0.53-1.69)	0.875	0.489
T ^e			0.92	(0.66-1.27)			1.22	(0.98-1.52)			0.93	(0.70-1.22)		
NR3C2_rs11099681														
TT	6,116	46	1.00	(ref)	11,427	98	1.06	(0.72-1.56)	8,957	70	0.91	(0.59-1.41)	0.849	
CT	4,930	39	0.99	(0.62-1.58)	9,871	72	0.90	(0.60-1.36)	6,543	60	1.04	(0.67-1.60)	0.876	
CC	781	6	1.08	(0.43-2.72)	1,881	15	0.99	(0.52-1.86)	1,480	13	1.01	(0.52-1.98)	0.428	0.861
C ^e			1.02	(0.70-1.50)			0.92	(0.71-1.18)			1.08	(0.82-1.43)		

Suppl Table S3.2 (continued).

SNPs in RAAS genes	Categories of fluid intake											
	Low (<1.75 l/d)				Medium (≥1.75, <2.25 l/d)				High (≥2.25 l/d)			
	Person years ^a	RCC Cases	HR ^b	95% CI	Person years ^a	RCC Cases	HR ^b	95% CI	Person years ^a	RCC Cases	HR ^b	95% CI
NR3C2_rs1879829												
TT	3,129	30	1.00	(ref)	6,254	44	0.68	(0.41-1.13)	4,905	41	0.73	(0.43-1.25)
CT	5,877	44	0.74	(0.45-1.24)	11,263	93	0.80	(0.50-1.26)	8,146	77	0.86	(0.53-1.40)
CC	2,821	17	0.62	(0.33-1.19)	5,681	48	0.83	(0.50-1.39)	3,969	25	0.57	(0.32-1.04)
C ^c			0.78	(0.56-1.08)			1.11	(0.90-1.38)			0.91	(0.71-1.15)
REN_rs2887284												
CC	7,315	63	1.00	(ref)	15,073	118	0.87	(0.62-1.22)	10,564	88	0.88	(0.61-1.27)
AC + AA	4,512	28	0.75	(0.47-1.21)	8,126	67	0.95	(0.65-1.38)	6,457	55	0.88	(0.58-1.33)
A ^e			0.77	(0.48-1.25)			1.08	(0.78-1.49)			0.98	(0.67-1.43)
REN_rs5705												
AA	9,021	77	1.00	(ref)	17,917	140	0.89	(0.65-1.21)	12,927	112	0.92	(0.66-1.29)
AC + CC	2,807	14	0.65	(0.36-1.19)	5,282	45	0.99	(0.67-1.48)	4,073	31	0.80	(0.50-1.28)
C ^e			0.63	(0.34-1.16)			1.11	(0.77-1.61)			0.87	(0.56-1.35)

^a in the subcohort; ^b hazard ratio adjusted for age at baseline (y), sex (men/women), BMI (kg/m²), hypertension (yes/no), smoking status (never or ex/current), smoking duration (y), smoking intensity (cig/d), alcohol consumption (g ethanol/d) and total energy intake (kcal/d); ^c dose-response relationships of categories of fluid intake are tested for each genotype separately; ^d using Wald χ^2 test for interaction; ^e trend for genotype based on the allelic model and tested for each category of fluid intake separately. ACE = angiotensin-converting enzyme, AGT = angiotensinogen, AGTR1 = Type 1 angiotensin II receptor, AGTR2 = Type 2 angiotensin II receptor, CYP11B2 = aldosterone-synthesizing enzyme, NLCS = Netherlands Cohort Study, NR3C2 = mineralocorticoid receptor, RAAS = renin-angiotensin-aldosterone system, RCC = renal cell cancer, REN = renin-binding protein, SNPs = single nucleotide polymorphisms.

CHAPTER 4

Gene-environment interactions in ion transport mechanisms in relation to renal cell cancer risk

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Submitted

ABSTRACT

Introduction

Gene-environment interactions help to unravel mechanisms underlying complex diseases, such as renal cell cancer (RCC). Ion transport mechanisms (ITMs) are related to renal homeostasis, the pathogenesis of hypertension and might be involved in carcinogenesis. We investigated ITMs as potential disease mechanism in RCC etiology using gene-environment interactions between candidate single nucleotide polymorphisms (SNPs) in ITM genes and associated environmental factors, including dietary intakes of sodium, potassium and fluid, hypertension and diuretic medication.

Methods

A literature-based selection of 13 SNPs in ten ITM genes were successfully genotyped in toenail DNA of 3,583 subcohort members and 503 RCC cases from the Netherlands Cohort Study (NLCS) using the AGENA MassARRAY® iPLEX platform. Diet and lifestyle were measured with baseline questionnaires. Cox regression analyses were conducted for the main effects of each SNP and for gene-environment interactions.

Results

*ADD1*_rs4961 was significantly associated with RCC risk, showing a hazard ratio (HR) of 1.24 (95% confidence interval (CI): 1.01-1.53) for the GT+TT (*versus* GG) genotype. Four of 65 tested gene-environment interactions were statistically significant. Three of these interactions clustered in *SLC9A3*_rs4957061, including the interactions with fluid intake, potassium intake, and diuretic medication. For high *versus* low fluid intake, the RCC risk was significantly lower in participants with the CC genotype [HR(95%CI): 0.47(0.26-0.86)], but not in participants with the CT+TT genotype (*P*-interaction=0.002).

Conclusion

Data show first indications of involvement of *ADD1*_rs4961 and *SLC9A3*_rs4957061 in RCC susceptibility, but do not support the general hypothesis that ITMs are underlying disease mechanisms in RCC etiology.

INTRODUCTION

Several modifiable risk factors have been confirmed to increase the risk of renal cell cancer (RCC), including obesity, cigarette smoking and hypertension, while increasing evidence suggests that moderate alcohol consumption may be inversely associated with RCC risk [1]. In addition to environmental factors, genetic susceptibility to RCC has been evaluated in numerous candidate-gene association studies, in which several single nucleotide polymorphisms (SNP) from relevant biologic pathways have been associated with RCC risk [1]. In addition, genome-wide association studies (GWAS) have identified several novel susceptibility loci for RCC [2-5]. However, in complex diseases such as RCC, a substantial part of the phenotypic variation may be explained by the modifying effects of genetic variations on lifestyle risk factors (gene-environment interactions) [6]. In addition, gene-environment interactions help to unravel mechanisms underlying carcinogenesis [7].

Recently, we reported that high sodium intake was associated with a higher risk of RCC [8] and that this association may differ by genetic variation in genes involved in the renin-angiotensin-aldosterone system (RAAS) [9], which is a hormonal mechanism that regulates blood pressure and renal sodium homeostasis [10]. Renal sodium homeostasis and the homeostatic balance of other solutes is maintained through renal reabsorption and secretion. This process of ion transport is facilitated by protein carriers or ion-specific channels and is essential, as free diffusion of ions through the renal tubules is limited [10]. These ion channels, transporters, exchanger pumps and associated enzymes are collectively referred to as ion transport mechanisms (ITMs) and have recently been put forward as potential novel mechanism underlying carcinogenesis [11]. Perhaps, genetic variation in ITMs may be associated with RCC risk or modify effects of sodium intake in relation to RCC risk.

Ion transport not only involves regulation of sodium homeostasis. Throughout the kidney, renal sodium reabsorption is linked to the reabsorption or secretion of other solutes, such as potassium [10]. Moreover, ITMs are a major focus in the pathogenesis of hypertension, as several forms of antihypertensive therapy intervene on various aspects of ion transport [12]. For example, diuretic medication increases the excretion of fluid and sodium from the kidneys. Therefore, potassium and fluid intake, hypertension and the use of diuretic medication are, in addition to sodium intake, relevant environmental factors in the context of gene-environment interactions in ITMs.

In the present study, we used data from the prospective Netherlands Cohort Study on diet and cancer (NLCS) to investigate the association between candidate SNPs in ITM genes, their interplay with associated environmental factors and RCC risk.

METHODS

Study design, study population and follow-up

The NLCS is a prospective cohort study initiated in 1986 when 58,279 men and 62,573 women between the ages of 55-69 years were included [13-15]. The case-cohort design was used for efficiency in questionnaire processing and follow-up. Cases were derived from the entire cohort, whereas a subcohort of 5,000 subjects was randomly sampled at baseline to estimate person years at risk [16]. Subcohort members were regularly followed up for vital status information. All cohort members were followed up for cancer occurrence through computerized record linkage with the Netherlands cancer registry and the Dutch pathology registry (PALGA) [17]. The coverage of cohort members by the Netherlands cancer registry and PALGA to establish cancer follow-up is estimated to be over 96% [18]. Cases and subcohort members with prevalent cancer (excluding skin cancer) at baseline were excluded. After 20.3 years of follow-up, 608 microscopically confirmed RCC cases were identified [International Classification of Diseases for Oncology 3 (ICD-O-3): C64].

Toenail DNA for genotyping

Approximately 90,000 participants provided toenail clippings at baseline. DNA was isolated from these toenails as described previously [19]. To increase our case sample, for 67 RCC cases without toenail clippings, DNA was isolated from formalin-fixed paraffin-embedded (FFPE) normal kidney tissues, as described by van Houwelingen *et al.* [20] and added to the sample collection of toenail DNA. Toenail DNA has shown to be a valid source of DNA for the genotyping of a limited set of SNPs when using the AGENA® MassARRAY® platform using the iPLEX™ assay (Agena, Hamburg, Germany) [21,22].

SNP selection and multiplex design

Genes were of interest if they were involved in ITMs in the kidney and have been associated with the regulation of blood pressure or hypertension. The following candidate genes were included: adducin1 (*ADD1*), ATPase Na⁺/K⁺ transporting alpha and beta 1 polypeptide (*ATP1A1* and *ATP1B1*), guanine nucleotide binding protein (G protein), beta polypeptide 3 (*GNB3*), regulator of G-protein signaling 5 (*RGS5*), sodium channel non-voltage-gated 1 beta and gamma subunit (*SCNN1B* and *SCNN1G*), solute carrier family 9, subfamily A (NHE3, cation proton antiporter 3) member 3 (*SLC9A3*), serine threonine kinase 39 (*STK39*) and WNK lysine deficient protein kinase 1 (*WNK1*). For these genes, SNPs were selected through extensive literature search considering the number of previously reported associations with i) the risk of RCC, ii) the risk of hyper- or hypotension iii) systolic or diastolic blood pressure, iv) enzymes or proteins in the renin-angiotensin-aldosterone system and v) salt handling, ion transport or salt sensitivity. This search strategy rendered over 100 potential SNPs of

interest. A maximum of 40 SNPs could be included in the multiplex design. We gave priority to SNPs with more than one previously reported association, a minor allele frequency (MAF) $\geq 20\%$ in Caucasians and to SNPs that were not in high linkage disequilibrium (LD) with each other ($r^2 < 0.8$). SNPs with the highest priority were first allocated to the assay. Nevertheless, due to potential sequence incompatibilities among primers, not all high priority SNPs could be included in the design. For example, we included *WNK1*_rs10849563 (A>G) as a proxy for our high priority SNP *WNK1*_rs765250 (T>C, $r^2 = 1.0$ and $D' = 1.0$), which has been associated with essential hypertension and blood pressure, but could not be combined with other SNPs on the multiplex. Subsequently, SNPs with less priority were used to fill the multiplex. Our final multiplex design included 31 SNPs.

SNP genotyping

For one SNP, *ADD1*_rs17833172, the genotyping completely failed for unknown reasons. The remaining 30 SNPs were successfully genotyped: 96% of the samples had a call rate of at least 90% and the genotype concordance between duplicate toenail samples and between toenail samples and FFPE healthy tissues samples was respectively 99.6% and 99.1% on average. For 4,067 samples corresponding to 3,583 subcohort members and 503 RCC cases, complete genotyping data were available for further analyses. The entire multiplex included SNPs in >20 genes involved in several pathways. For this study, 13 SNPs in ten ITM genes were successfully genotyped. All SNP call rates were at least 99.9%, except for *SCNN1G*_rs4299163, *SLC9A3*_rs4957061 and *WNK1*_rs10849563, which had a SNP call rate of 95.4%, 94.0% and 98.7%, respectively (Table 4.1).

Baseline questionnaire

All participants returned a mailed, self-administered, baseline questionnaire on diet and other risk factors for cancer. This baseline questionnaire included a 150-item, semi-quantitative food frequency questionnaire (FFQ), which was used for the assessment of dietary habits. Participants with an incomplete or inconsistent FFQ were excluded, leaving 3,379 subcohort members and 480 cases eligible for further analyses [13]. The FFQ ranked individuals adequately according to dietary intakes when compared to a 9-day dietary records [13], and reflected nutrient intakes for at least 5 years [14]. Nutrient intakes were calculated using the Dutch food composition table 1986–87 [23]. The daily sodium, potassium and fluid intakes were defined as intakes through both foods and beverages per day, including amounts naturally present in foods and beverages plus amounts added during food processing by food manufacturers. Sodium and potassium intake were adjusted for total energy intake by using the residual-mean method [24] and modelled as sex-specific tertile distributions. Fluid intake was modelled as categorical variable using the categories low (≤ 1.75 l/d), moderate (1.75–2.25 l/d) and high (> 2.25 l/d) intake.

Table 4.1 Characteristics of SNPs in ITM genes in the subcohort members in the NLCS, 1986-2006.

SNPs in ITM genes	Chr. location	Reference alleles	Minor allele	MAF	Genotypes						HWE ^b
					11 ^a		12 ^a		22 ^a		
					n	%	n	%	n	%	
<i>ADD1</i> _rs4961	4p16.3	G/T	T	0.212	2,232	62.33	1,182	33.01	167	4.66	0.52
<i>ATP1A1</i> _rs850602	1p21	C/G	G	0.236	2,089	58.35	1,290	36.03	201	5.61	0.93
<i>ATP1B1</i> _rs2901029	1q24	A/G	G	0.432	1,176	32.83	1,716	47.91	690	19.26	0.15
<i>GNB3</i> _rs1129649	12p13	C/T	C	0.351	1,536	42.87	1,582	44.15	465	12.98	0.07
<i>GNB3</i> _rs4963516	12p13	A/C	C	0.148	2,607	72.76	894	24.95	82	2.29	0.60
<i>RGSS</i> _rs2815287	1q23.1	A/C	C	0.377	1,371	38.27	1,718	47.96	493	13.76	0.23
<i>RGSS</i> _rs2999967	1q23.1	C/T	C	0.461	1,057	29.51	1,750	48.86	775	21.64	0.31
<i>SCNN1B</i> _rs11645151	16p12.2-p12.1	C/T	C	0.251	1,993	55.70	1,371	38.32	214	5.98	0.31
<i>SCNN1B</i> _rs239345	16p12.2-p12.1	A/T	A	0.252	1,994	55.67	1,368	38.19	220	6.14	0.51
<i>SCNN1G</i> _rs4299163	16p12	C/G	C	0.207	2,193	64.01	1,050	30.65	183	5.34	<0.001
<i>SLC9A3</i> _rs4957061	5p15.3	C/T	T	0.470	942	28.13	1,664	49.69	743	22.19	0.89
<i>STK39</i> _rs6749447	2q24.3	G/T	G	0.268	1,928	53.88	1,379	38.54	271	7.57	0.27
<i>WNK1</i> _rs10849563	12p13.3	A/G	G	0.306	1,748	49.45	1,413	39.97	374	10.58	<0.001

^a Genotypes 11, 12 and 22 refer to the homozygote common, heterozygote and homozygote rare genotype, respectively; ^b P-value for HWE. *ADD1* = adducin1, *ATP1A1* = ATPase Na⁺/K⁺ transporting alpha 1 polypeptide, *ATP1B1* = ATPase Na⁺/K⁺ transporting beta 1 polypeptide, Chr. = chromosome, *GNB3* = guanine nucleotide binding protein (G protein) beta polypeptide 3, HWE = Hardy-Weinberg equilibrium, ITM = ion transport mechanisms, NLCS = Netherlands Cohort Study, *RGSS* = regulator of G-protein signaling 5, *SCNN1B* = sodium channel non-voltage-gated 1 beta subunit, *SCNN1G* = sodium channel non-voltage-gated 1 gamma subunit, SNPs = single nucleotide polymorphisms, *STK39* = serine threonine kinase 39, *WNK1* = WNK lysine deficient protein kinase 1.

In addition to the FFQ, the baseline questionnaire included specific questions on salt added during home-preparation and before consumption (i.e. discretionary salt intake), which could not be measured using the 150 food items [8,25]. Furthermore, the baseline questionnaire included two questions on the saltiness of restaurant food and soups from package or can (ranging from “not salty enough” to “much too salty”), which were used to assess salt preference, as this may influence total salt (sodium) intake. Finally, participants were asked to report whether they had ever been diagnosed with ‘high blood pressure’ by a physician, whether they used any medication for more than six months and, if so, for which medical condition they used what kind of medication. Diuretic medication was defined according to the Anatomical Therapeutic Chemical (ATC) classification of the World health organisation (WHO) Collaborative Centre for Drug Statistical Methodology [26,27].

Statistical analyses

All analyses were conducted using Stata version 12 (Stata Corp., College Station, TX). Hazard ratios (HR) and 95% confidence intervals (CI) for the independent main effects of each SNP on RCC risk were evaluated in a crude model and an age and sex adjusted model using Cox proportional hazards analyses adjusted for the case-cohort design [28]. Genotypes were analysed in a dominant model to maintain optimal power and in an allelic model. In subcohort members, we evaluated whether SNPs in genes that have previously been associated with taste responses in mice, including *SCNN1B* and *SCNN1G* [29], were associated with perceived saltiness at baseline using the χ^2 test.

Gene-environment interactions including the candidate SNPs and the intakes of sodium, potassium and fluid, hypertension and the use of diuretic medication were tested using the Wald χ^2 test by including the interaction term into the model. Risk estimates were only presented for the significant gene-environment interactions. Interaction analyses were performed in a multivariable-adjusted model, including *a priori* selected potential confounders: age at baseline (y), sex (men/women), total energy intake (kcal/d), body mass index (BMI, kg/m²), smoking status (never or ex/current), smoking duration (y), smoking intensity (cigarettes/d), alcohol consumption (g ethanol/d), hypertension (no/yes) and use of diuretic medication (no/yes). Analyses including sodium intake were additionally adjusted for discretionary salt intake (g/d) in sensitivity analyses.

For all the models including covariates, the proportional hazards assumption was tested using the scaled Schoenfeld residuals. A violation was apparent for age, which was therefore modelled as time dependent covariate. All tests were two-sided and a *P*-value<0.05 was considered statistically significant.

RESULTS

Genotype and allele frequencies of SNPs in ITM genes in subcohort members of the NLCS are presented in Table 4.1. Two SNPs, *SCNN1G*_rs4299163 and *WNK1*_rs10849563, showed a deviation from Hardy-Weinberg Equilibrium (HWE), as tested with Pearson χ^2 test ($P < 0.001$ and $P < 0.001$, respectively). Despite our priority criteria for multiplex design, i.e. SNPs with $MAF \geq 20\%$ in Caucasians and SNPs that were not in high LD ($r^2 < 0.8$), some of the SNPs had a low MAF (*GNB3*_rs4963516; $MAF = 0.148$) or a high LD (*SCNN1B*_rs239345 and *SCNN1B*_rs11645151; $r^2 = 0.93$, $D' = 0.96$;) in this study population (Haploview, version 4.2 [30]).

At baseline, cases and subcohort members did not substantially differ in dietary intakes (Table 4.2). However, the proportion of men, cigarette smokers, participants with a diagnosis of hypertension and users of diuretic medication, was higher among cases than among subcohort members. Perceived saltiness, an inverse indicator of salt preference, was higher in cases than in subcohort members. Among subcohort members, there was no association between the three candidate SNPs in two genes that have previously been associated with taste responses in mice, including *SCNN1B* and *SCNN1G* [29], and perceived saltiness, as tested with the χ^2 test (range P -values = 0.516–0.846).

Table 4.2 Baseline characteristics of subcohort members and renal cell cancer cases in the NLCS, 1986–2006.

Baseline characteristics (mean (SD))	Subcohort members	RCC Cases
Total (n)	3379	480
Dietary intakes (in g/d)		
Sodium ^a	2.3 (0.6)	2.5 (0.7)
Discretionary salt ^{b,c}	2.8 (2.7)	2.8 (3.0)
Perceived saltiness (%)		
Not salty enough	1.9	2.0
Good	41.9	36.7
A little too salty	41.6	41.2
Much too salty	14.7	20.2
Potassium ^a	3.5 (0.6)	3.6 (0.6)
Fluid (l/d)	2.1 (0.5)	2.1 (0.5)
Potential confounders		
Age (y)	61.3 (4.2)	61.0 (4.0)
Male sex (%)	49.8	65.2
Total energy intake (kcal/d)	1925 (506.2)	1994.0 (515.9)
Alcohol consumption (g ethanol/d) ^d	13.7 (15.0)	15.7 (16.1)
BMI (kg/m ²) ^c	25.0 (3.1)	25.5 (3.0)
Current smokers (%)	27.4	31.9
Smoking duration (y) ^e	38.7 (8.7)	39.6 (8.5)
Smoking intensity (cig/d) ^e	14.7 (8.7)	15.9 (8.7)
Hypertension (%)	26.2	31.9
Use of diuretic medication (%)	10.8	13.1

^a Intakes are energy-adjusted; ^b Salt intake refers to sodium-chloride intake (sodium-chloride = sodium*2.54);

^c n does not correspond with the overall n, due to missing values; ^d In consumers only; ^e In current smokers. BMI = body mass index, cig = cigarettes, NLCS = Netherlands Cohort Study, RCC = renal cell cancer, SD = standard deviation.

Out of the 13 investigated candidate SNPs, only *ADD1*_rs4961 was significantly associated with RCC risk (Table 4.3). Results were similar for the crude and age- and sex-adjusted model. In the latter model, the HR(95%CI) for the GT+TT (*versus* GG) genotype was 1.24(1.01-1.53), whereas the HR(95%CI) for each T allele was 1.19(1.00-1.41). We tested 65 gene-environment interactions between the candidate SNPs and the exposures under study, including the dietary intakes of sodium, potassium and fluid, hypertension status and the use of diuretic medication, in relation to RCC risk (Table 4.4). Four gene-environment interactions were significant. In fact, the number of significant gene-environment interaction was not much higher than expected based on chance. Additional adjustment for discretionary salt intake in sensitivity analyses did not change the results. However, three out of the four observed significant gene-environment interactions included the same SNP, i.e. *SLC9A3*_rs4957061. RCC risk estimates by genotype of this SNP are presented in Table 4.5. A significantly lower RCC risk was observed for the highest (*versus* the lowest) category of fluid intake in participants with the CC genotype [HR(95%CI): 0.47(0.26-0.86)], whereas no such lower RCC risk was observed for participants with the highest fluid intake and the CT+TT genotype [HR(95%CI): 1.03(0.64-1.65), *P*-interaction=0.002]. This is in line with results from continuous analysis (*P*-interaction=0.031). For potassium intake, in continuous analyses, the RCC risk for each additional gram of potassium intake per day showed a subtle difference between the CC and CT+TT genotypes [HR(95%CI): 0.77(0.54-1.12) and 1.18(0.94-1.49), respectively; *P*-interaction=0.028)]. However, the interaction could not be confirmed in the analyses using tertiles (*P*-interaction=0.978). Moreover, the interaction between *SLC9A3*_rs4957061 and the use of diuretic medication was statistically significant (*P*-interaction=0.013). The RCC risk was highest for participants with the CC genotype who used diuretic medication [HR(95%CI): 2.15(1.23-3.75)]. High sodium intake (tertiles and increment of 1 g/d) and hypertension were associated with a higher risk of RCC, regardless of the *SLC9A3*_rs4957061 genotype (*P*-interaction=0.808, 0.827 and 0.399, respectively).

Table 4.3 Associations between SNPs in ITM genes and renal cell cancer risk in the NLCS, 1986-2006.

SNPs in ITM genes	Person years ^a	RCC cases	Crude model		Age- and sex-adjusted model	
			HR	95% CI	HR	95% CI
<i>ADD1</i> _rs4961						
GG	32,542	240	1.00	(ref)	1.00	(ref)
GT + TT	19,590	178	1.23	(1.00-1.52)	1.24	(1.01-1.53)
T ^b	52,133	418	1.17	(0.99-1.38)	1.19	(1.00-1.41)
<i>ATP1A1</i> _rs850602						
CC	30,051	239	1.00	(ref)	1.00	(ref)
CG + GG	22,064	179	1.02	(0.83-1.25)	1.03	(0.84-1.27)
G ^b	52,115	418	1.06	(0.89-1.26)	1.07	(0.90-1.27)

Table 4.3 (continued)

SNPs in ITM genes	Person years ^a	RCC cases	Crude model		Age- and sex-adjusted model	
			HR	95% CI	HR	95% CI
<i>ATP1B1</i> _rs2901029						
AA	17,296	135	1.00	(ref)	1.00	(ref)
AG + GG	34,847	283	1.04	(0.84-1.30)	1.07	(0.85-1.33)
G ^b	52,143	418	0.97	(0.84-1.12)	0.99	(0.86-1.14)
<i>GNB3</i> _rs1129649						
TT	22,427	192	1.00	(ref)	1.00	(ref)
CT + CC	29,729	227	0.89	(0.73-1.10)	0.90	(0.73-1.10)
C ^b	52,156	419	0.94	(0.81-1.10)	0.96	(0.82-1.12)
<i>GNB3</i> _rs4963516						
AA	38,191	293	1.00	(ref)	1.00	(ref)
AC + CC	13,965	126	1.18	(0.94-1.47)	1.15	(0.92-1.45)
C ^b	52,156	419	1.18	(0.96-1.43)	1.16	(0.95-1.42)
<i>RGS5</i> _rs2815287						
AA	20,255	1514	1.00	(ref)	1.00	(ref)
AC + CC	31,887	267	1.12	(0.91-1.39)	1.13	(0.91-1.40)
C ^b	52,142	418	1.12	(0.96-1.30)	1.12	(0.96-1.30)
<i>RGS5</i> _rs2999967						
TT	15,541	111	1.00	(ref)	1.00	(ref)
CT + CC	36,594	308	1.18	(0.94-1.49)	1.23	(0.97-1.56)
C ^b	52,135	419	1.10	(0.95-1.27)	1.13	(0.98-1.31)
<i>SCNN1B</i> _rs11645151						
TT	28,984	235	1.00	(ref)	1.00	(ref)
CT + TT	23,084	184	0.98	(0.80-1.21)	0.99	(0.80-1.22)
C ^b	52,069	419	1.04	(0.87-1.23)	1.04	(0.87-1.24)
<i>SCNN1B</i> _rs239345						
TT	29,081	233	1.00	(ref)	1.00	(ref)
AT + AA	23,075	186	1.01	(0.82-1.24)	1.01	(0.82-1.24)
A ^b	52,156	419	1.04	(0.88-1.23)	1.04	(0.87-1.23)
<i>SCNN1G</i> _rs4299163						
GG	31,805	248	1.00	(ref)	1.00	(ref)
GC + CC	17,990	146	1.04	(0.84-1.29)	1.05	(0.86-1.38)
C ^b	49,795	394	0.99	(0.83-1.18)	0.99	(0.83-1.17)
<i>SLC9A3</i> _rs4957061						
CC	13,797	109	1.00	(ref)	1.00	(ref)
CT + TT	35,129	297	1.07	(0.85-1.35)	1.09	(0.86-1.38)
T ^b	48,926	406	0.96	(0.84-1.11)	0.97	(0.84-1.12)
<i>STK39</i> _rs6749447						
TT	28,255	240	1.00	(ref)	1.00	(ref)
GT + GG	23,819	179	0.89	(0.72-1.09)	0.88	(0.71-1.08)
G ^b	52,075	419	0.90	(0.76-1.06)	0.90	(0.76-1.06)
<i>WNK1</i> _rs10849563						
AA	25,065	200	1.00	(ref)	1.00	(ref)
AG + GG	26,333	213	1.01	(0.82-1.25)	1.01	(0.82-1.24)
G ^b	51,398	413	0.97	(0.83-1.12)	0.97	(0.83-1.12)

^a In the subcohort; ^b Trend for genotype based on the allelic model. *ADD1* = adducin1, *ATP1A1* = ATPase Na⁺/K⁺ transporting alpha 1 polypeptide, *ATP1B1* = ATPase Na⁺/K⁺ transporting beta 1 polypeptide, *GNB3* = guanine nucleotide binding protein (G protein) beta polypeptide 3, ITM = Ion transport mechanisms, NLCS = Netherlands Cohort Study, RCC = renal cell cancer, *RGS5* = regulator of G-protein signaling 5, *SCNN1B* = sodium channel non-voltage-gated 1 beta subunit, *SCNN1G* = sodium channel non-voltage-gated 1 gamma subunit, SNPs = single nucleotide polymorphisms, *STK39* = serine threonine kinase 39, *WNK1* = WNK lysine deficient protein kinase 1.

Table 4.4 Summary of *P*-values for interaction between dietary intakes, hypertension and use of diuretic medication and SNPs in ITM genes for renal cell cancer risk in the NLCS, 1986-2006.

SNPs in ITM genes ^c	<i>P</i> -interaction ^{a,b}				
	Sodium intake (g/d) ^d	Potassium intake (g/d) ^d	Fluid intake (l/d)	Use of diuretic medication	Doctors' diagnosis of hypertension
<i>ADD1</i> _rs4961	0.101	0.279	0.346	0.875	0.990
<i>ATP1A1</i> _rs850602	0.336	0.684	0.847	0.535	0.091
<i>ATP1B1</i> _rs2901029	0.854	0.363	0.710	0.605	0.187
<i>GNB3</i> _rs1129649	0.355	0.942	0.706	0.276	0.026
<i>GNB3</i> _rs4963516	0.518	0.529	0.895	0.633	0.552
<i>RGS5</i> _rs2815287	0.353	0.061	0.701	0.863	0.692
<i>RGS5</i> _rs2999967	0.742	0.723	0.356	0.285	0.356
<i>SCNN1B</i> _rs11645151	0.481	0.275	0.797	0.326	0.498
<i>SCNN1B</i> _rs239345	0.507	0.268	0.647	0.699	0.376
<i>SCNN1G</i> _rs4299163	0.233	0.069	0.860	0.059	0.109
<i>SLC9A3</i> _rs4957061	0.827	0.028	0.031	0.013	0.399
<i>STK39</i> _rs6749447	0.279	0.751	0.498	0.147	0.338
<i>WNK1</i> _rs10849563	0.496	0.727	0.881	0.290	0.068

^a Using Wald χ^2 test for interaction; ^b Interaction adjusted for age at baseline (y), sex (men/women), total energy intake (kcal/d), smoking status (never or ex/current), smoking intensity (cig/d), smoking duration (y), BMI (kg/m²), alcohol intake (g ethanol/d), hypertension (no/yes) and use of diuretic medication (no/yes); ^c SNPs are analyzed in a dominant model; ^d Intakes are energy-adjusted by using the residual mean method. *ADD1* = adducin1, *ATP1A1* = ATPase Na⁺/K⁺ transporting alpha 1 polypeptide, *ATP1B1* = ATPase Na⁺/K⁺ transporting beta 1 polypeptide, *GNB3* = guanine nucleotide binding protein (G protein) beta polypeptide 3, ITM = Ion transport mechanisms, NLCS = Netherlands Cohort Study, *RGS5* = regulator of G-protein signaling 5, *SCNN1B* = sodium channel non-voltage-gated 1 beta subunit, *SCNN1G* = sodium channel non-voltage-gated 1 gamma subunit, *SLC9A3* = solute carrier family 9, subfamily A (NHE3, cation proton antiporter 3) member 3, SNPs = single nucleotide polymorphisms, *STK39* = serine threonine kinase 39, *WNK1* = WNK lysine deficient protein kinase 1

Table 4.5 Interactions between dietary intakes, hypertension and use of diuretic medication and *SLC9A3_rs4957061* for renal cell cancer risk in the NLCS, 1986-2006.

	Person years	SLC9A3_rs4957061						P-interaction ^b
		CC			CT + TT			
		Cases	HR ^a	95% CI	Cases	HR ^a	95% CI	
Sodium intake ^{c,d}								
T1	16,402	30	1.00	(ref)	85	1.19	(0.77-1.87)	0.808
T2	16,621	34	1.13	(0.66-1.91)	92	1.25	(0.80-1.95)	
T3	15,903	45	1.69	(1.02-2.79)	120	1.67	(1.09-2.56)	
Increment of 1 g/d	48,926	109	1.33	(0.91-1.93)	297	1.20	(0.99-1.45)	
Potassium intake ^{c,d}								
T1	15,969	36	1.00	(ref)	87	1.07	(0.70-1.63)	0.978
T2	16,642	33	1.03	(0.62-1.72)	104	1.11	(0.73-1.68)	
T3	16,316	40	1.06	(0.65-1.73)	106	1.20	(0.79-1.81)	
Increment of 1 g/d	48,926	109	0.77	(0.54-1.12)	297	1.18	(0.94-1.49)	
Fluid intake								
Low	11,281	28	1.00	(ref)	59	0.82	(0.50-1.34)	0.002
Moderate	21,686	58	1.01	(0.61-1.67)	120	0.81	(0.51-1.28)	
High	15,959	23	0.47	(0.26-0.86)	118	1.03	(0.64-1.65)	
Increment of 1 l/d	48,926	109	0.60	(0.36-1.02)	297	1.10	(0.83-1.46)	
Hypertension								
No	36,292	68	1.00	(ref)	204	1.18	(0.88-1.59)	0.399
Yes	12,635	41	1.57	(1.01-2.42)	93	1.49	(1.03-2.15)	
Use of diuretic medication								
No	43,944	85	1.00	(ref)	267	1.25	(0.96-1.62)	0.013
yes	4,982	24	2.15	(1.23-3.75)	30	1.16	(0.71-1.87)	

^a HRs are adjusted for age at baseline (y), sex (men/women), total energy intake (kcal/d), smoking status (never or ex/current), smoking intensity (cig/d), smoking duration (y), BMI (kg/m²), alcohol intake (g ethanol/d), hypertension (no/yes) and use of diuretic medication (no/yes); ^b Using Wald χ^2 test for interaction; ^c Tertile distribution is sex-specific and based on subcohort members; ^d Intakes are energy-adjusted by using the residual mean method. BMI = body mass index, cig = cigarettes, NLCS = Netherlands Cohort Study, *SLC9A3* = solute carrier family 9, subfamily A (NHE3, cation proton antiporter 3) member 3

DISCUSSION

In the present population-based cohort study, we investigated 13 candidate SNPs in ten ITM genes in relation to RCC risk. Data does not support the general hypothesis that genetic variation in ITM genes may influence RCC susceptibility, because only one of these SNPs, *ADD1_rs4961*, was significantly associated with RCC risk. We additionally investigated potential gene-environment interactions between these genes and exposures that may also be implicated in ion transport in the kidney, including dietary intakes of sodium, potassium and fluid, hypertension and the use of diuretic medication. Although significant gene-environment interactions clustered in one SNP, *SLC9A3_rs4957061*, data does not support the general hypothesis that gene-environment interactions related to ITMs are of interest in RCC etiology.

Of the 13 investigated candidate SNPs, only *ADD1*_rs4961 was significantly associated with RCC risk. The *ADD1* gene encodes for α -adducin, a ubiquitously expressed cytoskeleton protein implicated in the formation of actin-spectrin complex, actin polymerization and cell signal transduction [31,32] and has, to our knowledge, not previously been investigated in relation to RCC risk. Lin *et al.* concluded that the G allele of *ADD1*_rs4961 might modulate the decline of renal function in healthy elderly Chinese [33]. In contrast, we observed the highest RCC risk for the GT+TT (*versus* GG) genotype. The *ADD1* gene was selected as candidate gene, because genetic variations in this gene have been associated with hypertension [34-36]. We observed no significant gene-environment interaction between the candidate SNP, *ADD1*_rs4961, and hypertension in relation to RCC risk. In addition, the variant genotype of *ADD1*_rs4961 is believed to induce proximal tubule sodium reabsorption [37], but we observed no significant interactions between this SNP and sodium intake.

The observed significant gene-environment interactions were all clustered in one SNP, i.e. *SLC9A3*_rs4957061. The *SLC9A3* gene encodes the Na⁺/H⁺ exchanger 3 (NHE3), which is the major sodium transporter on the apical membrane of the renal proximal tubule cells [38]. This is of particular importance for RCC, as the majority of RCC tumors are thought to arise from the proximal renal tubule [39]. Animal studies, as well as studies in hypertensive patients suggest that this gene is important in the control of blood pressure through its effect on sodium transport [40-42]. It is however remarkable that the observed gene-environment interactions for *SLC9A3*_rs4957061 did not include hypertension or sodium intake, but potassium intake, fluid intake and use of diuretic medication. In Cox regression analyses, RCC risk estimates for fluid intake were similar in both continuous and categorical analyses; the highest intake was associated with a lower RCC risk for participants with the *SLC9A3*_rs4957061 reference genotype and not for participants with the variant genotype. Previously, we reported that there was no overall association between fluid intake and RCC risk present in this study population, after 17.3 years of follow-up [8].

Some of the candidate SNPs investigated in the present study are SNPs in *SCNN1B* and *SCNN1G*. These genes may be involved in the salty taste transduction pathway in the KEGG pathway database [43] and have previously been associated with salt taste responses in mice [29]. Therefore, these genes may be of particular importance in the association between sodium intake and RCC risk. We hypothesised that SNP-related interindividual variation in the *SCNN1B* and *SCNN1G* genes may determine one's perceived saltiness and ultimately the actual sodium intake. We could not confirm our hypothesis, because none of the SNPs in *SCNN1B* and *SCNN1G* were associated with perceived saltiness in the subcohort.

Several methodological considerations should be noted as to interpret the results of the present study. Given the prospective study design and the high ascertainment of

RCC cases, selection bias and information bias are unlikely in the NLCS. The long follow-up provided sufficient number of cases for the current analyses, but may also have introduced some random misclassification regarding dietary intakes, which were calculated from a single measurement at baseline. In addition, it is possible that the proportions of participants with hypertension and diuretic medication use may slightly be underestimated using this single measurement. Both might have resulted in attenuation of the investigated gene-environment interactions. Moreover, different action mechanisms of various diuretics (e.g. potassium sparing) may have influenced the results. Information on the exact type of diuretic medication was not available. Finally, the results of two SNPs in particular, i.e. *SCNN1G*_rs4299163 and *WNK1*_rs10849563, should be interpreted with care, as they were not in HWE in our subcohort.

For the present study, we used a candidate SNP approach and selected SNPs based on prior knowledge from current literature. SNPs were not selected based on GWAS results, as these SNPs tend to have a relatively lower MAF and higher penetrance, while SNPs with high MAF and low penetrance may be the ones to be more likely involved in gene-environment interactions [6]. Our initial SNP selection rendered too many potential SNPs of interest for the limited space available on the multiplex design. The 13 SNPs in ten ITM genes that were successfully genotyped cover only a fraction of the large and complex network of genetic and environmental factors in which the investigated SNPs are included. As a result, true effects might still be diluted or masked by effects of those SNPs not included in the present study. In addition, the null results in the present study do not exclude the possibility that other SNPs in these genes or SNPs in other ITM genes may influence RCC susceptibility.

In conclusion, results from the present study show some first indications of involvement of *ADD1*_rs4961 and *SLC9A3*_rs4957061 in RCC susceptibility. However, results do not support the general hypothesis that the ITMs are a type of underlying disease mechanism in RCC etiology, since SNPs in ITM genes and gene-environment interactions among these SNPs with related exposures were mostly not associated with RCC risk.

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PART III

Tumor heterogeneity

CHAPTER 5

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

Introduction

Sodium intake, but not potassium and fluid intake, has been associated with higher renal cell cancer (RCC) risk. However, risk factors may differ by molecular subtypes of the tumor. In renal physiology, electrolyte and water homeostasis is facilitated by ion transport mechanisms (ITMs). 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 PCR was used to analyze promoter CpG island methylation in tumor 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 tumors with no, low (1 gene) and high (≥ 2 genes) methylation.

Results

Sodium intake (high *versus* low) increased ccRCC risk particularly in tumors with a high methylation index [HR(95%CI): 2.04(1.16-3.58)], while 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 (*versus* low) potassium intake was low for unmethylated tumors [HR(95%CI): 0.60(0.36-1.01)], but high for tumors 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).

Conclusion

Our findings suggest for the first time a joint influence of dietary intakes and ITM gene-specific promoter methylation in relation to ccRCC risk, indicating a role for ITMs in ccRCC etiology.

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 (ITMs), 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, ITMs 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 etiological mechanisms of human cancers the molecular characterization of the tumor 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 tumor types [5-7]. Few studies have focused on promoter CpG island methylation of genes involved in ITMs in relation to RCC [8,9]. Promoter CpG island methylation of genes involved in ITMs may be particularly useful for the molecular characterization of RCC when studying risk factors related to ITMs, 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 tumors 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 5,000 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) tumor tissues from ~50 pathology laboratories throughout the Netherlands. Overall, 79.8% of the FFPE tumor tissues were retrieved (n=453). Inclusion was lowest for cases identified during the first 5-years of follow-up (71.3%), because the Dutch pathology registry (PALGA) had incomplete coverage during this period and some pathological laboratories had problems to locate older tissues in particular. The inclusion over the 5-year follow-up periods 1991-1995 and 1996-2000 was 87.7% and 84.6%, respectively. In the period between 2001 and 2006 the inclusion was 73.7%, because we were unable to retrieve tissues from two pathology laboratories in our recent efforts to expand the collection. In total, the collection of FFPE tumor tissues was unsuccessful for 115 cases; 46 cases had no surgery, for 24 cases the availability or location of their tissues was unknown from the Dutch pathology registry (PALGA) and, for 45 cases the pathological laboratory refused or was unable to send their tissues. There were no differences on potential confounders and exposures under study (i.e. age, sex, BMI, smoking, alcohol intake, hypertension, energy intake and dietary sodium, potassium and fluid intake) between eligible incident RCC cases with and without successful DNA collection. Haematoxylin and eosin (HE)-stained slides of all the collected FFPE tumor tissues were assessed by an experienced genitourinary pathologist (Marcella MLL Baldewijns) to confirm tumor histology based on the WHO-classification of tumors [15]. Based on this assessment, our collection of RCC cases includes 365 (80.6%) clear-cell (cc)RCC cases, 60 (13.3%) papillary (p)RCC cases, 15 (3.3%) chromophobe (chr)RCC cases and 13 (2.8%) other or undefined RCC cases. Additional classification of pRCC cases, resulted in 35 (7.7%) type 1 pRCC, 24 (5.3%) type 2 pRCC and 1 (0.2%) undefined pRCC.

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-administered, 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 4,439 subcohort members and 434 cases eligible for analyses [16]. The FFQ ranked individuals adequately according to dietary intakes when compared to a nine-day dietary records [16], and reflected nutrient intakes for at least 5 years [17]. Average daily nutrient intakes were calculated using the Dutch food composition table 1986-87 [18] 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 [3,19].

Gene selection

The KEGG pathway database was used to identify candidate genes involved in ITMs [20]. Genes were of interest if exhibiting 1) promoter CpG island methylation (-2000 to +500 bp from transcription start site) in ccRCC cell lines (SKRC1, SKRC10, SKRC52 and SKRC59) and not in a normal kidney cell line (HK-2) in a genome-wide methylation screen (MBD-affinity massive parallel sequencing; data not shown) and 2) cancer-specific down-regulation among ccRCC and adjacent normal tissues in publically available expression data. Using this selection strategy we selected: Rho GDP dissociation inhibitor gamma (*ARHGDI3*), 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 signaling pathway.

Promoter methylation

Promoter CpG island hypermethylation, in short promoter methylation, was analyzed using nested methylation-specific 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 Suppl Table S5.1. MSP analyses of *ARHGDI3*, *ATP1A1*, *SCNN1B* and *SLC8A3* were performed successfully for 98.5%, 97.8%, 92.7% and 96.7% out of the 453 RCC cases, respectively.

A methylation index was calculated combining all four individual genes into three subgroups representing tumors with no (0 of 4 genes methylated), low (1 of 4 genes methylated) and high (≥ 2 of 4 genes methylated) methylation. Proportions of promoter methylation are shown in Table 5.1. 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.

Table 5.1 Proportions of promoter methylation of *ARHGDIG*, *ATP1A1*, *SCNN1B* and *SLC8A3* in renal cell tumors and in histological subtypes of renal cell tumors, NLCS 1986-2006.

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

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

Statistical analyses

All analyses were conducted in Stata version 12 (Stata Corp., College Station, TX). Associations between sodium, potassium and fluid intake and RCC risk, were performed 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 at baseline (y), sex (men/women), total energy intake (kcal/d), body mass index (BMI; kg/m²), smoking status (never or ex/current), smoking duration (y), smoking

intensity (cigarettes/d), self-reported doctors' diagnosis of hypertension and/or use of antihypertensive medication (no/yes) and alcohol consumption (g ethanol/d). Analyses regarding sodium intake (g/d) 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 tumors 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. A P -value<0.05 is considered statistically significant.

RESULTS

The prevalence of promoter methylation of *ARHGDIG*, *ATP1A1*, *SCNN1B* and *SLC8A3* in ccRCC tumors was 23.1%, 15.9%, 46.0% and 18.2%, respectively (Table 5.1). To verify these proportions, probes matching the MSP regions were identified in The Cancer Genome Atlas (TCGA) ccRCC database. For *SLC8A3* a probe in the same region as the MSP amplicon indicated a prevalence of 16.6-22.5%. Neighboring probes were identified for *ARHGDIG* and *SCNN1B*, with estimated prevalence's 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, though these differences only statistically significant for *ARHGDIG* and *SLC8A3* (P -value=0.005 and 0.004, respectively). The methylation index did not significantly differ across the histological subtypes (P -value=0.109).

At baseline, ccRCC cases and subcohort members are relatively similar, except that ccRCC cases were more frequently male (Table 5.2). Among ccRCC cases, those with a high methylation index had a higher baseline potassium intake, a higher BMI and less frequently hypertension, compared to those without promoter methylation.

The overall associations between sodium, potassium and fluid intake and ccRCC risk showed a higher HR for high (*versus* 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 5.3). There was no association between potassium and fluid intake and ccRCC risk. Associations regarding pRCC and chrRCC are shown in Suppl Table S5.2.

Heterogeneous associations between dietary intakes and ccRCC risks for tumors with a different methylation index were particularly observed for potassium intake (P -heterogeneity=0.008, Table 5.3). In tumors without promoter methylation, high (*versus* 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 tumors with a high methylation index, high

(*versus* 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 tumors with a different methylation index (*P*-heterogeneity=0.54), though HRs for high (*versus* low) fluid intake varied between tumors without promoter methylation and tumors 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 (*versus* low) sodium intake was associated with a higher ccRCC risk particularly in tumors 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 (*versus* low) sodium intake was also associated with a higher ccRCC risk for tumors without promoter methylation and for tumors 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 and not significant compared to tumors with a high methylation index.

Table 5.2 Baseline characteristics of subcohort members, total clear-cell renal cell cancer cases and clear-cell renal cell cancer cases according to a methylation index in the NLCS, 1986-2006.

Baseline characteristics (mean (SD))	Subcohort members	Total ccRCC	Methylation index ^{a,b}			<i>P</i> -value ^c
			No methylation	Low methylation	High methylation	
Total (n)	3980	306	110	74	90	
Age at baseline (y)	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 (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 (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 (ml/d)	2,087 (487)	2,103 (471)	2,077 (525)	2,102 (361)	2,139 (455)	0.50
Potassium (mg/d) ^d	3,529 (602)	3,607 (651)	3,490 (633)	3,614 (674)	3,742 (636)	0.02
Alcohol (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 (kcal/d)	1,926 (515)	1,987 (507)	2,024 (581)	1,946 (446)	1,978 (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
Current smokers (%)	27.3	29.4	28.2	33.8	30.0	0.72
Smoking duration (y) ^g	38.7 (9.7)	39.7 (7.7)	40.3 (7.8)	41.0 (7.9)	37.7 (8.4)	0.16
Smoking intensity (cig/d) ^g	15.2 (8.8)	15.9 (7.8)	17.3 (8.1)	16.3 (8.5)	13.6 (6.5)	0.24
Hypertension (%)	32.1	35.6	44.6	35.1	25.6	0.02
Low-salt diet (%)	7.6	9.5	10.0	8.1	8.9	0.91

^a The methylation index of a tumor is based on frequencies of methylation in *ARHGDI*G, *ATP1A1*, *SCNN1B* and *SCL8A3*; no, low and high methylation index correspond 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 = 3,033); ^g In current smokers. *ARHGDI*G = Rho GDP dissociation inhibitor gamma, *ATP1A1* = ATPase Na⁺/K⁺ transporting alpha 1 polypeptide, BMI = body mass index, ccRCC = clear-cell renal cell cancer, cig = cigarettes, NLCS = Netherlands Cohort Study, *SCNN1B* = sodium channel non-voltage-gated 1 beta subunit, SD = standard deviation, *SCL8A3* = solute carrier family 8 member 3.

The contribution of each of the four individual genes to the methylation index is evaluated in single gene analyses (Tables 5.4 and 5.5). High (*versus* low) sodium intake increased ccRCC risk, regardless of promoter methylation of any of the genes, but in tumors with *ATP1A1* promoter methylation, the ccRCC risk for high (*versus* 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 (*versus* low) potassium intake and ccRCC risk by the methylation index was largely determined by the promoter methylation of *SCNN1B* (*P*-heterogeneity<0.001). In tumors without *SCNN1B* methylation, high (*versus* low) potassium intake decreased the ccRCC risk [HR(95%CI): 0.59(0.38-0.91), *P*-trend=0.02)], whereas in tumors with *SCNN1B* methylation high (*versus* 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 (*versus* low) potassium intake were observed by promoter methylation of the three other genes, yet only for *ATP1A1* heterogeneity was significant (*P*-heterogeneity=0.02). For high (*versus* low) fluid intake, differential ccRCC risks were only, though not significantly, observed by promoter methylation of *SCNN1B* [HR(95%CI): 0.65(0.40-1.05) and 1.58(0.90-2.78) with *P*-trend=0.08 and 0.12 in tumors without and with *SCNN1B* methylation, respectively].

Table 5.3 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 NLCS, 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-trend				0.016			0.016			0.287		0.099				0.011	
Increment 1 g/d		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-trend				0.799			0.909			0.050		0.972				0.063	
Increment 1 g/d		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-trend				0.881			0.730			0.146		0.793				0.218	
Increment 1 l/d		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

^a The methylation index of a tumor is based on frequencies of methylation in *ARHGDI1*, *ATP1A1*, *SCNN1B* and *SCL8A3*; no, low and high methylation index correspond to methylation in 0, 1 and ≥2 genes, respectively; ^b HRs are adjusted for age at baseline (y), sex (men/women), BMI (kg/m²), smoking status (never or ex/current), smoking duration (y), smoking intensity (cig/d), hypertension status (no/yes), alcohol intake (g ethanol/d) and total energy intake (kcal/d); ^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/d. *ARHGDI1* = Rho GDP dissociation inhibitor gamma, *ATP1A1* = ATPase Na⁺/K⁺ transporting alpha 1 polypeptide, BMI = body mass index, ccRCC = clear-cell RCC, cig = cigarettes, NLCS = Netherlands Cohort Study, RCC = renal cell cancer, *SCNN1B* = sodium channel non-voltage-gated 1 beta subunit, *SCL8A3* = solute carrier family 8 member 3, T1-3 = tertile 1-3.

Table 5.4 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 NLCS, 1986-2006.

Dietary intakes	ARHGDIG					ATP1A1						
	Unmethylated		Methylated		P-value ^b	Unmethylated		Methylated		P-value ^b		
	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)	97	1.23	(0.90-1.69)	26	3.19	(1.43-7.12)
P-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)		1.16	(0.94-1.43)		1.36	(0.96-1.93)
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)	78	0.86	(0.63-1.19)	25	1.51	(0.78-2.92)
P-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)		0.96	(0.74-1.23)		1.30	(0.84-2.02)
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)	81	0.86	(0.58-1.26)	18	1.57	(0.58-4.26)
P-trend			0.502			0.523			0.443			0.421
Increment 1 l/d		0.91	(0.66-1.26)		1.18	(0.68-2.06)		0.92	(0.67-1.26)		1.23	(0.66-2.28)
						0.843						0.256

^a HRs are adjusted for age at baseline (y), sex (men/women), BMI (kg/m²), smoking status (never or ex/current), smoking duration (y), smoking intensity (cig/d), hypertension status (no/yes), alcohol intake (g ethanol/d) and total energy intake (kcal/d); ^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/d. *ARGDIG* = Rho GDP dissociation inhibitor gamma, *ATP1A1* = ATPase Na⁺/K⁺ transporting alpha 1 polypeptide, BMI = body mass index, cig = cigarettes, NLCS = Netherlands Cohort Study, T1-3 = tertile 1-3.

Table 5.5 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 NLCS, 1986-2006.

Dietary intakes	SCNN1B						SLC8A3							
	Unmethylated			Methylated			P-value ^b	Unmethylated			Methylated			
	n	HR ^a	95% CI	n	HR ^a	95% CI		n	HR ^a	95% CI	n	HR ^a	95% CI	P-value ^b
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-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-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-trend			0.075			0.117				0.511			0.119	
Increment 1 l/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

^a HRs are adjusted for age at baseline (y), sex (men/women), BMI (kg/m²), smoking status (never or ex/current), smoking duration (y), smoking intensity (cig/d), hypertension status (no/yes), alcohol intake (g ethanol/d) and total energy intake (kcal/d); ^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/d. BMI = body mass index, cig = cigarettes, NLCS = Netherlands Cohort Study, *SCNN1B* = sodium channel non-voltage-gated 1 beta subunit, *SLC8A3* = solute carrier family 8 member 3, T1-3 = tertile 1-3.

DISCUSSION

To our knowledge, this is the first study that reported on dietary sodium, potassium and fluid intake and a methylation index of four ITM genes in relation ccRCC risk. We previously reported the overall associations between these dietary intakes and RCC risk in the same study population, only 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, while 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, an epigenome-wide screen and an exhaustive search in publically 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 tumors and their adjacent normal tissues promoter methylation was present in the tumor, but not in the normal tissues, indicating that methylation of these genes may be indeed cancer-specific (data not shown). We are the first to report the prevalence of promoter methylation of these genes in a population-based series of RCC tumors. For *SCNN1B*, hyper-methylation and down-regulation in ccRCC has previously been reported in tissues [8].

Although potassium intake was not associated with the overall ccRCC risk, we show significant differential associations between potassium intake and ccRCC risk by the methylation index. This highlights the crucial role of molecular characterization of the tumor to better understand etiology [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 hypokalemia [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].

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 tumors 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 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 nine-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 tumor. It is plausible that gene-specific promoter methylation, as early event in carcinogenesis, sensitizes the renal environment to the dietary intakes under study, so promoting the development of the ccRCC tumor. However, we cannot exclude that the observed promoter methylation of the carefully selected genes 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 the dietary intakes may have caused gene-specific promoter methylation in the tumor, 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 tumor 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; risk factors and potential confounders did not differ between cases with and without

tumor material. This study is among the largest prospective studies on RCC and, so far, the only prospective study including FFPE tumor 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 on the dietary intakes, introduced by the long follow-up and single baseline measurement, even though intakes were rather stable during the first five years of follow-up [17].

In conclusion, potassium intake was differentially associated with ccRCC risk only when the methylation index of the investigated genes was considered, indicating a joint influence of specific dietary intakes and gene-specific promoter methylation in relation to ccRCC risk. Using this approach of molecular pathological epidemiology, this study provides the first evidence of a biological rationale for the role of ITMs in the development of ccRCC. However, the temporal relationship between the dietary intakes and promoter methylation and the exact mechanism by which such ion-transporters may be involved ccRCC etiology remains to be determined.

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Suppl Table S5.1 Primer sequences and sequence condition used to analyze promoter methylation of *ARHGDIG*, *ATP1A1*, *SCNN1B* and *SLC8A3* in renal cell tumors in the NLCS, 1986-2006.

Gene	Primer sequence	Tm (°C)	Cycles (n)	Length (bp)
<i>ARHGDIG</i>				
Flanking primer, upstream	GGGGTAGTTTTGTGGTTAGAGT	56	35	23
Flanking primer, downstream	ACCAAAACCCRCACAAAAA	56	35	20
Unmethylated sense strand	TTGGTTAGAGTTGGGTATGGGT	66	30	23
Methylated sense strand	GGTTAGAGTCGGGTACGGGTC	66	30	21
Unmethylated antisense strand	CCACACAAAAACCCCAACA	66	30	21
Methylated antisense strand	CACAAAAACGCCCGAACG	66	30	18
<i>ATP1A1</i>				
Flanking primer, upstream	AGGAGGYGGATAYGTGGTAATAG	56	35	23
Flanking primer, downstream	CCAAATAAAAAAACTAAACTACC	56	35	25
Unmethylated sense strand	GTGGTAATAGTGGTAGTAGTTTGGGT	64	35	26
Methylated sense strand	GTAATAGCGGTAGTAGTTCGGGC	64	35	23
Unmethylated antisense strand	AAAAAACTAAACTACCACAAAACA	64	35	26
Methylated antisense strand	AAACTAAACTACCGCGAAACG	64	35	22
<i>SCNN1B</i>				
Flanking primer, upstream	TYGGGTGTTTAGTGTTATTAATATT	56	35	26
Flanking primer, downstream	AAACCRAATACRAACCAAAATTC	56	35	23
Unmethylated sense strand	GTGTTTAGTGTTATTAATATTGGTTGTT	64	35	30
Methylated sense strand	GTTTTAGTGTTATTAATATTCGGTCGTC	64	35	28
Unmethylated antisense strand	AAATTCACCATCATACCTACTCAAACA	64	35	27
Methylated antisense strand	GCCGTCATACCTACTCGAACG	64	35	21
<i>SLC8A3</i>				
Flanking primer, upstream	YGGTAGTTTAATGGGTAAGTTTTT	56	35	25
Flanking primer, downstream	CCTAACTTTCCTAACTAAAAAC	56	35	25
Unmethylated sense strand	GTAGTTTAATGGGTAAGTTTTTTGTTT	68	35	29
Methylated sense strand	AGTTTAATGGGTAAGTTTTTCGTTC	68	35	26
Unmethylated antisense strand	TAACTAAAAACACACCAATCTCCA	68	35	26
Methylated antisense strand	AAACGCGCCGAATCTCCG	68	35	18

ARHGDIG = Rho GDP dissociation inhibitor gamma, *ATP1A1* = ATPase Na⁺/K⁺ transporting alpha 1 polypeptide, NLCS = Netherlands Cohort Study, *SCNN1B* = sodium channel non-voltage-gated 1 beta subunit, *SLC8A3* = solute carrier family 8 member 3.

Suppl Table S5.2 Hazard ratios and 95% confidence intervals of papillary renal cell cancer risk and chromophobe renal cell cancer risk for intakes of sodium, potassium and fluid in the NLCS, 1986-2006.

Exposure	Person years ^a	pRCC cases			chrRCC cases		
		n	HR ^b	95% CI	n	HR ^b	95% CI
Sodium intake ^{c,d}							
T1	22,584	8	1.00	(ref)	6	-	-
T2	22,548	25	3.10	(1.39-6.93)	1	-	-
T3	22,115	13	1.70	(0.70-4.10)	5	-	-
<i>P</i> -trend				0.252			-
Increment 1 g/d			1.45	(0.90-2.35)		0.84	(0.32-2.22)
Potassium intake ^{c,d}							
T1	21,775	19	1.00	(ref)	2	-	-
T2	22,941	9	0.44	(0.20-0.99)	6	-	-
T3	22,531	18	0.88	(0.45-1.70)	4	-	-
<i>P</i> -trend				0.722			-
Increment 1 g/d			0.99	(0.62-1.59)		1.37	(0.82-2.30)
Fluid intake ^e							
Low	15,841	10	1.00	(ref)	3	-	-
Moderate	29,740	19	1.03	(0.48-2.23)	5	-	-
High	21,667	17	1.17	(0.51-2.67)	4	-	-
<i>P</i> -trend				0.699			-
Increment 1 l/d			0.82	(0.43-1.57)		0.49	(0.17-1.46)

^a In the subcohort; ^b HRs are adjusted for age at baseline (y), sex (men/women), BMI (kg/m²), smoking status (never or ex/current), smoking duration (y), smoking intensity (cig/d), hypertension status (no/yes), alcohol intake (g ethanol/d) and total energy intake (kcal/d); ^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/d. BMI = body mass index, chrRCC = chromophobe RCC, cig = cigarettes, NLCS = Netherlands Cohort Study, pRCC = papillary RCC, RCC = renal cell cancer, T1-3 = tertile 1-3.

CHAPTER 6

Alcohol and dietary folate intake and gene promoter methylation in clear-cell renal cell cancer

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Submitted

ABSTRACT

Introduction

High alcohol intake and low folate intake are hypothesized to be associated with promoter CpG island hypermethylation. We investigated whether alcohol and dietary folate intakes were associated with promoter methylation in clear-cell renal cell cancer (ccRCC).

Methods

The Netherlands Cohort Study with case-cohort design included 120,852 subjects aged 55-69 years in 1986. Diet was measured with a food-frequency questionnaire. After 20.3 years of follow-up, paraffin-embedded tumor blocks were collected. Methylation-specific PCR was used to analyze promoter methylation of eleven, frequently methylated genes in ccRCC. The ccRCC cases were classified into low, intermediate and high methylation, representing a methylation frequency of 0-19%, 20-39% and more than 40%, respectively. Multivariable Cox regression analyses were stratified according to this methylation index and included 3,980 subcohort members and 297 ccRCC cases.

Results

Higher intakes of alcohol were associated with a decreased ccRCC risk in all cases, but multivariable adjusted hazard ratios (HR) and 95% confidence intervals (CI) were not statistically significant [HR(95%CI) for ≥ 30 g/d *versus* 0 g/d: 0.78(0.48-1.24), P -trend=0.46]. Dietary folate intake was not associated with ccRCC risk [HR(95%CI) for high *versus* low intake: 1.07(0.70-1.64), P -trend=0.59]. There was no significant heterogeneity in ccRCC risk for alcohol and dietary folate intake according to categories of the methylation index and there was no effect-modification of alcohol and dietary folate intake on ccRCC risk.

Conclusion

Our findings do not support the hypothesis that alcohol and dietary folate intake are involved in ccRCC carcinogenesis through promoter methylation of our methylation index.

INTRODUCTION

Cigarette smoking, excess body weight, a diagnosis of hypertension and familial cancer syndromes are established risk factors of renal cell cancer (RCC) [1]. Recent evidence suggests that alcohol consumption may be inversely associated with RCC risk [1-4], although the mechanism explaining the inverse association has not been established yet.

One of the suggested mechanisms that may link alcohol to cancer is DNA methylation [5]. Aberrant promoter CpG island hypermethylation, in short promoter methylation, has been observed in many cancers, including RCC [6,7]. It has been suggested that there is a specific subtype of cancer that is characterized by extensive promoter methylation of tumor suppressor and DNA repair genes and referred to as CpG island methylator phenotype (CIMP) [8]. CIMP has been investigated extensively in colorectal cancer, but may also exist in other cancers, such as RCC [8,9]. However, no definition for CIMP in RCC has been established so far.

An adequate folate metabolism is necessary for maintaining DNA methylation and a high intake of alcohol is associated with folate reduction by different mechanisms. High alcohol intake decreases the absorption of folate from the intestinal lumen [10,11]. Alcohol may also increase urinary excretion of folate and influences proteins involved in folate metabolism [10,11]. Heavy abusers of alcohol often have a diet with a deficient folate intake [10]. The combination of high alcohol intake and a low dietary folate intake, may be a specific subgroup of the population that is at risk for adverse effects of folate deficiency [10-13].

In a previous analysis we investigated whether alcohol intake was associated with promoter methylation in the Von Hippel-Lindau (*VHL*) gene in RCC [14]. Alcohol intake was inversely associated with the RCC subtype without *VHL* promoter methylation, however, the number of RCC cases with *VHL* promoter methylation was too low for reliable point estimates.

If high alcohol and low folate intake are associated with promoter methylation in RCC, the association of these dietary exposures with RCC risk might differ by molecular subtypes of RCC defined by promoter methylation status. The overall association of alcohol with RCC risk is inverse, but this might not be true in the subgroup of RCC characterized by a high promoter methylation status. We therefore investigated whether associations between alcohol and dietary folate intake are different in RCC subgroups defined by levels of promoter methylation of CpG islands in a specifically selected gene panel in a large prospective study.

METHODS

Study design and study population

The Netherlands Cohort Study on Diet and Cancer (NLCS) is a prospective cohort study that was initiated in 1986 and included 120,852 participants aged 55-69 years at baseline [15]. 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 5,000 subjects was randomly sampled at baseline to estimate person years at risk for the entire cohort [15]. 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 the Netherlands Pathology Registry (PALGA) [16]. Only one male subcohort member was lost to follow-up. The completeness of cancer follow-up by the Netherlands cancer registry and PALGA is estimated to be over 96% [17]. Cases and subcohort members who reported prevalent cancer (excluding skin cancer) at baseline were excluded. A unique population-based collection of DNA isolated from formalin-fixed paraffin-embedded (FFPE) tissues 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 [18], yet recently the collection was expanded to 20.3 years of follow-up.

Tissue collection and DNA isolation

A total of 608 RCC cases were identified within the NLCS during 20.3 years of follow-up (between 1986 and 2006). Only histologically confirmed epithelial RCC cases (n=568) were eligible for the collection of FFPE tumor tissues from ~50 pathology laboratories throughout the Netherlands. Tumor blocks of approximately 80% of the eligible cases could be retrieved. The RCC cases were classified according to the World Health Organization (WHO) Classification of Tumors of 2004 [19] by two experienced urogenital pathologists (C.A. Hulsbergen-van de Kaa and M.M.L.L. Baldewijns). Overall, 81% of the cases were classified as clear-cell RCC (ccRCC) [19].

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 [18]. For the 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-administered, baseline questionnaire, including a 150-item, semi-quantitative food-frequency questionnaire (FFQ), which

concentrated on habitual consumption of foods and beverages in the year before the start of the study.

Consumption of alcoholic beverages was addressed by questions on beer, red wine, white wine, sherry, other fortified wines, liqueur, and liquor. Respondents who consumed alcoholic beverages less than once a month were considered non-users. Four items from the questionnaire (i.e., red wine, white wine, sherry, and liqueur) were combined into one wine variable, since these items were substantially correlated [20]. Mean daily alcohol consumption was calculated using the Dutch food composition table [21]. Based on a pilot study, 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 grams of alcohol, respectively.

Mean daily intakes of other nutrients were calculated by multiplying the frequencies and portion sizes of all food items and their tabulated nutrient contents from the Dutch Food Consumption table from 1986 [21]. Folate data were derived from a validated liquid chromatography trienzyme method [22] used to analyze the 125 most important Dutch foods contributing to folate intake [23]. Although the FFQ contained questions on the use of dietary supplements, dietary supplement use of folic acid was not accounted for in the analyses, as dietary supplements in general did not contain folic acid in the Netherlands in the late 1980s. Participants with incomplete or inconsistent dietary questionnaires were excluded [24]. The FFQ ranked individuals adequately according to dietary intakes when compared to a nine-day dietary records [24], and reflected nutrient intakes for at least 5 years [25].

Gene selection and promoter methylation

To date, no definition for CpG island methylator phenotype (CIMP) in ccRCC has been established. Therefore, we carefully selected CpG islands of interest in ccRCC as a measure for promoter methylation. Besides the *VHL*-gene [14], genes were selected exhibiting 1) promoter CpG island methylation (-2000 to +500 bp from transcription start site) in ccRCC cell lines (SKRC1, SKRC10, SKRC52 and SKRC59) and not in a normal kidney cell line (HK-2) in a genome-wide methylation screen (MBD-affinity massive parallel sequencing) [26] and 2) cancer-specific down-regulation among ccRCC in publically available expression data from The Cancer Genome Atlas. Using this selection strategy we selected eleven genes: Cysteine Dioxygenase type 1 (*CDO1*); Follistatin (*FST*); Frizzled Class Receptor 10 (*FZD10*); Gremlin 1, DAN family BMP antagonist (*GREM1*); Ladinin 1 (*LAD1*); Neurofilament, heavy polypeptide (*NEFH*); Neuralized E3 Ubiquitin Protein Ligase 1 (*NEURL*); Ras-specific Guanine Nucleotide-releasing Factor 2 (*RASGRF2*); Stratifin (*SFN*); Secreted Frizzled-Related Protein 1 (*SFRP1*) and *VHL* as candidate markers.

Promoter methylation of the CpG islands was analyzed using nested methylation-specific PCR (MSP), as previously described elsewhere [27-29]. MSP primer design was based on the MBD-affinity massive parallel sequencing data. Primer sequences and MSP conditions are shown in Suppl Table S6.1. From 45 cases, MSP-analysis was repeated in order to measure reproducibility. After excluding missing values due to genotyping failures, only one discrepancy in one gene was recorded (99.7% reproducibility).

A sum score representing promoter methylation was calculated combining promoter methylation in all eleven individual genes dividing the ccRCC cases into three categories that were approximately the same size. The categories low, intermediate and high methylation represent cases with a methylation frequency of 0-19%, 20-39% and more than 40%, respectively.

Statistical analyses

All analyses were conducted in Stata version 11 (Stata Corp., College Station, TX). Associations between alcohol and folate intake and RCC risk were performed for total RCC and for ccRCC. Hazard ratios (HR) and 95% confidence intervals (CI) were estimated using Cox proportional hazards analyses adjusted for the case-cohort design [30]. The proportional hazards assumption was tested using the scaled Schoenfeld residuals and by introducing time-covariate interactions into the models. The proportional hazards assumption in the analyses using time on study as timescale for the variable age was not fulfilled. Therefore, all models were carried out using age as the timescale. Analyses were performed for a simple model (adjusted for sex and year of birth) and a multivariable adjusted model, including *a priori* selected potential confounders: year of birth (1916-20, 1921-25 and 1926-31), sex (men/women), total energy intake (kcal/d), body mass index (BMI; kg/m²), smoking status (never or ex/current), smoking intensity (cigarettes/d), smoking duration (y), and self-reported doctors' diagnosis of hypertension and/or use of antihypertensive medication (no/yes). In a previous publication, we investigated the association between alcohol intake and RCC risk after 11.3 years of follow-up [14]. Current analyses were split into <11.3 and ≥11.3 years of follow-up, to compare the results with the previous publication [14].

The *P*-interaction was assessed by including cross-product terms in the models and performing a Wald χ^2 test. Because of the limited number of cases in stratified interaction analyses, alcohol and folate intake were each categorized into two categories (referred to as high *versus* low intake); alcohol intake was categorized into <15 and ≥15 g/d and folate intake into higher and lower than median intake in the subcohort (median intake in men 212.07 µg/d and in women 188.71 µg/d).

Tests for heterogeneity were performed to evaluate differences across tumors 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 [31,32]. All tests were two-sided. A P -value <0.05 is considered statistically significant.

RESULTS

After exclusion of cohort members with incomplete and/or inconsistent food-frequency questionnaires or with missing data on confounders, 3,980 subcohort members and 498 RCC cases were available for analysis. For 297 ccRCC cases promoter methylation could be genotyped of at least four genes.

Of the 297 ccRCC cases, MSP analyses of gene promoter regions were successful for 91% (*RASGRF2*) to 99% (*NEURL*). Proportions of promoter methylation per investigated gene are shown in Table 6.1. This proportion was on average 36%, but varied from 8% (*VHL*) to 97% (*SFN*). In 112 cases, less than 20% of the eleven investigated genes were methylated (referred to as low methylation), in 81 cases between 20% and 40% of the genes were methylated (intermediate methylation) and in 104 cases more than 40% of the genes were methylated (high methylation).

The proportion of non-users of alcohol was slightly higher in the subcohort (23.8%) than in the total group of RCC cases (22.3%) (Table 6.2). Mean alcohol intake in users was similar in subcohort and RCC cases (15.1 and 15.0 g/d). Intake of folate was slightly lower in the subcohort than in RCC cases (211.8 and 218.6 µg/d). RCC cases were more often men, had a slightly higher BMI, were more often smokers, and had more often a diagnosis of hypertension or diabetes at baseline than subcohort members.

Multivariable adjusted HRs for categories of alcohol intake and RCC risk were all lower than one, but not statistically significant (Table 6.3). The multivariable adjusted HR(95%CI) for participants with 30+ g/d alcohol intake was 0.79(0.54-1.16) compared to non-users and the P -trend was 0.27. When analyses were restricted to ccRCC, multivariable HRs were comparable. When ccRCC cases were stratified according to methylation status, heterogeneity of associations between alcohol intake and ccRCC risk was not statistically significant (P -heterogeneity=0.29).

Table 6.1 Descriptive data on methylation in clear-cell renal cell cancer cases in the NLCS, 1986-2006.

Gene	Successfully Genotyped ^a		Proportion of methylation		Proportion of methylation by methylation index ^b			
					Low		Intermediate	
	n	%	n	%	n	%	n	%
Total	297		297		112		81	
							104	
<i>FZD10</i>	277	93.3	146	52.7	20	20.4	50	66.7
<i>FST</i>	290	97.6	36	12.4	0	0.0	4	5.1
<i>SFRP1</i>	290	97.6	55	19.0	0	0.0	4	5.1
<i>SFN</i>	284	95.6	274	96.5	90	90.0	81	100.0
<i>CDO1</i>	279	93.9	114	40.9	2	2.0	27	35.1
<i>NEURL</i>	295	99.3	121	41.0	10	9.1	26	32.1
<i>GREM1</i>	285	96.0	114	40.0	4	3.9	26	32.9
<i>NEFH</i>	270	90.9	76	28.2	2	2.0	13	18.6
<i>LAD1</i>	288	97.0	87	30.2	5	4.8	18	22.5
<i>RASGFR2</i>	269	90.6	63	23.4	0	0.0	4	5.4
<i>VHL</i>	292	98.3	23	7.9	1	0.9	3	3.8

^a Only ccRCC cases with at least four genes successfully genotyped; ^b Categories of the methylation index low, intermediate and high represent 0-19%, 20-39% and 40% or more methylation, respectively. ccRCC = clear-cell renal cell cancer, *CDO1* = Cysteine Dioxygenase type 1, *FST* = Follistatin, *FZD10* = Frizzled Class Receptor 10, *GREM1* = Gremlin 1, DAN family BMP antagonist, *LAD1* = Ladinin 1, *NEFH* = Neurofilament, heavy polypeptide, *NEURL* = Neuralized E3 Ubiquitin Protein Ligase 1, NLCS = Netherlands Cohort Study, *RASGFR2* = Ras-specific Guanine Nucleotide-releasing Factor 2, *SFN* = Stratifin, *SFRP1* = Secreted Frizzled-Related Protein 1, *VHL* = von Hippel-Lindau.

The HR(95%CI) of ccRCC risk by low, intermediate and high methylation for ≥ 30 versus 0 g alcohol/d was 1.29(0.65-2.58) with P -trend=0.97, 0.46(0.17-1.25) with P -trend=0.25 and 0.55(0.24-1.27) with P -trend=0.76, respectively. There was no heterogeneity with respect to the association between the alcohol intake from beer, wine and liquor and risk of ccRCC, overall or stratified according to methylation (Suppl Table S6.2).

Multivariable adjusted HRs of dietary folate intake and RCC risk were all close to one (Table 6.3). In ccRCC, the multivariable HR(95%CI) for participants with the highest quintile of folate intake was 1.07(0.70-1.64) compared to the lowest quintile (P -trend=0.59). In addition, there was no indication for heterogeneity according to methylation status (P -heterogeneity=0.80). The HR(95%CI) of ccRCC risk by low, intermediate and high methylation for high versus low dietary folate intake was 1.09(0.56-2.13) with P -trend=0.77, 1.15(0.52-2.57) with P -trend=0.74 and 0.93(0.45-1.93) with P -trend=0.72, respectively.

Table 6.2 Baseline characteristics of subcohort members and renal cell cancer cases according to the methylation index in the NLCS, 1986-2006.

Baseline characteristics (mean (SD))	Subcohort (n=3,980)	RCC (n=498)	ccRCC (n=306)	ccRCC by methylation index ^a		
				Low (n=112)	Intermediate (n=81)	High (n=104)
Intake of alcohol (n (%) non-users)	947 (23.8)	111 (22.3)	73 (23.9)	24 (21.4)	22 (27.2)	26 (25.0)
Intake of alcohol (g/d) ^b	13.5 (15.1)	14.9 (15.0)	15.1 (15.0)	16.2 (18.4)	13.0 (12.4)	15.8 (12.4)
Intake of folic acid (µg/d),	211.8 (72.5)	218.6 (76.0)	216.8 (79.8)	212.9 (86.6)	224.8 (85.7)	215.3 (67.3)
Male sex (n (%))	1,954 (49.1)	329 (66.1)	195 (63.7)	64 (57.1)	52 (64.2)	74 (71.2)
Age at baseline (y)	61.3 (4.2)	60.9 (3.9)	60.7 (3.9)	60.5 (3.9)	61.0 (3.7)	60.8 (3.9)
Body mass index (kg/m ²)	25.0 (3.1)	25.4 (3.0)	25.5 (3.0)	25.0 (2.8)	25.8 (3.4)	25.7 (2.8)
Current smokers (n (%))	1,086 (27.3)	153 (30.7)	90 (29.4)	33 (29.5)	24 (29.6)	32 (30.8)
Smoking intensity (cig/d) ^b	15.3 (10.3)	17.0 (12.2)	16.6 (11.6)	15.4 (10.3)	17.7 (10.5)	17.0 (13.6)
Smoking duration (y) ^b	31.6 (12.2)	31.9 (12.0)	31.6 (11.9)	30.1 (13.2)	32.1 (11.7)	32.8 (10.8)
Hypertension (n (%))	1,059 (26.6)	157 (31.5)	95 (31.1)	39 (34.8)	27 (33.3)	26 (25.0)
Use of anti-hypertensive medication (n (%))	829 (20.8)	119 (23.9)	75 (24.5)	31 (27.7)	23 (28.4)	19 (18.3)
Diabetes mellitus (n (%))	134 (3.4)	22 (4.4)	12 (3.9)	4 (3.6)	2 (2.5)	6 (5.8)
Family history of RCC (n (%))	42 (1.1)	5 (1.0)	3 (1.0)	-	2 (2.5)	1 (1.0)
Energy intake (kcal/d)	1,926 (515)	2,005 (514)	1,987 (507)	2,009 (533)	2,016 (528)	1,949 (465)
Sodium intake (g/d) ^c	2.3 (0.6)	2.5 (0.7)	2.5 (0.6)	2.3 (0.6)	2.6 (0.7)	2.5 (0.6)
Fluid intake (l/d)	1.4 (0.4)	1.4 (0.4)	1.4 (0.4)	1.4 (0.4)	1.4 (0.4)	1.4 (0.4)

^a Categories of the methylation index low, intermediate and high represent 0-19%, 20-39% and 40% or more methylation, respectively. ^b among users/consumers only. ^c residuals. (cc)RCC= (clear-cell) renal cell cancer, cig = cigarettes, NLCS = Netherlands Cohort Study, SD = standard deviation.

Table 6.3 Hazard ratios for the association between alcohol and folate intake and the risk of renal cell cancer according to molecular subtypes in the NLCS, 1986-2006.

Dietary intakes (median)	Person years	RCC cases			ccRCC cases			ccRCC cases by methylation index ^a						P-value ^c		
		n	HR ^b	95% CI	n	HR ^b	95% CI	Low		Intermediate		High				
								n	HR ^b	n	HR ^b	n	HR ^b			
Alcohol intake (g/d)																
0 (0)	15,882	111	1.00	(ref)	73	1.00	(ref)	24	1.00	(ref)	22	1.00	(ref)	26	1.00	(ref)
0.1-4.9 (1.8)	19,946	132	0.91	(0.69-1.20)	76	0.79	(0.56-1.11)	33	1.06	(0.62-1.81)	19	0.66	(0.35-1.23)	20	0.58	(0.32-1.05)
5-14 (9.3)	15,026	102	0.77	(0.57-1.03)	62	0.72	(0.50-1.03)	23	0.87	(0.48-1.56)	17	0.66	(0.34-1.26)	19	0.56	(0.30-1.04)
15-29 (21.8)	10,544	100	0.92	(0.67-1.25)	63	0.92	(0.63-1.34)	16	0.79	(0.40-1.55)	17	0.82	(0.41-1.63)	30	1.08	(0.60-1.94)
30+ (39.9)	5,849	53	0.79	(0.54-1.16)	32	0.78	(0.48-1.24)	16	1.29	(0.65-2.58)	6	0.46	(0.17-1.25)	9	0.55	(0.24-1.27)
P-trend				0.27			0.46			0.97			0.25		0.76	
Continuous per 10 g	67,247	498	0.96	(0.90-1.03)	306	0.97	(0.89-1.06)	112	1.05	(0.93-1.19)	81	0.86	(0.72-1.03)	104	0.96	(0.84-1.10)
Folate intake (μg/d) ^d																
Q1 (160/139)	13,336	100	1.00	(ref)	66	1.00	(ref)	26	1.00	(ref)	16	1.00	(ref)	23	1.00	(ref)
Q2 (190/166)	13,522	87	0.85	(0.62-1.17)	52	0.76	(0.51-1.12)	21	0.85	(0.47-1.53)	16	0.95	(0.44-2.03)	12	0.46	(0.22-0.95)
Q3 (212/188)	13,500	101	1.01	(0.74-1.39)	59	0.89	(0.60-1.32)	22	0.95	(0.52-1.76)	13	0.80	(0.36-1.77)	22	0.84	(0.44-1.63)
Q4 (241/213)	13,560	96	0.94	(0.68-1.30)	58	0.86	(0.58-1.28)	20	0.90	(0.49-1.65)	16	0.94	(0.44-2.04)	21	0.75	(0.38-1.48)
Q5 (298/267)	13,329	114	1.13	(0.80-1.61)	71	1.07	(0.70-1.64)	23	1.09	(0.56-2.13)	20	1.15	(0.52-2.57)	26	0.93	(0.45-1.93)
P-trend				0.36			0.59			0.77			0.74		0.72	
Continuous per 100 μg	67,247	498	1.08	(0.89-1.30)	306	1.08	(0.85-1.39)	112	1.03	(0.65-1.62)	81	1.22	(0.77-1.93)	104	1.01	(0.71-1.44)

^a Categories of the methylation index low, intermediate and high represent 0-19%, 20-39% and 40% or more methylation, respectively. ^b Cox proportional hazards model adjusted for sex (men/women), year of birth (1916-20, 1921-25 and 1926-31), BMI (kg/m²), smoking status (never or ex/current), smoking duration (y), smoking intensity (cig/d), hypertension (no/yes; including use of anti-hypertensive medication), total energy intake (kcal/d), methionine (μg/d), vitamin B2 (μg/d) and vitamin B6 (μg/d) and using age as time-scale. ^c Test for heterogeneity. ^d median intake for men/women. BMI = body mass index, ccRCC = clear-cell renal cell cancer, cig = cigarettes, NLCS = Netherlands Cohort Study, Q1-Q5 = Quintile 1-5.

In interaction analyses on the combined effect of alcohol and folate intake in Table 6.4, we observed that the ccRCC risk for high alcohol/low folate consumers was not increased compared to low alcohol/high folate consumers [multivariable HR(95%CI): 0.87(0.56-1.35), P -interaction=0.08]. The HR(95%CI) of ccRCC risk stratified according to low, intermediate and high methylation for high alcohol/low folate consumers compared to low alcohol/high folate consumers was 0.73(0.36-1.46) with P -interaction=0.32, 0.89(0.39-2.04) with P -interaction=0.47 and 1.09(0.51-2.33) with P -interaction=0.16, respectively.

When the analyses of alcohol intake and RCC risk were split according to follow-up duration (<11.3 and ≥ 11.3 years of follow-up), heterogeneity was observed (Table 6.5). During the first 11.3 years of follow-up, HRs of alcohol consumers were inversely associated with risk of RCC overall and ccRCC, compared to non-users of alcohol. Analyses using data from 11.3 until 20.3 years of follow-up, showed that alcohol consumption was positively associated with risk of RCC overall and ccRCC. In ccRCC, the HR(95%CI) for >15 g alcohol/d was 0.59(0.39-0.91) with P -trend=0.02 and 1.91(1.02-3.57) with P -trend=0.04 for the first 11.3 years and remaining follow-up years, respectively. Analyses of ccRCC stratified according to strata of promoter methylation showed similar patterns as in ccRCC overall. There was no heterogeneity in the association between dietary folate intake and RCC risk according to period of follow-up (data not shown).

Table 6.4 Hazard ratios for the effect-modification of the association of alcohol intake and dietary intake of folate with risk of renal cell cancer according to methylation status in the NLCS, 1986-2006.

Dietary intakes (median) ^{c,d}	Person years	RCC cases			ccRCC cases			ccRCC cases by methylation index ^a						P-heterogeneity		
		n	HR ^b	95% CI	n	HR ^b	95% CI	Low		Intermediate		High				
								n	HR ^b	n	HR ^b	n	HR ^b		n	HR ^b
low alcohol, high folate	25,380	174	1.00	(ref)	103	1.00	(ref)	38	1.00	(ref)	28	1.00	(ref)	32	1.00	(ref)
low alcohol, low folate	25,474	171	0.99	(0.77-1.26)	108	1.06	(0.78-1.44)	42	0.95	(0.58-1.57)	30	1.17	(0.69-1.98)	33	1.16	(0.67-2.00)
high alcohol, high folate	8,136	88	1.14	(0.85-1.53)	56	1.29	(0.89-1.87)	18	1.17	(0.65-2.10)	13	1.11	(0.53-2.29)	25	1.74	(0.96-3.17)
high alcohol, low folate	8,257	65	0.83	(0.59-1.17)	39	0.87	(0.56-1.35)	14	0.73	(0.36-1.46)	10	0.89	(0.39-2.04)	14	1.09	(0.51-2.33)
P-interaction				0.15			0.08			0.32			0.47			0.16

^a Categories of the methylation index low, intermediate and high represent 0-19%, 20-39% and 40% or more methylation, respectively. ^b Cox proportional hazards model adjusted for sex (men/women), year of birth (1916-20, 1921-25 and 1926-31), BMI (kg/m²), smoking status (never or ex/current), smoking duration (y), smoking intensity (cig/d), hypertension (no/yes; including use of anti-hypertensive medication), total energy intake (kcal/d), methionine (μg/d), vitamin B2 (μg/d) and vitamin B6 (μg/d) and using age as time-scale. ^c Low alcohol intake, 0-14 g/d; high alcohol intake, 15+ g/d. ^d Low folate intake, < median intake in subcohort; high folate intake ≥ median intake in subcohort (median intake: 212 and 188 μg/day for men and women, respectively). BMI = body mass index, ccRCC = clear-cell renal cell cancer, cig = cigarettes, NLCS = Netherlands Cohort Study.

Table 6.5 Hazard ratios for the association between alcohol intake and the risk of renal cell cancer according to duration of follow-up by molecular subtypes in the NLCs, 1986-2006.

	Person years	RCC cases			ccRCC cases			ccRCC cases by methylation index ^a								
		n	HR ^b	95% CI	n	HR ^b	95% CI	Low			Intermediate			High		
								n	HR ^b	95% CI	n	HR ^b	95% CI	n	HR ^b	95% CI
Follow-up 0-20.3 years																
Alcohol intake (g/d)																
0	15,882	111	1.00	(ref)	73	1.00	(ref)	24	1.00	(ref)	22	1.00	(ref)	26	1.00	(ref)
0.1-14	34,972	234	0.85	(0.66-1.08)	138	0.76	(0.57-0.03)	56	0.98	(0.60-1.59)	36	0.68	(0.40-1.15)	39	0.58	(0.35-0.96)
15+	16,393	153	0.88	(0.66-1.18)	95	0.88	(0.62-1.25)	32	0.98	(0.55-1.75)	23	0.70	(0.37-1.36)	39	0.90	(0.51-1.60)
P-trend				0.47			0.56			0.95			0.33			0.95
Follow-up 0-11.3 years																
Alcohol intake (g/d)																
0	15,882	75	1.00	(ref)	56	1.00	(ref)	17	1.00	(ref)	18	1.00	(ref)	20	1.00	(ref)
0.1-14	34,972	133	0.72	(0.54-0.97)	83	0.61	(0.43-0.86)	28	0.66	(0.36-1.21)	22	0.54	(0.29-1.02)	28	0.55	(0.31-1.00)
15+	16,393	90	0.74	(0.53-1.05)	52	0.59	(0.39-0.91)	15	0.56	(0.28-1.14)	12	0.45	(0.20-1.02)	24	0.73	(0.36-1.47)
P-trend				0.12			0.02			0.12			0.06			0.48
Follow-up 11.3-20.3 years																
Alcohol intake (g/d)																
0	15,882	36	1.00	(ref)	17	1.00	(ref)	7	1.00	(ref)	4	1.00	(ref)	6	1.00	(ref)
0.1-14	34,972	101	1.11	(0.74-1.66)	55	1.28	(0.73-2.23)	28	1.74	(0.74-4.11)	14	1.26	(0.42-3.77)	11	0.65	(0.24-1.74)
15+	16,393	63	1.21	(0.76-1.94)	43	1.91	(1.02-3.57)	17	2.13	(0.80-5.70)	11	1.82	(0.55-6.05)	15	1.51	(0.55-4.12)
P-trend				0.43			0.04			0.13			0.30			0.30

^a Categories of the methylation index low, intermediate and high represent 0-19%, 20-39%, and 40% or more methylation, respectively. ^b Cox proportional hazards model adjusted for sex (men/women), age at baseline (y), BMI (kg/m²), smoking status (never or ex/current), smoking duration (y), smoking intensity (cig/d), hypertension (no/yes; including use of antihypertensive medication), total energy intake (kcal/d), dietary folate intake (quintiles) and using time on study as time-scale. BMI = body mass index, ccRCC = clear-cell renal cell cancer, cig = cigarettes, NLCs = Netherlands Cohort Study.

DISCUSSION

In this large prospective cohort study, alcohol intake was associated with a decreased RCC risk, although not statistically significant, and folate intake was not associated with RCC risk. There was no heterogeneity between alcohol or folate intake and ccRCC risk stratified according to categories of the methylation index.

Most prospective studies that investigated the association between alcohol intake and RCC risk have observed an inverse association with a clear dose-response effect [3,4,14,33,34]. Data from the NLCS were included in two previous publications with a shorter follow-up [4,14]. In the analysis of the Pooling Project of Prospective Studies on Diet and Cancer [4] 7.3 years of follow-up, and in the other [14] 11.3 years of follow-up were used. In the current analysis, with longer follow-up, the inverse association is weaker. When the analysis was split according to duration of follow-up, heterogeneity was observed. Alcohol consumption was associated with a decreased risk during the first 11.3 years of follow-up and with an increased risk after 11.3 years of follow-up. This heterogeneity may be explained by the fact that alcohol intake was measured only once at baseline and that lifestyle, including alcohol intake, of participants may have changed during follow-up. Alternatively, the latency period of the inverse association between alcohol intake and the occurrence of RCC could be short (i.e. $\sim <10$ years). It is also possible that the decreased HRs in the first period and the increased HRs in the second period of follow-up are due to chance.

Several studies have investigated the association between folate intake and RCC risk. A hospital-based case-control study, with a limited number of RCC cases, observed a statistically significant inverse association between folate intake and RCC risk [35]. Other studies, including two case-control studies [36,37] and a previous publication from the NLCS with shorter follow-up [38], did not observe an association between folate intake and RCC risk, although most point estimates were lower than one. In a nested case-control study in a cohort of Finnish smokers, serum levels of folate were not statistically significantly associated with RCC risk [39]. However, participants with the lowest levels of serum folate were at increased risk, compared to participants with higher serum folate levels [39]. In the hospital-based case-control study, in which questionnaires were used, participants with the lowest dietary intake of folate were also at increased risk [36]. In a large Central and Eastern European hospital-based case-control study, polymorphisms in genes involved in folate metabolism [methylenetetrahydrofolate reductase (*MTHFR*) and solute carrier family 19 (folate transporter), member 1 (*SLC19A1*)] were associated with increased risk of RCC, especially in participants with the lowest tertile of vegetable intake, an important source of folate [40,41]. The observations from these studies may be explained by a threshold effect in which only participants with the lowest intake of folate have an

increased risk. However, in the current study no association (including no indication of a threshold effect) between dietary folate intake and RCC risk was observed.

The aim of this study was to investigate whether alcohol and folate intake play a role in promoter methylation in genes frequently methylated in ccRCC. It has been suggested that there is a specific subtype of cancer that is characterized by extensive promoter methylation of tumor suppressor and DNA repair genes and referred to as CpG island methylator phenotype (CIMP) [8]. CIMP has been investigated extensively in colorectal cancer, but may also exist in other cancers, such as ccRCC [8,9]. Since no definition for CIMP in ccRCC has been established so far, we used a methylation index of CpG islands frequently methylated in ccRCC as a measure for general promoter methylation. Although we recognize that the Infinium 450K platform would for example be a more comprehensive way to analyse promoter methylation of CpG islands in ccRCC, our selection strategy to include particular genes of interest is much more efficient in large epidemiological studies.

Different cancer phenotypes may be associated with different pathways of carcinogenesis, including etiology. However, the current study did not uncover such pathway, because no heterogeneity was observed in associations between alcohol and folate intake and different molecular subtypes of ccRCC defined by the methylation index of selected genes inactivated by promoter methylation in ccRCC. There are several explanations for the absence of heterogeneity. First, the participants of the NLCS reported moderate alcohol consumption and less than 10% of the subcohort reported a consumption of more than 30 grams of alcohol per day. It is conceivable that only a high level of alcohol intake is associated with promoter methylation, and that the subgroup of high alcohol consumers in the current study is too small to demonstrate this. Second, since there is no clear definition of CIMP in ccRCC or in other cancers, we developed a methylation index to define subgroups of ccRCC defined by promoter methylation. Our definition of 'high methylation' included ccRCC cases with promoter methylation of more than 40% of the eleven selected genes. This subgroup constituted 35% of the ccRCC cases in our series. In the study of Arai *et al.* 14 out of 104 cases (13.5%) were characterized by high promoter methylation, using DNA methylation levels of 801 CpG islands [9]. More research is needed to confirm whether CIMP exists in ccRCC and what panel of markers can be used to classify CIMP accurately. Third, risk estimates may be attenuated due to random misclassification of dietary intakes, caused by a single baseline measurement and the long follow-up, even though intakes were rather stable during the first five years of follow-up [25]. Finally, only dietary folate intake was investigated. Other nutrients, such as choline, vitamin B and methionine, are also involved in folate metabolism [13,42], and might influence adequate methylation status as well.

The current study also has several strengths. Loss to follow-up was very limited and cancer follow-up was very complete (>96%) [17]. Furthermore, we collected tumor samples from ~80% of the cases. This, combined with the prospective study design, makes selection and information bias unlikely. The study is among the largest prospective studies on RCC and, so far, the only prospective study including FFPE tumor tissues.

In conclusion, in this study we did not observe an association between alcohol, folate intake and promoter CpG island hypermethylation in ccRCC using a methylation index as tool to define methylation-specific subgroups ccRCC. Therefore, this study does not support the hypothesis that alcohol and folate intake are involved in ccRCC carcinogenesis through promoter methylation of our methylation index.

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Suppl Table S6.1 Primer sequences and sequence condition used to analyse promoter CpG island methylation of selected genes in clear-cell renal cell cancer in the NLCS, 1986-2006.

Gene	Primer sequence	Tm (°C)	Cycles (n)
<i>CDO1</i>			
Flanking primer, upstream	GGGAGAGATTGYGYGGAGTTTA	56	35
Flanking primer, downstream	AAAACCACCCAAAAAATAAC	56	35
Unmethylated sense strand	GATTTTGGGATGTTGGAGATAAT	64	30
Methylated sense strand	TTTTTGGGACGTCGGAGATAAC	64	30
Unmethylated antisense strand	AAAACAAAAAACCTACAAACACA	64	30
Methylated antisense strand	CGAAAAAACCTACGAACACG	64	30
<i>FST</i>			
Flanking primer, upstream	TGTAAAGTTAGGTATTTATAGATTAGATATA	56	35
Flanking primer, downstream	CRCTCCAAAAAACTTTTACAAC	56	35
Unmethylated sense strand	TTTGTGTTTAGGTTGTGGGTGTTT	66	30
Methylated sense strand	CGTTTAGGTTGCGGGTGTTT	66	30
Unmethylated antisense strand	TACAAACAACCAAAAAACCCCA	66	30
Methylated antisense strand	AAACGACCGAAAAACCCCG	66	30
<i>FZD10</i>			
Flanking primer, upstream	GGGTTGTTTGGGGTTGG	56	35
Flanking primer, downstream	CCRCCRCTACTTTACATAAAAAAAC	56	35
Unmethylated sense strand	TGTGGGTGGGATTAGGTGTTGT	64	35
Methylated sense strand	GGCGGGATTAGGCGTTGC	64	35
Unmethylated antisense strand	TTTACATAAAAAACACAACAACCCA	64	35
Methylated antisense strand	TAAAAAACGCAACGACCCG	64	35
<i>GREM1</i>			
Flanking primer, upstream	GGGGTTTTTTTGTGAGGT	56	35
Flanking primer, downstream	CTACTACCACCAACCAAAAAAC	56	35
Unmethylated sense strand	TTTTTTTGTGAGGTTGTGGATATT	65	30
Methylated sense strand	TTTGTGAGGTCGCGGATATC	65	30
Unmethylated antisense strand	CAAAAACATATTCAAAAACCTCCA	65	30
Methylated antisense strand	AAAACGTATTTCGAAAACCTCCG	65	30
<i>LAD1</i>			
Flanking primer, upstream	ATTGGTTTTGAGTAGGAAGTTTTT	56	35
Flanking primer, downstream	CCRCATTAATTACAACCCAAA	56	35
Unmethylated sense strand	GAGTAGGAAGTTTTTTGTTTTGTTTT	63	35
Methylated sense strand	GTAGGAAGTTTTTCGTTTTCGTTTC	63	35
Unmethylated antisense strand	AACCCAAACCAACCCCAACCA	63	35
Methylated antisense strand	CCAAACCGACCCGACCG	63	35
<i>NEFH</i>			
Flanking primer, upstream	TTTATTGTTAAGGGGTTGGATT	56	35
Flanking primer, downstream	CRAAACTCATATACTAAACAAATAC	56	35
Unmethylated sense strand	GTTAAGGGGTTGGATTGTTGT	62	35
Methylated sense strand	GGGGTTGGATTTCGGTCGC	62	35
Unmethylated antisense strand	CATCATACTAAACAAATACACAAAACCA	62	35
Methylated antisense strand	AACTAAACAAATACGCGAAACCG	62	35

Suppl Table S6.1 (continued)

Gene	Primer sequence	Tm (°C)	Cycles (n)
<i>NEURL</i>			
Flanking primer, upstream	GGTTTAGGGTTTTGTTTGTTG	56	35
Flanking primer, downstream	CCTCAATTCCTTTCTCTAACTT	56	35
Unmethylated sense strand	TTGTTTGTTGTTTTGTTTTGTTAT	65	35
Methylated sense strand	GTTTGTGGTTTTTCGTTTCGTTAC	65	35
Unmethylated antisense strand	CTTCTCTAACTTCACAATCCTTAACA	65	35
Methylated antisense strand	TCTAACTTCGCGATCCTTAACG	65	35
<i>RASGFR2</i>			
Flanking primer, upstream	TATTTTTTGAGGGTTGTAGTTG	56.1	35
Flanking primer, downstream	CCCTAATCCCRAATTAAAA	56.2	35
Unmethylated sense strand	AACGTTTCGCGTTATTTAGGGTC	60.3	30
Methylated sense strand	GAATGAATGTTTGTTATTAGGGTT	60.3	30
Unmethylated antisense strand	ATTAACGACAAAAAATACGACG	60.1	30
Methylated antisense strand	CAAATTAACAACAAAAAATACAACA	60.1	30
<i>SFN</i>			
Flanking primer, upstream	GTTYGGAGGAGAAGGGGTT	56	35
Flanking primer, downstream	CTTCATCTTCAAATAAAAAACCC	56	35
Unmethylated sense strand	GGGGTTTGAGGTGTGTGAGTATT	64	35
Methylated sense strand	GGTTCGAGGTGCGTGAGTATC	64	35
Unmethylated antisense strand	CAAATAAAAAACCCAATCTCAACA	64	35
Methylated antisense strand	AAAAAACCCGACTCTCGACG	64	35
<i>SFRP1</i>			
Flanking primer, upstream	TTTAGTTTGTAGTTTTTGGAGTTAG	56	35
Flanking primer, downstream	CCCCRACCAATAACRACCTC	56	35
Unmethylated sense strand	GTTTTGTAGTTTTTGGAGTTAGTGTGTGT	66	25
Methylated sense strand	TGTAGTTTTCGGAGTTAGTGTGCGCG	66	25
Unmethylated antisense strand	CTCAACCTACAATCAAAAACAACACAAACA	66	25
Methylated antisense strand	CCTACGATCGAAAACGACGCGAACG	66	25
<i>VHL</i>			
Flanking primer, upstream	GGAGGGYGAGGAGATTGGGA	56	35
Flanking primer, downstream	CCTCRACRCCCAATTCCTCC	56	35
Unmethylated sense strand	AATTGGGATGAGGTTGAGGTAGGT	66	30
Methylated sense strand	GGGACGAGGTCGAGGTAGGC	66	30
Unmethylated antisense strand	TTCCTCAAACCAAACTCTTCCA	66	30
Methylated antisense strand	CTCCGA ACCGAA CTCTCCG	66	30

CDO1 = cysteine dioxygenase type 1, *FST* = follistatin, *FZD10* = frizzled class receptor 10, *GREM1* = gremlin 1, DAN family BMP antagonist, *LAD1* = Ladinin 1, *NEFH* = neurofilament, heavy polypeptide, *NEURL* = neuralized E3 ubiquitin protein ligase 1, NLCS = Netherlands Cohort Study, *RASGFR2* = ras-specific guanine nucleotide-releasing factor 2, *SFN* = stratifin, *SFRP1* = secreted frizzled-related protein 1, *VHL* = von Hippel-Lindau.

Suppl Table S6.2 Hazard ratios for the association between alcohol from different types of beverages and the risk of renal cell cancer according molecular subtypes in the NLCS, 1986-2006.

	Person years	RCC cases			ccRCC cases			ccRCC cases by methylation index ^a					
		n	HR ^b	95% CI	n	HR ^b	95% CI	Low			Intermediate		
								n	HR ^b	95% CI	n	HR ^b	95% CI
Alcohol from beer (g/d)													
No alcohol from beer	45,541	293	1.00	(ref)	184	1.00	(ref)	70	1.00	(ref)	48	1.00	(ref)
0.1-4.9	14,243	130	0.95	(0.74-1.23)	72	0.87	(0.63-1.21)	27	0.93	(0.56-1.54)	19	0.94	(0.50-1.76)
>=5	7,464	75	0.94	(0.69-1.28)	50	1.05	(0.72-1.52)	15	0.92	(0.50-1.69)	14	1.15	(0.54-2.42)
P-linear trend				0.67			1.00			0.76			0.79
Increment per 10g	67,247	498	0.92	(0.77-1.09)	306	0.99	(0.80-1.21)	112	1.11	(0.81-1.51)	81	0.78	(0.55-1.10)
Alcohol from wine (g/d)													
No alcohol from wine	26,746	214	1.00	(ref)	132	1.00	(ref)	40	1.00	(ref)	37	1.00	(ref)
0.1-4.9	23,917	166	0.99	(0.79-1.24)	99	0.94	(0.71-1.25)	45	1.36	(0.86-2.15)	23	0.77	(0.44-1.32)
>=5	16,584	118	0.96	(0.75-1.23)	75	0.98	(0.72-1.33)	27	1.09	(0.65-1.81)	21	1.02	(0.59-1.77)
P-linear trend				0.73			0.85			0.60			0.91
Increment per 10g	67,247	498	1.02	(0.90-1.15)	306	1.01	(0.87-1.17)	112	1.08	(0.86-1.35)	81	0.87	(0.64-1.18)
Alcohol from liquor (g/d)													
No alcohol from liquor	44,008	194	1.00	(ref)	182	1.00	(ref)	68	1.00	(ref)	52	1.00	(ref)
0.1-4.9	10,619	89	0.86	(0.65-1.13)	55	0.89	(0.64-1.25)	21	1.04	(0.62-1.74)	13	0.71	(0.37-1.37)
>=5	12,620	115	0.82	(0.63-1.06)	69	0.85	(0.61-1.16)	23	0.87	(0.51-1.49)	16	0.66	(0.36-1.21)
P-linear trend				0.12			0.29			0.65			0.16
Increment per 10g	67,247	498	0.94	(0.85-1.05)	306	0.94	(0.83-1.07)	112	1.00	(0.83-1.22)	81	0.88	(0.66-1.18)

^a Categories of the methylation index low, intermediate and high represent 0-19%, 20-39% and 40% or more methylation, respectively. ^b Cox proportional hazards model adjusted for sex (men/women), year of birth (1916-20, 1921-25 and 1926-31), BMI (kg/m²), smoking status (never or ex/current), smoking duration (y), smoking intensity (cig/d), hypertension (no/yes; including use of antihypertensive medication), total energy intake (kcal/d), dietary folate intake (quintiles), mutually adjusted for alcohol intake from other beverages (g/d) and using age as time-scale. BMI = body mass index, ccRCC = clear-cell renal cell cancer, cig = cigarettes, NLCS = Netherlands Cohort Study.

CHAPTER 7

Promoter methylation of *CDO1* identifies clear-cell renal cell cancer patients with poor survival outcome

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ABSTRACT

Introduction

In this era of molecular diagnostics, prediction of clear-cell renal cell cancer (ccRCC) survival requires optimization, as current prognostic markers fail to determine individual patient outcome. Epigenetic events are promising molecular markers. Promoter CpG island methylation of cysteine dioxygenase type 1 (*CDO1*), which was identified as prognostic marker for breast cancer, is studied as potential marker for ccRCC survival.

Methods

We collected primary tissues of 365 ccRCC cases identified within the prospective Netherlands Cohort Study (NLCS). In this population-based series, *CDO1* promoter methylation was observed in 124 of 324 (38.3%) patients with successful methylation-specific PCR analysis. Kaplan-Meier curves and Wilcoxon tests were used to evaluate 10-year ccRCC-specific survival. Cox regression analysis was used to obtain crude and multivariate hazard ratios (HR) and 95% confidence intervals (CI). The relative prognostic value of multivariate models with and without *CDO1* promoter methylation was compared using Likelihood-Ratio tests.

Results

Patients with *CDO1* promoter methylation have a significantly poorer survival than those without (Wilcoxon $P=0.006$). Differences in survival were independent of other prognostic factors, including age and sex [HR(95%CI)=1.66(1.12-2.45)] and TNM stage, tumor size and Fuhrman grade [HR(95%CI)=1.89(1.25-2.85)]. Multivariate models performed better with than without *CDO1* promoter methylation status (Likelihood-Ratio $P=0.003$). Survival curves were validated in an independent series of 280 ccRCC cases from the Cancer Genome Atlas (TCGA; Wilcoxon $P<0.001$).

Conclusion

CDO1 promoter methylation may not substitute common prognostic markers to predict ccRCC survival, but offers additional, relevant prognostic information, indicating that it could be a novel molecular marker to determine ccRCC prognosis.

INTRODUCTION

Major improvements in the understanding of the molecular biology of tumors increased the possibilities for targeted therapies, such as tyrosine kinase inhibitors (TKIs) and mammalian target of rapamycin (mTOR) inhibitors in clear-cell renal cell cancer (ccRCC) patients. In this era of personalized therapies and molecular diagnostics, current prognostic parameters, such as patient performance status, tumor size, cancer stage, and nuclear grade, fail to determine individual patient outcome and optimization of prognostic parameters is thus necessary.

Promoter CpG island methylation markers are promising tools for early detection of tumors, therapeutic stratification and to determine clinical prognosis. Promoter CpG island methylation occurs early and frequently in carcinogenesis, providing selective advantage to neoplastic cells and, therefore, contributing to both disease development and progression [1-4]. For ccRCC, genetic and epigenetic alterations in the Von Hippel-Lindau (*VHL*) tumor suppressor gene (TSG) appeared to have little value in the prediction of ccRCC prognosis, even though inactivation of the *VHL* gene is an early and key event in the development of ccRCC [5].

Recently, promoter CpG island methylation of cysteine dioxygenase type 1 (*CDO1*) has been found in primary tissues of multiple tumor types, including colon, breast, esophagus, ovary, lung, bladder, pancreas and stomach tumors [6,7], cholangiocarcinoma [8] and lung squamous cell carcinoma [9]. In addition, it has been identified as prognostic marker for breast cancer [7,10,11], but *CDO1* promoter CpG island methylation and its potential association with prognosis has not yet been investigated in ccRCC.

CDO1 is thought to be involved in carcinogenesis, as it plays a role in oxidative stress response of cancer cells by reducing antioxidant capacity [7]. Moreover, *CDO1* is a key enzyme in the taurine biosynthetic pathway, in which it oxidizes cysteine into cysteine sulfinic acid (CSA), eventually converting into taurine (2-aminoethanesulfonic acid) [12-14]. Taurine is a major intracellular amino acid and is involved in many physiological and biological processes in the kidney, including osmoregulation and regulation of renal blood flow and renal cell cycle and apoptosis [15]. Given that, epigenetic silencing of *CDO1* through promoter CpG island methylation may be of particular interest for renal carcinogenesis.

In the present study, we investigated the prognostic utility of *CDO1* promoter CpG island methylation in a population-based series of ccRCC patients embedded within the prospective Netherlands Cohort Study (NLCS) and in an independent series of ccRCC patients from The Cancer Genome Atlas (TCGA).

METHODS

Study population

The population-based series of ccRCC cases is derived from the prospective NLCS, which was initiated in 1986 and included 120,852 men and women in the ages of 55-69 years at baseline [16]. After 20.3 years of follow-up, 608 incident microscopically confirmed RCC cases (ICD-O: M8010-8119, 8140-8570) were identified within the NLCS using computerized record linkage with the Netherlands Cancer registry and the Dutch Pathology registry PALGA [17,18]. Only histologically confirmed RCC cases (n=568) were eligible for the collection of formalin-fixed paraffin-embedded (FFPE) tumor tissues from ~50 pathology laboratories throughout the Netherlands. The collection was conducted in two phases; initially only cases from the first 11.3 years of NLCS follow-up were included [19], however recent efforts were made to expand the series. This study was approved by the review board of Maastricht University (Maastricht, the Netherlands).

Tissue collection

Tumor tissues were available for 79.8% of the RCC cases (n=453). Inclusion ranged over 5-years follow-up periods from 71.3% to 87.7%. For cases identified between 1986 and 1990 retrieval was lowest, because the Dutch pathology registry (PALGA) had incomplete coverage during this period and some pathological laboratories had problems to locate older tissues in particular. In total, the retrieval was unsuccessful for 115 cases; 46 cases had no surgery, for 24 cases the availability or location of their tissues was unknown from the Dutch pathology registry (PALGA) and, for 45 cases the pathological laboratory refused or was unable to send their tissues. Hematoxylin and eosin (HE) stained slides of tumor tissues were assessed by an experienced genitourinary pathologist to confirm tumor histology based on the WHO classification of tumors and Fuhrman grade [20,21]. The entire series of collected tumor tissues included 80.6% (n=365) tumors with clear-cell histology. Information on patient and tumor characteristics, such as age at diagnosis, gender, TNM stage, tumor size and initial treatment was available from the pathological reports and cancer registries. Follow-up was accomplished by record linkage to the municipal population registries and the causes of death registry from Statistics Netherlands. ccRCC cases of which tissues were collected after autopsy were excluded. In total, 356 ccRCC cases were eligible for survival analyses.

DNA isolation and *CDO1* promoter CpG island methylation

Methods used for DNA isolation of material from ccRCC cases included in the initial, 11.3 years follow-up collection have been described previously [19]. For recently added cases, vital tumor areas of the FFPE tumor tissues were dissected before DNA

isolation. DNA was isolated using the QIAamp DNA Mini Kit for isolation of Genomic DNA from Tissue (Qiagen), according to manufacturer's instructions.

CDO1 promoter CpG island hypermethylation, in short *CDO1* promoter methylation, was analyzed by nested methylation-specific PCR (MSP), as previously described in detail elsewhere [22-24]. All PCRs were done with controls for unmethylated alleles [DNA from normal lymphocytes or DNA from human umbilical vein endothelial cells (HUVEC)], methylated alleles [normal lymphocyte DNA treated in vitro with SssI methyltransferase (New England Biolabs, Ipswich, MA)], and a no template control. Reproducibility of MSP analysis in 53 cases was 89%. Primer sequences and MSP conditions are provided in Suppl Table S7.1. MSP analyses were performed successfully for 324 out of 356 cases (91.0%).

Statistical analyses

Statistical analyses were conducted in STATA version 12 (STATA Corp., College Station, TX). Patient and tumor characteristics of ccRCC cases with and without *CDO1* methylation were compared using the Kruskal-Wallis tests (for continuous variables) or χ^2 tests (for categorical variables). Survival was defined as the time from diagnosis until death or until the end of follow-up. However, for some cases follow-up up to 20 years has been established. As we do not consider such long follow-up relevant for ccRCC-specific survival, follow-up was truncated after 10 years. Kaplan-Meier curves and Wilcoxon tests were used to evaluate 10-year survival of ccRCC cases with and without *CDO1* promoter methylation. In addition, Cox proportional hazards analyses were used to obtain hazard ratios (HR) and corresponding 95% confidence intervals (CI) for the association between *CDO1* promoter methylation and 10-year ccRCC-specific survival. HRs were adjusted for age at diagnosis (y) and sex (men/women) and, in a multivariable-adjusted model, additionally for a set of *a priori* selected confounders known for their prognostic value in ccRCC survival; TNM stage [I-II, III, IV; 4th version (1987)], tumor size (mm) and Fuhrman grade (I, II, III, IV). Cases with missing data on any of the confounders were excluded for Cox proportional hazards analyses (n=17). The relative prognostic value of *CDO1* promoter methylation compared to the known prognostic factors was assessed using Likelihood-Ratio tests for nested models and the Akaike information criterion (AIC) for non-nested models. In general, a lower AIC is considered a better model. Tests using Schoenfeld residuals and visual inspection of $-\ln(-\ln(\text{survival}))$ plots showed no indications of violation of the proportional hazard assumption. In sensitivity analyses, all analyses were repeated after exclusion of patients with distant metastasis (TNM stage IV), patients with (neo)adjuvant treatment and patients with sarcomatoid differentiation. In addition, in subgroup analyses, analyses were conducted by categories of age at diagnosis; ≥ 55 to < 65 years, ≥ 65 to < 75 years and ≥ 75 years.

To independently validate the findings obtained in the population-based ccRCC series, all Kaplan-Meier curves and Wilcoxon tests were re-evaluated in 280 ccRCC cases from the TCGA series with full data available on patient and tumor characteristics and survival, only then using a maximum of 5-year survival, as longer follow-up was not available. In the TCGA data, promoter methylation was measured on the Illumina Human Methylation 450K platform as a quantitative trait. We identified three probes near our primer region and determined a composite *CDO1* methylation level by taking the maximum beta value of those three probes. We defined methylated samples as those with a composite methylation level of at least 30%. A two-sided *P*-value of 0.05 or less was considered statistically significant.

RESULTS

The median ccRCC-specific survival in the population-based series was 4.5 years. For 13.7% of the patients a distant metastasis was reported (TNM stage IV, Table 7.1). The majority of patients (94.1%) had no (neo)adjuvant treatment. *CDO1* promoter methylation was observed in 124 out of 324 patients (38.3%). *CDO1* promoter methylation was more frequent in male patients (*P*-value=0.001) and in those with a longer duration and a higher intensity of cigarette smoking (*P*-value=0.002 and 0.002). However, age at diagnosis and other lifestyle indicators including body mass index (BMI), hypertension and alcohol intake, did not differ by *CDO1* promoter methylation. Tumors with *CDO1* promoter methylation were similar in TNM stage and tumor size compared to those without, yet they showed a significantly higher Fuhrman grade (*P*-value<0.001). Interestingly, for none of the tumors without *CDO1* promoter methylation sarcomatoid differentiation was observed, whereas 6.5% of tumors with *CDO1* promoter methylation had signs of such sarcomatoid differentiation.

To independently validate our findings from the population-based series of ccRCC patients, we used data of ccRCC patients from the TCGA (Table 7.2). Compared to the population-based series, ccRCC patients in the TCGA series had a lower mean age at diagnosis, but with a considerably larger standard deviation (70.6±5.9 and 61.5±12.0 years, respectively), and a shorter median survival time (4.5 and 2.8 years). In the TCGA series, 54 out of 280 patients (19.3%) showed a distant metastasis (TNM stage IV) and 7 out of 280 patients (2.5%) had a history of (neo)adjuvant treatment. *CDO1* promoter methylation was present in 122 out of 280 ccRCC patients (43.6%). Patients with *CDO1* promoter methylation were significantly older (*P*-value<0.001), more frequently male (*P*-value=0.03) and their tumor characteristics, i.e. TNM stage and Fuhrman grade were less favorable compared to those without (*P*-value<0.001 and <0.001, respectively).

Table 7.1 Characteristics of clear-cell renal cell cancer patients overall and by promoter methylation of *CDO1* in the NLCS, 1986-2006.

Characteristics (mean (SD))	Total ccRCC (n=324)	<i>CDO1</i> promoter methylation		
		No (n=200)	Yes (n=124)	<i>P</i> -value ^a
Patient				
Age at diagnosis (y)	70.6 (5.9)	70.6 (5.9)	70.6 (6.1)	0.78
Male sex (%)	194 (59.9)	105 (52.5)	89.9 (71.8)	0.001
ccRCC-specific survival (y)	5.9 (5.0)	6.43 (5.1)	5.0 (4.8)	0.005
Tumor				
TNM stage ^b (%; n=321)				
Stage 1 and 2	189 (58.9)	124 (62.0)	65 (53.7)	
Stage 3	88 (27.4)	50 (25.0)	38 (31.4)	
Stage 4	44 (13.7)	26 (13.0)	18 (14.9)	0.33
Tumor size (mm; n=309)	67.6 (32.9)	66.5 (32.2)	69.4 (34.0)	0.48
Fuhrman grade (%)				
Grade 1	44 (13.9)	39 (19.5)	5 (4.0)	
Grade 2	129 (39.8)	85 (42.5)	44 (35.5)	
Grade 3	103 (31.8)	55 (27.5)	48 (38.7)	
Grade 4	48 (14.8)	21 (10.5)	27 (21.8)	<0.001
<i>CDO1</i> methylation (%)	124 (38.3)	0 (0.0)	124 (100.0)	-
Sarcomatoid differentiation (%)	8 (2.5)	0 (0.0)	8 (6.5)	<0.001
Eosinophilic variant (%; n=155)	2 (1.3)	1 (1.0)	1 (1.8)	1.0
No (neo)adjuvant treatment (%; n=322)	303 (94.1)	185 (93.4)	118 (95.2)	0.63
Lifestyle				
Body mass index (kg/m ² , n=309)	25.5 (2.9)	25.4 (2.9)	25.7 (16.2)	0.30
Hypertension (%)	110 (34.0)	71 (35.5)	39 (31.5)	0.45
Current smokers (%)	105 (32.6)	62 (31.2)	43 (35.0)	0.48
Smoking duration (y, n=318) ^c	23.3 (17.6)	20.8 (18.0)	27.4 (16.2)	0.002
Smoking intensity (cig/d, n=306) ^c	11.9 (12.3)	10.0 (10.3)	14.9 (14.4)	0.002
Alcohol intake (g ethanol/d, n=307)	11.4 (15.1)	10.5 (15.4)	12.7 (14.6)	0.22

^a *P*-value tested with Kruskal-Wallis test (for continuous variables) or χ^2 test (for categorical variables); ^b TNM stage as defined in 1987; ^c In current smokers. ccRCC = clear-cell renal cell cancer, *CDO1* = cysteine dioxygenase type 1, cig = cigarettes, SD = standard deviation.

Figure 7.1 shows the Kaplan-Meier curve for *CDO1* promoter methylation and 10-year ccRCC-specific survival in our population-based series of ccRCC patients. We observed a significantly poorer survival for patients with *CDO1* promoter methylation compared to patients without *CDO1* promoter methylation (Wilcoxon *P*=0.006). In Cox proportional hazards analyses, *CDO1* promoter methylation was also associated with unfavorable patient outcome [HR(95%CI): 1.66(1.13-2.44), Table 7.3]. HRs did not substantially change after adjustment for age at diagnosis and sex [HR(95%CI): 1.66(1.12-2.45)] and after additional adjustment for relevant clinicopathological parameters, including TNM stage, tumor size and Fuhrman grade [HR(95%CI): 1.89(1.25-2.85)]. In sensitivity analyses, both Kaplan-Meier curves (Figure 7.2) and multivariable-adjusted Cox proportional hazards analyses (Table 7.3) showed that the exclusion of TNM stage IV patients resulted in a slight attenuation of the association between *CDO1* promoter methylation and ccRCC-specific 10-year survival [Wilcoxon *P*=0.09, HR(95%CI): 1.44(0.90-2.30)] and that the restriction to patients without (neo)adjuvant treatment did not substantially change survival [Wilcoxon *P*=0.007;

HR(95%CI): 1.86(1.22-2.86)]. However, after excluding patients with sarcomatoid differentiation, the association between *CDO1* promoter methylation and ccRCC survival was in the multivariable adjusted model stronger than in the total population [HR(95%CI): 1.94(1.27-2.97)].

Table 7.2 Characteristics of clear-cell renal cell cancer patients overall and by promoter methylation of *CDO1* – The Cancer Genome Atlas (TCGA).

Characteristics (mean (SD))	Total ccRCC (n=280)	<i>CDO1</i> promoter methylation		
		No (n=158)	Yes (n=122)	<i>P</i> -value ^a
Patient				
Age at diagnosis (y)	61.5 (12.0)	59.3 (12.6)	64.3 (10.6)	<0.001
Male sex (%)	85 (66.1)	96 (60.8)	89 (73.0)	0.033
Overall survival (y)	2.8 (2.3)	2.7 (2.3)	2.9 (2.2)	0.38
Tumor				
TNM stage (%)				
Stage 1 and 2	153 (54.6)	115 (72.8)	38 (31.2)	
Stage 3	73 (26.1)	25 (15.8)	48 (39.3)	
Stage 4	54 (19.3)	18 (11.4)	36 (29.5)	<0.001
Tumor size (mm)	-	-	-	-
Fuhrman grade (%; n=277)				
Grade 1	5 (1.8)	5 (3.2)	0 (0.0)	
Grade 2	113 (40.8)	82 (52.6)	31 (25.6)	
Grade 3	111 (40.1)	58 (37.2)	53 (43.8)	
Grade 4	48 (17.3)	11 (7.1)	37 (30.6)	<0.001
<i>CDO1</i> methylation (%)	122 (43.6)	0 (0.0)	122 (100.0)	-
Sarcomatoid differentiation (%)	-	0.0	6.5	-
Eosinophilic variant (%)	-	1.0	1.8	0.67
No neoadjuvant treatment(%)	273 (97.5)	152 (96.2)	121 (99.2)	0.14

^a *P*-value tested with Kruskal-Wallis test (for continuous variables) or χ^2 test (for categorical variables). ccRCC = clear-cell renal cell cancer, *CDO1* = cysteine dioxygenase type 1, SD = standard deviation.

In line with the results from the population-based series, Kaplan-Meier curves in the TCGA series showed a significantly poorer 5-year survival for patients with *CDO1* promoter methylation compared to those without (Wilcoxon $P<0.001$, Figure 7.2A). In sensitivity analyses, the 5-year survival attenuated after the exclusion of TNM stage IV patients, but was still significantly different by *CDO1* promoter methylation status (Wilcoxon $P=0.047$, Figure 7.2B). In addition, the 5-year survival did not change after restricting the population to patients without (neo)adjuvant treatment (Wilcoxon $P<0.001$, Figure 7.2C).

Figure 7.1 Ten-year survival of clear-cell renal cell cancer patients from a population-based case series according to *CDO1* methylation A) in the total population, B) in patients with TNM stage I-III and C) in patients without (neo)adjuvant treatment.

In Table 7.4 we show the goodness-of-fit of five relevant models, in order to indicate each model's prognostic value. In the population-based series, *CDO1* methylation status alone (Model 1) is better in predicting prognosis than the models including age at diagnosis and sex (Model 2 and 3). In fact, all models with *CDO1* methylation status performed significantly better in predicting survival than the same models without *CDO1* methylation status (e.g. Model 4 versus 5, P -value=0.003). In the TCGA series, *CDO1* promoter methylation only improved the model including age at diagnosis and

sex (Model 2 versus 3, P -value=0.001) and not the model including all other prognostic variables (Model 4 versus 5, P -value=0.40).

Figure 7.2 Five-year survival of clear-cell renal cell cancer patients from the TCGA A) in the total population, B) in patients with TNM stage I-III and C) in patients without (neo)adjuvant treatment.

In our population-based series of ccRCC patients, subgroup analyses by categories of age at diagnosis showed substantially different Kaplan-Meier curves; a poorer survival by *CDO1* promoter methylation was observed for patients aged ≥ 55 to < 65 years at diagnosis (Wilcoxon $P=0.009$), but not for patients aged ≥ 65 to < 75 or > 75 years at

diagnosis (Wilcoxon $P=0.20$ and 0.20 , respectively; Suppl Figure S7.1). Although there is no significant interaction between age at diagnosis and *CDO1* promoter methylation in relation to survival in multivariable-adjusted analyses (P -interaction= 0.68), risk estimates of unfavorable patient outcome were also substantially different by categories of age at diagnosis. For ccRCC patients aged ≥ 55 to <65 years at diagnosis the HR(95%CI) was 3.21(1.25-8.23) in the age- and sex-adjusted model and, as a result of limited statistical power, 2.81(0.67-11.71) in the multivariable-adjusted model. In older patients, none of risk estimates were statistically significant. In the validation series, subgroup analyses by categories of age at diagnosis showed the strongest association between *CDO1* promoter methylation and 5-year survival in the youngest patients (<55 years at diagnosis, Wilcoxon $P<0.013$, Suppl Figure S7.2). In all other age categories, the observed difference in Kaplan-Meier curves was not significant.

Table 7.3 Hazard ratios and 95% confidence intervals of 10-year clear-cell renal cell cancer survival for promoter methylation of *CDO1* in the NLCS, 1986-2006.

Population	Crude model		Age- and sex-adjusted model ^a		Multivariable adjusted model ^b	
	HR	(95% CI)	HR	(95% CI)	HR	(95% CI)
Total population (n=307)	1.66	(1.13-2.44)	1.66	(1.12-2.45)	1.89	(1.25-2.85)
TNM stage I-III (n=268)	1.55	(0.98-2.44)	1.56	(0.98-2.48)	1.44	(0.90-2.30)
No (neo)adjuvant treatment (n=291)	1.76	(1.17-2.64)	1.76	(1.16-2.66)	1.86	(1.22-2.86)
No sarcomatoid differentiation (n=300)	1.61	(1.09-2.39)	1.61	(1.08-2.41)	1.94	(1.27-2.97)
Total population (n=307) ^c						
Age at diagnosis >55 , ≤ 65 (n=40)	3.10	(1.24-7.77)	3.21	(1.25-8.23)	2.81	(0.67-11.71)
Age at diagnosis >65 , ≤ 75 (n=183)	1.34	(0.81-2.23)	1.34	(0.80-2.25)	1.43	(0.81-2.54)
Age at diagnosis >75 (n=84)	1.73	(0.77-3.85)	1.62	(0.72-3.66)	2.01	(0.81-5.01)

^a Adjusted for age at diagnosis (y) and sex (men/women); ^b Adjusted for age at diagnosis (y), sex (men/women), TNM stage (I-II, III, IV; 1987), tumor size (mm) and Fuhrman grade (I-IV); ^c P -interaction= 0.68 in multivariable adjusted model. *CDO1* = cysteine dioxygenase type 1, NLCS = Netherlands Cohort Study.

Table 7.4 Model fit among five models including prognostic factors in relation to survival of clear-cell renal cell cancer patients.

Models	NLCS				TCGA			
	n	Df	AIC	P -value ^a	n	Df	AIC	P -value ^a
Model 1 ^b	307	1	1117.76		277	1	832.65	
Model 2 ^c	307	2	1126.05		277	2	842.77	
Model 3 ^d	307	3	1121.76	0.012	277	3	834.24	0.001
Model 4 ^e	307	8	1073.19		277	6	775.80	
Model 5 ^f	307	9	1066.26	0.003	277	7	777.40	0.40

^a P -value for Likelihood-Ratio test, current model compared to model above; ^b Model including *CDO1* methylation (no/yes); ^c Model including age at diagnosis (y) and sex (men/women); ^d Model including age at diagnosis (y), sex (men/women) and *CDO1* methylation (no/yes); ^e Model including age at diagnosis (y), sex (men/women), TNM stage (I-II, III, IV) and Fuhrman grade (I-IV). The NLCS uses the 1987 version of the TNM classification and includes also tumor size (mm); ^f Model including age at diagnosis (y), sex (men/women), TNM stage (I-II, III, IV), Fuhrman grade (I-IV) and *CDO1* methylation (no/yes). The NLCS uses the 1987 version of the TNM classification and includes also tumor size (mm). AIC = Akaike information criterion, *CDO1* = cysteine dioxygenase type 1, Df = degrees of freedom, NLCS = Netherlands Cohort Study, TCGA = The Cancer Genome Atlas.

DISCUSSION

In the present study, we reported for the first time that *CDO1* promoter CpG island methylation is associated with poorer survival in a population-based series of ccRCC patients and validated these finding in an independent series of ccRCC patients from the TCGA. Therefore, data suggests that *CDO1* promoter methylation may be a relevant marker for ccRCC prognosis.

The investigated association between *CDO1* promoter methylation and ccRCC survival remained significant after adjustment for age at diagnosis, sex and other clinicopathological parameters, including TNM stage, tumor size and Fuhrman grade. Thus, the association appeared to be independent of current prognostic factors. Moreover, based on results from the population-based series, *CDO1* promoter methylation status may be included in the model as prognostic marker to assess survival, in addition to common clinicopathological parameters. The additional value of *CDO1* promoter methylation in the model including sex and age, but not in the extended model, was confirmed in ccRCC patients from the TCGA. Thus, although *CDO1* promoter methylation status by itself is not suitable to substitute common clinicopathological parameters in the prediction of ccRCC survival, it offers additional, relevant prognostic information. It has been suggested that prognostic risk models, such as the University of California Los Angeles integrated staging system (UISS) and the Stage Size Grade Necrosis (SSIGN) risk score, which combine independent prognostic factors, may be more accurate in predicting survival than TNM stage and Fuhrman grade alone [25]. However, the UISS requires information on patient performance stage and the SSIGN risk score requires information on necrosis, which are both not available in the NLCS.

Several conditions can be hypothesized that might influence the association between *CDO1* promoter methylation and ccRCC survival, which were addressed in sensitivity and subgroup analyses. First, it can be argued that in patients with distant metastases, i.e. TNM stage IV, poor prognosis may be predominantly determined by the presence of the distant metastasis rather than by their *CDO1* promoter methylation status. We observed a slight attenuation in HRs in both series when excluding these patients. Second, the choice of treatment may, on the one hand, influence a patient's survival regardless of the *CDO1* promoter methylation status and thus conceal the potential effect of *CDO1* promoter methylation on ccRCC survival. On the other hand, the choice of treatment may be based on the expected patient's survival (e.g. adjuvant treatment for patients with the worst prognostic profile) and bias the association under study. However, no substantial changes in the results were observed after restricting the population to patients without (neo)adjuvant treatment. It should, however, be noted that surgery has been the primary and only treatment with curative intent for long, as ccRCC tumors are refractory to cytotoxic chemotherapy

and immunotherapy, and that consequently the proportion of patients with (neo)adjuvant treatment is very low. Moreover, the inclusion of patients depended on the availability of FFPE tumor tissues. We were able to evaluate this phenomenon of selection during the inclusion process in the population-based series by comparing ccRCC cases with and without tissue material. Indeed, we observed that the proportion of patients with TNM stage IV or with (neo)adjuvant treatment was lower for the ccRCC cases for which tissue retrieval was successful. Third, the presence of a sarcomatoid differentiation in the ccRCC tumor portends a worse prognosis [26]. Information on sarcomatoid differentiation was only available in the population-based series. As we observed that for all ccRCC tumors with a sarcomatoid component *CDO1* promoter methylation was present, poor prognosis may be predominantly determined by the presence of the sarcomatoid differentiation rather than by the *CDO1* promoter methylation itself. However, in contrast to this hypothesis, the association between *CDO1* promoter methylation and ccRCC-specific survival was stronger when excluding cases with sarcomatoid differentiation. Excluding these cases did not substantially change Kaplan-Meier curves (Wilcoxon $P=0.01$).

Finally, it has been hypothesized that global promoter methylation is associated with age [27]. As both series had a considerably different age range at diagnosis, we evaluated the Kaplan-Meier curves by categories of age at diagnosis. Generally, the association between *CDO1* promoter methylation and ccRCC survival was stronger in the lowest age category. For the TCGA data, it is plausible that smaller effects of *CDO1* methylation may be observed in older patients, as in this series survival was defined as overall survival rather than RCC-specific survival and RCC is likely a smaller contributor to total deaths in the elderly population than in the younger one. However, in the population-based series, survival was RCC-specific. For this series, we cannot exclude the possibility that confounding factors have influenced differential observations by categories of age at baseline, as the multivariable-adjusted interaction between *CDO1* promoter methylation and the age categories was not statistically significant.

Although both series of ccRCC patients included in this study generally showed the same results, some methodological differences are of particular interest. The population-based series of ccRCC patients, which is derived from the NLCS, has the advantage of including ~80% of all incident cases from a representative base population, hereby limiting the likelihood of selection bias. Any selection criterion for the inclusion of patients runs the risk of overestimating the effect of a particular marker, in this case *CDO1* promoter methylation. Indeed, associations between *CDO1* promoter methylation and ccRCC survival were stronger in the hospital-based TCGA series compared to the population-based series. In addition, in the population-based series, cancer stage was classified according to the 1987 version of the TNM classification [28]. A uniform cancer stage increases consistency within the series over

time, as the TNM classification regularly changes. However, old TNM classifications lack important information that allows recoding into more recent versions. The difference in tumor size between the 1987 version and the more recent versions of the TNM classification was addressed by the inclusion of the tumor size as covariate with TNM stage in the statistical models.

Promoter methylation of the *CDO1* gene has previously been associated with down-regulation of *CDO1* mRNA expression [7]. Moreover, in the TCGA dataset a negative correlation was observed for all three individual CpG's present at the Infinium human methylation 450k platform, with only one CpG being significantly correlated (Pearson $r=0.12$; P -value=0.036). Interestingly, the expression of exon 1 was significantly lower in the methylated samples when adhering to the binary classification applied in the paper by integrating the β -values of all three CpG's (P -value=0.020). *CDO1* catalyzes the oxidation of cysteine to cysteine sulfinic acid (CSA) [29], and is thus crucial for the removal of excess cysteine in the body. Deficient *CDO1* activity and subsequent high cysteine levels may be cytotoxic and have been associated with multiple human diseases, such as Parkinson's and Alzheimer's disease [30] and rheumatoid arthritis [31]. Importantly, high cysteine levels may also be related to cancer, as higher cysteine levels have been found in prostate cancer tissues compared to benign prostate tissues [32]. Furthermore, *CDO1* may indirectly regulate intracellular redox homeostasis [14]. Dominy *et al.* demonstrated that glutathione levels, a major antioxidant molecule, and its major substrate, intracellular cysteine, were lowered in presence of *CDO1* [29], suggesting that *CDO1* may be able to diminish antioxidant capacity. Loss of *CDO1* function in cancer cells may thus help these cells to escape oxidative damage and cell death due to enhanced antioxidant capacity, as cancer cells tend to develop enhanced antioxidant capacity under excessive and toxic amounts of reactive oxygen species (ROS) [33]. The principle of increased ROS detoxification capacity and its advantage for cancer cell survival as a result of DNA methylation of the *CDO1* gene was demonstrated by Jeschke *et al.* in breast cancer cells [7]. Whether this mechanism may also apply for ccRCC cells has not been elucidated.

CDO1 promoter methylation status may, in addition to its prognostic value, also have predictive value and have clinical implications for cancer therapy. In breast cancer, *CDO1* methylation is suggested to be a useful marker for prediction of resistance to the commonly used treatment with anthracyclines, as reactivation of epigenetically silenced *CDO1* sensitized breast cancer cells to anthracycline therapy [7]. Interestingly, cytotoxic chemotherapy, including anthracyclines, was never found to be effective in ccRCC [34]. It has been suggested that the resistance of ccRCC to chemotherapeutic agents may partly be due to increased levels of the transcription factor nuclear factor- κ B [35,36], yet *CDO1* promoter methylation may play a role too.

In conclusion, *CDO1* promoter methylation could be a relevant marker for ccRCC prognosis as it is a significant predictor of poor ccRCC-related survival in two independent series of ccRCC patients. However, further research needs to evaluate potential age-related differences in this association and the potential use of *CDO1* promoter methylation in therapeutic stratification.

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Suppl Table S7.1 Primer sequences and PCR conditions used to analyze promoter methylation of *CDO1* in a population-based series of clear-cell renal cancer patients in the NLCS, 1986-2006.

<i>CDO1</i> primer	Primer sequence	Tm (°C)	Cycles (n)
Flanking primer, upstream	GGGAGAGATTGYGYGGAGTTTA	56	35
Flanking primer, downstream	AAAACCACCCAAAAAATAAC	56	35
Unmethylated sense strand	GATTTTGGGATGTTGGAGATAAT	64	30
Methylated sense strand	TTTTTGGGACGTCGGAGATAAC	64	30
Unmethylated antisense strand	AAAACAAAAAACCTACAAACACA	64	30
Methylated antisense strand	CGAAAAACCTACGAACACG	64	30

CDO1 = cysteine, dioxygenase type 1, NLCS = Netherlands Cohort Study.

Suppl Table S7.2 Genomic information of the *CDO1* probes selected from TCGA data.

Probe ID	Chromosome	Probe start	Distance to TSS	CpG Island
cg08516516	5	115152491	160	yes
cg11036833	5	115152493	158	yes
cg23180938	5	115152484	167	yes

CDO1 = cysteine dioxygenase type 1, TCGA = The Cancer Genome Atlas.

Suppl Figure S7.1 Ten-year survival of clear-cell renal cell cancer patients from the population-based series according to *CDO1* methylation in patients A) aged ≥ 55 to < 65 years, B) aged ≥ 65 to < 75 years and C) aged ≥ 75 years at diagnosis.

Suppl Figure S7.2 Five-year survival of clear-cell renal cell cancer patients from the TCGA according to *CDO1* methylation in patients A) aged <55 years, B) aged ≥ 55 to <65 years, C) aged ≥ 65 to <75 years and D) aged ≥ 75 years at diagnosis.

CHAPTER 8

General discussion

This final chapter first summarizes the main findings of all chapters in this thesis. In addition, the most important methodological issues are discussed, in order to interpret these main findings and draw overall conclusions. Finally, our view on future directions in renal cell cancer (RCC) research is described.

SUMMARY OF MAIN FINDINGS

In this thesis, we identified that the dietary intake of sodium, but not fluid and potassium, is a potential novel risk factor for RCC, particularly if fluid consumption is low (**Chapter 2**). We found no indications for modifying or mediating effects of hypertension in this association. Furthermore, we found evidence that genes involved in the renin-angiotensin-aldosterone (RAAS) system may influence RCC risk (**Chapter 3**), because we showed that *AGTR1*_rs1492078 and *AGTR1*_rs5186, two single nucleotide polymorphisms (SNPs) in RAAS genes, were associated with RCC risk and that hypertension modified the association between *AGT*_rs3889728 and RCC risk. Interactions between SNPs in RAAS genes and sodium, potassium and fluid intake were not significant. However, sodium intake was differentially associated with RCC risk defined by genotypes of several SNPs. In **chapter 4** we investigated the role of gene-environment interactions in ion transport mechanisms (ITMs) as potential cancer-causing disease mechanism in RCC etiology. We describe a association between *ADD1*_rs4961 and RCC risk and a few significant gene-environment interactions between SNPs and dietary intakes of sodium, potassium and fluid, hypertension and diuretic medication, particularly in *SLC9A3*_rs4957061. Compared to the relatively large the number of tests performed, this evidence was too weak to support the general hypothesis that ITMs are important disease mechanisms in RCC etiology. In **chapter 5** we found that the intake of potassium, but not sodium or fluid, was differentially associated with molecular subtypes of clear-cell RCC. In this study clear-cell RCC was classified according to a promoter CpG island hypermethylation index of genes specifically selected for their involved in ITMs. However, promoter methylation in a larger, more general panel of genes was not influenced by dietary intakes that may be linked to the general mechanism of methylation, i.e. alcohol and folate intake, in clear-cell RCC in **Chapter 6**. Finally, in **chapter 7**, we demonstrated that patients with promoter methylation of cysteine dioxygenase type 1 (*CDO1*) gene have a significantly poorer clear-cell RCC-specific 10-year survival than those without and that this association was independent of other prognostic factors.

METHODOLOGICAL CONSIDERATIONS

Results of observational studies might be influenced by several types of bias [1]. Before interpreting the overall results of this thesis, the most important methodological issues potentially influencing exposures and outcomes will be discussed.

Assessment of dietary exposures

Throughout this thesis, the food frequency questionnaire (FFQ) was the primary method used to assess habitual intake of dietary exposures. The FFQ has long been considered the appropriate dietary assessment instrument in large-scale epidemiological studies due to its ability to rank participants at reasonable costs [2]. Moreover, compared to other dietary assessment measures, such as the 24h-recall method, the FFQ has the advantage to be less sensitive to day-to-day variations. Therefore, the FFQ may better determine long-term dietary habits, which is particularly important when investigating complex diseases such as cancer that may develop over longer time [3]. Nevertheless, dietary exposure assessment using the FFQ may be influenced by two types of measurement errors, i.e. random measurement error and systematic measurement error [1]. Random measurement error may result in underestimation of the associations under study, whereas systematic measurement error may result in either over- or underestimation of the associations under study [1]. Random dietary measurement error is a potential source of bias, because participants may not accurately remember their average intake of food items of the preceding year. In addition, it has been shown that persons with high body mass index systematically tend to underestimate (unhealthy) food intake compared to persons with normal weight [3]. In order to minimize the effects of this systematic dietary measurement error, we have adjusted the results for energy intake and other variables that have been shown to influence self-report of dietary intakes [3].

In addition to these general limitations of the FFQ, the combination of the single baseline measurement in the NLCS and the long follow-up used in this thesis, may have introduced some misclassification of the exposure. Although a reproducibility study showed that dietary intakes in the NLCS were rather stable during the first 5 years of follow-up [4], it is possible that intakes may have changed in the later follow-up. Given the prospective design of the NLCS and the fact that participants are generally unaware of the amount of nutrients they consume, it is most likely that changes in diet may have occurred independently of the outcome (i.e. at random). If random classification error was present in our data, it might have resulted in an underestimation of the reported associations [1]. In order to evaluate this potential

underestimation, we separately investigated early and late follow-up in chapter 2 and confirmed that risk estimates were much higher in early compared to late follow-up.

In particular, it has been recognized that exposure to sodium is difficult to measure accurately. The method of multiple 24h-urine collection is considered the gold standard, as this biomarker method is suitable to assess the absolute amount of intakes of a population and to capture total salt intake, including sodium intake from foods as well as added salt intake [5]. Generally, FFQs may underestimate the total amount of sodium intake, because they do not consider added salt intake [5]. The NLCS is one of the few prospective cohort studies that have a special salt module included in its questionnaire to assess added salt intake and salt preference. Therefore, we obtained more comprehensive data on salt consumption than the only other study investigating dietary sodium intake and RCC risk [6].

An important issue to consider when investigating sodium intake, is the phenomenon of reverse causation (i.e. when the outcome influences the exposure). Reverse causation typically occurs in a cross-sectional setting in populations that are at high disease risk prior to the start of the study [1]. Even though our study is prospective and population-based, reverse causation between the exposure (salt intake) and intermediate factor (hypertension) may have influenced the baseline RCC risk of some participants. Indeed, we observed that, at baseline, participants with hypertension were overrepresented in the lowest category of added salt intake. Because hypertension is an established risk factor for RCC [7], participants in the lowest category of added salt intake may have had a high baseline RCC risk. Therefore, we cannot exclude the possibility that reverse causation may have resulted in a u-shaped association between added salt intake and RCC risk.

Selection of genes and methods to assess gene-environment interactions

Many approaches can be used to study gene-environment interactions, ranging from agnostic to hypothesis driven approaches [8]. Traditionally, most gene-environment interaction studies are candidate gene studies, starting from an established association with an environmental factor and proceeding to explore genes in pathways of interest [9]. Currently, more and more gene-environment interactions are studied using an agnostic approach in a genome-wide setting [i.e. genome-wide environmental interaction studies (GWEIS)], as advances in technologies make genotyping and phenotyping more easily available at reasonable costs [10]. Because our aim was to unravel potential novel pathways underlying the association between sodium, potassium and fluid intake and RCC risk, we chose a hypothesis-based, candidate gene approach and we selected genes and SNPs based on currently available literature. We considered the RAAS and ITMs as two potentially cancer-

causing, biologic mechanisms of interest, because they have been implicated in both renal (patho)physiology and carcinogenesis. Our final selection included 13 SNPs in seven RAAS genes and 13 SNPs in ten ITM genes.

When aiming to explore genes in pathways of interest, helpful tools to select genes are pathway databases, such as the KEGG database [11]. Although our search strategy initially resulted in more than 100 potential SNPs of interest, we could only include a limited number of SNPs in our final selection. Since *the* candidate gene selection strategy does not exist, the selection process is subject to carefully considered, pre-defined selection criteria. The criteria we used and their consequences have been discussed in chapters 3 and 4 of this thesis and included, amongst others, the cut-off value for the minor allele frequency (MAF). In order to warrant optimal power, we only included SNPs with MAF of at least 20%. However, since populations differ in genetic make-up, and because reference data on the genetic make-up of the Dutch population are not yet available, the MAF of some SNPs in our population turned out to be lower than expected based on other Caucasian populations. We tried to minimize the problem of limited power by analyzing these SNPs under the assumption of a dominant mode of inheritance (homogeneous common genotype *versus* heterogeneous and homozygote rare genotype). Consequently we could have missed gene-environment interactions with SNPs that for example have a recessive mode of inheritance (homogeneous common and heterogeneous genotype *versus* homozygote rare genotype).

In addition to the mode of inheritance, our decisions regarding the type of interaction may also have influenced the results of this thesis. Many types of interaction may be distinguished depending on the context, including statistical interaction, quantitative interaction, qualitative interaction, public health synergy and biological interaction [9]. Biological interaction results from physical interactions between biomolecules (e.g. DNA, RNA, proteins, enzymes, etc.) and occurs at the cellular level in an individual, whereas statistical interaction occurs at the population level and is realized when there is inter-individual variation in DNA sequences [12]. In addition to these pathophysiologic models, different measurement scales should be considered; the multiplicative scale is generally used to study disease etiology, whereas the additive scale may best be used to predict the number of cases in a population [13]. The question of which scale should be used to define interaction has been intensely debated upon. Since we were in this thesis mainly interested in inter-individual variation in DNA sequences in RCC etiology, we tested for statistical interaction on a multiplicative scale. Therefore, we cannot exclude the possibility that we might have missed interesting findings related to other types of interactions.

In candidate gene-environment interaction studies, several limitations and pitfalls have been recognized a main challenge, including limited power and multiple testing

[9]. One way to overcome these issues is by using a sum score of unfavorable alleles, because such approach will limit the number of associations tested and optimize the power. Moreover, it is biologically more plausible to study the relationship between a complex pathway and a multidimensional disease as a whole. However, currently available evidence should be used to appoint these unfavorable alleles and avoid 'overfitting' of the sum score. Due to the explorative nature of our study, the candidate SNPs have rarely been investigated in relation to RCC. Instead, our selection of candidate genes was mainly based on associations with blood pressure, hypertension, and sodium handling and, therefore, no external information was available to construct a solid sum score of unfavorable alleles. Alternatively, we explored the potential of multifactor dimensionality reduction (MDR) to fit a better model to our complex data than we could achieve by using a single-marker approach. MDR is a non-parametric, data mining method that allows for higher-order interactions, including all SNPs and exposures of interest [12,14]. This approach simultaneously limits the concerns regarding sample size and multiple testing. Moreover, these types of methods do not assume a mode of inheritance [12]. Although we were faced with major challenges with respect to our case-cohort design and the interpretation of the results while using MDR at this stage, we feel that this type of machine learning methods are worthwhile to consider in future research, particularly given the developments in GWEIS.

Taken together, it should be noted that a certain degree of inevitable subjectivity in the selection of our approach, the genes and SNPs and the analyses techniques, is a major challenge in gene-environment interaction studies and that, despite all our careful considerations, we cannot exclude the possibility that we missed some important observations. Given the hypothesis-generating nature of studies in this thesis, this may even outweigh common criticism on candidate gene studies on false-positive findings.

Selection bias and classification of the RCC tumor in molecular (pathological) epidemiology

In general, studies in the NLCS using traditional epidemiological methods are unlikely to be influenced by selections bias, because of the prospective design, which assures that participants are not included as a consequence of their disease status, and the limited loss-to-follow-up [1]. However, in molecular (pathological) epidemiological studies, the incomplete collection of tumor material for histological and molecular classification, if differential, may lead to selection bias. There are several reasons why tumor tissue may not be available in molecular epidemiological studies [15]. In our study, we observed that for a reasonable amount of the cases without tissues material no material exists. As the primary therapy for RCC is surgery, it can be expected that material is available to large extent [16]. However, the percentage of surgical

procedures is lower for stage IV tumors. To minimize the potential effects of these stage IV tumors, we excluded this subgroup in sensitivity analyses regarding survival (chapter 7). Secondly, no record linkage with the Dutch Pathology Register (PALGA) may have been possible, due to record linkage errors or incompleteness of PALGA before 1991 [17] and, thirdly, particularly older tissues may have been lost or destroyed. In our study, inclusion was indeed slightly lower for cases identified during the first 5-years of follow-up. Nevertheless, we were able to collect tumor material of an exceptional amount of eligible histologically confirmed epithelial RCC cases (i.e. 80%). In addition, our collection of RCC cases is embedded within the NLCS and thus population-based. Therefore, we considered the likelihood of selection bias in our series of RCC cases to be low.

The collection of tumor material provided the opportunity to conduct molecular pathological analyses separately for each histological subtype (i.e. clear-cell RCC, papillary RCC and chromophobe RCC). Haematoxylin and eosin (HE)-stained slides of all the collected FFPE tumor tissues were assessed by two experienced genitourinary pathologist to confirm tumor histology based on the WHO-classification of tumors [18]. Based on this assessment, our collection of RCC cases includes 365 (80.6%) clear-cell (cc)RCC cases, 60 (13.3%) papillary (p)RCC cases, 15 (3.3%) chromophobe (chr)RCC cases and 13 (2.8%) other or undefined RCC cases. This is important when investigating molecular tumor heterogeneity, because different molecular pathways are thought to be involved in different subtypes [19]. RCC cases of the clear-cell subtype were further classified according to the degree to which promoter methylation in a set of candidate genes was present in the tumor. However, there is no accepted way to classify tumors according to these genes in particular and for RCC in general, such as CpG island methylator phenotype (CIMP) in colorectal cancer [20]. Therefore, it is possible that the selected genes intended to represent CIMP may not be generalizable to general promoter methylation status. Neither can we exclude the possibility that our panel of genes involved in ITMs may represent CIMP instead.

Multidimensional approach to study ion transport mechanisms (ITMs)

In order to investigate the role of ITMs in the associations between sodium, potassium and fluid intake and RCC risk, we used a multidimensional approach, including molecular data on both SNPs (for gene-environment interactions) and promoter CpG island methylation (for tumor heterogeneity). As previously explained, *the* candidate gene selection strategy does not exist and thereby the selection process is subject to carefully considered, pre-defined selection criteria. Different criteria are applicable when assessing gene-environment interactions compared to molecular tumor heterogeneity. As a result, in both types of molecular analyses, the final gene panel differed. For example, not all genes exhibited CpG islands in or near the promoter region and could not be included by definition, when studying tumor heterogeneity by

means of promoter CpG island methylation. Inversely, genes of interest for studying promoter CpG island methylation do not necessarily have SNPs of interest to study gene-environment interactions.

In addition to the differences in genes selected, this multidimensional approach highlights two distinct mechanisms by which the genes involved in ITMs may be aberrantly regulated or silenced, including a genetic (SNPs) and epigenetic (promoter methylation) mechanism. SNPs may influence the expression of the gene modestly, but potentially bidirectional mechanism (higher and lower expression), whereas promoter methylation tends to silence the whole gene (no expression). Moreover, SNPs are germline variations that may provide information on genetic predisposition, which may be relevant for the development of RCC, whereas promoter methylation may both influence the development and the progression of RCC. Together this multidimensional approach provides a broader view of the role of ITMs in RCC carcinogenesis.

OVERALL INTERPRETATION OF THE RESULTS

The aims of this thesis were to evaluate dietary intakes of sodium, potassium and fluid as novel risk factors for RCC and to unravel novel disease pathways implicated in renal (patho)physiology in the etiology and prognosis of RCC. After extensive considerations of the most important methodological issues, we conclude that the present thesis provides the first evidence that sodium intake is a novel risk factor for RCC, particularly if fluid consumption is low. This interaction between sodium and fluid intake supports our hypothesis that the link between sodium and fluid in renal physiology, and perhaps renal (patho)physiology in general, is important in RCC etiology. However, replication of these findings in other prospective studies is warranted.

Moreover, we hypothesized that dietary sodium intake may influence RCC risk, through hypertension. In this thesis, we found no indications for modifying or mediating effects of hypertension in the association between dietary sodium intake and RCC risk. This suggests that sodium intake may increase RCC risk independently of hypertension. Additionally, we observed that the RCC risk for participants with 'no added salt intake' was different between those with and without hypertension, suggesting that hypertension may specifically influence the association between added salt intake and RCC risk. As discussed previously, we cannot exclude the possibility that this association may be influenced by reverse causation. Importantly, we also found evidence that hypertension modified the association between some SNPs in RAAS genes, particularly *AGT* and *AGTR1*, and RCC risk. Given the key role of the RAAS in both sodium homeostasis and hypertension, we conclude that it is

worthwhile to further investigate the exact role of hypertension in the association between sodium intake and RCC risk.

In the second part of this thesis, two biological mechanisms involved in renal physiology, including the RAAS and ITMs, have been put forward as potential cancer-causing mechanisms that may explain the association between high sodium intake and RCC risk or that may be involved in RCC etiology otherwise. The RAAS regulates blood pressure and sodium homeostasis in the kidney and may, therefore, reflect a mechanism potentially underlying associations between sodium intake and RCC risk. As discussed previously, we tested for statistical interaction on a multiplicative scale. None of the SNP-diet interactions were statistically significant. Nevertheless, several polymorphisms in RAAS genes, particularly in *ACE*, showed suggestive interaction in the association between sodium intake and RCC risk. These results in combination with the results regarding RAAS SNPs and hypertension have led us to conclude that the RAAS is a likely candidate pathway in RCC etiology.

This thesis shows differential results regarding the role of ITMs in RCC carcinogenesis. As previously discussed, given the differences regarding the nature of the events studied (genetic *versus* epigenetic) and the selected genes, a one-to-one comparison of the results of the studies investigating the ITMs (Chapter 4 and 5) is not possible. However, more importantly, this multidimensional approach provides a broader view on the role of ITMs in RCC carcinogenesis. In chapter 4, evidence regarding gene-environment interactions with SNPs in ITM genes was too weak to support the hypothesis that ITMs are important in RCC etiology. However, in chapter 5, the intake of potassium was differentially associated with molecular subtypes of clear-cell RCC classified according to a promoter methylation index of ITM genes. Together, these results regarding ITMs suggest that promoter methylation plays a more dominant role in RCC than SNPs. This is plausible because promoter methylation tends to silence the whole gene (no expression), while SNPs may influence the expression of the gene only modestly and potentially bidirectional (higher and lower expression). Moreover, we were not able to include all potential interesting SNPs in our analyses and might have missed some interesting findings with other SNPs in ITM genes involved. Overall, we concluded that promoter methylation status may be used to classify the RCC tumor in terms of molecular heterogeneity and that ITMs may play a role in the etiology of RCC.

In the third part of the thesis, we studied promoter methylation in a larger, more general context in the etiology of RCC and in relation to RCC survival. We could not show that promoter methylation is a potential cancer-causing mechanism in the association between dietary intakes of folate and alcohol and RCC risk. Therefore, we concluded that the associations between alcohol and folate intake and RCC risk are not influenced by promoter methylation. However, the complexity of the relationship

under study and the generalizability of the gene panel towards CIMP, may have contributed to this negative finding. Moreover, promoter methylation is thought to contribute to both cancer development and progression [21-24]. Therefore, we hypothesized that promoter methylation may, in addition to RCC etiology, also play a role in the prognosis of RCC. We demonstrated that promoter methylation of cysteine dioxygenase type 1 (*CDO1*) gene did matter in relation to RCC survival, as patients with *CDO1* promoter methylation had a significantly poorer clear-cell RCC-specific 10-year survival than those without.

FUTURE DIRECTIONS IN RENAL CELL CANCER RESEARCH

Based on the results of this thesis, we recommend future research to focus, firstly, on reproducing the association between dietary sodium intake and RCC risk in other populations as well as on the exact role of hypertension in this association. To further elucidate the role of the RAAS and ITMs underlying these associations, future gene-environment interaction studies may benefit from current developments in molecular epidemiology, such as advanced bioinformatics analysis techniques, which enable the complexity of these mechanisms to be incorporated into the analyses. As a result, future gene-environment interaction studies may want to combine hypothesis-based pathway selection with a more agnostic approach to select SNPs and genes. In addition to the biological mechanisms explored in this thesis, other pathways are suggested to play a role in the development of RCC, such as the oxygen-sensing pathway. Future research may focus on the role of the oxygen-sensing pathway in RCC etiology, as it has been recognized that loss of function of the Von Hippel-Lindau (*VHL*) gene, which is involved in the oxygen-sensing pathway [25], is an important event in the development of clear-cell RCC [26].

More generally, to advance in the field of RCC research and gain insight into RCC etiology and prognosis, future RCC research should benefit from the identification of genetic and epigenetic subtypes. In large measure, the genetic basis of the RCC tumor has been established [27-34], yet before this information can be used in future research and in a clinical setting, efforts should be made to integrate this genetic data with information on lifestyle, tumor characteristics, treatment and prognosis. In this context, the emerging field of molecular pathological epidemiology (MPE), which integrates molecular subgroups into traditional epidemiological research [35], provides great opportunities to explain observed differences in risk factors across populations and studies and to increase our knowledge on the underlying biologic pathways [35]. A better understanding of tumor heterogeneity is crucial to eventually develop tools that may be used for early detection and to guide clinical decisions on treatment and eventually move closer towards personalized cancer medicine and prevention.

CONCLUDING REMARKS

This thesis describes, for the first time, that dietary sodium intake and renal (patho)physiological mechanisms (i.e. the RAAS and ITMs) were associated RCC risk. Thus, this thesis shows that (molecular) epidemiological research in RCC may benefit from a more renal (patho)physiologic perspective. We illustrated that molecular (pathological) epidemiology is a promising new field of research that can be used to provide insight into underlying biologic pathways of disease. While faced with several limitations regarding exposure, outcome and analyses, this is not an easy task. Nevertheless, the population-based nature of our collection of RCC cases and the availability of extensive information on demographic and dietary characteristics of the patients, tumor characteristics and survival status, make this series, to date, the best available infrastructure in the world to study molecular pathological epidemiological research questions in the field of RCC.

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Summary

Several risk factors have been established to increase the risk of renal cell cancer (RCC). However, the effects that these factors have on RCC risk are rather modest and, in general, the etiology of RCC is still poorly understood. In order to advance in the field of RCC and gain insight into its etiology and prognosis, it is important to search beyond current perspectives and generate new hypothesis for future research. Therefore, the overall aim of this thesis is to unravel novel risk factors and disease pathways implicated in renal (patho)physiology in the etiology and prognosis of RCC.

In **part 1 (chapter 2)** of this thesis, we investigated the role of sodium intake, potassium intake and fluid intake in relation to RCC risk using a traditional epidemiological approach. We identified that sodium intake is a novel risk factor for RCC, particularly if fluid consumption is low. This interaction between sodium and fluid intake supports our hypothesis that, given the link between sodium and fluid homeostasis in renal physiology, the balance between sodium and fluid intake is important in RCC etiology. We found no indications for modifying or mediating effects of hypertension. This suggests that sodium intake may increase RCC risk independently of hypertension. In addition, we observed that fluid and potassium intake were not associated with RCC risk.

In **part two (chapters 3 and 4)** of this thesis, we investigated the role of single nucleotide polymorphisms (SNPs) in candidate genes and their potential interplay with the environment in RCC etiology using a molecular-epidemiological approach. **Chapter 3** focused on the renin-angiotensin-aldosterone system (RAAS) as potential disease mechanism. We showed that two candidate SNPs in *AGTR1* were associated with RCC risk; the homozygote rare genotype of *AGTR1*_rs1492078 decreased RCC risk, whereas the homozygote rare genotype of *AGTR1*_rs5186 increased RCC risk. Hypertension modified the association between *AGT*_rs3889728 and RCC risk. Interactions between dietary intakes (sodium, potassium and fluid) were not significant. However, sodium intake was differentially associated with RCC risk defined by genotypes of several SNPs in genes involved in the RAAS. These results indicate a biologic interaction instead of a statistical interaction. In **chapter 4** we investigated the role of gene-environment interactions in ion transport mechanisms (ITMs) as potential disease mechanism. We tested main effects of 13 candidate SNPs in ITM genes and gene-environment interactions between these SNPs and associated environmental factors, including dietary intakes of sodium, potassium and fluid, hypertension and diuretic medication. Only *ADD1*_rs4961 was significantly associated with RCC risk in the main effects analyses. Additionally, three out of four significant gene-environment interactions clustered in *SLC9A3*_rs4957061, which indicates involvement of *SLC9A3*_rs4957061 in RCC etiology. Nevertheless, the body of evidence is too weak to support the hypothesis that the mechanism of ion transport is a disease mechanism in RCC etiology.

In **part 3 (chapters 5-7)** of this thesis, we classified the clear-cell RCC tumor according to promoter CpG island hypermethylation of a subset of genes to study molecular subtype-specific etiology (**chapters 5 and 6**) and prognosis (**chapter 7**) using the approach of molecular pathological epidemiology (MPE). In **chapter 5** we classified the clear-cell RCC tumor according to a promoter methylation index of genes specifically selected for their involvement in the mechanism of ion transport. We investigated dietary intakes that are linked to ITMs, i.e. sodium, potassium and fluid intake, as potential risk factors for clear-cell RCC using these molecular subtypes. We found that the intake of potassium, but not sodium or fluid, was differentially associated with the molecular subtypes of clear-cell RCC. High *versus* low potassium intake was associated with a lower clear-cell RCC risk for unmethylated tumors and a higher clear-cell RCC risk for tumors with a high promoter methylation index. Thus, this study illustrates that risk factors may differ according to molecular subtypes of the tumor. In addition, we showed the first evidence for a potential role of ITMs in RCC etiology. In **chapter 6**, we determined promoter CpG island hypermethylation status for a larger, more general panel of genes and classified the clear-cell RCC tumor accordingly. We investigated dietary intakes that may be linked to the general mechanism of methylation, i.e. alcohol and folate intake, in relation to these molecular subtypes. We did not observe differential associations between alcohol and folate intake and molecular subgroups of the clear-cell RCC tumor defined by promoter CpG island hypermethylation. Therefore, this study does not support the hypothesis that promoter CpG island hypermethylation of this gene panel may be the disease mechanism linking alcohol and folate intake to clear-cell RCC risk. In **chapter 7**, we classified the clear-cell RCC tumor by the presence of promoter CpG island hypermethylation of the cysteine dioxygenase type 1 (*CDO1*) gene and evaluated these molecular subgroups in a prognostic setting. Results demonstrated that patients with promoter CpG island hypermethylation of *CDO1* have a significantly poorer clear-cell RCC-specific 10-year survival than those without. Differences in survival were independent of common prognostic makers, including age at diagnosis, sex, TNM stage, tumor size and Fuhrman grade. Thus, promoter CpG island hypermethylation of *CDO1* offers additional, relevant, prognostic information, but may not substitute common prognostic makers to predict clear-cell RCC-specific 10-year survival. Results were validated in clear-cell RCC cases from The Cancer Genome Atlas (TCGA).

Finally, this thesis concludes with a summary and interpretation of main findings, a general discussion and implications for future RCC research (**Chapter 8**). After extensive considerations of the most important methodological issues, we concluded that this thesis provides the first evidence that dietary sodium intake and renal (patho)physiological mechanisms (i.e. the RAAS and ITMs) were implicated in RCC etiology. Thus, this thesis shows that (molecular) epidemiological research in RCC may benefit from a more renal (patho)physiologic perspective. Moreover, we illustrated

that molecular (pathological) epidemiology is a promising new field of research which can be used to provide insight into underlying biologic pathways of disease.

Samenvatting

Jaarlijks wordt er in Nederland bij ongeveer 10 per 100,000 mensen nierkanker gediagnostiseerd. Daarmee staat nierkanker op de 7^e plaats van meest voorkomende vormen van kanker. Nierkanker komt vaker voor bij mannen dan bij vrouwen en naar mate men ouder wordt. Het is bekend dat ook andere factoren naast geslacht en leeftijd het risico op nierkanker kunnen verhogen; zoals overgewicht, roken, hoge bloeddruk en familiale aanleg. Tegelijkertijd zouden een gematigde inname van alcohol en het eten van voldoende groentes en fruit het risico op nierkanker juist verlagen. Toch is het tot op heden niet precies bekend hoe nierkanker ontstaat.

In dit proefschrift maken we gebruik van een nieuwe invalshoek om het ontstaan van nierkanker te onderzoeken om op deze manier tot nieuwe inzichten te komen ten aanzien van de ontwikkeling van deze kanker. Het doel van dit proefschrift is het ontdekken van nieuwe risicofactoren en biologische mechanismen, welke enerzijds gerelateerd zijn aan de nierfysiologie en anderzijds het ontstaan van nierkanker of de overleving van patiënten met nierkanker kunnen beïnvloeden.

We hebben deze vraagstelling onderzocht in een groot, prospectief, cohort onderzoek onder gezonde mensen uit de algemene Nederlandse bevolking, namelijk in de Nederlandse Cohort Studie naar voeding en kanker (NLCS). In 1986 retourneerden 120,852 deelnemers een vragenlijst over hun voedingsinname en verschillende andere leefstijlfactoren. Ook verzamelden we teennagels die we later hebben kunnen gebruiken voor het bepalen van de genetische variatie tussen de deelnemers. We volgden deze deelnemers tot 2006, dus meer dan 20 jaar. In totaal kregen 608 van de deelnemers tijdens deze 20 jaar een vorm van nierkanker. In de analyses hebben we de deelnemers met nierkanker vergeleken met een willekeurige subgroep van 5.000 deelnemers uit het totale cohort.

In het eerste deel van dit proefschrift (hoofdstuk 2) stond de relatie tussen de inname van zout, kalium en vocht, en het risico op nierkanker centraal. Met een traditioneel epidemiologische benadering konden we aantonen dat er een verband is tussen een hoge inname van zout en nierkanker. Dit verband leek sterker wanneer er tegelijkertijd ook sprake was van een lage inname van vocht. Deze bevinding suggereert dat de balans tussen zout- en vochtinname mogelijk belangrijk is bij het ontstaan van nierkanker. We konden in dit hoofdstuk geen verband aantonen tussen een hoge inname van kalium en het risico op nierkanker. Het is bekend dat zout het risico op hoge bloeddruk vergroot, terwijl hoge bloeddruk weer een risicofactor is van nierkanker. Echter, het verband dat wij vonden voor hoge zoutinname en nierkanker werd niet verklaard door hoge bloeddruk.

In het tweede deel van dit proefschrift (hoofdstuk 3 en 4) hebben we genetische variatie onderzocht in twee fysiologische systemen die in de nier een belangrijke rol spelen bij het reguleren van de bloeddruk en de zout- en vochtbalans van ons lichaam,

namelijk het renine-angiotensine-aldosteron systeem (RAAS) en ion-transport mechanismen (ITM). Genetische variaties in deze fysiologische systemen zouden een potentiële verklaring kunnen zijn voor het verband tussen een hoge zoutinname en het risico op nierkanker dat we eerder al hadden gevonden. Om hiervoor aanwijzingen te vinden hebben we zogenaamde gen-omgeving interacties in het risico op nierkanker onderzocht tussen 26 verschillende genetische varianten aan de ene kant en het dieet (bijvoorbeeld zoutinname) aan de andere kant. Hiervoor gebruikten we een moleculair-epidemiologische benadering. Door middel van deze zogenaamde gen-omgeving interacties konden we bewijzen dat RAAS wellicht een rol speelt bij het ontstaan van nierkanker. We vonden namelijk voor meerdere genetische varianten dat het risico op nierkanker bij een hoge zoutinname voor deelnemers met de genetische variant anders was dan voor deelnemers zonder de genetische variant. Daarnaast vonden we dat een bepaalde genetische variant het risico op nierkanker bij aanwezigheid van een hoge bloeddruk veranderde. In tegenstelling tot deze resultaten in het RAAS, konden we niet aantonen dat ook ITM een mogelijke rol spelen bij het ontstaan van nierkanker.

In het derde deel van dit proefschrift (hoofdstuk 5-7) onderzochten we tumorheterogeniteit zowel in relatie tot de ontwikkeling van nierkanker, als in relatie tot de overleving van patiënten met nierkanker. Het vermogen om genen aan- en uit te zetten, onderdeel van de epi-genetica, kan een kenmerk zijn van hoe een tumor is ontstaan, maar ook van hoe een tumor zich zou kunnen ontwikkelen. In twee studies hebben we onderzocht of we met behulp van een bepaalde vorm van deze epi-genetica, namelijk DNA methylering, groepen van niertumoren met verschillen in risicofactoren zouden kunnen onderscheiden. In de ene studie onderzochten we DNA methylering in genen die gerelateerd zijn aan ITM en in de andere studie gebruikten we een groep genen met verschillende functies. De eerste studie liet zien dat een hoge kaliuminname verband houdt met een verlaagd risico op nierkanker in de groep zonder DNA methylering in de ITM genen (wanneer deze genen dus ‘aan’ staan) en een verhoogd risico op nierkanker in de groep met veel DNA methylering in de ITM genen (wanneer deze genen dus ‘uit’ staan). Deze bevinding is niet in lijn met de bevindingen van hoofdstuk 2, waarin we juist geen verband konden aantonen tussen een kaliuminname en het risico op nierkanker. Hiermee bewijzen we dus dat risicofactoren kunnen verschillen op basis van de tumorheterogeniteit tussen niertumoren. Bovendien vonden we, in tegenstelling tot ons eerder onderzoek in hoofdstuk 4, dat ook ITM een mogelijke rol spelen bij het ontstaan van nierkanker. In de tweede studie over tumorheterogeniteit op basis van de groep genen met verschillende functies konden we geen groepen van niertumoren onderscheiden met verschillen in risicofactoren (alcohol- en folaatinname). Tenslotte, hebben we in de laatste studie onderzocht of we met behulp van DNA methylering in een specifiek gen, het cysteine dioxygenase type 1 (*CDO1*) gen, nierkankerpatiënten met verschillen in overleving kunnen onderscheiden. In de literatuur staat al beschreven dat DNA

methylering van het *CDO1* gen borstkankerpatiënten met verschillen in overleving kan onderscheiden. Onze studie laat nu zien dat ook nierkankerpatiënten met DNA methylering van het *CDO1* gen na 10 jaar een lagere overlevingskans hebben dan nierkankerpatiënten zonder DNA methylering in het *CDO1* gen.

We kunnen dus concluderen dat we met dit proefschrift een hoge zoutinname, in het bijzonder in combinatie met een lage vochtinname, als nieuwe risicofactor voor het ontstaan van nierkanker hebben geïdentificeerd. Daarnaast hebben we aanwijzingen gevonden voor de rol van twee fysiologische systemen in de nier, namelijk RAAS en ITM, bij het ontstaan van nierkanker. Daarmee hebben we laten zien dat toekomstig onderzoek naar het ontstaan van nierkanker inderdaad zou kunnen profiteren van onze nieuwe fysiologische invalshoek. Bovendien hebben we aangetoond dat het noodzakelijk is de tumorheterogeniteit tussen niertumoren te includeren in onderzoek naar de risicofactoren en de overleving deze kanker.

Valorisation addendum

In addition to the scientific value of this thesis described in Chapters 2-7, the results of the research presented also have societal and economic value. In this addendum we try to put the results of this thesis into a broader perspective and highlight its potential for clinicians and policymakers and in a public health setting.

DIETARY SODIUM INTAKE INTO PERSPECTIVE

Average sodium intakes around the world are much higher than the 0.2 to 0.5 grams sodium per day that people physiologically need [1]. In fact, most populations around the world are consuming between 3.0 and 6.0 grams sodium per day [7.5 to 15.0 grams of salt (sodium-chloride) per day] [2,3]. In 2012, men and women in the Netherlands consumed 4.0 and 3.0 grams of sodium per day, respectively [4]. Moreover, sodium intakes ranged between 3.0 and 4.0 grams per day in neighbouring countries, including Belgium, Denmark, Germany and the UK [5]. In our study population, the mean sodium intake was measured in 1986 and estimated to be 3.5 grams per day.

It has been established that a chronic excess of sodium may be related to multiple diseases, including hypertension, heart failure and kidney failure. In this thesis, we provide the first evidence that a high sodium intake, particularly in combination with low fluid intake, may also increase the risk of renal cell cancer. Before this finding has any additional value in a public health setting, confirmation of this association in more and other populations is crucial. In addition, lowering of the sodium intake will help to prevent hypertension, heart failure and kidney failure at the population level. Reduction of sodium intake is for example the most used strategy to prevent hypertension. It has been shown in a number of systematic reviews on randomized controlled trials that decreased sodium intake relative to usual or higher intake results in lowered blood pressure in adults with or without hypertension [6-8]. Now, the reduction of sodium intake is a highly prioritized recommendation to prevent all kinds of non-communicable diseases such as cardiovascular diseases, but also diabetes mellitus type 2 [9]. Recently, the World Health Organization (WHO) adjusted the recommendation on sodium consumption for adults, which is now 2.0 g sodium per day [10].

The question remains how much reduction of the sodium intake at the individual level is desirable. The optimal exposure level of sodium is currently under debate. Firstly, WHO recommendations are based on cardiovascular disease risk and not cancer risk. Moreover, the type of relationship between sodium intake and these disease risks is still unclear, as increasing evidence suggests that the association between sodium intake and cardiovascular events might be J-shaped instead of linear [11-16]. This suggests that high sodium intake is associated with morbidity and mortality, while a

very low sodium intake (less than 2.3 grams per day) is not associated with health benefits or, alternatively, may be related to a higher cardiovascular risk [17]. This thesis supports, in part, the concerns on too low sodium intake levels, given the observed J-shaped association between added salt intake and renal cell cancer risk. However, it remains unclear whether this higher risk in the lowest sodium consumers is a real physiological risk or perhaps may reflect one's ability to reduce added salt intakes to prevent or control hypertension. Before any further recommendations on lowering sodium intake can be adopted, it is essential to clarify and exclude potential risks of a too low sodium intake for the individual.

Given the particularly high intakes of sodium in many populations to date, the subsequent higher risks of diseases and the positive effects of reducing sodium intake on reducing these disease risks, we feel that the quest to reduce the sodium content of food products and thereby the sodium intake on the population level is still justified, at least as long as the uncertainty on the effects of a very low sodium consumption on the individual level remains.

A number of strategies can potentially be used to decrease the average sodium intake of a population. The simplest and safest strategy to reduce sodium intake is to consume less foods that have high sodium content. Sodium is naturally present in a variety of foods and food groups, which are consumed depending on culture and population [10]. The highest sodium content is found in soups and soy sauce, but fair amounts are also found in for example cheese, milk and shellfish. Moreover, sodium is often found in high amounts in processed foods such as breads, crackers, processed meats and snack foods. To a large extent, people are unaware of the sodium content in particular food items, although recent efforts seemed to increase the awareness of the high sodium content in processed foods. However, awareness interventions on the content of sodium that is naturally present in unprocessed foods, may also increase an individual's ability to consciously choose which type of foods to consume and enables them to better monitor their overall sodium intake.

In addition to foods and food groups, common table salt, including the chemical components sodium and chloride, is often used for seasoning during and after cooking. Because, these habits are particularly influenced by attitudes and beliefs, a systematic review concerning advice to reduce sodium consumption concluded that intensive behavior change interventions targeting decreasing sodium intake are necessary to successfully reduce blood pressure in adults with or without hypertension [18]. However, the authors also concluded that environmental changes (e.g. reduction of sodium in processed foods) would facilitate a greater reduction in sodium consumption and, therefore, have a greater impact. Lobby groups have made multiple efforts to convince policymakers, governments and food industries to reduce

the amount of sodium in food products. To some extent, small successes to reduce the sodium content in foods have been booked already [19].

THE ROLE OF HYPERTENSION

In the thesis we found evidence both in favor of and against the role of hypertension in the association between sodium intake and renal cell cancer. Therefore, we concluded that it is worthwhile to further investigate its exact role. From a public health perspective it is crucial to elucidate the potential influence of hypertension. If hypertension would be the driving force of the higher risk of renal cell cancer, preventive strategies to reduce hypertension, other than reduction of sodium intake may be opted for by health promoters and policymakers. One such potential strategy is to normalize the sodium-to-potassium ratio in the body by promoting the intake of potassium rich foods and so increasing the average amount of potassium intake of a population instead of decreasing the amount of sodium intake [1]. Potassium is naturally present in fruits and vegetables, potatoes and nuts, and is recognized to lower the blood pressure [20]. Food manufactures are investigating the opportunities to use potassium chloride instead of sodium chloride for preservation purposes. However, if hypertension does not influence the association between sodium intake and renal cell cancer, reduction of sodium intake would be the best strategy for renal cell cancer prevention after all.

GENE-ENVIRONMENT INTERACTIONS

In the second part of this thesis (chapters 3 and 4) we studied gene-environment interactions in relation to two biological systems influencing blood pressure and sodium handling, i.e. the renin-angiotensin-aldosterone system (RAAS) and ion-transport mechanisms (ITMs) and the risk of renal cell cancer. In gene-environment interaction research there are no direct opportunities to use any findings in a clinical, economical or societal setting at this point. However, these gene-environment interaction studies may help to identify individuals at high disease risk based on their genetic profile and their dietary consumption pattern. We observed, for example, that sodium intake was differentially associated with renal cell cancer risk defined by several genetic variations in genes involved in the RAAS. For the future, this type of information may be used to tailor interventions in so-called precision medicine. For example, salt-sensitive individuals may benefit more from low salt interventions to reduce disease risk than individuals who do not have this sensitivity. However, before we can use information on genetic profiles in a public health setting for prevention, the ethical, scientific and economic issues need to be solved first.

TUMOR HETEROGENEITY

In the third part of this thesis (chapters 5-7) we investigated the influence of tumor heterogeneity within the renal tumor. In the clinical setting, a better understanding of inter-tumor heterogeneity and the identification of renal cell cancer subtypes can be used to better classify a renal cell cancer patients' need in terms of disease management and to move closer towards personalized cancer medicine and personalized cancer prevention. For example, a better understanding of tumor heterogeneity and associated subtype-specific risk factors or subtype-specific prognosis is crucial to eventually develop tools that may be used for early detection and to guide clinical decisions on treatment. Currently, patients with advanced cancer experience complex physical, psychological and social consequences not only from the disease itself but also from the treatment. For the majority of advanced cancers, the standard of care still consists of 'trial and error' therapy. This means that a significant subset of patients will not benefit from the therapy but needlessly suffer from the side effects of the drug. Tools to select the most effective anti-cancer therapy and sparing patients the toxicity associated with the treatment are urgently needed to avoid unnecessary reduction of the quality of life. Particularly for renal cell cancer, molecular-targeted agents have successfully been developed and targeted therapies, such as sunitinib, sorafenib, everolimus and bevacuzimab, are currently being used for the treatment of renal cell cancer patients. However, tools to personalize these treatments and guide clinical decision-making are not yet available. Integrating the tumor heterogeneity into (molecular) epidemiological research is a step forward, although it should be recognized that such tools are still far away. However, it is inevitable that guided decision-making instead of 'trial and error' therapy will have a major impact on the quality of life of renal cell cancer patients and may also be attractive from a health economical perspective as it will lead to less useless treatment and ill-spent costs.

In conclusion, this chapter illustrates how the scientific findings described throughout this thesis can potentially be used by clinicians, policymakers and in a public health setting, to create societal and economic value.

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About the author

Ivette Antoinette Gerarda Deckers was born on January 22nd, 1987 in Nijmegen, the Netherlands. After completing secondary school (Athenaeum) at St Ursula in Horn, the Netherlands in 2005, she studied Health Sciences at Maastricht University, in Maastricht, the Netherlands. Three years later in 2008, Ivette obtained her BSc. degree and enrolled in a two year, English-taught Health Sciences Research Masters' program with specialization in clinical epidemiology at Maastricht University. She obtained her MSc. degree in 2010. During her one-year the Masters' internship at the Department of Epidemiology, Maastricht University, she spent a five month period abroad at the Center for Population Health Sciences in Edinburgh, United Kingdom and first-authored a systematic review of epidemiological studies in the field of allergy, which was published in *PLoS ONE*. After graduating, Ivette started a PhD project at the Department of Epidemiology and GROW, School for Oncology and Developmental Biology of Maastricht University under supervision of promotores prof. dr. Piet A van den Brandt and prof. dr. Manon van Engeland and copromotor dr. Leo J Schouten. The research, as described in this thesis, was conducted within the framework of the Netherlands Cohort Study. From October 2014 onwards, Ivette works as research consultant and data analyst at the Dutch Pathology Registry (PALGA).

List of publications

1. **Deckers IAG**, Schouten LJ, Van Neste L, van Vlodrop I, Soetekouw PMMB, Baldewijns MMLL, Jeschke J, Ahuja N, Herman JG, van den Brandt PA and van Engeland M. Promoter methylation of *CDO1* identifies clear-cell renal cell cancer patients with poor survival outcome. *Clin Cancer Res* 2015 Apr (Epub ahead of print)
2. **Deckers IAG**, van den Brandt PA, van Engeland M, van Schooten FJ, Godschalk RWL, Keszei AP and Schouten LJ. Polymorphisms in genes of the renin-angiotensin-aldosterone system and renal cell cancer risk: interplay with hypertension and dietary intakes of sodium, potassium and fluid. *Int J Cancer* 2015, 136(5):1104–1116
3. **Deckers IAG**, van den Brandt PA, van Engeland M, Soetekouw PMMB, Baldewijns MMLL, Goldbohm RA and Schouten LJ. Long-term dietary sodium, potassium and fluid intake; exploring potential novel risk factors for renal cell cancer in the Netherlands Cohort Study on diet and cancer. *Br J Cancer* 2014, 110(3):797-801
4. **Deckers IAG**, McLean S, Linssen S, Mommers M, van Schayck CP and Sheikh A. Investigating International Time Trends in the Incidence and Prevalence of Atopic Eczema 1990–2010: A Systematic Review of Epidemiological Studies. *PLoS ONE* 2012, Jul;7(7)

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1. **Deckers IAG**, van den Brandt PA, van Engeland M, van Schooten FJ, Godschalk RWL, Keszei AP and Schouten LJ. Gene-diet interactions in ion transport mechanisms in relation to renal cell cancer risk.
2. **Deckers IAG**, van Engeland M, van den Brandt PA, Van Neste L, Soetekouw PMMB, Aarts MJB, Baldewijns MMLL, Keszei AP and Schouten LJ. Promoter CpG island methylation in ion transport mechanisms and associated dietary intake jointly influence the risk of clear-cell renal cell cancer.
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