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Chapter 4.3

Interaction between calcium and variations in the calcium concentrations SNP's and the risk of colorectal cancer risk: The Rotterdam study.

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ABSTRACT

Objectives

Previous studies showed that high calcium intake may be associated with reduced colorectal cancer (CRC) risk, but results were inconclusive. We evaluated whether calcium intake from diet and supplements, as well as the calcium levels itself were associated with CRC risk in middle-aged and older individuals. Also we evaluated whether these associations were modified by genetic variation of calcium homeostasis.

Design

This study was embedded in The Rotterdam Study, a prospective cohort study among adults aged 55 years and older without CRC at baseline, from the Ommoord district of Rotterdam, The Netherlands (N=10,941). Effect modification by a pre-defined genetic risk score (GRS) from seven loci known to be associated with calcium concentrations, was evaluated.

Results

Relative to the recommended dietary calcium intake, only higher than the recommended dietary calcium intake (≥1,485 mg/day) was associated with a reduced risk of CRC (HR: 0.66; 95% CI: 0.44 – 1.00). All seven loci for serum calcium concentrations, as well as the GRS, were not associated with CRC but showed effect modification by the GRS in the association between calcium intake and CRC (p for interaction=0.08). After stratification of GRS into low, intermediate and high, we found a lower CRC risk for low weighted GRS per increase in calcium intake.

Conclusion

The results from this study demonstrate that there is no consistent association between calcium indices on CRC. However, the association between calcium intake and CRC may be modified by genetic variation associated with serum calcium concentrations that deserves further replication in other studies with different population.

Keywords: dietary calcium, calcium supplements, colorectal cancer, calcium SNPs, prospective cohort

INTRODUCTION

Colorectal cancer (CRC) is a growing public health concern worldwide, with over 1.8 million new cases in 2018 globally (1). Several lifestyle factors such as physical inactivity, low dietary fiber intake, high red and processed meat intake and high alcohol intake are associated with an increased risk of developing CRC (2).

Previous studies have demonstrated that high dietary calcium intake (from dairy products) may be associated with reduced CRC risk, but the evidence for the association for non-dairy calcium and CRC is still inconclusive (2). These differential results may be explained by differences in bioavailability, for example some of the green vegetables have a low bioavailability of calcium due to the presence of oxalate (3, 4).

Several biological mechanisms may explain a potential protective role of calcium in the development of CRC. For example, experimental studies in animals and humans showed that calcium may protect against CRC development by binding to bile acids and fatty acids in the gastrointestinal tract, and subsequently protecting the colon mucosa from these potentially toxic products (5, 6). Also, calcium may influence differentiation and apoptosis of colonic epithelial cells and might reduce inflammation and oxidative damage in these cells (7).

Moreover, a potential role of the calcium-sensing receptor (CASR) in influencing carcinogenesis of colon epithelium and mediating antineoplastic effect of calcium was suggested (8). In this study, it was found that higher calcium intake was associated with a lower risk of CASR-positive tumours but not with CASR-negative tumours. Besides CASR, seven other Single Nucleotide Polymorphisms (SNPs) associated with serum calcium have been identified (i.e. rs1801725, rs10491003, rs1550532, rs1570669, rs7336933, rs7481584, rs780094); these may be relevant in CRC etiology as well (9). However, inconsistent results on (colorectal) cancer have been reported for calcium-related SNPs so far (e.g. related to CASR or Vitamin D Receptor (VDR) genes) (8, 10, 11). Also, the association of these SNPs and CRC, and the effect modification of these SNPs on the association of calcium indices and CRC have not been investigated yet. Our working hypothesis is that higher intake of calcium could decrease the risk of CRC but that this depends on the genetic variability related to calcium homeostasis. Therefore, the aim of the present study was to determine whether calcium intake from diet and supplements as well as total calcium levels was associated with CRC risk in middle-aged and older individuals. Furthermore, we aimed to assess whether 7 loci for serum calcium concentration are associated with CRC and whether the association

between calcium indices and colorectal cancer risk differs according to pre-specified SNPs involved in calcium homeostasis.

Subjects and methods

The Rotterdam Study

This study was embedded in The Rotterdam Study, an ongoing prospective population-based cohort study originally designed to investigate the occurrence and determinants of common age-related diseases (12). Briefly, The Rotterdam Study is composed of four cohorts. We used the existing data from the first and second cohort of The Rotterdam Study (RS-1 and RS-II). Individuals aged 55 years and older, who were living in the Ommoord district of Rotterdam, the Netherlands were recruited for RS-I, between 1990 and1993 (n=7,983). The study was extended in 2001 with 3,011 participants in RS-II. Baseline data, were obtained by a home interview and two subsequent visits to the research center in Ommoord. Follow-up examinations were repeated approximately every three to four years, with a response rate of 78%, which is in line. Clinical outcomes such as morbidity and mortality were continuously monitored throughout the study period. All participants provided written informed consent, and ethical approval was obtained from the Medical Ethical Committee of the Erasmus Medical Center (12, 13).

Calcium

Dietary calcium intake data were obtained by using semi-quantitative food frequency questionnaires (FFQ) at baseline (between 1989 and 1993 in RS-I-1 and between 2000 and 2001 in RS-II-1), managed by a trained dietician at the study center (12, 14). Portion size of each food item was specified in standardized units, household measures or grams, and the frequency of each item was collected in times per day, week or month. Food items were coded using the corresponding NEVO-code (Dutch Food Composition Table) (15). Dietary intake of nutrients (incl. total energy and calcium) was calculated using the Dutch Food Composition database (NEVO) (15). Dietary calcium intake was adjusted for total energy intake using the residual method to adjust for measurement error and residual confounding (16, 17). In a validation study (*n*=80) of The Rotterdam Study nutrient intake assessed with the FFQ was validated against multiple food records (18). The validation study showed a good correlation for calcium intake (Pearson's correlation after adjustment for age, sex, energy intake, and within-person variation: 0.72) (18).

In addition, drug *use of participants of The Rotterdam Study was continuously monitored since January 1, 1991, through computerized records from the pharmacies in the Ommoord district. The pharmacy data included the Anatomical Therapeutical Chemical (ATC)-code, the dispensing date, the total number of drug units per prescription, the prescribed daily number of units, and product name of the drugs. On the basis of this information, the number of calcium dispensings was extracted (A12AA and A12AX) (19).*

Serum calcium levels were determined at baseline using a cresolphthalein complexone method (Merck Diagnostica, Amsterdam, the Netherlands) with a Kone auto-analyser (Kone Diagnostics, Espoo, Finland) (12).

Single Nucleotide Polymorphisms (SNPs) selection

The Rotterdam Study RS-I and II consist of 8.448 DNA samples at baseline, and from all Rotterdam Study samples the genotypes of SNPs are being estimated using the basis Illumina 500 K SNP dataset configurations in each subject (12). The selection of SNPs in this study, was pre-specified using the seven loci (six new regions) known to be associated with serum calcium based on literature and based on our hypothesis only for the included participants of RS-I and RS-II (9). The selected SNPs were rs1801725, rs10491003, rs1550532, rs1570669, rs7336933, rs7481584, rs780094.

Colorectal Cancer (CRC)

Diagnosis of incident cancer was based on medical records of general practitioners (including hospital discharge letters) and furthermore through linkage with Dutch Hospital Data (Landelijke Basisregistratie Ziekenhuiszorg), histology and cytopathology registries in the region (PALGA), and the Netherlands Cancer Registry. Cancer diagnoses were coded independently by two physicians and classified according to the International Classification of Diseases, 10th revision (ICD-10) (20). In case of discrepancy, consensus was sought through consultation with a physician specialised in internal medicine. In these analyses, only pathology proven CRC were used from baseline of the cohort until the end of follow up on December 31, 2014. Date of diagnosis was based on date of biopsy or—if unavailable—date of hospital admission or hospital discharge letter. Codes C18-C20 about malignant neoplasms of the colon, recto-sigmoid junction and rectum, were used to classify CRC diagnosis.

Covariates

The following characteristics of the study population and other information were assessed during the home interviews and the visit to the research center at baseline of the cohort between 1990 and 2001: gender, age, education level, income, smoking status, other dietary variables (including intake of alcohol, dietary fiber and processed red meat), height, weight, waist circumference, history of diabetes mellitus type II, serum total cholesterol levels and serum total calcium levels. Level of education and net monthly household income were used as indicators of socioeconomic status. Highest attained educational level was classified according to the International Standard Classification of Education using the following categories: primary education, lower/intermediate general and lower vocational education, higher general and intermediate vocational education, and higher vocational education and university (21). For the present study, education level was categorized into two categories: low education (primary education solely) and intermediate to high education (secondary education and higher). Income of the participants was expressed in net monthly household income. For the present study, income was categorized into low to intermediate income (<2,400 gulden/per month) and intermediate to high income (≥2,400 gulden/per month) based on net modal household income of the study population. Smoking status was categorized into two categories: never or ever smokers and current smokers.

Intake of energy (kcal/day), alcohol (g/day), dietary fiber (g/day) and processed red meat (g/day) were assessed with the FFQ as described previously. All dietary nutrient intake were adjusted for total energy intake using the residual method (17).

Height and weight were measured at the research center, and Body Mass Index (BMI) was calculated by weight in kilograms divided by the square of height in meters (kg/m^2) (22). Waist circumference was measured midway between the lowest rib and the iliac crest using a measuring tape (12). Diabetes mellitus type II was defined as fasting plasma glucose concentrations of ≥7 mmol/l or the use of glucose lowering drugs or insulin using the World Health Organization (WHO) and American Diabetes Federation (ADA) guideline (23, 24). Serum total cholesterol levels were determined with blood samples using an automated enzymatic procedure (25).

Physical activity and vitamin D level were obtained during the visit to the research center at the third follow-up visit of the cohort between 1997 and 2001. Physical activity was determined by means of an adapted version of the Zutphen Physical Activity Questionnaire (ZPAQ) (26). The ZPAQ was previously validated with a test-retest reliability of 0.93. The correlation of ZPAQ with doubly labeled water which

is the golden standard measurement of physical activity was 0.60 (27). The adapted questionnaire consisted of questions about walking, cycling, gardening, diverse sports, hobbies and housekeeping. The Metabolic Equivalent of Task (MET) was used to express the intensity of physical activity of each activity. MET-values were based on the metabolic rate for that specific activity compared to the resting metabolic rate using the 2011 Compendium of Physical Activities (28). Vitamin D status was assessed with plasma concentrations of 25-hydroxyvitamin D (25OHD) (nmol/l) from non-fasting blood samples using electrochemiluminescence immunoassay (COBAS, Roche Diagnostics GmbH, Germany (12).

Statistical Analyses

Continuous variables with a normal distribution were expressed as mean with its standard deviation (SD), and continuous variables with a skewed distribution were expressed as median with its interquartile range (IQR). Categorical variables were presented in frequencies and relative percentages.

Cox regression analyses were performed to determine the associations between calcium diet, supplements, calcium level andCRC risk separately. Follow-up time (in years) was used as underlying timescale in the analyses. The proportional hazard assumption was explored by performing an interaction test of exposure with time in the Cox proportional hazard models. The proportional hazard assumption is assumed to hold when the *P*-value of the interaction between exposure and time is >0.05 (29). The association between prescribed calcium supplement intake and CRC was analyzed using Cox regression analysis with prescribed calcium supplement intake as a time-dependent covariate. In these analyses the prescriptions of calcium supplement was compared with non-prescriptions of calcium supplements at the same time point. Time since first calcium dispensing was used as underlying timescale (30).

Dietary calcium intake was first analyzed continuously (per 200 mg). Subsequently, dietary calcium intake was analyzed as a categorical variable after stratification into four categories on the basis of the Recommended Dietary Allowance (RDA) for dietary calcium intake in the Netherlands (1,100 mg/day) (31) ± standard deviation (SD) of the study population. The category that included the RDA was used as reference group for further analyses. To assess linear trends between dietary calcium intake and CRC risk, tests for trend were calculated using the categorical variable as a continuous variable in the Cox proportional hazard models. In addition, prescribed calcium supplement intake were analyzed continuously (per each prescription of calcium supplements) and dichotomously (yes/no) for the analyses. The category that included

no prescribed supplement intake was used as reference group for the dichotomous analyses. Because calcium homeostasis is affected by albumin concentrations, serum total calcium level was adjusted for serum albumin level in a subgroup (available only in RS-I) with the use of the following formula: 0.8 (4.0 – serum albumin level) + serum calcium level (32). Serum total calcium level was analyzed continuously (per each mmol/L) and in quartiles (first quartile as reference).

Potential confounders were added to the sex- and age-adjusted model (Model 2). We also included another model in which we additionally adjusted for BMI and waist circumference (Model 3).

The weighted Genetic Risk Score (GRS) was calculated from seven SNPs for calcium concentration (80% of variance is explained by these SNPs) by multiplying with the effect estimate of each SNP from GWAS on calcium concentration (9). After that, we summed all the scores of all seven SNPs (33). The association between seven SNPs separately as well as the GRS was analyzed by a Cox proportional hazard model. Furthermore, we tested the effect modification by weighted GRS on the association between calcium intake and CRC (*P*-value for statistically significantly interaction <0.10). Additionally, the associations of calcium level and calcium intake were tested (in Model 3) for effect modification by serum 25(OH)D level. The associations were stratified by 25(OH)D level (< 50 and ≥ 50 nmol/l) if the interaction term was below 0.10.

To reduce bias associated with missing data, the multiple imputation procedure according to the fully conditional specification method was used (*n*=10 imputations) (**Supplemental tables S1 and S2**) (34). The percentage of missing data of variables varied from 5.1% for smoking status to 42.8 % for history of diabetes type II (**Supplemental table S2**).

Results are presented as hazard ratios (HRs) and 95% confidence intervals (Cis). The pooled results from the multiple imputation procedure are given for all analyses. Statistical significance was set at $P < 0.05$. All analyses were performed with IBM SPSS Statistics version 25 for Windows.

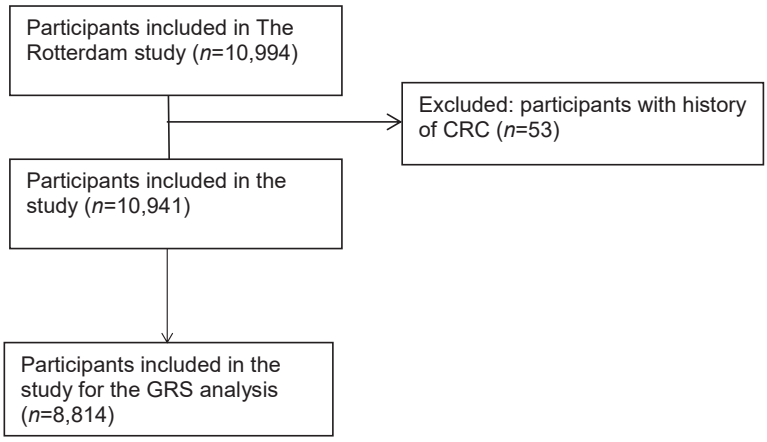

Figure 1. Flowchart of the included study participants

RESULTS

Population characteristics

Baseline characteristics of the study population are presented in **table 1**. Of the 10,941 subjects included in the study (**figure 1**), 427 subjects (3.9 %) were diagnosed with CRC. The incidence rate was 2.9 per 1,000 person-years. The median age of the study population was 67.4 [IQR: 61.0-76.0 years]. The mean intake of dietary calcium intake was 1,116.7 (±390.0) mg/day (unadjusted for energy). Dispensed calcium supplement intake was reported by 17.3% of the study population. The mean of serum total calcium level was 2.4 (±0.1) mmol/L. As shown in **table 1**, the median (IQR) serum 25(OH)D level of the study population was 45.8 [IQR: 29.2-67.7].

Dietary calcium intake and CRC risk

Associations between dietary calcium intake and CRC risk are shown in **table 2**. Dietary calcium was only significantly associated with higher CRC risk in the crude model, when analyzed continuously (HR: 0.93; 95% CI: 0.87-0.99). When analyzed the dietary calcium intake in categories with using the RDA as reference, lower risk was found for high dietary calcium intake (≥1,485 mg/day), compared to the RDA (≥1,100-1,485 mg/day; HR: 0.66; 95% CI: 0.44-1.00 in fully adjusted model).

Table 1. Baseline characteristics of the study population (*n*=10,941)

Characteristics	
CRC cases, n (%)	427 (3.9)
Follow-up, years	13.6 (7.5)
Age, years[c]	67.4 [61.0-76.0]
Women, n (%)	6,543 (59.8)
Education level, n (%)	
Primary solely	6,094 (55.7)
Secondary and higher	4,847 (44.3)
Income, n (%)	
Low to intermediate (<2,400)	5,049 (46.1)
Intermediate to high (≥2,400)	5,892 (53.9)
Total energy intake, kcal/d	1,954.5 (552.4)
Total dietary calcium intake[b], mg/d	1,116.7 (393.0)
Total dietary fiber intake[b], g/d	26.3 (79.4)
Total processed red meat[b], g/d	101.1 (79.4)
Total alcohol intake g/d	9.7 (14.9)
Smoking status, n (%)	
Never/ever	8,679 (79.3)
Current	2,262 (20.7)
History of diabetes mellitus type II, n (%)	1,326 (12.1)
Physical activity[a], MET hours per week	80.8 (44.4)
Body mass index, kg/m2	26.5 (3.9)
Waist circumference, cm	91.4 (11.5)
25(OH)D status[a], nmol/l, median[c]	45.8 [29.2-67.7]
Serum total cholesterol levels, mmol/l	6.4 (1.2)
Serum total calcium level, mmol/l	2.4 (0.1)
Prescribed calcium supplement intake, n (%)	
Once or more	17.3
No	82.7

Values presented as means (SD), unless noted otherwise
[a] Measured during the third follow-up, not at baseline
[b] Adjusted for energy intake
[c] Median [IQR]

Prescribed calcium supplementation and CRC risk

Associations between dispensed calcium supplement intake and CRC risk are shown in **table 3**. No associations were found between dispensed calcium supplement and CRC risk when analyzed continuously or dichotomously (**Table 3**).

Table 2. The association between dietary calcium intake and CRC risk

	CRC cases	Model 1[a] HR (95% CI)	Model 2[b] HR (95% CI)	Model 3[c] HR (95% CI)
Dietary calcium intake - Continuous	322	**0.93 (0.87-0.99)***	0.95 (0.89-1.02)	0.95 (0.89-1.02)
Dietary calcium intake (mg/day) in categories				
Category 1 (≤ 715)	59	**1.47 (1.06-2.04)***	1.32 (0.93-1.89)	1.33 (0.93-1.89)
Category 2 (715-1,100)	120	0.85 (0.65-1.10)	0.83 (0.63-1.09)	0.82 (0.62-1.09)
Category 3 (1,100-1,485)	111	Reference	Reference	Reference
Category 4 (≥ 1,485)	31	**0.65 (0.44-0.97)***	**0.66 (0.44-0.99)***	**0.66 (0.44-1.00)***
***P*-trend[d]**		**0.01***	0.06	0.07

Continuous: per each 200 mg
Categories: on the basis of the Recommended Dietary Allowance and standard deviation
[a]Model 1 was adjusted for cohort, age (years) and sex
[b]Model 2 was adjusted for age (years), sex, education (primary solely, secondary or higher), income (low to intermediate, intermediate to high), history of diabetes type II (no/yes), smoking status (never/ever, current), alcohol intake (g/day), dietary fiber intake (g/day), red meat intake (g/day), serum total cholesterol levels (mmol/l) and physical activity (hours/day)
[c]Model 3 was additionally adjusted for BMI (kg/m^2) and waist circumference (cm)
[d]Test for trend were carried out by entering the categorical variables as continuous variables in Model 3 of the Cox's proportional hazard models
*p-value of < 0.05

Table 3. The association between prescribed calcium supplement and CRC risk

	CRC cases	Model 1[a] HR (95% CI)	Model 2[b] HR (95% CI)	Model 3[c] HR (95% CI)
Prescribed calcium supplement	453			
- Continuous		0.99 (0.98-1.01)	0.99 (0.96-1.01)	0.99 (0.97-1.01)
- Dichotomous (y/n)		0.91 (0.70-1.17)	0.91 (0.58-1.43)	0.94 (0.59-1.49)

Continuous (prescribed calcium intake): per each prescription of calcium supplements
Dichotomous: yes/no
[a]Model 1 was adjusted for cohort, age (years) and sex
[b]Model 2 was adjusted for age (years), sex, education (primary solely, secondary or higher), income (low to intermediate, intermediate to high), history of diabetes type II (no/yes), smoking status (never/ever, current), alcohol intake (g/day), dietary fiber intake (g/day), red meat intake (g/day), serum total cholesterol levels (mmol/l) and physical activity (hours/day)
[c]Model 3 was additionally adjusted for BMI (kg/m^2) and waist circumference (cm)
*p-value of < 0.05

Calcium concentration and CRC risk

The association between total serum calcium level and CRC in RS-I and RS-II is depicted in **table 4**. Total serum calcium level were not associated with CRC risk (**Table 4**), and no linear trend was found (P-trend value >0.19). However, in the sensitivity analysis in RS-I population, where serum total calcium level was adjusted for serum albumin level in a subgroup (RS-I), we found a statistically significantly reduced CRC risk for total serum calcium level and a significantly increased CRC risk for higher albumin-adjusted calcium level **(Supplemental table S3)**.

Table 4. The association between serum total calcium level and CRC risk

	CRC cases	Model 1[a] HR (95% CI)	Model 2[b] HR (95% CI)	Model 3[c] HR (95% CI)
Serum total calcium level - Continuous	257	0.49 (0.19-1.25)	0.47 (0.14-1.51)	0.48 (0.15-1.57)
Serum total calcium level - Categorical				
Quartile 1 (≤ 2.31)	63	Reference	Reference	Reference
Quartile 2 (2.31-2.39)	56	0.82 (0.59-1.15)	0.81 (0.57-1.15)	0.82 (0.58-1.16)
Quartile 3 (2.39-2.46)	40	0.85 (0.60-1.21)	0.80 (0.55-1.16)	0.81 (0.55-1.14)
Quartile 4 (>2.46)	48	0.84 (0.60-1.18)	0.77 (0.53-1.13)	0.78 (0.54-1.14)
P-trend[d]	50	0.35	0.19	0.21

Continuous: per each mmol/l
Categorical: quartiles (mmol/l)
[a]Model 1 was adjusted for cohort, age (years) and sex
[b]Model 2 was adjusted for age (years), sex, education (primary solely, secondary or higher), income (low to intermediate, intermediate to high), history of diabetes type II (no/yes), smoking status (never/ever, current), alcohol intake (g/day), dietary fiber intake (g/day), red meat intake (g/day), serum total cholesterol levels (mmol/l) and physical activity (hours/day)
[c]Model 3 was additionally adjusted for BMI (kg/m^2) and waist circumference (cm)
[d]Test for trend were carried out by entering the categorical variables as continuous variables in Model 3 of the Cox's proportional hazard models
*p-value of < 0.05

Effect modification by calcium concentrations SNP's

The association between 7 SNPs separately as well as GRS is shown in **table 5**: we found no statistically significantly associations.

After evaluating the effect modification by weighted GRS from the calcium concentrations SNPs, we found effect modification with dietary calcium intake by the GRS on CRC risk (p=0.08). No statistically significant effect modification by SNP with serum

Table 5. The association between GRS, 7 SNPs for calcium concentrations and CRC risk (Cox analysis in Model 3)

SNPs of calcium concentration	HR (95% CI)
rs1801725	1.06 (0.78; 1.32)
rs1550532	1.06 (0.87; 1.30)
rs780094	0.96 (0.80; 1.16)
rs10491003	0.93 (0.68; 1.28)
rs7336933	1.14 (0.90; 1.45)
rs1570669	1.13 (0.93; 1.38)
rs7481584	0.99 (0.81; 1.20)
GRS	0.42 (0.02; 7.68)

calcium level and calcium supplementation for CRC risk was found (*P* for interaction 0.56 and 0.98, respectively). After stratification of GRS in low, intermediate and high weighted GRS in the association between dietary calcium intake and CRC risk, we found a significant lower CRC risk for the participants with lower GRS (HR= 0.78 per increase in calcium intake; 95%CI: 0.67-0.92, **table 6**).

Table 6. The association between dietary calcium intake and CRC risk, stratified by GRS score (low, intermediate and high GRS) (n=8,814)

Dietary calcium intake	CRC cases	HR (95% CI)
Low GRS	108	**0.78 (0.67-0.92)***
Intermediate GRS	124	0.94 (0.82-1.08)
High GRS	109	1.05 (0.94-1.18)

Sensitivity analyses on effect-modification by 25(OH)D status

Serum 25(OH)D level was a significant effect-modifier in the association between calcium intake and CRC (p=0.001) and in the association between calcium level and CRC (p=0.04). After stratification for serum 25(OH)D level, dietary calcium intake was associated with lower risk of CRC in subgroup of 25(OH)D level <50 nmol/l, and calcium level was associated with lower risk of CRC in subgroup of 25(OH)D level ≥ 50 nmol/l. (**Supplemental table S4**).

The association of calcium concentration and calcium supplementation with CRC risk was not modified by serum 25(OH)D level (*P* for interaction 0.13 and 0.72, respectively). Finally, a list of results from this study and comparison with literature has been added on **supplemental table S5**.

DISCUSSION

Main findings

In this prospective population-based cohort study, we did not find a consistent association between calcium intake from diet or supplements or total serum calcium level and CRC risk. However, our findings suggest that the association between dietary calcium intake and CRC risk may be modified by the weighted GRS for SNPs for calcium concentrations, with calcium intake associated with a lower CRC risk for those with a low GRS.

Comparison with literature

Our results regarding dietary calcium intake and CRC risk are not fully in line with previous studies. Some prospective cohort studies found inverse associations of dietary calcium intake on CRC risk (8, 35). Moreover, results of combined prospective cohort studies showed a linear association; each 300 mg/day intake of total calcium was inversely associated with approximately 8% reduced CRC risk (36). In our study, we found an inverse association between dietary calcium intake and risk of CRC (Table 2). The discrepancy between our results and those from previous studies may be explained by differences in average dietary calcium intake. The average intake of total calcium intake was below 800 mg/day for the previously studies (8, 16, 36), whereas our study population had a relatively high dietary calcium intake (1,116.7) mg/day).

In contrast to previous findings of studies of the association between dietary intake of calcium and CRC, a meta-analysis of randomized trials found no association between calcium supplement intake and CRC risk over a period of four years (3). It may be argued that the duration of the included trials was too short and probably lacked power to detect effects on CRC risk. As we know, calcium from diet mainly contains calcium phosphate, whereas calcium from supplements generally contains other compounds such as calcium citrate malate. Calcium from supplements has a higher bioavailability than calcium from diet (10).The duration in our study was longer, however, the percentage of calcium prescriptions was around 17%, which is also low powered to discover any association. Moreover, we had no reliable data of the dosage, frequencies of the prescribed supplementation and over-the-counter calcium supplementation.

In our study, serum total calcium level was also not associated with CRC risk. Most studies on serum calcium levels and CRC have been conducted in selected patient group, where hypercalcemia is a well-known characteristic of various malignancies

(19). One previous study showed that serum total calcium levels were associated with a slightly higher risk of CRC risk in women (37). Another study showed that lower serum calcium levels may be a prognostic factor for CRC development (38). Most of the previous studies were conducted using serum calcium levels uncorrected for albumin. Sensitivity analysis in this study showed interestingly a higher risk for CRC for albumin-adjusted calcium levels, which is an important result and were also found in another study (37). This finding suggests that the true association between calcium and colorectal cancer may depend on other factors regulating calcium homeostasis. Differences may also be explained by suggested potential role of SNPs of calcium concentrations in influencing carcinogenesis of colon epithelium and mediating antineoplastic effect of calcium (8). CASR could be associated with CRC survival (39); however, others showed no statistically significant effect modification investigating genome-wide SNPs, associated with calcium level and risk of CRC (40). In the present study, we evaluated whether the seven loci known to be associated with serum calcium concentration, discovered from genome-wide study (9), were associated with CRC. In our study, we found no association between seven SNPs as well as weighted GRS score with CRC risk. Furthermore, only effect modification by the weighted GRS score in the association between total dietary calcium intake and CRC risk was found, suggesting that the protective effect may differ according to different genetic variability for altered calcium levels .

Additionally, the association of dietary calcium intake or calcium level with CRC risk was modified by 25(OH)D status. It is well known from previous evidence that vitamin D can modify the association between calcium level and CRC (41, 42). Previous study showed that 25-hydroxyvitamin D levels were associated with reduced CRC risk for concentrations of >80 nmol/l (43). Stratification by 25(OH)D status, showed that the association between dietary calcium intake and CRC risk appeared to be lower in subjects with a serum 25(OH)D level below 50 nmol/l suggesting that high calcium intake may inhibit the adverse impact of vitamin D deficiency. Besides, the association between calcium level and CRC risk was lower in subjects with a serum 25(OH)D level above 50 nmo/l. Vitamin D and calcium are interrelated. As described previously, vitamin D is important for the absorption of calcium in the gut (41). Like calcium, vitamin D plays an important role in growth restraining, controlling differentiation and apoptosis in cells of the intestines (41). Based on this, the association between calcium intake and CRC risk is hypothesized to become weaker for higher levels of 25(OH)D status (5).

Potential mechanisms

We hypothesized that calcium may be associated with CRC risk through several mechanisms. First, calcium may influence cell growth. Calcium has growth inhibiting properties on normal and tumor intestinal cells and may thereby influence CRC development (5). Also, in *vivo* and in *vitro* studies on human colonic epithelial cells showed that calcium suppresses proliferation and induces apoptosis in the lining of the colon, and thereby protects against CRC development (5, 42). Furthermore, experimental studies in animals and humans showed that calcium may bind to bile acids and fatty acids in the gastrointestinal tract, forming insoluble complexes, such as calcium soaps that protect the lining of the colon, and thereby reduces the risk of CRC (5, 6).

Moreover, we hypothesized that individual common genetic variants of calcium concentrations do modify the association between dietary calcium intake and CRC risk, and indeed we observed such an effect modification by the GRS on the association of calcium intake with CRC. To investigate effect modification by other genetic variants, larger studies with sequence data and genome-wide studies of calcium and CRC risk are needed.

Strengths and limitations

Our study has several strengths and limitations. One of the strengths is the prospective study design, which minimizes recall bias associated with CRC diagnosis. Also, this study had a long follow-up period, which is important because of the long latency period of CRC (44) and it may reduce the influence of reverse causation. Another strength of this study is the large study sample from a population-based setting, which increases the generalizability of the results.

Several potential limitations of our study need to be considered. First, information on dietary intake was obtained by self-report and at baseline of the study. Although diet in middle-aged and older individuals remains fairly consistent over time (45) and we adjusted our analyses for total energy intake to reduce potential measurement error (18), misclassification in calcium intake may still have occurred. Also, measurement error may have occurred since not all dietary supplement intakes were specified in dosages and frequency of usage. Furthermore, calcium homeostasis is affected by i.e. albumin concentrations. Unfortunately, we were only able to perform albumin-adjusted calcium level analysis in a subgroup. Finally, the association between calcium intake and CRC risk may differ in parts of the colon or rectum (46), we could not evaluate potential differences because of a limited number of CRC cases.

CONCLUSION

In this prospective population-based cohort study, we did not find a consistent association between calcium intake from diet , supplements or total serum calcium levels and CRC risk. However, on the basis of SNPs related to calcium concentrations, we observed effect modification of the weighted GRS on the association between dietary calcium intake and CRC risk, with lower risk of CRC by increasing calcium intake in subjects with low weighted GRS score. Considering the increasing incidence of CRC, it is important to further investigate other factors regulating calcium homeostasis and its role on CRC etiology.

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Abbreviations used

BMI: body mass index, CRC: colorectal cancer, FFQ: food frequency questionnaire, HR: hazard ratio, IQR: interquartile range, RDA: Recommended Dietary Allowance, SD: standard deviation

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SUPPLEMENTARY DATA

Table S1. Details of the multiple imputation modelling

	Multiple imputation procedure
Software used	SPSS 25.0 for Windows
Imputation method	Fully conditional specification (Markov chain Monte Carlo method)
Maximum iterations	10
Imputed datasets created	10
Exposures and outcomes (not imputed, used in model as predictor)	CRC, calcium level and dietary calcium intake
Covariates (imputed)	
Additional predictors	Sex, cohort, packyears, alcohol, smoking
Treatment of non-normally distributed variables	Predictive mean matching
Treatment of binary/ categorical variables	Logistic regression models

Number of missing for the variables imputed can be found in supplementary table S2.

Table S2: Basic characteristics before and after multiple imputation

Characteristics	N	Missing (n)	Original data	Imputed data
CRC cases, n (%)	10,941	0	427 (3.9)	*No missing*
Follow-up, years Age, years[c]	10,941	0	13.6 (7.5) 67.4 [61.0-76.0]	*No missing*
Women, n (%)	10,941	0	6,543 (59.8)	*No missing*
Education level, n (%)	8,263	2,687		
Primary solely			4,348 (39.7)	6,094 (55.7)
Secondary and higher			3,915 (35.8)	4,847 (44.3)
Income, n (%)	8,912	2,029		
Low to intermediate (<2,400)			3,808 (34.8)	5,049 (46.1)
Intermediate to high (≥2,400)			5,104 (46.7)	5,892 (53.9)
Total energy intake, kcal/d	6,638	4,303	1,968.7 (549.7)	1,954.5 (552.4)
Total dietary calcium intake[b], mg/d	6,188	4,753	1,116.7 (393.0)	*Not Imputed*
Total dietary fiber intake[b], g/d	6,207	4,734	26.3 (75.9)	26.3 (79.4)
Total processed red meat[b], g/d	6,207	4,734	101.1 (78.0)	101.1 (79.4)
Total alcohol intake[b], g/d	6,404	4,537	9.9 (15.0)	9.7 (14.9)
Smoking status, n (%)	10,379	562		
Never/ever			8,217 (75.1)	8,679 (79.3)
Current			2,162 (19.8)	2,262 (20.7)
History of diabetes mellitus type II, n (%)	6,263	4,678	827 (7,6)	1,326 (12.1)
Physical activity[a], MET hours per week	7,273	3,668	80.8 (44.4)	80.8 (44.4)
Body mass index, kg/m2	9,545	1,396	26.6 (3.9)	26.5 (3.9)
Waist circumference, cm	8.954	1,987	91.4 (11.5)	91.4 (11.5)
25(OH)D status[a], nmol/l, median[c]	6,269	4,672	49.1 [32.4-71.1]	45.8 [29.2-67.7]
Serum total cholesterol levels, mmol/l	9,591	1,350	6.4 (1.2)	6.4 (1.2)
Serum total calcium level, mmol/l	6,636	4,308	2.4 (0.1)	*Not Imputed*

[a]presented as mean (SD); [b]presented as median [interquartile range]

Table S3. The association between serum total calcium level (unadjusted and adjusted for albumin level) and CRC risk in RS-I

	CRC cases	Model 1[a] HR (95% CI)	Model 2[b] HR (95% CI)	Model 3[c] HR (95% CI)
Albumin-adjusted calcium level	356	1.03 (0.95-1.11)	**1.11 (1.00-1.23)***	**1.11 (1.00-1.23)***
Total serum calcium level	356	0.44 (0.16-1.22)	**0.24 (0.06-0.94)***	**0.26 (0.06-1.03)* (p=0.054)**

Continuous: per each mmol/l
[a]Model 1 was adjusted for age (years) and sex
[b]Model 2 was adjusted for age (years), sex, education (primary solely, secondary or higher), income (low to intermediate, intermediate to high), history of diabetes type II (no/yes), smoking status (never/ever, current), alcohol intake (g/day), dietary fiber intake (g/day), red meat intake (g/day), serum total cholesterol levels (mmol/l) and physical activity (hours/day)
[c]Model 3 was additionally adjusted for BMI (kg/m^2) and waist circumference (cm)
*p-value of < 0.05

Table S4. The association between dietary calcium intake and CRC risk stratified by serum 25(OH)D (< and ≥ 50 nmol/l)

Dietary calcium intake	CRC cases	HR (95% CI)	P=0.001**
25(OH)D <50 nmol/l	299	**0.88 (0.79-0.97)***	
25(OH)D ≥50 nmol.l	181	1.01 (0.90-1.12)	
Calcium level			**P=0.04****
25(OH)D <50 nmol/l	299	0.94 (0.21-4.25)	
25(OH)D ≥50 nmol.l	181	**0.06 (0.01-0.65)***	

Continuous: per each 200 mg
*p-value of < 0.05 **p-value of <0.10

Table S5. List of Results from literature

Results from our study	In line with the following studies	In contrast to the following studies
Low CRC risk for high dietary calcium intake (≥1,485 mg/day), compared to the RDA (≥1,100-1,485 mg/day)	Garland et al., 1985 (1) Flood et al., 2005 (2) Abid et al., 2014 (3) Park & Kim, 2015 (4) Zhang et al., 2016 (5) Yang et al., 2018 (6) Meng et al., 2019 (7)	
No association between dispensed calcium supplement and CRC risk	Bristow et al., 2013 (8)	Flood et al., 2005 (2) Barry et al., 2019 (9)
No association between serum calcium level and CRC risk		Fuszek et al., 2004 (10)
A higher CRC risk for higher albumin-adjusted calcium level in a subgroup analysis	Proctor et al., 2010 (11) Wulaningsih et al., 2013 (12)	
No association between 7 SNPs separately as well as GRS and CRC risk	Mahmoudi et al., 2014 (1 of the SNPs) (13)	Jacobs et al., 2010 (some of the SNPs) (14) Zhu et al., 2017 (some of the SNPs) (15)
Effect modification by weighted GRS from calcium concentrations SNPs were found on the association between dietary calcium level and CRC risk. After stratification, a lower CRC risk was found for the participants with lower GRS	Park & Kim, 2015 suggest that gene-diet interactions may possibly alter the associations among dietary intake, genetic polymorphisms, and CRC risk (4)	Figueiredo et al., 2011 (16)
Serum 25(OH)D was also an effect-modifier in the association between calcium intake and calcium level and CRC. After stratification for serum 25(OH)D level, dietary calcium intake was associated with lower risk of CRC in subgroup of 25(OH)D level <50 nmol/l, and calcium level was associated with lower risk of CRC in subgroup of 25(OH)D level ≥ 50 nmol/l.	Ng et al., 2014 (17) Park et al., 2007 (18)	

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