Author Manuscript Published OnlineFirst on February 13, 2019; DOI: 10.1158/1940-6207.CAPR-18-0279
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Calcium intake and risk of colorectal cancer according to tumor infiltrating T cells

Running title: Calcium, immunity, and colorectal cancer

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This is the author's manuscript of the article published in final edited form as:
Yang, W., Liu, L., Keum, N., Qian, Z. R., Nowak, J. A., Hamada, T., Song, M., Cao, Y., Nosho, K., Smith-Warner, S. A., Zhang, S., Masugi, Y., Ng, K., Kosumi, K., Ma, Y., Garrett, W. S., Wang, M., Nan, H., Giannakis, M., … Zhang, X. (2019). Calcium Intake and Risk of Colorectal Cancer According to Tumor-infiltrating T Cells. Cancer Prevention Research. https://doi.org/10.1158/1940-6207.CAPR-18-0279

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Author contributions: M.J.S., W.C.W., C.S.F., A.T.C., K.Ng., S.O., R.N., and X.Z. obtained the funding; X.Z. and S.O. conceived and designed the study; W.Y. and L.L. analyzed the data; W.Y. and X.Z. drafted the paper; All authors interpreted the data, edited or commented, and approved the final manuscript.

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Acknowledgements: We would like to thank the participants and staff of the Nurses' Health Study and Health Professionals Follow-up Study for their valuable contributions as well as the following state cancer registries for their help: AL, AZ, AR, CA, CO, CT, DE, FL, GA, ID, IL, IN, IA, KY, LA, ME, MD, MA, MI, NE, NH, NJ, NY, NC, ND, OH, OK, OR, PA, RI, SC, TN, TX, VA, WA, WY. The authors assume full responsibility for analyses and interpretation of these data.

Abbreviations: CI, confidence interval; FFQ, food frequency questionnaire; HPFS, Health Professionals Follow-up Study; HR, hazard ratio; METS, metabolic equivalent task score; MSI, microsatellite instability; NHS, Nurses' Health Study.

Use of standardized official symbols: We use HUGO (Human Genome Organisation)-approved official symbols (or root symbols) for genes and gene products, including *CASR, CD3, CD8, FOXP3, IL6, IL23, LTA*, and *PTPRC*, all of which are described at www.genenames.org. The official symbols are italicized to differentiate from non-italicized colloquial names that are used along with the official symbols. This format enables readers to familiarise the official symbols for genes and gene products together with common colloquial names.

Funding: This work was supported by the National Institutes of Health (P01 CA87969, UM1 CA186107 to M.J. Stampfer; P01 CA55075, UM1 CA167552 to W.C. Willett; U01 CA167552 to W.C. Willett and L.A. Mucci; P50 CA127003, R01 CA118553, R01 CA169141, R01 CA137178, K24 DK098311 to A.T.C.; R01 CA205406 to K.Ng.; R01 CA151993, R35 CA197735 to S.O.; K07 CA190673 to R.N.; and R03 CA176717, K07 CA188126 to X.Z.); Nodal Award (to S.O.) from the Dana-Farber Harvard Cancer Center; Research supported by a Stand Up To Cancer Colorectal Cancer Dream Team Translational Research Grant (Grant Number: SU2C-AACR-DT22-17) to C.S.F. Stand Up To Cancer is a division of the Entertainment Industry Foundation. Research grants are administered by the American Association for Cancer Research, the Scientific Partner of SU2C; and by grants from The Project P Fund for Colorectal Cancer Research, The Friends of the Dana-Farber Cancer Institute, Bennett Family Fund, and the Entertainment Industry Foundation through National Colorectal Cancer Research Alliance. W.Y. and L.L. were supported by scholarship grants from Chinese Scholarship Council. L.L. was also supported by a fellowship grant from Huazhong University of Science and Technology. K.K. was supported by the JSPS Overseas Research Fellowships grant from the Japan Society for the Promotion of Science (JP2017-775). The content is solely the responsibility of the authors and does not necessarily represent the official views of NIH. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Role of the sponsors: The funders had no role in design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript.

Conflict of interest: The authors have no competing interests to disclose.

Abstract

Calcium intake has been associated with a lower risk of colorectal cancer. Calcium signaling may enhance T cell proliferation and differentiation, and contribute to T-cell mediated antitumor immunity. In this prospective cohort study, we investigated the association between calcium intake and colorectal cancer risk according to tumor immunity status to provide additional insights into the role of calcium in colorectal carcinogenesis. The densities of tumour-infiltrating T-cell subsets (*CD3*$^+$, *CD8*$^+$, CD45RO (*PTPRC*)$^+$, or *FOXP3*$^+$ cell) were assessed using immunohistochemical and computer-assisted image analysis in 736 cancer cases that developed among 136,249 individuals in two cohorts. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated using Cox proportional hazards regression. Total calcium intake was associated with a multivariable HR of 0.55 (comparing ≥1200 vs. <600 mg/day, 95% CI, 0.36–0.84; p_{trend}=0.002) for *CD8*$^+$ T-cell-low but not for *CD8*$^+$ T-cell-high tumours (HR=1.02, 95%CI, 0.67–1.55; p_{trend}=0.47). Similarly, the corresponding HRs (95 CIs) for calcium for low vs. high T-cell infiltrated tumours were 0.63 (0.42–0.94; p_{trend}=0.01) and 0.89 (0.58–1.35; p_{trend}=0.20) for *CD3*$^+$; 0.58 (0.39–0.87; p_{trend}=0.006) and 1.04 (0.69–1.58; p_{trend} =0.54) for *CD45RO*$^+$; and 0.56 (0.36–0.85; p_{trend}=0.006) and 1.10 (0.72–1.67; p_{trend}=0.47) for *FOXP3*$^+$, although the differences by subtypes defined by T cell density were not statistically significant. These potential differential associations generally appeared consistent regardless of sex, source of calcium intake, tumor location, and tumor microsatellite instability status. Our findings suggest a possible role of calcium in cancer immunoprevention via modulation of T cell function.

Keywords: calcium; cancer epidemiology; colorectal cancer; diet; cancer prevention

Introduction

Research on calcium intake and colorectal neoplasia has important public health implications. Calcium is a simple, modifiable, inexpensive agent, and approximately 43% of U.S. adults use supplemental calcium (1). Further, most epidemiological studies (2-4) have reported an inverse association between higher calcium intake and risk of developing colorectal adenoma and cancer. However, evidence from the randomized controlled trials of calcium supplementation has been inconsistent (5,6). Partly because of these discrepant findings, the Institute of Medicine called for more targeted research on calcium and colorectal cancer (7). Most previous studies have investigated total colorectal cancer, but this tumor comprises a group of heterogeneous subtypes (8), and the association with calcium intake may therefore differ by specific molecular subtypes (9). Hence, integrating host factors (such as diet) and tumor molecular features (such as immunity status) may enhance our understanding of the mechanisms through which calcium may act on colorectal carcinogenesis.

Accumulating evidence suggests that effector or cytotoxic (*CD3*$^+$ cells and *CD8*$^+$ cells), memory [CD45RO (*PTPRC*)$^+$ cells], and regulatory (*FOXP3*$^+$ cells) T cells play an important role in colorectal cancer development and prognosis (10-12). Calcium acts as second messenger in lymphocytes that enhances T cell proliferation and regulates its differentiation, and gene expression (13,14). Hence, it is plausible that calcium may influence colorectal carcinogenesis through immunity. In fact, human trials showed that supplementation with calcium could reduce several tumor-promoting inflammation biomarkers (15-17), and reverse the upregulation of expression of genes involved in inflammation and immune response induced by Western-style diet which is low in calcium (18). In light of the biological evidence, we hypothesized that the association between calcium intake and colorectal cancer risk might differ by tumor immunity

status defined by densities of infiltrated T cells in the tumor microenvironment.

To test this hypothesis, we conducted an immunologic molecular pathological epidemiology study (8) by integrating data on calcium intake, colorectal cancer outcomes, and tumor pathological immunity status from two large U.S.-nationwide prospective cohorts, the Nurses' Health Study (NHS) and the Health Professionals Follow-up Study (HPFS). We examined the association between calcium intake and risk of colorectal cancer according to the T cell densities in tumor tissue.

Materials and Methods

Study Population

The study population included 121 700 female participants from NHS and 51 529 male participants from HPFS (19,20). Briefly, for NHS, the recruitment of 121 700 U.S. female registered nurses aged 30 to 55 years was completed in 1976. For HPFS, the recruitment of 51 529 U.S. male professionals aged 40 to 75 years was completed in 1986. In both cohorts, questionnaires were administrated biennially to collect and update information on demographic characteristics, lifestyle factors, and medical history, with follow-up rates over 90% in each cohort. This study was approved by the institutional review boards of the Brigham and Women's Hospital and Harvard T.H. Chan School of Public Health. In this study, we excluded participants with a history of cancer (except for non-melanoma skin cancer), polyposis syndrome, ulcerative colitis/Crohn's disease, implausible energy intakes at baseline (<600 or >3500 kcal/day for women, or <800 or >4200 kcal/day for men), or with no reports of calcium intake. After exclusion, a total of 136 249 participants (88 509 women and 47 740 men) were

included in the present analysis. A flow chart showing how the study population for analysis was developed is presented in Supplementary Figure 1.

Assessments of calcium intake and other dietary factors

Details on assessments of calcium intake as well as other dietary factors were described previously (2,9,21). In brief, we used validated (22,23) semi-quantitative food frequency questionnaires (FFQs) to collect dietary information at baseline and every 4 year thereafter. The energy-adjusted correlation coefficients of total calcium intake comparing the FFQs and the average of multiple 1-week diet records were 0.61 for men (22) and 0.63 for women (23). The correlation coefficients for dietary calcium intake were 0.60 for men (22) and 0.70 for women (23). We also collected information on dietary factors including intakes of alcohol, vitamin D, folate, red meat and processed meat (22,24).

Assessments of covariates

We collected information on potential colorectal cancer risk factors including height, adult body weight, physical activity (metabolic equivalent task score [METS]-hours/week), cigarette smoking, sigmoidoscopy/colonoscopy screening, family history of colorectal cancer, aspirin use, and menopausal status, and use of menopausal hormones on the baseline and updated in biennial follow-up questionnaires.

Ascertainment of colorectal cancer cases

The incident colorectal cancer cases were defined as a primary tumor with International Classification of Diseases-9 codes of 153 and 154. Participants from the two cohorts were

asked for written permission to obtain medical records and pathological reports if they reported colorectal cancer on biennial questionnaires. We searched state vital statistics records, the National Death Index, to identify additional unreported cancer deaths. For all deaths attributable to colorectal cancer, we requested permission from next-of-kin to review medical records. All possible cancer cases were further confirmed through review of medical and pathological records. A study physician who was blinded to exposure data abstracted information on tumor anatomic location, stage, and histology type. We included colon and rectal carcinoma cases based on the colorectal continuum model (25,26).

Tumor immunity and molecular analyses

We constructed tissue microarray (27), and assessed *CD3*$^+$ cell, *CD8*$^+$ cell, CD45RO (*PTPRC*)$^+$ cell, and *FOXP3*$^+$ cell densities in tumor tissue using immunohistochemistry. We used image analysis through an automated scanning microscope and the Ariol image analysis system (Genetix, San Jose, California, USA) to calculate the average density (cells per mm^2) of each T cell subset in tissue microarray cores, as previously reported (10). We classified each of the T-cell densities (cells per mm^2) into quartiles (Q1–Q4) and divided cases into two groups: low (Q1–Q2) or high (Q3–Q4) in the analyses for statistical efficiency. We also analyzed tumor microsatellite instability (MSI) status and calcium sensing receptor (*CASR*) expression as previously reported (9,28,29). DNA from paraffin-embedded tissue was extracted. The status of MSI was determined by analyzing variability in the length of the microsatellite markers from tumor DNA compared to normal DNA, including D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67, and D18S487 (29). As previously described (9), we constructed tissue microarrays from colorectal cancer blocks, and conducted immunohistochemistry for CASR. CASR expression levels in all cases were reviewed by Y.M. For agreement study, selected tumors (n=118) were independently examined by a second

observer (Z.R.Q.), and the concordance between the two observers (Y.M. and Z.R.Q) was reasonable with a weighted κ of 0.71 (95% CI: 0.61–0.82) (9).

Statistical analysis

Age-adjusted and multivariable-adjusted cohort-specific hazard ratios (HRs) and 95% confidence intervals (CIs) for each colorectal cancer subtype according to the densities of tumor-infiltrated T-cell subsets (i.e., *CD3*$^+$ cells, *CD8*$^+$ cells, CD45RO$^+$ cells, and *FOXP3*$^+$ cells) were calculated using the duplication method Cox proportional hazards regression model (30). This method permits the estimation of separate regression coefficients for the exposure stratified by CRC subtype defined by the densities of tumor-infiltrated T-cell subsets (30). The model was stratified simultaneously by age (in months) and year of questionnaire return (every two year since baseline questionnaire), accounting for the finest possible control of confounding for age and secular trends. Person-years of follow-up were calculated from the date of baseline questionnaire return to the date of diagnosis of colorectal cancer, date of death, loss to follow-up, or the end of follow-up (June 1, 2012 for the NHS and January 31, 2012 for the HPFS), whichever came first. Cancer cases without tumor immunity data were censored at diagnosis. We used the energy-adjusted(31) cumulative average intake of total calcium as reported on all available questionnaires up to the start of each 4-year follow-up interval as the main exposure (2), to minimize within-person variation and to better reflect long-term intake. Likewise, we used cumulative average for covariates and modeled them as time-varying variables when appropriate to allow for potential changes over follow-up periods. The adjusted covariates as well as their categorizations in the multivariable models were shown in Table 1 and Table 2 footnotes. We found no violation of proportional hazard assumption.

Our primary hypothesis testing was the heterogeneity test on the subtype-specific associations (statistical linear trends) of calcium intake with risk of colorectal cancer subtypes classified by densities of tumor-infiltrating T-cells. Considering multiple hypothesis testing for our four primary hypotheses associated with 4 immunity variables (i.e., densities of *CD3*$^+$ cells, *CD8*$^+$ cells, CD45RO$^+$ cells, and *FOXP3*$^+$ cells), we adjusted α level to 0.01 (≈0.05/4) by Bonferroni correction. All other analyses including evaluations of individual HRs and evaluations of a statistical linear trend in a specific stratum represent secondary analyses. We examined the statistical significance of the differences in association according to cancer subtypes using the likelihood ratio test that compared the model fit that allowed separate associations by different tumor immunity status with the model fit that assumed a common effect (30). Trend tests were conducted using the median of each category of total calcium intake as a continuous variable. To maximize statistical power, we combined the results from the two cohorts since we did not observe any significant heterogeneity between sex ($p_{heterogeneity}$ for sex=0.16).

In secondary analyses, we examined the associations between calcium intake and colorectal cancer risk according to the densities of tumor-infiltrated T cells by sex, tumor location, and source of calcium intake. We also explored a time-lagged analysis (2) using 8-year time latency. To account for potential confounding by tumor MSI status, we further evaluated these associations jointly by tumor-infiltrated T cells and MSI status. Lastly, we assessed the associations stratified by tumor *CASR* status because we speculated that *CASR* may partially mediate the potential effect of calcium on colorectal cancer immunoprevention (9). All analyses were performed using the SAS software (SAS Institute, Version 9.2, Cary, NC).

Results

During up to 32 years of follow-up of 136 249 participants (88 509 women and 47 740 men) in these prospective cohorts, we identified 3079 colorectal adenocarcinoma cases. Among cases with available tissue specimens, we could assess T-cell infiltration in the tumor microenvironment for 736 cases (472 women and 264 men). The included colorectal cancer cases with immunity data were comparable to all eligible colorectal cancer patients without immunity data (Supplementary Table 1). Participants with lower total calcium intake were more likely to be current smokers, consumed more alcohol, and tended to have higher intake of red meat, processed meat, and fat, but less vitamin D and folate (Table 1).

As shown in Table 2, we found that higher calcium intake appeared to be associated with a lower risk of colorectal carcinomas containing low densities of *CD8*$^+$ cells (p_{trend}=0.002) but not with risk of carcinoma containing high densities of CD8$^+$ cells (p_{trend}=0.47), although the difference was not statistically significant ($p_{heterogeneity}$=0.06, with the adjusted α of 0.01 by Bonferroni correction). Specifically, compared to calcium intake of <600 mg/day, calcium intake of ≥1200 mg/day was associated with a multivariable HR of 0.55 (95% CI, 0.36–0.84) for *CD8*$^+$ T-cell-low tumors and of 1.02 (95% CI, 0.67–1.55) for *CD8*$^+$ T-cell-high tumors. Similarly, the corresponding HRs (95 CIs) for low vs. high T-cell tumours were 0.63 (0.42–0.94; p_{trend} =0.01) and 0.89 (0.58–1.35; p_{trend}=0.20) for *CD3*$^+$ ($p_{heterogeneity}$=0.30); 0.58 (0.39–0.87; p_{trend}=0.006) and 1.04 (0.69–1.58; p_{trend} =0.54) for *CD45RO*$^+$ ($p_{heterogeneity}$=0.09); and 0.56 (0.36–0.85; p_{trend}=0.006) and 1.10 (0.72–1.67; p_{trend}=0.47) for *FOXP3*$^+$ ($p_{heterogeneity}$=0.04),although the differences by subtypes defined by T cell density were not statistically significant for any of the T cells examined.

Though statistical power was generally limited, the stronger inverse associations of calcium intake with tumours infiltrated with low densities of T cells but not high generally appeared consistent regardless of sex (Supplementary Table 2 and Supplementary Table 3), source of

calcium intake (Table 3), tumor location (Supplementary Table 4), tumor MSI status (Supplementary Table 5), and time-lagged analyses (Supplementary Table 6). Interestingly, the potential differential associations appeared slightly stronger in *CASR* positive tumors (Supplementary Table 7).

Discussion

In these two large prospective cohort studies, we found that higher calcium intake appeared to be primarily associated with lower risk of colorectal cancer infiltrated with low, but not high, densities of T cells regardless of the type of T cell examined, although the differences in the associations by subtype were not statistically significant for any of the T cells examined. These suggestive differential associations generally persisted regardless of sex, source of calcium intake, tumor location, and tumor microsatellite instability status. Our findings suggest a possible role of calcium in colorectal cancer immunoprevention (32) through modulation of T cells.

The role of immunity in cancer development and progression is becoming increasingly recognized (33-36). In this study, we investigated whether the potential anti-cancer effect of calcium on colorectal cancer differs by immune status in the tumor microenvironment. The observed differential associations by tumor immunity status suggest potential crosstalk between calcium intake and host immunity in affecting colorectal carcinogenesis. In the immune system, calcium is essential for diverse cellular functions including proliferation, differentiation, and effector function (37). Changes in the flux of calcium ions (Ca^{2+}) through Ca^{2+} channels in lymphocyte membranes play an important role in the regulation of T cell function and immunity (13,14,38). Of note, dysregulated Ca^{2+} responses are critical for T cell-mediated autoimmunity and inflammation including inflammatory bowel disease (38,39), a risk factor for colorectal

cancer (15). In line with experimental studies showing a potential effect of calcium on immunity, clinical trials have shown that supplementation with calcium reduces several tumor-promoting inflammation biomarkers (15-17). Furthermore, a recent human crossover trial (18) showed that consumption of a Western-style diet (characterized by low calcium and vitamin D) modestly upregulated genes (e.g., human leukocyte antigen class genes) which are involved in inflammation and immune response. In contrast, supplementation of calcium (but not vitamin D) to Western-style diet reversed these deleterious effects, and upregulated genes in the anti-inflammatory interferon signaling and the *IL23* pathways (18).

It is also possible that calcium exerts its immunomodulatory effect partially via *CASR*. The *CASR*, a calcium-binding G protein-coupled receptor, is expressed in the entire intestinal epithelium and plays a key role in the preservation of gut microbiota and immune homeostasis (40-42). The *CASR* is also functionally expressed in human T lymphocytes (43). Evidence shows that intestinal epithelial *CASR* deficiency enhances permeability of the epithelial barrier, leading to the translocation and dissemination of luminal bacteria and activation of local and systemic innate and adaptive proinflammatory immune responses (44). In addition, calcium may promote T lymphocyte function through activation of *CASR* to secrete cytokines including *IL6* and *LTA* (TNF-β) (43), which may play important roles in immune defense as well as systemic inflammatory response. Collectively, our data support that calcium exerts its immunomodulatory effect partially via *CASR* as the differential associations we observed by immunity status appeared slightly stronger in *CASR*-positive tumors than in *CASR*-negative tumors (see Supplementary Table 7). However, the exact mechanisms underlying these differential associations remain unclear. We emphasize that our study remains hypothesis generating and requires confirmation from independent studies.

Our study also suggests a different role of host immunity in mediating the effect of calcium and vitamin D in colorectal cancer chemoprevention because we previously found that the inverse association for plasma 25(OH)D was stronger for risk of colorectal cancer subtypes with intense immune reactions (35). Consistently, the aforementioned human crossover trial found that supplementing the Western-style diet with $1,25(OH)_2D_3$ upregulated genes involved in immune response and inflammation pathways, whereas calcium supplementation largely abrogated these changes (18).

Recent studies showed that MSI-high colorectal cancers were sensitive to immune checkpoint blockade (45,46), indicating an important interplay between MSI status and immune cells. MSI-high tumors have frame shift mutations in coding sequences throughout the genome, which may elicit intense and more diverse immune responses and improve cancer survival (47,48). In the current study, however, the observed differential associations appeared to be independent of MSI status. This suggests that MSI status is not the sole determinant of tumor immune response since the levels of T cell infiltrates overlap considerably between MSI-high and non-MSI-high tumors, though are on average higher in MSI-high cancers (10).

Our present study has several strengths, including prospective cohort design, high follow-up rates, validated colorectal cancer outcomes, and the use of repeated measures of calcium and other covariates during follow-up of the cohorts. The integration of tumor immunology analyses into the framework of molecular pathological epidemiology is an emerging research area (49)(50), which enabled us to better understand etiological heterogeneity according to tumor molecular and immune features. However, several limitations should be noted. First, despite the overall large sample size of the cohorts, we had a limited number of cases with tumor tissue data on T cell infiltration for the secondary analyses by anatomic subsites, sources of calcium intake, tumor MSI or *CASR* status. Second, the inclusion of cancer cases with available tissue

specimen may introduce potential selection bias. However, cases that provided tumor tissue were comparable to all eligible cases with regard to a number of demographic, dietary and lifestyle factors. Third, because most of participants in our study are Caucasian U.S. health professionals, the generalizability of our findings to the general population is limited. However, little heterogeneity across diverse populations has been suggested in the association between calcium intake and risks of colorectal cancer (3). Lastly, we cannot rule out residual confounding although we have adjusted for a wide range of known risk factors for colorectal cancer.

In summary, we found inverse associations between calcium intake and risk of colorectal cancers with low densities of T cell infiltration, but not with risk of colorectal cancers with high densities of T cell infiltration, although the differences by subtypes defined by T cell density were not statistically significant for any of the T cells examined. Our results suggest a possible immunomodulatory effect of calcium in colorectal carcinogenesis. Future studies are warranted to confirm our findings and elucidate the underlying mechanisms for colorectal cancer immunoprevention by calcium.

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Table 1. Baseline characteristics of participants by frequency of total calcium intake in the Nurses' Health Study (1980) and Health Professionals Follow-up Study (1986)

	Total calcium intake (mg/d)				
	<600	**600-799**	**800-999**	**1000-1199**	**≥1200**
Women (Nurses' Health Study)					
No.	34,137	24,290	14,732	8,325	7,022
Age, years*	46.5(7.0)	46.8(7.2)	46.8(7.3)	46.7(7.4)	47.1(7.4)
White, %	96.4	98.0	98.3	98.4	98.2
Body mass index, kg/m²	24.0(4.2)	24.0(4.2)	24.1(4.1)	24.2(4.1)	24.4(4.4)
Activity, METS-hours/week	12.5(18.0)	14.2(19.7)	15.2(21.5)	15.3(21.0)	16.2(26.4)
Family history of colorectal cancer, %	7.9	7.8	7.9	7.9	7.7
Regular aspirin use (2 or more tablets/week), %	33.1	33.4	32.7	31.4	30.6
Past smoking, %	25.3	28.8	29.2	28.9	28.5
Current smoking, %	32.0	27.5	26.4	26.0	26.1
Multivitamin use, %	28.5	33.8	37.9	40.6	45.0
History of sigmoidoscopy/endoscopy, %	9.9	10.0	10.0	10.6	10.4
Postmenopausal status, %	45.0	44.0	44.2	43.9	44.8
Postmenopausal hormone use, %	18.3	18.5	18.8	19.3	19.2
Total energy intake, kcal/day	1573(513)	1546(484)	1565(518)	1602(481)	1569(497)
Dietary calcium intake, mg/day	457(97)	691(61)	883(71)	1078(88)	1376(263)
Dairy calcium intake, mg/day	211(94)	413(97)	595(113)	791(132)	1082(287)
Supplemental calcium intake**, mg/day	358(434)	372(426)	382(424)	392(433)	402(456)
Alcohol, g/day	7.8(12.5)	6.2(9.6)	5.5(8.8)	4.8(8.1)	3.9(7.2)
Total folate intake, μg/day	311(228)	365(236)	399(253)	417.2(262)	503(504)
Total vitamin D, IU/day	238(227)	309(238)	378(252)	451(268)	606(489)
Red meat, servings/week	3.2(2.3)	2.4(1.8)	2.1(1.7)	1.9(1.5)	1.5(1.4)
Processed meat, servings/week	1.3(1.9)	1.2(1.8)	1.0(1.6)	0.9(1.6)	0.7(1.2)
Total fat, g/day	73.6(14.3)	69.9(12.6)	66.9(12.8)	64.9(12.8)	61.9(13.6)
Total fiber, g/day	15.4(5.6)	17.4(6.2)	18.1(6.9)	17.9(6.9)	17.6(7.4)
ω-3 polyunsaturated fatty acids, g/day	0.2(0.1)	0.2(0.2)	0.2(0.2)	0.2(0.2)	0.2(0.2)
ω-6 polyunsaturated fatty acids, g/day	6.4(2.5)	6.3(2.4)	6.1(2.4)	6.0(2.4)	5.9(2.6)
Men (Health Professional Follow-up study)					

No.	10,817	13,820	9,049	5,328	8,726
Age, years*	54.0(9.6)	53.9(9.7)	54.5(9.9)	54.6(9.9)	55.8(9.8)
White, %	93.3	95.8	96.7	97.0	97.3
Body mass index, kg/m²	25.6(3.4)	25.6(3.3)	25.5(3.2)	25.4(3.3)	25.4(3.3)
Activity, METS-hours/week	18.3(27.3)	20.8(28.5)	22.3(31.7)	21.8(31.5)	22.6(30.5)
Family history of colorectal cancer, %	8.7	8.3	8.3	8.6	8.5
Regular aspirin use (2 or more tablets/week), %	26.9	28.9	30.1	31.0	31.3
Past smoking, %	43.5	42.9	40.9	40.5	39.6
Current smoking, %	12.4	9.7	8.2	8.9	8.1
Multivitamin use, %	50.6	57.5	62.0	67.3	74.2
History of sigmoidoscopy/endoscopy, %	24.0	26.2	26.9	26.7	27.1
Total energy intake, kcal/day	1957(638)	1994(605)	1956(632)	2111(631)	1959(583)
Dietary calcium intake, mg/day	500(76)	683(78)	845(115)	982(109)	1180(395)
Dairy calcium intake, mg/day	201(76)	357(98)	506(136)	643(206)	838(409)
Supplemental calcium intake, mg/day	7(22)	21(55)	52(103)	118(180)	423(550)
Alcohol, g/day	15.5(19.3)	11.8(14.9)	9.6(13.3)	9.9(14.2)	8.2(12.1)
Total folate intake, μg/day	381(210)	447(227)	497(251)	529(287)	612(363)
Total vitamin D, IU/day	272(241)	338(253)	407(279)	488(291)	637(371)
Red meat, servings/week	2.2(1.9)	1.9(1.6)	1.6(1.5)	1.7(1.5)	1.4(1.4)
Processed meat, servings/week	1.4(2.0)	1.3(1.8)	1.1(1.8)	1.2(1.9)	1.0(1.7)
Total fat, g/day	73.5(14.6)	72.2(13.3)	70.2(13.7)	70.6(13.8)	68.6(14.5)
Total fiber, g/day	19.1(6.4)	21.1(6.5)	22.3(7.1)	21.6(7.6)	21.8(7.9)
ω-3 polyunsaturated fatty acids, g/day	0.3(0.3)	0.3(0.3)	0.3(0.3)	0.3(0.3)	0.3(0.2)
ω-6 polyunsaturated fatty acids, g/day	12.2(3.8)	12.0(3.5)	11.6(3.4)	11.5(3.4)	10.9(3.4)

Values are means (SD) or percentages and are standardized to the age distribution of the study population.
* Value is not age adjusted.
** Calcium supplement data of NHS were based on questionnaires returned in 1986.

Table 2. Total calcium intake and risk of colorectal cancer according to densities of tumor-infiltrating T-cell subsets in the Nurses' Health Study (1980-2012) and Health Professionals Follow-up Study (1986-2012)

	Total calcium intake (mg/d)						
	<600	600-799	800-999	1000-1199	≥1200	p_{trend}*	$p_{heterogeneity}$¶
Total colorectal cancer							
Person-years (n=3,663,039)	617,339	889,849	809,364	596,191	750,297		
No. cases (n=736)	116	207	176	121	116		
Age-adjusted HR (95% CI)	1 (ref)	1.10 (0.87-1.38)	1.00 (0.79-1.27)	0.94 (0.72-1.21)	0.68 (0.53-0.89)	0.0002	
Multivariable HR (95%CI) §	1 (ref)	1.12 (0.89-1.42)	1.04 (0.80-1.34)	1.01 (0.76-1.34)	0.80 (0.60-1.08)	0.04	
CD3$^+$							
Low							
No. cases (n=347)	64	103	73	58	49		
Age-adjusted HR (95% CI)	1 (ref)	1.01 (0.74-1.39)	0.78 (0.56-1.10)	0.85 (0.59-1.22)	0.55 (0.38-0.80)	0.0004	0.34
Multivariable HR (95% CI)§	1 (ref)	1.02 (0.74-1.40)	0.80 (0.56-1.14)	0.89 (0.61-1.31)	0.63 (0.42-0.94)	0.01	0.30
High							
No. cases (n=350)	48	98	91	56	57		
Age-adjusted HR (95% CI)	1 (ref)	1.22 (0.86-1.73)	1.20 (0.84-1.71)	1.00 (0.67-1.48)	0.76 (0.52-1.13)	0.03	
Multivariable HR (95% CI)§	1 (ref)	1.24 (0.88-1.77)	1.24 (0.86-1.78)	1.07 (0.71-1.61)	0.89 (0.58-1.35)	0.20	
CD8$^+$							
Low							
No. cases (n=339)	59	93	86	55	46		
Age-adjusted HR (95% CI)	1 (ref)	0.95 (0.68-1.32)	0.91 (0.65-1.27)	0.77 (0.53-1.11)	0.48 (0.33-0.72)	<0.0001	0.06
Multivariable HR (95% CI)§	1 (ref)	0.95 (0.68-1.33)	0.92 (0.65-1.30)	0.80 (0.54-1.18)	0.55 (0.36-0.84)	0.002	0.06
High							
No. cases (n=344)	47	104	79	57	57		
Age-adjusted HR (95% CI)	1 (ref)	1.38 (0.97-1.95)	1.16 (0.80-1.67)	1.17 (0.79-1.74)	0.90 (0.60-1.33)	0.14	
Multivariable HR (95% CI)§	1 (ref)	1.40 (0.98-1.98)	1.18 (0.81-1.72)	1.24 (0.82-1.87)	1.02 (0.67-1.55)	0.47	
CD45RO$^+$							
Low							
No. cases (n=348)	65	98	80	57	48		
Age-adjusted HR (95% CI)	1 (ref)	0.91 (0.66-1.24)	0.81 (0.58-1.13)	0.79 (0.55-1.13)	0.50 (0.35-0.74)	0.0002	0.11
Multivariable HR (95% CI)§	1 (ref)	0.92 (0.67-1.27)	0.84 (0.59-1.18)	0.84 (0.57-1.23)	0.58 (0.39-0.87)	0.006	0.09
High							
No. cases (n=359)	47	101	89	60	62		
Age-adjusted HR (95% CI)	1 (ref)	1.35 (0.96-1.92)	1.25 (0.87-1.79)	1.14 (0.77-1.68)	0.89 (0.61-1.31)	0.12	
Multivariable HR (95% CI)§	1 (ref)	1.38 (0.97-1.96)	1.29 (0.89-1.86)	1.22 (0.81-1.82)	1.04 (0.69-1.58)	0.54	

FOXP3^{+}							
Low							
No. cases (n=336)	61	89	89	55	42		
Age-adjusted HR (95% CI)	1 (ref)	0.91 (0.65-1.26)	0.98 (0.70-1.36)	0.81 (0.56-1.17)	0.47 (0.32-0.71)	0.0001	0.04
Multivariable HR (95% CI)§	1 (ref)	0.92 (0.66-1.29)	1.01 (0.72-1.43)	0.87 (0.59-1.28)	0.56 (0.36-0.85)	0.006	0.04
High							
No. cases (n=337)	45	95	74	59	64		
Age-adjusted HR (95% CI)	1 (ref)	1.30 (0.91-1.85)	1.05 (0.72-1.53)	1.16 (0.78-1.72)	0.94 (0.64-1.39)	0.29	
Multivariable HR (95% CI)§	1 (ref)	1.32 (0.92-1.89)	1.07 (0.73-1.59)	1.23 (0.81-1.86)	1.10 (0.72-1.67)	0.87	

CI, confidence interval; HR, hazard ratio.
Duplication-method Cox proportional cause-specific hazards regression for competing risks data was used to compute HRs and 95% CIs.
All analyses were stratified by **age** (in month), **year of questionnaire return** and **sex**.
*: Linear trend test using the median intake of each category.
¶: The likelihood ratio test was used to test for the heterogeneity of the association between total calcium intake and colorectal cancer risk by densities of tumor-infiltrating T-cell subsets.
§: Multivariable hazard ratios were adjusted for **age** (in month), **race** (Caucasian vs. non-Caucasian), **adult BMI** (< 25, 25 -< 27.5, 27.5 -< 30, or ≥ 30 kg/m^2), smoking (0, 1-10, or > 10 pack-years), **history of colorectal cancer in a parent or sibling** (yes or no), **history of sigmoidoscopy/colonoscopy** (yes or no), **physical activity** (< 3, 3-< 27, ≥ 27 MET-hrs/wk), **regular aspirin use** (yes, no), **alcohol consumption** (0 -< 5, 5 -< 15, or ≥ 15 g/d), energy-adjusted total intake of **folate**, **vitamin D**, **red meat** and **processed meat** (all in tertiles).

Table 3. Intake of dietary calcium, dairy calcium and calcium supplement and risk of colorectal cancer according to densities of tumor-infiltrating T-cell subsets in the Nurses' Health Study (1980-2012) and Health Professionals Follow-up Study (1986-2012)

	Dietary calcium intake (mg/d)					
	<600	**600-749**	**750-899**	**≥900**	p_{trend}*	$p_{heterogeneity}$¶
Total colorectal cancer						
Person-years (n=3,663,039)	1,006,115	1,018,393	769,809	868,723		
No. cases (n=736)	205	210	160	161		
Age-adjusted HR (95% CI)	1 (ref)	0.97 (0.80-1.17)	0.98 (0.79-1.20)	0.86 (0.69-1.05)	0.15	
Multivariable HR (95%CI) §	1 (ref)	1.00 (0.82-1.23)	1.04 (0.83-1.30)	0.96 (0.76-1.21)	0.74	
$CD3^+$						
Low						
No. cases (n=347)	110	97	62	78		
Age-adjusted HR (95% CI)	1 (ref)	0.84 (0.64-1.11)	0.74 (0.54-1.01)	0.80 (0.60-1.08)	0.12	0.78
Multivariable HR (95% CI)§	1 (ref)	0.87 (0.66-1.15)	0.77 (0.56-1.06)	0.88 (0.64-1.20)	0.36	0.74
High						
No. cases (n=350)	90	102	87	71		
Age-adjusted HR (95% CI)	1 (ref)	1.05 (0.79-1.39)	1.16 (0.86-1.56)	0.82 (0.60-1.12)	0.25	
Multivariable HR (95% CI)§	1 (ref)	1.08 (0.81-1.45)	1.23 (0.90-1.67)	0.90 (0.65-1.26)	0.63	
$CD8^+$						
Low						
No. cases (n=339)	101	100	72	66		
Age-adjusted HR (95% CI)	1 (ref)	0.94 (0.71-1.25)	0.91 (0.67-1.23)	0.74 (0.54-1.01)	0.06	0.36
Multivariable HR (95% CI)§	1 (ref)	0.97 (0.73-1.28)	0.94 (0.69-1.29)	0.80 (0.58-1.12)	0.20	0.36
High						
No. cases (n=344)	91	98	77	78		
Age-adjusted HR (95% CI)	1 (ref)	1.01 (0.76-1.35)	1.05 (0.77-1.43)	0.91 (0.67-1.24)	0.53	
Multivariable HR (95% CI)§	1 (ref)	1.04 (0.78-1.39)	1.10 (0.80-1.51)	0.99 (0.71-1.37)	0.93	
$CD45RO^+$						
Low						
No. cases (n=348)	106	94	66	82		
Age-adjusted HR (95% CI)	1 (ref)	0.82 (0.62-1.09)	0.77 (0.56-1.05)	0.81 (0.60-1.09)	0.18	0.62
Multivariable HR (95% CI)§	1 (ref)	0.85 (0.64-1.13)	0.82 (0.59-1.12)	0.90 (0.66-1.23)	0.52	0.57
High						
No. cases (n=359)	93	108	83	75		
Age-adjusted HR (95% CI)	1 (ref)	1.11 (0.84-1.47)	1.13 (0.84-1.52)	0.91 (0.67-1.24)	0.51	

Multivariable HR (95% CI)§	1 (ref)	1.15 (0.87-1.53)	1.20 (0.88-1.64)	1.03 (0.74-1.42)	0.91	
FOXP3$^+$						
Low						
No. cases (n=336)	104	89	62	81		
Age-adjusted HR (95% CI)	1 (ref)	0.81 (0.61-1.08)	0.75 (0.55-1.04)	0.84 (0.62-1.12)	0.22	0.57
Multivariable HR (95% CI)§	1 (ref)	0.85 (0.64-1.14)	0.81 (0.59-1.12)	0.94 (0.68-1.28)	0.64	0.59
High						
No. cases (n=337)	82	102	85	68		
Age-adjusted HR (95% CI)	1 (ref)	1.18 (0.88-1.58)	1.30 (0.95-1.76)	0.93 (0.67-1.28)	0.68	
Multivariable HR (95% CI)§	1 (ref)	1.23 (0.91-1.65)	1.38 (1.01-1.90)	1.03 (0.73-1.45)	0.82	

	Dairy calcium intake (mg/d)					
	0-299	**300-499**	**500-699**	**≥700**		
Total colorectal cancer						
Person-years (n=3,663,039)	1,045,066	1,372,061	749,951	495,962		
Cases, No. (n=736)	223	266	149	98		
Age-adjusted HR (95% CI)	1 (ref)	0.90 (0.75-1.07)	0.90 (0.73-1.11)	0.87 (0.69-1.11)	0.25	
Multivariable HR (95%CI) §	1 (ref)	0.92 (0.76-1.10)	0.95 (0.76-1.19)	0.97 (0.75-1.26)	0.83	
CD3$^+$						
Low						
No. cases (n=347)	111	126	64	46		
Age-adjusted HR (95% CI)	1 (ref)	0.87 (0.67-1.12)	0.80 (0.59-1.09)	0.84 (0.59-1.19)	0.24	0.99
Multivariable HR (95% CI)§	1 (ref)	0.88 (0.68-1.14)	0.84 (0.61-1.15)	0.92 (0.64-1.32)	0.52	0.98
High						
No. cases (n=350)	107	124	76	43		
Age-adjusted HR (95% CI)	1 (ref)	0.86 (0.66-1.11)	0.93 (0.70-1.26)	0.78 (0.55-1.11)	0.24	
Multivariable HR (95% CI)§	1 (ref)	0.88 (0.68-1.14)	0.98 (0.72-1.33)	0.86 (0.59-1.25)	0.54	
CD8$^+$						
Low						
No. cases (n=339)	100	131	71	37		
Age-adjusted HR (95% CI)	1 (ref)	0.99 (0.76-1.28)	0.95 (0.70-1.29)	0.76 (0.52-1.10)	0.17	0.61
Multivariable HR (95% CI)§	1 (ref)	1.00 (0.77-1.30)	0.99 (0.72-1.36)	0.82 (0.55-1.23)	0.40	0.62
High						
No. cases (n=344)	109	118	66	51		
Age-adjusted HR (95% CI)	1 (ref)	0.81 (0.62-1.05)	0.82 (0.60-1.12)	0.91 (0.65-1.28)	0.50	
Multivariable HR (95% CI)§	1 (ref)	0.83 (0.63-1.08)	0.85 (0.62-1.17)	1.00 (0.70-1.42)	0.84	

CD45RO$^+$						
Low						
No. cases (n=348)	113	119	67	49		
Age-adjusted HR (95% CI)	1 (ref)	0.81 (0.62-1.05)	0.81 (0.60-1.10)	0.86 (0.61-1.21)	0.35	0.80
Multivariable HR (95% CI)§	1 (ref)	0.82 (0.63-1.07)	0.85 (0.62-1.16)	0.95 (0.66-1.35)	0.71	0.72
High						
No. cases (n=359)	104	132	77	46		
Age-adjusted HR (95% CI)	1 (ref)	0.93 (0.72-1.21)	0.99 (0.74-1.33)	0.88 (0.62-1.25)	0.56	
Multivariable HR (95% CI)§	1 (ref)	0.96 (0.74-1.25)	1.05 (0.77-1.43)	0.99 (0.69-1.44)	0.92	
FOXP3$^+$						
Low						
No. cases (n=336)	114	114	66	42		
Age-adjusted HR (95% CI)	1 (ref)	0.75 (0.58-0.98)	0.78 (0.58-1.06)	0.72 (0.50-1.02)	0.06	0.05
Multivariable HR (95% CI)§	1 (ref)	0.77 (0.59-1.01)	0.83 0.61-1.14)	0.79 (0.54-1.15)	0.21	0.05
High						
No. cases (n=337)	85	130	74	48		
Age-adjusted HR (95% CI)	1 (ref)	1.15 (0.87-1.51)	1.17 (0.85-1.60)	1.15 (0.80-1.64)	0.41	
Multivariable HR (95% CI)§	1 (ref)	1.18 (0.89-1.55)	1.22 (0.88-1.69)	1.27 (0.88-1.86)	0.19	

	Calcium supplement (mg/d)					
	0-199	**200-299**	**300-499**	**≥500**		
Total colorectal cancer						
Person-years (n=3,663,039)	2,505,441	321,408	428,893	407,297		
No. cases (n=736)	514	80	91	51		
Age-adjusted HR (95% CI)	1 (ref)	1.14 (0.89-1.45)	0.99 (0.78-1.25)	0.56 (0.42-0.75)	0.001	
Multivariable HR (95%CI) §	1 (ref)	1.22 (0.95-1.56)	1.10 (0.87-1.39)	0.67 (0.49-0.90)	0.09	
CD3$^+$						
Low						
No. cases (n=347)	251	32	41	23		
Age-adjusted HR (95% CI)	1 (ref)	0.93 (0.64-1.36)	0.93 (0.66-1.30)	0.52 (0.34-0.80)	0.006	0.52
Multivariable HR (95% CI)§	1 (ref)	0.99 (0.68-1.45)	1.03 (0.73-1.46)	0.62 (0.40-0.96)	0.07	0.49
High						
No. cases (n=350)	237	43	48	22		
Age-adjusted HR (95% CI)	1 (ref)	1.32 (0.94-1.84)	1.12 (0.81-1.55)	0.51 (0.33-0.80)	0.05	
Multivariable HR (95% CI)§	1 (ref)	1.43 (1.02-2.00)	1.25 (0.90-1.74)	0.62 (0.39-0.97)	0.37	

CD8$^+$						
Low						
No. cases (n=339)	233	39	47	20		
Age-adjusted HR (95% CI)	1 (ref)	1.09 (0.77-1.54)	0.99 (0.72-1.37)	0.43 (0.27-0.68)	0.003	0.39
Multivariable HR (95% CI)§	1 (ref)	1.16 (0.82-1.65)	1.10 (0.79-1.53)	0.51 (0.32-0.81)	0.05	0.38
High						
No. cases (n=344)	244	37	39	24		
Age-adjusted HR (95% CI)	1 (ref)	1.21 (0.85-1.72)	0.99 (0.70-1.41)	0.61 (0.40-0.94)	0.07	
Multivariable HR (95% CI)§	1 (ref)	1.30 (0.91-1.86)	1.10 (0.77-1.57)	0.73 (0.47-1.13)	0.42	
CD45RO$^+$						
Low						
No. cases (n=348)	258	34	37	19		
Age-adjusted HR (95% CI)	1 (ref)	1.01 (0.70-1.46)	0.85 (0.60-1.22)	0.43 (0.27-0.69)	0.0008	0.12
Multivariable HR (95% CI)§	1 (ref)	1.08 (0.75-1.56)	0.94 (0.65-1.34)	0.51 (0.31-0.82)	0.01	0.11
High						
No. cases (n=359)	237	44	49	29		
Age-adjusted HR (95% CI)	1 (ref)	1.27 (0.92-1.78)	1.07 (0.78-1.48)	0.65 (0.44-0.97)	0.15	
Multivariable HR (95% CI)§	1 (ref)	1.36 (0.97-1.90)	1.19 (0.86-1.65)	0.77 (0.52-1.16)	0.67	
FOXP3$^+$						
Low						
No. cases (n=336)	238	35	41	22		
Age-adjusted HR (95% CI)	1 (ref)	1.09 (0.76-1.57)	0.96 (0.68-1.36)	0.53 (0.34-0.82)	0.01	0.63
Multivariable HR (95% CI)§	1 (ref)	1.18 (0.82-1.70)	1.08 (0.76-1.53)	0.63 (0.40-1.00)	0.15	0.64
High						
No. cases (n=337)	226	44	42	25		
Age-adjusted HR (95% CI)	1 (ref)	1.35 (0.97-1.88)	0.99 (0.70-1.39)	0.59 (0.39-0.90)	0.06	
Multivariable HR (95% CI)§	1 (ref)	1.44 (1.03-2.02)	1.10 (0.78-1.55)	0.71 (0.46-1.09)	0.41	

CI, confidence interval; HR, hazard ratio.
Duplication-method Cox proportional cause-specific hazards regression for competing risks data was used to compute HRs and 95% CIs.
All analyses were stratified by age (in month), year of questionnaire return and sex.
*: Linear trend test using the median intake of each category.
¶: The likelihood ratio test was used to test for the heterogeneity of the association between total calcium intake and colorectal cancer risk by densities of tumor-infiltrating T-cell subsets.
§: Multivariable hazard ratios were adjusted for **age** (in month), **race** (Caucasian vs. non-Caucasian), **adult BMI** (< 25, 25 -< 27.5, 27.5 -< 30, or ≥ 30 kg/m^2), smoking (0, 1-10, or > 10 pack-years), **history of colorectal cancer in a parent or sibling** (yes or no), **history of sigmoidoscopy/colonoscopy** (yes or no), **physical activity** (< 3, 3-< 27, ≥ 27 MET-hrs/wk), **regular aspirin use** (yes, no), **alcohol consumption** (0 -< 5, 5 -< 15, or ≥ 15 g/d), energy-adjusted total intake of **folate**, **vitamin D**, **red meat** and **processed meat** (all in tertiles).