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Calcium, Colorectal Cancer, and Other Health Outcomes

By

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Doctor of Philosophy

Epidemiology

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Calcium, Colorectal Cancer, and Other Health Outcomes

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B. Med., Fudan University, 2010

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An abstract of

A dissertation submitted to the Faculty of the

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Abstract

Calcium, Colorectal Cancer, and Other Health Outcomes

By Baiyu Yang

Calcium is an essential nutrient for the human body. There is strong evidence that calcium may be protective against colorectal neoplasms. However, the mechanisms for calcium's chemopreventive properties are not fully understood. In addition, despite compelling evidence for an inverse association of calcium intake with colorectal cancer incidence, there are limited data regarding the impact of calcium on colorectal cancer survival. Furthermore, the association of calcium intake with other major causes of death, such as cardiovascular disease (CVD), needs to be investigated in order to comprehensively evaluate the benefits and harms of calcium intakes and better inform dietary recommendations.

In the first study, we tested the effect of calcium supplementation on plasma biomarkers of inflammation, oxidative stress, and gut permeability over a 4-month treatment period, among colorectal adenoma patients in a randomized, double-blinded, placebo-controlled clinical trial (n = 193); we observed no appreciable effects either overall or within strata of several major risk factors for colorectal carcinogenesis. In the second study, among 2,284 persons diagnosed with invasive, non-metastatic colorectal cancer, we observed lower all-cause mortality among those with higher post-diagnosis total calcium or milk intakes, and marginally lower colorectal cancer-specific mortality among those with higher post-diagnosis total calcium intakes. In the third study, among 132,823 participants in a large cohort initially free from cancer or CVD at baseline, we found that calcium intake in general was not associated with risk of mortality in this cohort, but high intake of supplemental calcium ($\geq$ 1,000 mg/d) in men may be associated with increased all-cause and CVD-specific mortality.

Overall, this dissertation contributes to a better understanding of the role of calcium in colorectal cancer development and progression, and adds to the limited evidence base regarding whether or not increasing calcium consumption would, on balance, be of public health benefit.

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CHAPTER 1. INTRODUCTION AND BACKGROUND

Introduction

Colorectal cancer is the third most common incident cancer and the third leading cause of cancer death in each sex in the United States.[1] Extensive evidence suggests that Western diet and lifestyle play an important role in the etiology of this disease.[2] There is strong biological plausibility and animal experimental and human observational evidence for protection against colorectal neoplasms by calcium,[3-7] and a major randomized controlled trial found statistically significantly reduced colorectal adenoma recurrence with calcium supplementation.[8]

There are at least three major hypotheses for how calcium may reduce risk for colorectal neoplasms: 1) calcium binds bile and fatty acids in the colon lumen, forming insoluble soaps and thus preventing their colonic toxicity (which occurs via an oxidative mechanism and results in an inflammatory response and increased proliferation);[4,9,10] 2) calcium has direct effects on colonic cell cycle,[11-17] including proliferation, differentiation, and apoptosis; and 3) calcium promotes E-cadherin expression and suppresses β-catenin/TCF activation.[18] Findings from our preliminary chemoprevention trial indicated that calcium may modulate multiple hypothesis-based tissue and circulating biomarkers of risk for colorectal neoplasms.[11,12,19-23] Although these findings were promising, the interpretations were limited by the relatively small sample size in this pilot trial; thus, further investigations in a larger, full-scale clinical trial is needed. My first objective for my dissertation is to test the effect of calcium supplementation on circulating biomarkers of risk for colorectal cancer, including biomarkers of inflammation, oxidative stress, and gut permeability, using data and blood samples from a previously-conducted full-scale randomized clinical trial among patients with previous colorectal adenoma.

Although calcium is generally considered to be inversely associated with colorectal cancer incidence, whether calcium is also favorably associated with colorectal cancer survival is

unclear. The overall 5-year survival rate for colorectal cancer patients is 65% in the United States. There are currently 1.2 million colorectal cancer survivors in the U.S.;[24] worldwide, the five-year prevalence (which captures patients within five years of diagnosis) is estimated to be 3.54 million.[25] Because colorectal cancer survivors will be actively seeking diet and lifestyle changes to improve their diagnosis, information on the role of modifiable factors in colorectal cancer survival is important to inform specific dietary guidelines for survivors. To date, there have been only four studies that evaluated the association of calcium intake with colorectal cancer survival.[26-29] All reported no association of pre-diagnosis calcium intake with mortality among colorectal cancer survivors, but none evaluated post-diagnosis calcium intake, which could be of stronger clinical relevance. My second dissertation objective is to evaluate the pre- and post-diagnosis intakes of calcium, vitamin D, and dairy products with mortality from all causes and specifically from colorectal cancer among patients diagnosed with invasive, non-metastatic colon or rectal cancer.

Although adequate calcium intake is important for bone health and several major physiologic functions,[30] and may prevent against colorectal cancer,[31] the effects of calcium on other health outcomes are largely unclear. Especially, the potential adverse effects of supplemental calcium on cardiovascular health have raised concerns. Several large prospective cohort studies, including the EPIC and NIH-AARP cohorts, reported that supplemental calcium was associated with adverse cardiovascular events,[32-34] although null or inverse associations were reported in a few others.[35-38] Also, several randomized clinical trials of calcium supplementation on non-cardiovascular disease (CVD) outcomes (such as bone health) monitored CVD events during the trial, and a meta-analysis of these trials reported that calcium supplementation with or without vitamin D increased myocardial infarction (MI) risk by 24%, and the risk of a composite of MI or stroke by 15%.[39] With regard to cancer, in addition to strong evidence supporting an inverse association of calcium intake with colorectal cancer, some evidence suggests that total or

dietary calcium may be associated with lower risk of breast cancer [40,41], and total calcium or dairy intake may be positively associated with risk of prostate cancer [42], but the World Cancer Research Fund considers the level of evidence "limited" for both types of cancer [43,44]. My third dissertation aim is to comprehensively evaluate the associations of calcium intake (total, dietary, and supplemental) and mortality from all causes, cancer, and CVD, in a large cohort of individuals with no histories of cancer or CVD at baseline.

Overall, this dissertation will improve understanding of the role of calcium consumption along the continuum of colorectal cancer, including its development and progression. This dissertation will also provide insights on whether calcium consumption, overall, can be of public health benefit, and may further inform personalized recommendations for the dietary intake of this important nutrient.

Background

Epidemiology of Colorectal Cancer

The large bowel consists of the cecum, ascending colon, hepatic flexure, transverse colon, splenic flexure, descending colon, sigmoid colon, and the rectum.[45] According to the anatomic distribution, the colon can be classified into proximal colon (which includes all parts up to the mid-transverse colon) and distal colon (which includes all parts after the mid-transverse colon).[46]

Colorectal cancer is the third most common incident cancer and the third leading cause of cancer deaths in each sex in the United States, with an estimated 132,700 incident cases and 49,700 deaths in 2015 combining both sexes.[1] Worldwide, it is estimated that 1,361,000 cases and 694,000 deaths occurred in 2012.[47] There is substantial international variation in colorectal cancer incidence, with the highest incidence in highly-industrialized regions such as Australia/New

Zealand, Europe, North America, and Eastern Asia, and the lowest in Africa.[25] Also, residents from lower-risk countries tend to acquire higher risk for colorectal cancer with westernization and migration into higher-risk countries,[2] suggesting that it is a disease largely related to Western diet and lifestyle.

The overall 5-year relative survival rate for colorectal cancer patients is 65% in the United States, and differs by tumor stage (90% for localized tumors, 70% for regional tumors, and 13% for metastatic tumors).[1] There are currently 1.2 million colorectal cancer survivors in the US;[24] worldwide, the five-year prevalence (which captures patients within five years of diagnosis) is estimated to be 3.54 million. [25]

Colorectal cancer has been categorized into sporadic, familial, and inherited types.[48] About 70% of colorectal cancer cases are sporadic cases, with no familial or inherited predisposition.[48] Fewer than 10% of cases are inherited cases (with inherited predisposition to colorectal cancer),[48] and there are two major types of inherited syndromes, namely, familial adenomatous polyposis (FAP, characterized by an inherited mutation of the *APC* gene) and hereditary nonpolyposis colorectal cancer (HNPCC, characterized by inherited mutations of mismatch repair genes).[45] In addition, up to 25% are familial cases, which develop too frequently to be considered sporadic cancer, but in a pattern inconsistent with inherited syndromes.[48]

Colon Carcinogenesis

Most colorectal cancers originate from adenomatous polyps, also known as adenomas.[49] While the prevalence of adenomas is high (35% to 60% in the U.S.),[50] only about 10% of adenomas develop into cancer.[51] Based on the earliest hypothesis by Hill *et al.*,[52] Fearon and Vogelstein proposed a multistep progression model from colorectal adenoma to cancer,[53] involving the progression from normal epithelium to hyper-proliferative epithelium, early/intermediate/late adenoma, carcinoma, and metastasis, accompanied by mutations of oncogenes and tumor

suppressor genes, such as *APC*, *KRAS*, and *p53*.[53] While this model has been widely-accepted, since then accumulating evidence also suggested several alternative mechanisms of colorectal carcinogenesis. The major pathways are summarized by Potter, as presented below:[54]

- APC-β-Catenin-Tcf-MYC Pathway (the adenoma-carcinoma sequence, primarily based on Fearon's model): this pathway is initiated by a mutation of the *APC* gene, which then loses its function of regulating β-Catenin signaling, cell-adhesion, and migration; subsequently, there is an increased concentration of β-Catenin and up-regulation of the downstream oncogene *c-myc*, followed by a series of genetic and epigenetic alterations, eventually transforming the normal epithelium into metastatic carcinomas. [54]
- Mismatch Repair Pathway: this is commonly found in HNPCC as well as sporadic tumors with microsatellite instability. This pathway involves mutations in DNA mismatch repair genes (MMR genes, e.g., *hMLH1* and *hMSH2*) or methylation specifically of *hMLH1*, leading to a loss of the DNA mismatch repair function and contributing to further microsatellite instability, not only of the MMR genes, but also other important genes such as *TGF-β* and *BAX* which control cell growth and apoptosis. [54]
- Ulcerative-colitis-dysplasia-carcinoma pathway: chronic inflammation in patients with ulcerative colitis results in genetic alterations and subsequent dysplasia without necessarily growing a polyp, and the pattern of genetic alterations are not well defined. [54]

Currently, colorectal endoscopy (sigmoidoscopy and colonoscopy) primarily targets adenomas, especially advanced adenomas.[55] However, recent research revealed that serrated polyps (traditionally considered non-malignant hyperplastic polyp subtypes) may also be of malignant potential.[56,57] Unlike the traditional adenoma-carcinoma sequence, a major role of *CIMP* and *BRAF* mutation has been proposed in the serrated pathway,[57] and this is supported by evidence that 55% of serrated polyps were *BRAF* mutation positive, and 26% were *CIMP*-high, as opposed to the

traditional adenoma (≤ 1% for both markers).[58] The risk factors for traditional adenomas and serrated polyps may also differ. For example, Burnett-Hartman *et al.* evaluated risk factors for colorectal adenomas and serrated polyps in a case-control study of 1,469 cases (628 with adenoma, 594 with serrated polyp, and 247 with both) and 1,037 polyp-free controls, and identified several factors (sex, smoking, and estrogen-only hormone replacement therapy) which had different associations with adenoma vs. serrated polyp.[59] The authors also reported (in a separate investigation) that previous endoscopy was associated with lower risk of advanced adenomas but not sessile serrated polyps, probably because the flat shape of the sessile serrated polyps makes it harder to identify these polyps, especially by general practitioners (as opposed to specialists).[60] Considering their malignant potential, more effective surveillance strategies for serrated polyps are needed.[57]

Molecular Subtypes of Colorectal Cancer

In order to enhance the understanding of causality and improve the clinical management of this disease, Jass proposed a new molecular classification system for colorectal cancer based on the type of genetic instability (microsatellite instability, i.e., MSI) and the level of DNA methylation (CpG island methylator phenotype, i.e., CIMP).[61] The five types, their proportion in colorectal cancer cases, and major features are summarized in Table 1.1 (adapted from Jass[61]).

It is important to recognize that colorectal cancer is not a single entity, but contains heterogeneous pathways.[61] This may help in the identification of risk factors and early chemoprevention targets.[61] For example, smoking is moderately associated with higher colorectal cancer incidence in general, but more strongly associated with MSI-high tumors,[62-68] CIMP-high tumors,[67,69] and BRAF-mutated tumors[66,67,69] (all of which are correlated).

Table 1.1. Molecular classification of colorectal cancer (Jass[61])

	Type 1 (sporadic MSI-H)	Type 2	Type 3	Type 4	Type 5 (Lynch syndrome)
Proportion	12%	8%	20%	57%	3%
MSI status	high	stable/low	stable/low	stable	high
CIMP	high	high	low	Negative	Negative
BRAF	+++	++	-	-	-
CIN	No	No	Yes	Yes	No
Origin in	serrated polyp	serrated polyp	serrated polyp/adenoma	adenoma	adenoma

Abbreviations: CIMP, CpG island methylator phenotype; CIN, chromosomal instability; MSI, microsatellite instability

Ogino and Goel subsequently proposed a slightly updated classification system, as shown below:[70]

Table 1.2. Molecular classification of colorectal cancer (Ogino and Goel[70])

	Type 1 (Sporadic MSI-H)	Type 2	Type 3	Type 4	Type 5	Type 6
Proportion	10%	5%	5-10%	5%	30-35%	40%
MSI status	High	High	Low/MSS	Low	MSS	Low/MSS
CIMP	High	Low/0	High	Low	Low	0
BRAF	+	?	+	?	?	-
CIN	-	-	-	?	-	+

Abbreviations: CIMP, CpG island methylator phenotype; CIN, chromosomal instability; MSI, microsatellite instability; MSS, microsatellite stable. "?" means the information was not mentioned in the paper.

Ogino's research group (Yamauchi *et al.*) further examined the frequency of major colorectal tumor characteristics along the bowel sub-sites and found that MSI-high, CIMP-high and BRAF mutations increase gradually from the rectum to the ascending colon, but dropped in the cecum.[71] These findings directly challenge the traditional dichotomous classification of colorectal cancer by site (proximal *vs*. distal).

Inflammation, Oxidative Stress, and Colorectal Cancer

Inflammation has long been linked to the etiology of cancer, particularly colon cancer.[72] That inflammatory bowel disease (IBD) is an established risk factor for colorectal cancer,[73] taking

nonsteroidal anti-inflammatory drugs (NSAIDs) has been consistently and strongly associated with lower risk of colorectal neoplasms,[74] and NSAIDs reduced colorectal adenoma recurrence in large randomized controlled trials,[75] strongly indicate that chronic inflammation plays a key role in colorectal carcinogenesis. In a recent review of eight prospective studies (including 1,159 colorectal cancer cases and 37,986 controls) C-reactive protein (CRP), a nonspecific marker of systemic inflammation, was statistically significantly associated with higher risk for colorectal cancer,[76] making it a potential biomarker of risk for colorectal neoplasms in chemopreventive trials. In addition, cytokines, broadly categorized as pro-inflammatory (*e.g.*, interleukin [IL]-6) and anti-inflammatory (*e.g.*, IL-10),[77] are important components that link inflammation and cancer,[78] which may have a role in all steps of tumorigenesis, including initiation, promotion, progression, and metastasis.[79] For sporadic colorectal cancer, epithelium cell mutations are usually initiated by environmental mutagens; then immune cells are recruited to the local microenvironment, and cytokines stimulate the production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) to induce additional mutations and epigenetic changes.[79] Cytokines can also serve as growth and survival factors to promote the transformation of a single premalignant cell to a fully developed tumor.[79] Serum levels of several pro-inflammatory cytokines, including vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF)-α, IL-6, and IL-8, were found to be higher in colorectal cancer cases than in controls.[80] Jung *et al*. reported that human colon epithelial cells express IL-8, monocyte chemotactic protein (MCP)-1, granulocyte macrophage colony-stimulating factor (GM-CSF) and TNF-α in response to bacteria invasion.[81] Among IBD patients, T helper 1 (Th1)/Th17 responses (mainly involving IL-12 and IL-23) may be crucial for Crohn's disease, while Th2 responses (mainly involving IL-13) may be crucial for ulcerative colitis; another group of cytokines, such as TNF-α, IL-1β, and IL-6, may bridge the Th1/Th17-Th2 spectrum and exert both "upstream" and "downstream" proinflammatory effects.[82] Anti-cytokine drugs have been used to treat patients with IBD, and together with other traditional treatments

(surgery, chemotherapy, radiation) they may be used to treat colorectal cancer patients as well, but have not been tested in clinical trials.[79]

We hypothesized that the colon is a major source of circulating cytokines, and since circulating levels of calcium are maintained in a very narrow range, we further hypothesized that if calcium reduces inflammation in the colon, it will be reflected in the circulation and unlikely be due to systemic actions of calcium. A few animal studies and clinical trials have been previously published regarding the effect of calcium on circulating biomarkers of inflammation. One animal study demonstrated that in mice with experimentally-induced inflammatory bowel disease (IBD), diet with calcium, active vitamin D, or both, led to lower severity of IBD and reduced secretion of TNF-α, the level of which is directly associated with IBD activity), thus suggesting that dietary calcium and vitamin D suppress IBD through inhibition of the TNF-α pathway.[83] Two other animal studies found that calcium together with vitamin D or dairy product consumption reduced IL-1β, TNF-α or IL-6 in mice.[84,85] In our preliminary clinical trial we found a reduction of circulating inflammatory biomarkers individually or combined as a z-score in response to 6 months of calcium supplementation.[23] To our knowledge, there are no other human studies regarding the effect of calcium on inflammation among subjects with high risk for colorectal polyps, but several other studies examined this effect among healthy individuals. Gannagé-Yared *et al.* reported no effect of 1 g/d calcium and 800 IU/d cholecalciferol on serum CRP, IL-6, and TNF-α among 47 healthy post-menopausal women during 12 weeks, but this may have been due to the very low levels of cytokines in healthy participants.[86] Similarly, Grey *et al.* reported no effect of 1 g/d of calcium on CRP level among 116 healthy post-menopausal women,[87] and Pittas *et al.* reported no effect of calcium plus vitamin D supplementation on CRP and IL-6 among non-diabetic adults.[88] In addition, three studies reported that a diet high in dairy products reduced the levels of CRP or TNF-α in overweight or obese adults.[85,89,90] Although dairy products are a rich source of calcium, these

studies were unable to distinguish whether the effects were due to calcium or other dairy components.

For this dissertation we chose a panel of markers to represent different aspects of the inflammatory response/immunomodulation in order to provide a more complete summary of the overall effect of calcium on inflammation. Categories of markers represented include mediators of natural and adaptive immunity (e.g., TNF-α and IL-4, respectively); inflammation promotion and inhibition (e.g., IL-6 and IL-10, respectively); cytokines originating from different cell sources, such as T, B, natural killer (NK), Th1, and Th2 cells, macrophages, fibroblasts, epithelial cells, and others; cytokines with different cell targets; and cytokines with different primary effects. It is noted that there is some overlap across and interactions among these categories and the representatives of them. We also considered known effects of specific markers. For example, TNF-α can activate the pro-inflammatory transcription factor NF-κB and contribute to all steps of carcinogenesis,[91,92] and IL-6 leads to an increase in the expression of several oncogenes and promotes tumor formation.[91] Finally, considering the complex functions and interactions of the different inflammation-related markers in colorectal carcinogenesis, as well as the weak associations between each individual cytokine and colorectal neoplasms, a comprehensive summary of cytokines, such as an inflammation z-score, or a ratio between pro-inflammatory/anti-inflammatory cytokines, may serve as a more appropriate biomarker of inflammation and risk for colorectal neoplasms.

Oxidative stress, primarily acting through reactive oxygen and nitrogen species (RONS), is likely another key factor in colorectal carcinogenesis.[93] RONS can induce damage in almost all cellular components, including oxidizing cellular lipids (lipid peroxidation),[94] which is believed to be one of the major determinants of oxidative stress-related colorectal carcinogenesis.[95,96] F_2-isoprostanes, formed via the peroxidation of arachidonic acid, has been recognized as the most reliable marker of lipid peroxidation *in vivo,* with great potential utility in human studies due to the

non-invasive method of quantification and sufficiently detectable levels in a wide range of biological fluids such as plasma and urine.[94,97] Associations of F_2-isoprostanes and/or its metabolites have been investigated in relation to several types of cancer, including breast,[98] lung,[99] and prostate[100] cancers, but not yet with colorectal cancer. One recent prospective cohort study (n = 425) found no overall association between F_2-isoprostanes and colorectal adenoma,[101] but in a case-control study by our research group, we found that those with serum F_2-isoprostane levels above the median were at statistically significant higher risk for colorectal adenoma.[102] The etiologic role of oxidative stress in colorectal cancer development warrants further study.

One source of RONS is their release from various immune cells that are activated during an inflammatory event.[91] On the other hand, oxidative stress can induce cellular damage, which further propagates the effects of inflammatory stimuli,[91] suggesting that oxidative stress and inflammation are two closely interrelated events. Results from our pilot clinical trial suggested that calcium may reduce oxidative DNA damage as measured by 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in the normal colorectal mucosa,[19] and unpublished data from two of our case-control studies suggest that there is an inverse association between calcium intake and plasma F_2-isoprostanes. From other groups, one animal study reported that treating mice with calcium reduced markers of oxidative stress (ROS production, NADPH oxidase mRNA and plasma malondialdehyde),[85] and a clinical trial among 20 obese or overweight adults reported that a diet high in dairy products reduced the levels of oxidative stress biomarkers;[90] the evidence from these studies supports a full-scale investigation of calcium supplementation on biomarkers of inflammation and oxidative stress.

Gut Permeability and Colorectal Cancer

The gastrointestinal tract has the largest mucosal surface in the body interacting with the environment. An intact gut barrier with selective permeability is key to the balance between absorption of nutrients and blocking harmful wastes, such as bacterial products, and the gut barrier

function is maintained by several key components: at the extracellular level, mucus forms an unstirred layer of fluid at the surface of epithelial cells and blocks direct contact with large particles, such as bacteria; at the cellular level, the apical junctional complex (mainly the tight junction and the adherens junction) seals the paracellular pathway, and in particular, the tight junction is the principle determinant of the gut permeability.[103]

There are several methods to measure gut permeability. Based on the existing literature, especially the papers by Farhadi,[104] Turner,[103] and Bornholdt,[105] I summarize the common measurements of gut permeability below:

a) Tight junction proteins
    i) Claudins: members of the claudin family are the most important components of the tight junction. Most claudins contribute to an enhanced gut barrier by sealing the junctions, such as claudin-1, -4, -5, -7, -8, -11, -14, and -19. Conversely, some claudins, such as claudin-2, -10 and -16, are involved in the formation of small pores and are associated with decreased epithelial tightness.
    ii) Other: the roles of other proteins such as occludin and zonulin are less well studied.
b) Probes
    i) Sugar probes, e.g., sucrose, mannitol, cellubiose, lactulose and sucralose. Typically after oral use of these sugar probes, the urinary level is measured, allowing for the calculation of several ratios, such as lactulose: mannitol ratio or sucralose: mannitol ratio.
    ii) Others, e.g., polyethylene glycol, ^{14}C mannitol, FITC-dextran, and ^{51}CrEDTA.
c) Bacterial antigens and immune responses against these antigens
    i) Circulating LPS (endotoxin) and LPS-binding protein
    ii) Anti-LPS and Anti-flagellin immunoglobulins
    iii) CRP and cytokines

iv) Bacterial translocation to mesenteric lymph node, liver and spleen

d) Others: transepithelial electric resistance, ruthenium red, Ussing chamber

For this dissertation, I chose to evaluate circulating levels of flagellin- and LPS-specific immunoglobulins (Igs) IgA and IgG in response to calcium supplementation. Circulating levels of flagellin- and LPS-specific IgA and IgG may serve as markers of long-term systemic exposure to flagellin and LPS and may indicate altered adaptive immune responses related to colonic hyperpermeability.[106-111] Of note, levels of anti-LPS and anti-flagellin Igs may reflect not only erosion of mucosal anatomic and immune barriers, but also gut bacteria composition and their ability to translocate across the gut, and immune responses against bacterial antigens. Although these Igs may not be the most direct measures of gut barrier functions or gut permeability, emerging evidence suggests a positive correlation of Igs against LPS and flagellin with serum fluorescein isothiocyanate–dextran, a direct measurement of intestinal barrier function,[110] thus supporting their role as biomarkers of gut permeability.

The direct role of gut hyperpermeability in the development of colorectal cancer has been investigated to a limited extent. An *in vivo* investigation reported that tight junctions (as measured by several parameters; e.g., the transepithelial electrical resistance [TEER]) of rats or human colon tumors was leakier than that of normal colon.[112] Several cross-sectional studies among human subjects reported that colon or colorectal tumor tissues, compared to normal tissues, had higher levels of permeability, but these studies had relatively small sample sizes.[105,113,114] The role of gut barrier dysfunction in colorectal cancer likely initiates in the early stages of colorectal carcinogenesis, as evidenced by reports that human colorectal adenoma tissues had defective mucin expression and disorganized tight junctions,[115] and individuals with higher plasma endotoxin (also known as lipopolysaccharides or LPS) concentrations were more likely to have prevalent colorectal adenomas.[116] Furthermore, there is emerging but limited evidence that the erosion of gut barrier

function, especially the loss of tight junction barrier function, may be associated with colorectal cancer recurrence, metastasis, and poorer survival.[117,118] To our knowledge, no reported epidemiologic study prospectively evaluated the association of gut permeability with colorectal cancer incidence or clinical outcomes.

Although evidence on the role of gut hyperpermeability in the etiology of colorectal cancer is limited, there is a substantial body of evidence that gut barrier dysfunction may be associated with several clinical conditions that could influence the risk of developing colorectal cancer. As previously reviewed, gut barrier dysfunction may contribute to inflammatory bowel diseases (Crohn's disease and ulcerative colitis),[119-122] which are established risk factors for colorectal cancer.[45] Gut barrier dysfunction has also been associated with several other gastrointestinal disorders, such as food allergy,[104] Celiac disease,[104] and short bowel syndrome.[106] Gut barrier dysfunction may also play an important role in obesity, diabetes, and other metabolic disorders, all of which are risk factors for colorectal cancer. In a hallmark study by Cani *et al.*, mice with induced metabolic endotoxemia (through infusion of LPS) for 4 weeks had an increase in fasted glycemia, insulinemia, and markers of inflammation, and also experienced whole-body, liver, and adipose tissue weight gain, thus providing strong evidence that metabolic endotoxemia, possibly as a consequence of gut permeability, triggers the onset of obesity and diabetes, possibly mediated by inflammatory responses.[123] Consistent with these results, there are several reports from population-based cross-sectional studies that LPS-binding protein (LBP) levels or immunoglobulins against bacterial products were significantly higher among obese/overweight individuals relative to those with normal weight, and LBP was also associated with low-HDL cholesterol, type 2 diabetes, and metabolic syndrome.[124-126]

The associations of gut permeability with demographic/diet/lifestyle factors have only been studied to a limited extent. One study reported that gut permeability, as measured by LBP,

increases with age, and is higher among smokers;[125] also, given the same amount of *in vivo* LPS exposure, male mice produce higher levels of LPS-binding protein and higher inflammation mediators than females, suggesting sex differences.[127] Several studies have assessed whether gut permeability is associated with selected dietary factors, or could be modified by diet. As reviewed by Ulluwishewa *et al.*, the tight junction, an important structure to support gut barrier function, can be strengthened by glutamine, and by extracts from black and green peppers, linden, star anise, *Arenga engleri*, and black tea; in contrast, it may be impaired by gliadin, food surfactants, tryptophan, and extracts from capsianoside, galangal, marigold, *Acer nikoense*, and hops.[128] There is also a large body of evidence that a high-fat diet may increase intestinal permeability, partially through a change in microbiota composition, and through epithelial erosion by bile acids.[129,130] An elemental diet (a chemically-defined liquid diet containing easily digestible nutrients) may reduce gut permeability among patients with Crohn's disease.[131,132] Treating eight healthy subjects by Western-style diet for one month increased plasma LPS levels, whereas a prudent-style diet reduced the LPS levels.[133] Vitamin D may also play a role in maintaining gut barrier integrity, as vitamin D-receptor (VDR) deficient mice had a loss of tight junction functions,[134] 1,25-dihydroxy-vitamin D_3 enhanced tight junction in cell cultures,[134] and 2,000 international units (IU)/d of vitamin D_3 supplementation for three months inhibited the increase of gut permeability in a randomized controlled trial among 27 Crohn's disease patients in remission.[135] In addition, a recent report suggested that two dietary emulsifiers increased gut permeability in mice, as measured by fluorescein isothiocyanate–dextran.[110]

Calcium is an agent that plausibly may play a role in modulating gut barrier function since calcium can bind bile and fatty acids in the colon lumen by forming insoluble soaps, thus preventing them from oxidatively damaging the colonic mucosa and consequently producing inflammation,[4,9,10] which, in turn, may help maintain the strength of the gut mucosal barrier. Bovee-Oudenhoven and colleagues conducted several controlled trials in rats, and reported that a high-

calcium diet reduced the translocation of *Salmonella*, inhibited the increase in intestinal permeability as measured by urinary chromium EDTA (CrEDTA), and improved resistance to intestinal infection;[136-138] they also found a similar effect of high-calcium milk relative to low-calcium milk against enterotoxigenic *Escherichia coli* (ETEC) infection in rats and a small group of men (n = 32),[139] but the potential interaction between calcium and other components in milk could not be excluded. Altogether, these findings support our hypothesis that calcium may favorably modulate gut barrier functions, but need to be replicated in a large, full-scale clinical trial among humans.

Factors Associated with Colorectal Cancer Incidence

Demographic factors

The risk of colorectal cancer increases with age, with about 90% of cancers developed after age 50 years.[45,140] Overall, colorectal cancer incidence and mortality rates are 30% to 40% higher in men than in women;[140] also, colorectal cancer location may differ by sex, with women more likely to develop colorectal cancer in the proximal colon than men.[45,140] In terms of race/ethnicity, black men and women have the highest rates overall (about 25% higher incidence rates and 50% mortality rates than those in whites); rates are the lowest in Asians/Pacific Islanders.[140]

Genetic predisposition

It is widely recognized that individuals with a family history of colorectal cancer are at higher risk for developing colorectal cancer. According to a recent meta-analysis of fifty-nine studies, the relative risk (RR) was 2.24 (95% confidence interval [CI] 2.06 – 2.43) for those with at least one first degree relative with colorectal cancer.[141] The RR is higher for those with two or more affected relatives,[141] or with relatives diagnosed before age 45.[142] Furthermore, individuals

with a family history of colorectal adenoma are also at a higher risk for colorectal cancer (RR 1.99, 95% CI 1.55 – 2.55).[142]

There are two major genetic syndromes, namely hereditary nonpolyposis colorectal cancer (HNPCC, also known as Lynch Syndrome) and familial adenomatous polyposis (FAP), which are established causes of colorectal cancer.[140] HNPCC accounts for about 5% of the cases; the age of onset is usually mid-forties (earlier than that for sporadic colorectal cancers), and the lifetime risk for developing colorectal cancer among HNPCC patients is approximately 50%.[140] Most HNPCC tumors have microsatellite instability and genetic alterations in mismatch repair genes; the occurrence of adenomas in HNPCC patients is uncommon, making early detection of a colorectal neoplasm in them difficult.[45] Notably, HNPCC is also a cause of many other types of cancer, such as endometrial, stomach, and ovarian cancers, although the associations with these types of cancer are not as strong as that for colorectal cancer.[140] FAP is characterized by the occurrence of hundreds to thousands of colorectal adenomas; the age of onset for this syndrome is typically 20s and 30s, and patients will very likely develop colorectal cancer by age 40 if not treated.[45,140]

In addition to high-penetrance genes (e.g., *APC* and mismatch-repair genes that may underlie genetic syndromes), low penetrance genes that were associated with increased predisposition to colorectal cancer were found in genome wide association studies (GWAS). Theodoratou *et al.* did a comprehensive evaluation of published genetic association studies of colorectal cancer up to 2012, and identified 16 independent variants at 13 loci (*MUTYH, MTHFR, SMAD7*, and common variants tagging the loci 8q24, 8q23.3, 11q23.1, 14q22.2, 1q41, 20p12.3, 20q13.33, 3q26.2, 16q22.1, and 19q13.1) to be most credibly associated with colorectal cancer, in addition to 23 variants with less credible evidence and 20 variants with limited evidence.[143] Most colorectal cancer susceptibility loci do not seem to interact with selected major risk factors for colorectal cancer (such as body mass index [BMI], smoking, and dietary factors).[144]

Medical history

Individuals with inflammatory bowel diseases (IBD, primarily ulcerative colitis and Crohn's disease) are at higher risk for colorectal cancer; the risk increases with earlier onset of IBD, longer duration of symptoms, and severity of the disease.[73] The mechanisms involve chronic inflammation and oxidative stress.[145] It is believed that carcinogenesis in IBD-related colorectal cancer follows a different sequence from that observed in sporadic colorectal cancer, although there is considerable overlap.[79,146]

Diabetes is another medical condition that may predispose an individual to higher colorectal cancer risk. A meta-analysis of 24 observational studies including 3,659,341 participants reported that diabetes was associated with higher risk of colorectal cancer (RR, 1.26; 95% CI, 1.20 – 1.31).[147] Even though diabetes and colorectal cancer may share several risk factors (e.g., obesity, Western diet, physical inactivity, and smoking), the association persists after accounting for these factors.[148] The associations between diabetes treatments and colorectal cancer are relatively poorly understood. There is evidence that insulin use may be associated with higher colorectal cancer incidence, whereas metformin may be chemopreventive against colorectal cancer; however, epidemiological studies to address these questions have produced conflicting results, partially due to methodological issues (such as confounding by indication), and thus this question needs to be more carefully addressed in future studies.[148]

Dietary and lifestyle factors

There is great potential for the primary prevention of colorectal cancer through targeting modifiable factors (including diet and lifestyle).[74] According to a comprehensive review by Chan and Giovannucci,[74] modifiable factors positively associated with colorectal cancer risk may include

red/processed meat, alcohol drinking, smoking, and obesity; and factors inversely associated with colorectal cancer risk may include calcium and vitamin D intakes, physical activity, and use of aspirin, COX-2 inhibitors, and post-menopausal hormone. In addition to single dietary components, dietary patterns such as the Mediterranean diet or the Paleolithic diet may be important to evaluate in the future.[74]

The World Cancer Research Funds/American Institute for Cancer Research summarized the associations of diet-related factors with colorectal cancer in 2011.[31]

The following factors may be associated with lower incidence of colorectal cancer:

- Probable: Garlic, milk, calcium
- Limited suggestive: Non-starchy vegetables, fruits, foods containing vitamin D

The following factors may be associated with higher incidence of colorectal cancer:

- Convincing: red and processed meat; alcoholic drinks (for men); body and abdominal fatness; adult attained height
- Probable: alcoholic drinks (for women)
- Limited suggestive: foods containing iron; cheese; foods containing animal fats; foods containing sugar

There is no conclusion regarding whether the following factors may be associated with the incidence of colorectal cancer: fish, glycemic index, folate, vitamin C, vitamin E, selenium, low fat diet, and dietary pattern.

Here in we review the evidence regarding the role of calcium in colorectal cancer prevention.

Calcium is an essential nutrient for the human body. About 99% of calcium in the human body is stored for bone formation and metabolism; the remaining 1% supports other critical functions, including vascular contraction/dilation, muscle function, cell signaling, nerve transmission, and hormone secretion.[149] Calcium is absorbed in the intestinal mucosa; the fractional calcium absorption can be approximately 60% as an infant, but decreases with age, and the average percentage among men and non-pregnant women is 25%.[149,150] Serum calcium is tightly regulated primarily by PTH (Parathyroid hormone), calcitriol (a vitamin D metabolite), and calcitonin, based on a homeostatic feedback mechanism, to maintain a level between 8.5 and 10.5 mg/dL.[149] Non-absorbed calcium is excreted mainly in urine and feces.[149]

Calcium can be found in a variety of foods. The main food sources of calcium are dairy products (e.g., milk, yogurt, and cheese), which account for 72% of calcium in the United States.[149] Other food sources include vegetables, grains, legumes, and so on.[149] In addition, several foods, including juice and cereals, can be fortified with calcium.[149] There are also dietary supplements that contain calcium, most commonly as calcium carbonate and calcium citrate.[149]

The Recommended Dietary Allowance (RDA) is defined as the level of intake that likely exceeds the requirement for 97.5% of the population.[149] According to the Institute of Medicine (IOM), the RDA for men is 1,300 mg if aged 9 - 18y, 1,000 mg if aged 19 - 70y, and 1,200 mg if aged > 70y; for women, it is 1,300 mg if aged 9 - 18y, 1,000 mg if aged 19 - 50y, and 1,200 mg if > 51y.[149] The choice of foods rich in calcium and vitamin D has been encouraged by the U.S. Department of Agriculture's (USDA) Dietary Guideline for Americans.[151] Calcium supplement use is common in the U.S. where an estimated 43% of the population uses calcium supplements; this proportion rises to 62% among subjects 71 years or older (56% of males and 65% of females).[152]

In the 1980s, Garland *et al*. first reported statistically significantly inverse associations of dietary vitamin D and calcium intakes with 19-year risk of colorectal cancer in a cohort of 1,954 men.[153] Since then, this association has been consistently found in large cohort studies, although the strength of association has been modest. Of note, data from the Cancer Prevention Study II Nutrition Cohort (which I am using for two of my three dissertation projects) previously revealed inverse associations of colorectal cancer with total calcium (especially calcium from supplements) and total vitamin D intakes, but no association with dairy products.[154] A pooled analysis from 10 large prospective cohort studies reported 15% lower colorectal cancer risk associated with higher milk intake (a rich source of dietary calcium), and 22% lower risk associated with higher total calcium intake (both results compared the highest to lowest categories, and were statistically significant).[6] A more recent dose-response meta-analysis of 15 large cohorts reported that every 300 mg/day increase of total calcium intake was associated with a statistically significant 8% lower risk of colorectal cancer.[155] Also, a meta-analysis found statistically significant inverse associations of colorectal cancer incidence with both total dairy and milk.[156] In addition, clinical trials conducted among patients with a previous colorectal adenoma found that daily treatment with calcium (ranging from 1.2 to 2.0 g/day), relative to placebo, reduced colorectal adenoma recurrence.[8,157,158] In contrast, the Women's Health Initiative clinical trial reported no effect of calcium plus vitamin D supplementation on colorectal cancer incidence during seven years of intervention period and five years post-intervention,[37,159,160] but there was evidence of some benefit among women who were not concurrently assigned to estrogen therapies[161] and those who were not taking personal calcium or vitamin D supplements.[162]

Based on knowledge that approximately 75% of calcium consumed is not absorbed and passes through the colon, several potential mechanisms have been proposed for the chemopreventive properties of calcium. The earliest hypothesis was that because calcium can bind bile and fatty acids in the colon lumen by forming insoluble soaps, it prevents their colonic toxicity

(which occurs via an oxidative mechanism), which prevents an inflammatory response and compensatory hyperproliferation).[4,9,10] It has also been long known from *in vitro* studies that calcium has direct effects on the cell cycle,[11-17] reducing proliferation and increasing differentiation and apoptosis, suggesting that it may likewise affect colorectal epithelial cells. In addition, calcium may promote E-cadherin expression and suppress β-catenin/TCF activation,[18] or lead to alterations in *KRAS* mutation.[163] Of note, one important pathway for the cell to sense extracellular change of calcium concentration is through the calcium sensing receptor (CaSR)[164]. Kallay *et al*. reported that the proliferative responses of colon cancer cells induced by low ambient calcium can be reverted by activating CaSR through using its agonist.[165] Lamprecht and Lipkin concluded in their review that CaSR may be a major molecular target for dietary calcium in inhibiting colorectal carcinogenesis.[164]

Factors Associated with Colorectal Cancer Survival

According to the College of American Pathologists Consensus Statement 1999, several pathological factors have been proven to be of prognostic value for colorectal cancer patients, including primary tumor stage, lymph node metastasis, vessel invasion, residual tumor after curative surgery, and pre-operative elevation of carcinoembryonic antigen elevation.[166] Other pathological factors that have been strongly suggested to be of prognostic value include tumor grade, histologic type, loss of heterozygosity at 18q, and MSI status, among other tumor characteristics.[166] Below I summarize recent novel findings regarding tumor molecular pathology in relation to colorectal cancer survival.

Tumor somatic mutations and epigenetic events

Microsatellite instability (MSI) refers to altered length of short repeat DNA sequences,[167] and results from a defective DNA mismatch repair (MMR) system.[168] A meta-analysis in 2005 of 32 studies with a combined total of 7,642 colorectal cancer cases reported favorable overall survival for patients with MSI-high tumors (hazard ratio [HR] 0.65, 95% CI 0.59-0.71), including subgroups of patients with a locally advanced tumor or those treated with adjuvant 5-fluorouracil (5-FU), although patients with MSI-high tumors did not benefit from adjuvant 5-FU (HR 1.24, 95% CI 0.72-2.14).[169] A more recent meta-analysis of 31 studies among 12,782 colorectal cancer patients reported a similar association of MSI with more favorable overall or disease-free survival.[170] **The CpG island methylation phenotype (CIMP)** is a unique phenotype in colorectal cancer that has been associated with MSI, as the promoter methylation and subsequent silencing of *MLH1* (a major mismatch repair gene) is a major cause of MSI.[70] The association of CIMP with colorectal cancer survival has been inconsistent across previous studies, but there is evidence that CIMP-high in non-MSI-high tumors likely is associated with poor prognosis.[171,172] ***BRAF*** mutation is independently associated with higher mortality among colorectal cancer patients: according to a recent review and meta-analysis of 26 studies involving 11,773 colorectal cancer patients, patients with a *BRAF*-mutated tumor has an over two-fold higher mortality after diagnosis (HR 2.25, 95% CI 1.82-2.83).[173] ***KRAS*** mutation has been assessed in several studies in relation to colorectal cancer survival, but results have been inconsistent.[174-177] The association between colorectal cancer survival and combinations of these tumor molecular characteristics has also been evaluated. The Colon Cancer Family Registry (CCFR) collected colorectal tumor samples from 2,050 participants and created five molecular subgroups following the scheme proposed by Jass;[61] compared to those with type 4 tumors (MSS or MSI-low, negative for CIMP, *BRAF* and *KRAS*), those with type 2 (CIMP and *BRAF* positive, otherwise same as type 4) or type 3 (*KRAS* positive, otherwise same as type 4) had statistically significant higher disease-specific mortality.[178] Because of the associations

of these tumor molecular characteristics with colorectal cancer outcome and the inter-correlation among these characteristics, Ogino *et al.* pointed out that a comprehensive understanding of the molecular correlates is necessary to identify confounding factors in association studies of molecular events and clinical cancer outcomes.[70]

Tumor immunity in the microenvironment

As reviewed by Ogino *et al*., enhanced immune responses in the tumor microenvironment may be independently associated with favorable survival among colorectal cancer patients.[179] A hallmark study by Galon *et al*. reported that the type, density, and location of immune cells in the colorectal tumor tissues were better predictors of survival than was tumor histological stage, and this finding was validated in two additional patient populations.[180] In addition, in the Nurses' Health Study and the Health Professional Follow-up Study, tumor-infiltrating CD45RO+-cell density and an overall lymphocytic reaction score were each associated with better survival independent of major tumor molecular characteristics (such as MSI).[181,182]

In contrast to the vast amount of studies on pathological prognosis factors, the roles of modifiable risk factors such as diet and lifestyle in the prognosis of colorectal cancer have only been investigated in a very limited number of studies.[183] The American Cancer Society nutrition and physical activity guidelines for cancer survivors suggested that colorectal cancer survivors should generally maintain a healthy weight, be physically active, and keep a balanced diet consistent with guidelines for chronic disease prevention,[184] but did not make specific recommendations for cancer survivors, due to the dearth of empirical evidence. Because cancer survivors actively seek information on diet and lifestyle changes that may influence prognosis and quality of life, it is important to contribute to this evidence base. Herein I briefly review the existing evidence of diet and lifestyle factors with colorectal cancer survival.

Body Mass Index (BMI)

According to a review published in 2010, based on 20 observational studies among colorectal cancer patients, BMI or body fatness either prior to or at the time of diagnosis may be positively associated with all-cause or colorectal cancer-specific mortality.[183] However, the association of BMI or weight change after colorectal cancer diagnosis with survival has only been reported by two studies, and both studies reported null associations.[185,186] In an updated review published in 2014,[187] the authors reported that pre-diagnosis adiposity was generally associated with reduced colorectal cancer survival; postdiagnosis adiposity was not associated with survival in studies using population-based databases, but was associated with higher mortality in observational studies nested in adjuvant chemotherapy trials. The authors of the review argued that the former type of study may be subject to confounding by weight loss. Overall, it is still unclear whether weight control interventions will improve prognosis in those with colorectal cancer.

Physical activity

In 2003, Dray *et al*. reported that among 148 colorectal cancer patients in France who had tumor resection, pre-diagnosis physical activity was not associated with five-year survival; however, physical activity was not the main exposure of interest, and thus was not examined in sufficient detail.[28] Haydon *et al.* examined the association of baseline physical activity with survival among 526 colorectal cancer cases in Australia during a 5.5-year follow-up, and found that exercise was associated with a statistically significantly higher disease-specific survival, primarily among stage II-III patients.[188] Meyerhardt and colleagues used previously validated physical activity questionnaires and assessed physical activity in the form of metabolic equivalent task (MET)-hours in a series of studies: among 832 patients with stage III colon cancer in trial CALGB 89803, physical activity assessed six months after treatment was associated with significantly lower mortality (disease-free, recurrence-free, or overall);[189] among 573 female patients and 668 male

patients with stage I to III colorectal cancer, respectively, those engaged in at least 18 (for female) or 27 (for male) MET-hours of physical activity after diagnosis had lower overall and colorectal cancer specific mortality,[190,191] but the inverse associations were only observed for those with tumors that expressed p27 (the cyclin-dependent kinase inhibitor) (p for interaction = 0.03)[192] or prostaglandin-endoperoxide synthase 2 (PTGS2).[193] More recently, the Cancer Prevention Study II (CPS-II) Nutrition Cohort and the Women's Health Initiative (WHI) both found that a higher amount of physical activity before and after colorectal cancer diagnosis was associated with lower mortality,[194,195] and CPS-II additionally found, for the first time, that leisure time spent sitting was associated with higher mortality in colorectal cancer patients.[194]

Non-steroidal anti-inflammatory drug (NSAID) use

In 2005, Fuchs *et al*. first reported that among 830 patients diagnosed with stage III colon cancer, consistent aspirin use after diagnosis was associated with improved outcomes, including recurrence-free, disease-free, and overall survival.[196] Chan *et al.* extended this investigation in 1,279 patients diagnosed with stage I, II or III colorectal cancer within the Nurses' Health Study and Health Professionals Follow-up Study, and observed an inverse association between post-diagnosis aspirin use and mortality (all-cause or colorectal cancer-specific), especially among patients who did not take aspirin before diagnosis, and patients whose tumor overexpressed cyclooxygenase 2 (COX-2).[197] In the same study population, Liao *et al*. recently found that aspirin may be beneficial for patients with mutated-PIK3CA colorectal cancer patients, regardless of aspirin use before diagnosis.[198] Using data from the Seattle Colon Cancer Family Registry, Coghill *et al.* found that NSAID use before diagnosis was associated with statistically significantly lower mortality from colorectal cancer after eight years of follow-up, and this association might depend on the duration of use;[199] the authors found similar results in a different population,[200] but a later analysis by the same authors using data from the Women's Health Initiative found that only women

who took NSAIDs at both baseline and year 3 had a lower risk of colorectal cancer mortality.[201] An inverse association of NSAID use with colorectal cancer survival was also reported in the California Teacher's Cohort (n = 621 women).[202] In contrast, two other research groups reported no association of NSAIDs with overall mortality,[203,204] although one of them found a weak inverse association of aspirin with colorectal cancer mortality.[204]

Smoking and alcohol

A recent meta-analysis of studies conducted among colorectal cancer patients that assessed smoking status before, at the time of, or after cancer diagnosis found higher risk of all-cause mortality among current smokers (summary HR 1.26, 95% CI 1.15 – 1.37) compared with never smokers.[205] One possible explanation might involve the role of tumor molecular phenotypes. Smokers may be at higher risk for colorectal cancer, especially for tumors that are MSI-high,[62,64-67] CIMP-high,[67,69,206] and *BRAF*-mutated,[66,67,69] all of which are inter-correlated. Although MSI-high tumors generally have a better prognosis,[169,170] *BRAF* mutation is independently associated with higher risk of mortality among colorectal cancer patients[173], and CIMP-high in non-MSI-high tumors likely predicts poor prognosis.[171,172] It is likely that at the time of diagnosis, smokers may bear more pathologically aggressive tumors that confer a worse prognosis. Three studies reported that the impact of smoking on colorectal cancer survival differs according to tumor molecular phenotype, although the patterns of association across tumor molecular phenotypes varied across studies and more research is needed to determine whether smoking specifically impacts on certain molecular phenotypes of colorectal carcinogenesis to influence patient prognosis.[207-209] Importantly, two studies reported changes in smoking status from pre- to post-diagnosis (particularly quitting after diagnosis) in relation to mortality risk, and the authors reported that current smokers who quit after diagnosis were at slightly lower risk of colorectal-cancer specific mortality or all-cause mortality than were those who continued smoking,[209,210] but a limitation for

both studies was that the reasons for quitting smoking after diagnosis were unknown and could have been associated with prognosis.

Only three studies investigated the association of overall alcohol drinking and survival among colorectal cancer patients: in the NIH-AARP study, moderate drinking was associated with statistically significant lower all-cause mortality (RR 0.82, 95% CI 0.71-0.93) and marginally lower colorectal cancer-specific mortality (RR 0.86, 95% CI 0.73-1.01) among colon cancer patients but not rectal cancer patients; heavy or moderate drinking was also associated with lower mortality from cardiovascular disease.[211] The other two studies reported null results.[209,212] However, another study reported that wine consumption (but not beer or liquor) was inversely associated with all-cause mortality in familial (but not sporadic) colorectal cancer cases, suggesting that this association may depend on the type of alcohol and the type of colorectal cancer.[27]

Diet

Dietary factors (either before or after diagnosis) in relation to mortality among colorectal cancer patients have only been examined in a few studies. Factors examined include the intakes of total energy, fiber, fat, protein, cholesterol, carbohydrate, red meat, alcohol, fruit and vegetables, cod liver oil, calcium, vitamins, dietary patterns, and blood concentration of some micronutrients, but the very limited amount of literature precludes any meaningful conclusions.[183] To our knowledge, there are only four published articles that investigated the association of colorectal cancer survival with calcium intake (only one of which has presented detailed results), five with vitamin D, and two with dairy products or milk, as reviewed below.

Calcium

Slattery *et al*. examined associations of various dietary factors with colorectal cancer survival among 411 colon cancer patients identified through the Utah Cancer Registry from two case-control studies between 1976 and 1981: pre-diagnosis calcium intake, assessed by a

quantitative food frequency questionnaire, was not associated with survival (the point estimate was not provided).[26]

Zell *et al*. investigated wine assumption with colorectal cancer survival among 141 familial and 358 sporadic CRC cases. Calcium intake one year before diagnosis, derived from the Block food frequency questionnaire (FFQ), was evaluated as a covariate, but removed from the model because it was not associated with survival (the point estimate was not provided).[27]

Dray *et al.* followed 148 colorectal cancer survivors for 10 years and evaluated a series of dietary factors in relation to their survival, and reported that the relative risks of death were, respectively, 0.73 and 0.69 for those in the 2nd and 3rd tertiles of dietary calcium intake relative to those in the lowest tertile, but neither estimate was statistically significant.[28]

Dik *et al*. reported no associations of prediagnosis dietary calcium intake with all-cause and colorectal cancer-specific mortality among 3,859 colorectal cancer survivals in the EPIC cohort. The RR comparing those in the highest vs. the lowest quartiles of dietary calcium intake was 1.01 for both outcomes, and did not differ by whether the calcium intake was from dairy or non-dairy sources.[29]

Vitamin D

Ng and colleagues reported in 2008 that among 304 colorectal cancer patients identified from two large U.S. cohorts, higher circulating levels of 25-hydroxyvitamin D_3 [25(OH)D] were associated with reduced all-cause mortality (RR: 0.52, 95% CI: 0.29 - 0.94, comparing those in the highest vs. the lowest quartiles).[213] As this study was limited by its sample size and having only a single measurement before diagnosis, the same group of authors conducted another study and created a prediction model for post-diagnosis, long-term 25(OH)D based on race, region of residence, vitamin D intake, BMI, and physical activity among 1,017 colorectal cancer patients,

and reported that the predicted value was associated with lower mortality both from all-causes (RR: 0.62, 95% CI: 0.42 - 0.93) and specifically from colorectal cancer (RR: 0.50, 95% CI: 0.26 - 0.95).[214]

Mezawa *et al*. directly measured serum 25(OH)D at surgery from 257 colorectal cancer patients in Japan, and reported that higher 25(OH)D level (as a continuous variable) was favorably associated with overall survival among these patients (RR: 0.91, 95% CI: 0.84 - 0.99), adjusted for month of blood collection, age at diagnosis, sex, cancer stage, and other factors.[215]

Fedirko *et al*. investigated an association of pre-diagnostic 25(OH)D with survival among 1,202 European colorectal cancer patients based on the EPIC cohort during a six-year follow-up, and found inverse associations with both overall mortality (RR: 0.67, 95% CI: 0.50 - 0.88) and colorectal cancer-specific mortality (RR: 0.69, 95% CI: 0.50 - 0.93) comparing those in the highest vs. lowest quintile. A potential interaction with pre-diagnostic dietary calcium intake was also found (associations were stronger for patients with higher pre-diagnosis dietary calcium intake).[216]

Tretil *et al*. examined serum 25(OH)D at the time of diagnosis in relation to disease-specific survival in a Norwegian population. The authors reported non-significant inverse associations between 25(OH)D and CRC-specific mortality, but the sample size was limited (n = 52 colorectal cancer survivors).[217]

Zgaga *et al*. reported that among 1,598 colorectal cancer patients (stage I - III) in the United Kingdom, higher postoperative plasma 25(OH)D level was associated with lower colorectal cancer-specific (RR: 0.68, 95% CI: 0.50 - 0.90) and all-cause (RR: 0.70, 95% CI: 0.55 - 0.89) mortality; furthermore, interactions were detected between vitamin D receptor genotypes and 25(OH)D concentration.[218]

Dairy or Milk

Dray *et al.* followed 148 colorectal cancer survivors for 10 years and reported that the relative risks of death after being diagnosed with colorectal cancer were, respectively, 0.53 and 0.63 for those in the 2nd and 3rd tertiles of dairy products intake, but neither of these estimates was statistically significant due to the relatively small sample size.[28]

Dik *et al*. investigated associations of pre-diagnosis dietary calcium intake with all-cause and colorectal cancer-specific mortality among 3,859 colorectal cancer survivors in the EPIC cohort, and reported null results for total dairy products, as well as for milk, yogurt, and cheese individually.[29]

Overall, evidence on associations of calcium intake with colorectal cancer survival is very limited, especially regarding post-diagnosis intakes of calcium or dairy products (the major food sources of calcium). It would be important to add to the evidence base to better inform the development of specific dietary guidelines for colorectal cancer survivors who may be actively seeking diet and lifestyle changes to improve their diagnosis.

Calcium and Other Health Outcomes

Cardiovascular disease (CVD)

Calcium may have a complex relationship with CVD pathogenesis: it has been proposed that calcium may favorably regulate cholesterol levels, blood pressure, and insulin sensitivity; on the other hand, calcium may also cause vascular calcification.[219] One of the earliest studies of calcium and risk of cardiovascular events was published in 1973, when Knox reported a lower risk of ischemic heart disease mortality with higher dietary calcium intake.[220] Subsequent investigations in this area suggested that the association of calcium with CVD may depend on the

source of calcium (foods or supplements). Some evidence suggests that ingesting calcium supplements, but not calcium-rich foods, may lead to an acute increase in serum calcium,[219,221,222] which may be positively associated with vascular calcification,[223-225] a risk factor for cardiovascular diseases.[226-230] Although not entirely consistent, dietary calcium generally appears to be weakly associated with lower risk of cardiovascular events (including incidence and mortality): according to a meta-analysis in 2010, the relative risk summarized from prospective cohorts comparing the extreme categories of dietary calcium intake was 0.92 for risk of coronary artery disease and 0.86 for stroke, but the confidence intervals for both risk estimates overlapped one;[219] a more recent meta-analysis published in 2015 reported that the RR for CVD mortality comparing the extreme levels of dietary calcium intake was 0.97 (0.89 – 1.07).[231] The associations of supplemental calcium use and CVD outcomes have been inconsistent in the current literature: supplemental calcium use was associated with adverse cardiovascular events in several large cohort studies, including the EPIC and NIH-AARP cohorts,[32-34] while null or inverse associations were reported in the Iowa Women's Health Study, Harvard Health Professional Study, and others.[35-38] Furthermore, secondary analyses from several randomized clinical trials (e.g., those with osteoporosis as the primary outcome, primarily among older women not concurrently taking vitamin D supplements) indicated that patients in the calcium arm compare to the placebo arm, either had no difference in cardiovascular outcomes[8,232,233] or a higher risk of MI,[234] coronary revascularization,[235] vascular disease mortality,[236] or a composite outcome (MI, stroke, or sudden death).[234] As summarized by Bolland *et al*. using data from nine clinical trials, calcium supplements taken with or without vitamin D increased the risk of MI by 24%, and the risk of the composite of MI or stroke by 15%, and both risk estimates were statistically significant.[39] Although the above trials were not primarily designed to assess the effect of calcium supplementation on cardiovascular events, the results from these secondary analyses raised concerns about the potential adverse effects of supplemental calcium on the cardiovascular system, and thus warrant further investigation.

Cancer

There is strong observational evidence that calcium is inversely associated with risk of colorectal cancer, and a major clinical trial reported statistically significant reduction of colorectal adenoma recurrence with calcium supplementation.[6-8] Other types of cancer have not been as extensively studied, but some evidence suggests that total or dietary calcium may be associated with lower risk of breast cancer,[40,41] and total calcium or dairy intake may be positively associated with risk of prostate cancer,[42] but the World Cancer Research Fund considers the level of evidence "limited" for both types of cancer.[43,44] In observational studies, dietary calcium is generally associated with lower overall cancer incidence and mortality, although the associations may be restricted to certain sub-populations (e.g., only women) or to a specific cancer site (e.g., gastrointestinal tract cancers).[13-15,74] For example, in the NIH-AARP cohort study, Park *et al.* found an inverse association of dietary calcium with total cancer incidence only in women, and an inverse association of total calcium with cancers of the digestive system in both sexes, but no association of calcium with total cancer mortality.[237] Supplemental calcium, in contrast, does not seem to be associated with total cancer incidence and mortality. Bristow *et al.* conducted a meta-analysis of randomized clinical trials, and found no association of calcium supplementation with total cancer risk (RR 0.95, 95% CI 0.76 - 1.18) or cancer mortality (RR 0.96, 95% CI 0.74 - 1.24).[16] However, these trials are usually designed for other primary outcomes, and not sufficiently powered to detect the effect of calcium on cancer incidence or mortality; also, the relatively short durations of the trials did not allow for evaluating outcomes with a longer latency.[16] Therefore, the associations of diet and supplemental calcium intakes with cancer incidence and mortality need to be further investigated in large, well-characterized cohorts, and specifically-designed clinical trials if feasible.

Hypotheses

1. I hypothesize that calcium supplementation (1.0 or 2.0 g/d) over a 4-month treatment period can favorably modulate plasma biomarkers of inflammation (C-reactive protein and a 10-plex panel of cytokines), oxidative stress (F_2-isoprostanes), and gut permeability (antibodies against flagellin and LPS), among patients with previous sporadic colorectal adenoma in a randomized controlled trial.

2. I hypothesize that intakes of calcium, vitamin D, and dairy products before and/or after diagnosis are associated with lower mortality among individuals diagnosed with invasive, non-metastatic colon or rectal cancer.

3. I hypothesize that among individuals initially free from cancer or CVD, high intake of supplemental calcium is associated with increased mortality from CVD, particularly in men; also, total or dietary calcium intake is not associated with mortality outcomes in men or women.

Objectives

My primary objective is to explore the mechanisms underlying the chemopreventive properties of calcium on colorectal neoplasms, and further investigate whether any benefits of calcium intake on the prevention of colorectal cancer incidence extends to prognosis and survival outcomes among those already diagnosed with colorectal cancer. Furthermore, I aim to investigate the role of calcium beyond colorectal cancer, and evaluate whether it is associated with several major causes of death, including CVD and cancer, which may inform future dietary recommendations.

Specific Aims

Aim 1: Estimate the effects of calcium supplementation (1 g/d or 2 g/d) on circulating biomarkers of inflammation, oxidative stress, and gut permeability over four months of treatment,

using data and blood samples from a previously conduced randomized controlled trial, among 193 patients with previous sporadic colorectal adenoma.

1a: Test the effects of calcium on circulating levels of C-reactive protein, cytokines (alone or in combination), and F_2-isoprostanes, all of which are putative biomarkers of risk for colorectal cancer.

1b: Test the effects of calcium on antibodies against flagellin and LPS (which may be involved in the development of metabolic diseases, gastrointestinal disorders, and cancer), and evaluate baseline association of these biomarkers with selected demographic, dietary, and lifestyle factors.

Aim 2: Investigate associations of pre- and post-diagnosis intakes of calcium (total, dietary, and supplemental), vitamin D (total and dietary), and dairy products (total dairy and milk only) with mortality from all causes and specifically from colorectal cancer, among 2,284 individuals diagnosed with invasive, non-metastatic colorectal cancer in the Cancer Prevention Study II Nutrition Cohort.

Aim 3: Investigate associations of total, dietary, and supplemental calcium intakes with mortality from all causes, cancer, CVD, and other causes, among 132,823 participants in the Cancer Prevention Study II Nutrition Cohort who were initially free from cancer or CVD at baseline (1992/1993).

Methods and Power Calculations

Aim 1:

We used data and blood samples from a chemoprevention trial conducted from 1990 to 1994 in the Minneapolis, MN metropolitan area.[17] Eligible patients with previous colorectal adenoma who consented to participate in this study (n = 193) were randomly assigned (stratified by sex) to one of three groups: a placebo control group (n = 66) and 1.0 g (n = 64) and 2.0 g (n = 63) elemental calcium supplementation groups. Blood samples were collected and biomarkers were measured at baseline and 4-month follow-up visit. The effects of calcium on biomarkers of inflammation, oxidative stress, and gut permeability were estimated using a mixed linear models procedure for repeated measures data as implemented in SAS Institute's Mixed Procedure (SAS version 9.4; SAS Institute, Cary, NC). We had 99% power to detect effect estimates equal to those estimated in the preliminary studies, and 80% power to detect effect estimates that are half the size or less than those found in our preliminary studies.

Aim 2:

Within the Cancer Prevention Study II Nutrition Cohort, we identified 2,284 persons diagnosed with invasive, non-metastatic colon or rectal cancer after baseline (1992 or 1993) and up to 2009 and following for their mortality outcomes through 2010. Dietary information was collected at baseline using a Block FFQ, and updated in 1999 and 2003 using a Willett FFQ. We estimated associations of pre- and post-diagnosis intakes of calcium, vitamin D, and dairy products with mortality from all causes, colorectal cancer, and CVD, using multivariable-adjusted Cox proportional hazards regression models, adjusted for age and tumor stage at diagnosis, sex, and pre- or post-diagnosis intakes of total energy and total folate. For the association between each exposure variable and all-cause mortality, the power is > 80% for detecting an RR of 0.80. We acknowledge that the analyses for the secondary outcomes (colorectal cancer and CVD) will have less power;

we feel these analyses are still worthwhile to explore and generate specific hypotheses concerning the associations, if any, between these dietary exposures and colorectal cancer survival.

Aim 3:

Within the Cancer Prevention Study II Nutrition Cohort, we identified 132,823 eligible participants initially free from cancer or CVD at baseline (1992/1993), and followed for their mortality outcomes through 2012. Dietary information was collected at baseline using a Block FFQ, and updated in 1999 and 2003 using a Willett FFQ. We assessed associations of total, dietary, and supplemental calcium intakes with mortality from all causes, cancer, CVD, and other causes, separately by sex, using multivariable-adjusted Cox proportional hazards regression models, with cumulative updating of the main exposure variables. We have sufficient power to detect a modest association between each type of calcium intake and mortality: for all-cause mortality, the power is $\geq$ 95% for detecting an RR of 0.95 or 1.05; for cancer- or CVD-specific mortality, the power is around 50% when the RR is 0.95 or 1.05, but rises to above 90% when the RR becomes 0.90 or 1.10.

CHAPTER 2. CIRCULATING BIOMARKERS OF GUT BARRIER FUNCTION: CORRELATES AND RESPONSES TO CALCIUM SUPPLEMENTATION AMONG SPORADIC COLORECTAL ADENOMA PATIENTS IN A DOSE-RESPONSE RANDOMIZED CONTROLLED TRIAL

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Abstract

Gut barrier dysfunction contributes to several gastrointestinal disorders, including inflammatory bowel disease and colorectal cancer, but factors associated with intestinal hyperpermeability have been minimally studied in humans. We evaluated factors associated with baseline circulating biomarkers of gut permeability, and tested the effects of two doses of calcium (1.0 or 2.0 g/d) on these biomarkers over a 4-month treatment period among colorectal adenoma patients in a randomized, double-blinded, placebo-controlled clinical trial (n = 193). Circulating levels of anti-flagellin and anti-lipopolysaccharide (LPS) immunoglobulins (Igs) as markers of colonic hyperpermeability were measured via ELISA. At baseline, mean levels of anti-flagellin IgA and anti-LPS IgA were, respectively, statistically significantly proportionately higher by 11.8% and 14.1% among men, 31.3% and 39.8% among those with a body mass index (BMI) $\geq$ 35 kg/m^2, and 19.9% and 22.0% among those in the upper relative to the lowest sex-specific tertile of waist circumference. A combined permeability score (the summed optical densities of all four permeability biomarkers) was 24.3% higher among women ($p_{trend} < 0.01$) who were in the upper tertile of plasma C-reactive protein, but not among men. We found no appreciable effects of supplemental calcium on anti-flagellin or anti-LPS Igs. Our results suggest that 1) men and those with a larger BMI or waist circumference may have greater gut permeability, 2) markers of gut permeability and systemic inflammation may be directly associated with one another, and 3) supplemental calcium may not modify circulating levels of biomarkers of gut permeability within four months.

Introduction

The gastrointestinal tract has the largest mucosal surface in the body interacting with the environment, and an intact gut barrier with selective permeability is key to balancing the absorption of nutrients and blocking harmful wastes, such as bacterial products.[103] Abnormal gut barrier function contributes to several gastrointestinal disorders, such as inflammatory bowel disease (IBD), Celiac disease, food allergies,[104] and colorectal cancer.[105,112,238] Factors associated with gut hyperpermeability have not been well-characterized, although evidence suggests that diet, among other factors, may impact gut permeability, based on animal studies and very limited human clinical trials.[104,128]

Calcium is a plausible agent that may play a role in modulating gut barrier function since calcium can bind bile and fatty acids in the colon lumen by forming insoluble soaps, thus preventing them from oxidatively damaging the colonic mucosa and consequently producing inflammation,[4,9,10] which, in turn, may help maintain the strength of the gut mucosal barrier. Our research group previously conducted a 6-month pilot randomized controlled trial among patients with previous colorectal adenoma, and found that among subjects treated with calcium (n = 23) compared to the placebo (n = 23), 8-hydroxydeoxyguanosine level (as a marker of oxidative DNA damage) in the normal-appearing colon tissue was reduced by 22%,[19] and a comprehensive summary z-score of multiple plasma biomarkers of inflammation was reduced by 48%.[23] Based on these data, we hypothesized that calcium may also favorably modulate gut permeability. The effect of calcium supplementation on gut permeability was previously tested in a very limited number of animal studies[136-138] and one pilot human clinical trial (n = 32),[139] and their results all support this novel hypothesis. However, to our knowledge, there are no reported full-scale clinical trials that directly tested the effect of calcium on gut permeability in humans.

To address these gaps in the literature, we measured circulating levels of flagellin- and lipopolysaccharides (LPS)-specific immunoglobulins (Igs) IgA and IgG among patients with previous colorectal adenomas in a full-scale, randomized, double-blinded, placebo-controlled clinical trial ("the Calcium Trial", n = 193). Circulating levels of flagellin- and LPS-specific IgA and IgG may serve as markers of long-term systemic exposure to flagellin and LPS and may indicate altered adaptive immune responses related to colonic hyperpermeability.[106-110] We evaluated factors associated with these circulating biomarkers of gut permeability at baseline (including major demographic, diet and lifestyle factors, and systemic inflammation levels) and tested whether biomarker levels were affected by calcium supplementation over four months of treatment.

Patients and Methods

This study was approved by the Committee on Use of Human Subjects in Research of the University of Minnesota. Written informed consent was obtained from each study participant.

Participant Population

Detailed information on study recruitment protocol, eligibility and exclusion criteria was published previously.[17] Briefly, subjects aged 30 – 74 years who were in general good health and had a history of pathology-confirmed adenomatous polyps within the previous five years were recruited from the patient population of a major private-practice gastroenterology group in Minneapolis-St. Paul, MN. Exclusion criteria included contraindications to calcium supplementation or rectal biopsies; medical conditions, habits, or medication usage that would otherwise jeopardize safety, adherence, or interpretation of the study results; and failure to take > 80% of the prescribed tablets in a 1-month placebo run-in trial.

Clinical Trial Protocol

Potential participants were first invited for an eligibility visit to complete questionnaires and provide blood samples, after which those who appeared eligible entered a 4-week placebo run-in trial. Only participants without substantial perceived side effects and who had taken > 80% of their tablets in the 4-week placebo run-in trial were eligible for randomized assignment. Eligible participants (n = 193) then underwent a baseline visit and were randomly assigned (stratified by sex) to one of three groups: a placebo control group (n = 66) and 1.0 g/d (n = 64) and 2.0 g/d (n = 63) elemental calcium supplementation groups. The supplement and placebo pills, prepared by SmithKline Beecham, Pittsburgh, PA, were identical in size, appearance, and taste. The calcium tablets were in the form of calcium carbonate and taken in two equally divided doses twice daily with food. The reasons for choosing calcium carbonate were described previously.[17]

The treatment period was 6 months, and participants attended follow-up visits at 1, 2, 4, and 6 months after random assignment (baseline). Pill-taking adherence was assessed at follow-up visits by questionnaire, interview, and pill count. Participants were instructed to remain on their usual diets during the study, and a Willett semi-quantitative food-frequency questionnaire was administered at baseline and again at the final follow-up visit. Factors hypothesized to be related to gut barrier function (such as interviewer-measured body mass index [BMI] and waist-hip ratio) were assessed at baseline, several were reassessed at each follow-up visit, and all factors were reassessed at the final follow-up visit.

Peripheral venous blood samples were collected at the baseline and 4-month follow-up visits, after the subject sat upright with his or her legs uncrossed for 5 minutes. Blood was drawn into pre-chilled Vacutainer tubes for plasma and serum, and then immediately placed on ice and shielded from light. Tubes were immediately processed, plasma and serum were aliquotted into

cryopreservation tubes, the air was displaced with nitrogen, and then the aliquots were immediately placed in a -80 ^{0}C freezer until analysis.

Laboratory Protocol

Levels of flagellin- and LPS-specific IgA and IgG were measured via a previously described custom-made ELISA at Georgia State University.[106,107,111] ELISA plates (Costar™) were coated overnight with laboratory-made flagellin (100 ng/well) or purified *E. coli* LPS (2 µg/well; from *E. coli* 0128: B12, Sigma, Catalog No. 2887). Plasma samples diluted 1:200 were applied to wells coated with flagellin or LPS. After incubation and washing, the wells were incubated either with IgG coupled to horseradish peroxidase (GE, Catalog No.375112) or, in the case of IgA-specific antibodies, with peroxidase-labeled IgA (KPL, Catalog No. 14-10-01). Quantitation of total immunoglobulins was performed using the colorimetric peroxidase substrate tetramethylbenzidine (TMB), and optical density (OD) was read at 450 nm and 540 nm (the difference was taken to compensate for optical interference from the plate), with an ELISA plate reader. Data are reported as OD corrected by subtracting background (determined by readings in blank samples) and are normalized to each plate's control sample, which was prepared in bulk, aliquotted, frozen, and thawed daily as used. Standardization was performed using preparations of known concentrations of IgA, and IgG. The technician was blinded to treatment group and treated all samples identically. Baseline and follow-up samples from each participant were included in the same batch. The laboratory previously performed assays of these biomarkers in replicates with a very low coefficient of variation (CV < 5%); therefore, our samples were analyzed in singleton to minimize costs and time. For quality control, two duplicate plasma samples were measured in each batch. The within-batch coefficient of variation was < 20%, and most frequently < 5%.

Plasma levels of the inflammation biomarkers were measured using electrochemiluminescence detection-based immunoassays in the Emory Multiplexed Immunoassay Core (EMIC). All biomarkers were measured in duplicate, according to the manufacturer's protocol, and the technicians were blinded to the treatment group assignment. We selected biomarkers with an average intra-assay coefficient of variation (CV) < 15% for further analysis, including C-reactive protein (CRP), interleukin (IL)-10, IL-12p40, IL-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF).

Statistical Analysis

Treatment groups were compared on baseline characteristics using analysis of covariance (ANCOVA) for continuous variables, and the chi-square or Fisher's exact test for categorical variables; sex was included as a covariate when appropriate. Pearson correlation coefficients were calculated for each pair-wise combination of the four gut permeability biomarkers. Associations of selected baseline demographic, diet and lifestyle factors, and circulating biomarkers of inflammation with gut barrier function biomarkers were assessed using ANCOVA, adjusted for sex and BMI as appropriate. To better present different aspects of inflammation, we created a baseline cytokine summary z-score, as the sum of the z values for each cytokine [$z = (x - \mu)/\delta$, where x is the natural log-transformed values for each individual marker, and μ and δ are the sex-specific mean and standard deviation of the natural log-transformed biomarker value, respectively, at baseline]. The z-score for IL-10 was included with a negative sign because of its anti-inflammatory properties.[239]

The primary analysis of the effects of calcium on gut barrier function biomarkers was based on random assignment of treatment group regardless of adherence (intent-to-treat). Because the biomarker values were normally distributed, they were not log-transformed before statistical testing. Treatment effects on the biomarkers from baseline to 4-month follow up across

the three treatment groups were compared using a mixed linear model for repeated measures data as implemented in SAS Institute's Mixed Procedure (SAS version 9.4; SAS Institute, Cary, NC). The model included as predictors the intercept, visit (baseline and 4-month follow-up), treatment groups (coded as dummy variables), and a treatment-by-visit interaction term. An absolute effect, obtained from the Mixed model, was defined as [(treatment group follow-up mean) - (treatment group baseline mean)] - [(placebo follow-up mean) - (placebo baseline mean)]. In order to provide a conservative estimate of the proportional change in the treatment group relative to that in the placebo group, we also calculated a relative effect, defined as (treatment group follow-up mean/treatment group baseline mean) / (placebo follow-up mean/placebo baseline mean). Its interpretation is somewhat analogous to that of an odds ratio (e.g., a relative effect of 1.10 would mean that the proportional change in the treatment group was 10% higher than that in the placebo group).

We first analyzed each gut permeability biomarker individually. Then, we created several combinations to better capture different aspects of gut barrier function, including anti-flagellin Igs (flagellin IgA + flagellin IgG), anti-LPS Igs (LPS IgA + LPS IgG), IgA (flagellin IgA + LPS IgA), IgG (flagellin IgG + LPS IgG), and all four biomarkers combined as a permeability score (flagellin IgA + flagellin IgG + LPS IgA + LPS IgG). These biomarkers were directly summed up because their optical density measurements were approximately on the same scale. To adjust for possible batch effects, we ran sensitivity analyses using batch-adjusted biomarker levels calculated as the original value divided by the mean level within the batch.

Results

The mean age of the study participants was 59 years, 63% were men, 99% were White, and 28% had a family history of colorectal cancer in a first-degree relative. The baseline

characteristics of the participants did not differ significantly across the three treatment groups (Table 2.1).

Among the 193 participants, measurements of the plasma biomarkers of gut permeability were available for 189 at baseline, and 174 at follow-up. The baseline gut permeability biomarkers were moderately to strongly correlated (Pearson correlation coefficients 0.20 – 0.67 for men and 0.37 – 0.80 for women), and the p-values for all pair-wise Pearson correlations were < 0.05 (Table 2.2). As shown in Table 2.3, the baseline levels of anti-flagellin IgA and anti-LPS IgA were, respectively, statistically significantly proportionately higher by 11.8% and 14.1% among men (p value < 0.05) relative to women, 31.3% and 39.8% among those who were very obese (BMI $\geq$35 kg/m^2) relative to those who were underweight/normal weight ($p_{trend} < 0.01$), and 19.9% and 22.0% among those in the upper relative to the lowest sex-specific tertile of waist circumference ($p_{trend} < 0.01$). A combined permeability score (the summed optical density measurements from all biomarkers) was 24.3% higher among women who were in the upper relative to the lowest tertile of plasma C-reactive protein concentrations ($p_{trend} < 0.01$), but not among men (Table 2.4). No associations of any of the gut barrier function biomarkers were found with age, waist-hip ratio, cigarette smoking, alcohol drinking, NSAID use, or adenoma characteristics (Table 2.3), nor with physical activity, vitamin/mineral supplement use, intakes of fat, red/processed meat, and fruit/vegetable, or a comprehensive oxidative balance score (OBS) [240,241] (data not shown). Batch-adjustment did not change the results (data not shown).

Overall adherence to visit attendance was 95.3%, and did not differ among the treatment groups. The mean percentage of pills taken in each group was 97%, and > 98% of all participants in each group took > 80% of their pills. Changes in the gut barrier function biomarkers, alone or in combination, for each calcium treatment group relative to the placebo group, are shown in Table 2.5. We found no appreciable or statistically significant treatment effects of either

supplemental calcium dose on any of the biomarkers, alone or in combination. The results were similarly null among categories of BMI, sex, age, OBS, NSAID use, adenoma characteristics, and usual pre-trial calcium intake, and when the analyses were restricted to participants with good treatment adherence (data not shown).

Discussion

Our results suggest that 1) men and participants with higher overall or abdominal adiposity may have higher levels of anti-flagellin and anti-LPS IgA, indicating greater gut permeability; 2) markers of gut permeability and systemic inflammation may be directly associated with one another, particularly among women; and 3) supplemental calcium at moderate and relatively higher doses has no substantial effect on levels of biomarkers of gut barrier function over four months among individuals with previously diagnosed colorectal adenoma.

We found higher levels of anti-LPS and anti-flagellin Igs in men than in women. Overall, levels of anti-LPS and anti-flagellin Igs may reflect erosion of mucosal anatomic and immune barriers, gut bacteria composition and their ability to translocate across the gut, and immune responses against bacterial antigens. Because men generally have lower innate and adaptive immune responses than women,[242] it is likely that men are systemically exposed to a higher level of bacterial products as a result of impaired gut barrier function and/or distinct microbiome profiles[243] potentially due to diet, lifestyle, or hormonal factors. Alternatively, there is evidence that given the same amount of *in vivo* LPS exposure, male mice produce higher levels of LPS-binding protein and higher inflammation mediators than female mice.[127] While the exact biological mechanisms require further investigation, future observational epidemiologic studies for the association of gut permeability with various health outcomes may need to consider sex as an important confounder and/or effect modifier.

Our findings that BMI and waist circumference, a reliable predictor of visceral fat, are positively associated with colonic permeability is largely consistent with previous literature. Evidence from several human cross-sectional studies supports a positive association of obesity (especially abdominal obesity) with several intestinal permeability measurements, such as the sucralose-to-*mannitol ratio*, IgG against bacterial antigens, and LPS-binding protein (LBP).[125,126,244] One possible explanation is that obese individuals may have different gut microbiota and/or gut microbiome patterns;[245] for example, obese individuals often consume a high-fat diet, which may favor the growth of gram-negative bacteria in the gut.[246] Gram-negative bacteria may have a greater ability to translocate across the gut mucosa into the circulation compared to gram-positive microbes.[108] Furthermore, LPS is a major component of the outer membrane of Gram-negative bacteria. Therefore, it is biologically plausible that obese individuals have higher levels of anti-LPS and anti-flagellin Igs. However, the temporal sequence of gut barrier dysfunction and obesity cannot be assessed in such cross-sectional studies. Results from a few animal and human trials suggested that gut barrier dysfunction and obesity could mutually influence each other. For example, mice with induced metabolic endotoxemia (through infusion of LPS) experienced weight gain in 4 weeks, suggesting that the LPS system may trigger the onset of obesity.[123] Conversely, mice with induced-obesity had significantly higher IgG against bacterial extracts,[126] and rats with transplanted visceral adipose tissue or that were injected with leptin had increased colonic epithelial permeability as measured by expression of trans-epithelial resistance and tight junction proteins, suggesting that obesity may induce gut barrier impairment.[247] In humans, plasma LPS levels were higher in obese individuals (n = 49) than in controls (n = 17), but they were reduced after bariatric surgery; however, reduced LPS levels were not found with a preoperative weight-loss intervention, and the postoperative LPS reduction was not correlated with a BMI reduction, suggesting mechanisms beyond weight loss.[248]

Our study provides some evidence that levels of systemic inflammation may be positively correlated with gut permeability. We previously hypothesized that oxidative damage and subsequent inflammatory responses in the gut result in damage to the gut barrier and increase gut permeability. Current evidence suggests that enhanced mucosal immune activities may also be a consequence of gut barrier dysfunction.[103] For example, Hollander *et al.* found that compared to healthy controls, patients with Crohn's disease and their clinically unaffected relatives had similarly increased gut permeability, suggesting that gut barrier dysfunction is not secondary to intestinal inflammation.[249] In experimental studies, translocation of flagellin across epithelia mediated Salmonella-induced mucosal inflammatory activities *in vitro*,[250] via activating basolaterally expressed Toll-like receptor 5 (TLR5),[251] and systematic injection of flagellin in mice induced the expression of a panel of pro-inflammatory mediators such as cytokines.[252] Gut permeability and inflammation are likely closely related in a complex manner, and may act together in the pathogenesis of metabolic disorders such as diabetes and obesity,[123,124,253] both of which are associated with the incidence of several types of cancer, including colorectal cancer.

The effect of calcium on gut permeability has rarely been studied before. Bovee-Oudenhoven and colleagues conducted several controlled trials in rats, and reported that a high-calcium diet reduced the translocation of *Salmonella*, inhibited the increase in intestinal permeability as measured by urinary chromium EDTA (CrEDTA), and improved resistance to intestinal infection;[136-138] they also found a similar effect of high-calcium milk relative to low-calcium milk against enterotoxigenic *Escherichia coli* (ETEC) infection in rats and a small group of men (n = 32),[139] but the potential interaction between calcium and other components in milk could not be excluded. We found no effects of calcium supplementation on immunoglobulins against selected bacterial products, possibly due to several reasons. First, calcium may simply have no important effect on gut permeability in humans. Second, the circulating biomarkers investigated in this study may not be the most direct measurement of gut permeability; however,

emerging evidence suggests a positive correlation of antibodies against LPS and flagellin with serum fluorescein isothiocyanate–dextran–dextran, a direct measurement of intestinal barrier function.[110] Third, although the treatment period of the original trial was 6 months, blood was only collected at baseline and month 4, since blood biomarkers were not the pre-specified primary outcomes of the trial. This treatment duration may be insufficient to observe an effect of calcium on these permeability markers, as antibodies against bacterial products can persist for several months,[254,255] and the effect of calcium on gut barrier function may not be immediately accompanied by a decrease of antibody levels. Fourth, the original trial was conducted in the 1990s, so it is possible that the samples deteriorated over the years; however, we did not find strong evidence to support this. The samples were immediately processed and stored with no additional freeze-thaw cycles since the original storage, the levels of the inflammation markers were comparable to those in another trial with more recently collected blood samples,[23] and anti-LPS and anti-flagellin Igs are stable over time (personal communication with A. Gewirtz) and, as described above, were associated with BMI as in other reported studies. Finally, chance remains a possible explanation.

Major strengths of our study include that it is a full-scale randomized, controlled trial with a dose-response component. Other strengths include the inclusion of novel gut permeability biomarkers and the excellent overall adherence to treatment. We also collected detailed questionnaire information and were able to evaluate associations of baseline demographic, diet, and lifestyle factors with gut permeability levels, which may provide insights for future epidemiological studies. Limitations of the study include the above-mentioned relatively short treatment period and long storage period of the blood samples. In addition, the gut permeability biomarkers were measured in singleton; however, based on previous assays on these same biomarkers we expect that our biomarker measurement reliability was high. The use of antibiotics may impact gut bacteria and subsequent immune responses against bacterial products.

We excluded patients who were on antibiotics at baseline but lacked data on the use of antibiotics during the trial or during the year prior to the trial (which may have a long-term effect on the gut microbiota); however, antibiotic use is expected to be balanced among the three groups due to randomization. Also, this study is based on a population of patients with a history of colorectal adenoma who were participating in a chemoprevention trial, and thus our findings may have limited external generalizability.

In conclusion, taken together with previous literature, our results suggest that those with greater adiposity may have greater gut permeability. Our results also suggest that men may have greater gut permeability and that markers of gut permeability and systemic inflammation may be directly associated with one another. Finally, supplemental calcium may not modify circulating levels of biomarkers of gut permeability, at least in sporadic colorectal adenoma patients, within a 4-month treatment period. Our findings may facilitate better understanding of the factors that influence gut permeability biomarkers to inform development of treatable biomarkers of risk for colorectal cancer and other health conditions and outcomes.

Tables and Figures

Table 2.1. Selected baseline characteristics of study participants in the Calcium Trial

Characteristics	Treatment group			P-value[1]
	Placebo (n = 66)	Calcium 1 g (n = 64)	Calcium 2 g (n = 63)	
Age, yrs.	60 (9)	60 (9)	58 (10)	0.37
Men (%)	64	63	62	0.98
White (%)	98	100	100	> 0.99
College graduate (%)	35	19	33	0.08
Employed (%)	52	45	56	0.48
Family history (%)	26	25	30	0.78
Take aspirin[2] (%)	21	27	16	0.34
Take non-aspirin NSAID[2] (%)	9	11	10	0.92
Currently smoke (%)	20	16	24	0.53
Alcohol intake, g/d	11 (19)	13 (20)	8 (13)	0.20
Body mass index, kg/m^2				
Men	28.0 (3.8)	29.0 (3.1)	28.8 (4.5)	0.47
Women	30.1 (5.2)	28.1 (8.4)	26.3 (4.4)	0.12
Vigorous/moderate physical activity, MET-hours/d	33 (21)	30 (22)	28 (21)	0.47
Dietary intakes				
Total energy, kcal/d	2,097 (753)	2,000 (627)	2,102 (633)	0.63
Total fat, g/d	64 (27)	62 (24)	70 (24)	0.19
Dietary fiber, g/d	24 (10)	22 (7)	22 (9)	0.33
Total vitamin D, IU/d	345 (251)	294 (268)	314 (207)	0.48
Total calcium, mg/d	884 (339)	787 (364)	855 (416)	0.33
Phosphorous, mg/d	1,359 (435)	1,248 (441)	1,327 (418)	0.34
Omega-3 fatty acids, g/d	0.2 (0.2)	0.2 (0.2)	0.2 (0.2)	0.41
Take any vitamin supplement(s) (%)	38	38	33	0.82

Abbreviations: MET, metabolic equivalents of task; NSAID, nonsteroidal anti-inflammatory drug

Note: unless otherwise specified, values presented are mean (standard deviation)

[1] P values calculated from ANCOVA for continuous variables, and chi-square or Fisher's exact test for categorical variables. Sex was included as a covariate when appropriate.

[2] Regularly take once or more a week

Table 2.2. Pearson correlation coefficients (r) for correlations between plasma concentrations of flagellin- and LPS-specific immunoglobulins IgA and IgG in the Calcium Trial

	Flagellin-IgA		Flagellin-IgG		LPS-IgA		LPS-IgG	
	Men	Women	Men	Women	Men	Women	Men	Women
Flagellin-IgA			0.45	0.41	0.67	0.80	0.20	0.37
Flagellin-IgG					0.31	0.43	0.30	0.46
LPS-IgA							0.28	0.55
LPS-IgG								

Abbreviations: Ig, immunoglobulin; LPS, lipopolysaccharide

Note: p value < 0.05 for all correlations

Table 2.3. Mean baseline plasma levels of gut permeability biomarkers by demographic and lifestyle factors in the Calcium Trial

		Flagellin IgA			Flagellin IgG			LPS IgA			LPS IgG			Permeability score[1]		
	N	Mean	SE	P_{value}	Mean	SE	P_{value}	Mean	SE	P_{value}	Mean	SE	P_{value}	Mean	SE	P_{value}
Age, yrs.																
< 55	61	1.55	0.06		1.64	0.06		1.32	0.07		0.87	0.05		5.37	0.18	
55 - 64	72	1.65	0.06		1.74	0.05		1.39	0.06		0.86	0.05		5.64	0.16	
≥ 65	56	1.66	0.07	0.21	1.67	0.06	0.68	1.40	0.07	0.37	0.81	0.05	0.42	5.54	0.18	0.49
Sex																
Male	119	1.71	0.04		1.73	0.04		1.46	0.05		0.83	0.03		5.73	0.12	
Female	70	1.53	0.06	0.01	1.65	0.05	0.22	1.28	0.06	0.02	0.86	0.04	0.62	5.32	0.16	0.05
BMI, kg/m^2																
< 25	49	1.47	0.07		1.63	0.06		1.18	0.07		0.76	0.05		5.04	0.19	
25 - 27.49	34	1.58	0.09		1.72	0.07		1.36	0.09		0.84	0.07		5.49	0.24	
27.50 - 29.99	41	1.61	0.08		1.70	0.07		1.40	0.08		0.94	0.06		5.64	0.22	
30 - 34.99	50	1.72	0.07		1.72	0.06		1.47	0.07		0.83	0.05		5.74	0.19	
≥ 35	15	1.93	0.13	< .01	1.70	0.11	0.34	1.65	0.13	< .01	0.98	0.10	0.08	6.26	0.35	< .01
Waist-hip ratio[2]																
Tertile 1	63	1.59	0.06		1.69	0.05		1.32	0.07		0.81	0.05		5.41	0.17	
Tertile 2	64	1.54	0.06		1.65	0.05		1.34	0.07		0.88	0.05		5.41	0.17	
Tertile 3	62	1.73	0.06	0.13	1.72	0.05	0.72	1.45	0.07	0.17	0.85	0.05	0.55	5.74	0.18	0.18
Waist circumference, cm^2																
Tertile 1	64	1.51	0.06		1.65	0.05		1.27	0.06		0.76	0.05		5.19	0.17	
Tertile 2	63	1.55	0.06		1.70	0.05		1.29	0.06		0.90	0.05		5.44	0.17	
Tertile 3	62	1.81	0.06	< .01	1.71	0.05	0.35	1.55	0.06	< .01	0.87	0.05	0.10	5.95	0.17	< .01
Current cigarette smoker																
Yes	38	1.66	0.08		1.67	0.07		1.43	0.08		0.79	0.06		5.55	0.22	
No	148	1.61	0.04	0.59	1.68	0.04	0.86	1.36	0.04	0.48	0.87	0.03	0.24	5.52	0.12	0.93

(Table continues on the next page)

		Flagellin IgA			Flagellin IgG			LPS IgA			LPS IgG			Permeability score[1]		
	N	Mean	SE	P_{value}	Mean	SE	P_{value}	Mean	SE	P_{value}	Mean	SE	P_{value}	Mean	SE	P_{value}
Regular NSAID user[3]																
Yes	54	1.69	0.07		1.74	0.06		1.48	0.07		0.80	0.05		5.71	0.18	
No	135	1.59	0.04	0.26	1.66	0.04	0.26	1.33	0.04	0.06	0.86	0.03	0.31	5.45	0.12	0.24
Alcohol drinks per day																
0	55	1.64	0.07		1.69	0.06		1.41	0.07		0.86	0.05		5.60	0.18	
0.1 - 2	44	1.61	0.07		1.74	0.06		1.38	0.07		0.87	0.06		5.60	0.20	
> 2	74	1.66	0.06	0.82	1.66	0.05	0.65	1.34	0.06	0.43	0.82	0.05	0.55	5.48	0.17	0.61
History of high-risk adenomas[4]																
Yes	95	1.57	0.05		1.64	0.04		1.36	0.05		0.78	0.04		5.36	0.14	
No	89	1.69	0.05	0.12	1.73	0.05	0.14	1.38	0.05	0.78	0.88	0.04	0.07	5.68	0.15	0.11

Abbreviations: Ig, immunoglobulin; LPS, lipopolysaccharide; SE, standard error; BMI, body mass index; NSAID, nonsteroidal anti-inflammatory drug

Note: All means, standard errors, and p values were calculated using analysis of covariance (ANCOVA). Models for all variables but sex were adjusted for sex (men/women). Models for all variables but body mass index, waist-hip ratio, and waist circumference were also adjusted for BMI (continuous). P value is for trend if the explanatory variable has > two categories. The unit for any permeability biomarker alone or in combination is optical density (OD).

[1] Defined as the sum of the optical densities of all permeability biomarkers

[2] Tertiles are sex-specific

[3] Regularly take once or more a week

[4] Defined as having a history of multiple adenoma (≥ 2) or at least one large (> 1 cm) or villous or tubulovillous adenoma

Table 2.4. Mean baseline plasma levels of gut permeability biomarkers by sex-specific tertiles of systemic inflammation biomarkers in the Calcium Trial

		Flagellin IgA			Flagellin IgG			LPS IgA			LPS IgG			Permeability score[2]		
Categories[1]	N	Mean	SE	P_{trend}	Mean	SE	P_{trend}	Mean	SE	P_{trend}	Mean	SE	P_{trend}	Mean	SE	P_{trend}
Men and women combined																
CRP																
tertile 1	62	1.62	0.06		1.72	0.05		1.36	0.07		0.81	0.05		5.51	0.17	
tertile 2	64	1.52	0.06		1.63	0.05		1.27	0.06		0.85	0.05		5.27	0.17	
tertile 3	63	1.73	0.06	0.18	1.71	0.05	0.92	1.48	0.06	0.18	0.88	0.05	0.36	5.80	0.17	0.22
Z-score[3]																
tertile 1	61	1.67	0.06		1.63	0.05		1.38	0.07		0.80	0.05		5.48	0.18	
tertile 2	64	1.57	0.06		1.68	0.05		1.30	0.06		0.84	0.05		5.38	0.17	
tertile 3	64	1.63	0.06	0.69	1.74	0.05	0.14	1.43	0.06	0.60	0.90	0.05	0.15	5.71	0.17	0.36
Men																
CRP																
tertile 1	39	1.76	0.07		1.86	0.07		1.49	0.08		0.88	0.06		5.99	0.21	
tertile 2	40	1.64	0.07		1.60	0.07		1.36	0.08		0.82	0.06		5.43	0.21	
tertile 3	40	1.75	0.07	0.95	1.73	0.07	0.18	1.53	0.08	0.70	0.80	0.06	0.34	5.80	0.21	0.55
Z-score[3]																
tertile 1	39	1.73	0.07		1.65	0.07		1.40	0.08		0.78	0.06		5.56	0.21	
tertile 2	40	1.63	0.07		1.76	0.07		1.34	0.08		0.82	0.06		5.55	0.21	
tertile 3	40	1.79	0.07	0.55	1.77	0.07	0.26	1.64	0.08	0.04	0.91	0.06	0.13	6.10	0.21	0.07
Women																
CRP																
tertile 1	23	1.43	0.11		1.50	0.08		1.20	0.11		0.68	0.07		4.81	0.29	
tertile 2	24	1.37	0.11		1.71	0.07		1.18	0.10		0.88	0.07		5.13	0.28	
tertile 3	23	1.78	0.11	0.03	1.72	0.08	0.06	1.47	0.11	0.08	1.01	0.07	< .01	5.98	0.29	< .01

(Table continues on the next page)

		Flagellin IgA			Flagellin IgG			LPS IgA			LPS IgG			Permeability score[2]		
Categories[1]	N	Mean	SE	P_{trend}	Mean	SE	P_{trend}	Mean	SE	P_{trend}	Mean	SE	P_{trend}	Mean	SE	P_{trend}
Z-score[3]																
tertile 1	22	1.63	0.12		1.64	0.08		1.43	0.11		0.84	0.08		5.54	0.31	
tertile 2	24	1.52	0.11		1.58	0.07		1.29	0.10		0.86	0.07		5.25	0.29	
tertile 3	24	1.43	0.11	0.23	1.72	0.08	0.44	1.13	0.11	0.07	0.87	0.08	0.81	5.15	0.30	0.39

Abbreviations: Ig, immunoglobulin; LPS, lipopolysaccharide; SE, standard error; BMI, body mass index; CRP, C-reactive protein

Note: All means, standard errors, and p values were calculated using analysis of covariance (ANCOVA). Models for all variables but sex were adjusted for sex (men/women). Models for all variables but body mass index, waist-hip ratio, and waist circumference were also adjusted for BMI (continuous). P value is for trend if the explanatory variable has > two categories. The unit for any permeability biomarker alone or in combination is optical density (OD).

[1] Tertiles are sex-specific

[2] Defined as the sum of the optical densities of all permeability biomarkers

[3] Summary z-score of pro- and anti-inflammatory cytokines (IL-6, IL-1β, TNF-α, IL-8, IL-12p40, VEGF, and IL-10) calculated as the summation of the z-value for each cytokine [$z = (x - \mu)/\delta$, where x is the natural log-transformed values for each individual marker, and μ and δ are the sex-specific mean and standard deviation of the natural log-transformed biomarker value, respectively, at baseline]. The z-value for IL-10 was included with a negative sign.

Table 2.5. Effects of calcium supplementation on plasma concentrations of gut barrier function biomarkers in the Calcium Trial

	Baseline			4-month follow-up			Absolute treatment effect[1]			Relative Effect[2]
	n	Mean	SE	n	Mean	SE	Mean	SE	p value	
Flagellin IgA										
Placebo	64	1.60	0.06	59	1.58	0.06				
Calcium 1 g	62	1.71	0.06	58	1.72	0.06	0.03	0.04	0.40	1.02
Calcium 2 g	63	1.64	0.06	57	1.67	0.06	0.05	0.04	0.19	1.03
Flagellin IgG										
Placebo	64	1.69	0.05	59	1.69	0.05				
Calcium 1 g	62	1.79	0.05	58	1.80	0.05	0.00	0.03	0.88	1.01
Calcium 2 g	63	1.62	0.05	57	1.64	0.05	0.02	0.03	0.61	1.01
LPS IgA										
Placebo	64	1.37	0.06	59	1.34	0.07				
Calcium 1 g	62	1.40	0.07	58	1.43	0.07	0.06	0.03	0.09	1.04
Calcium 2 g	63	1.44	0.07	57	1.44	0.07	0.04	0.03	0.26	1.02
LPS IgG										
Placebo	64	0.83	0.05	59	0.87	0.05				
Calcium 1 g	62	0.83	0.05	58	0.84	0.05	-0.02	0.04	0.54	0.97
Calcium 2 g	63	0.87	0.05	57	0.87	0.05	-0.03	0.04	0.43	0.95
(Flagellin + LPS) IgA										
Placebo	64	2.97	0.12	59	2.92	0.12				
Calcium 1 g	62	3.11	0.12	58	3.15	0.12	0.09	0.06	0.17	1.03
Calcium 2 g	63	3.08	0.12	57	3.11	0.12	0.08	0.06	0.18	1.03
(Flagellin + LPS) IgG										
Placebo	64	2.52	0.08	59	2.56	0.08				
Calcium 1 g	62	2.62	0.08	58	2.64	0.08	-0.02	0.06	0.72	0.99
Calcium 2 g	63	2.49	0.08	57	2.51	0.08	-0.01	0.06	0.81	0.99

(Table continues on the next page)

	Baseline			4-month follow-up			Absolute treatment effect[1]			Relative Effect[2]
	n	Mean	SE	n	Mean	SE	Mean	SE	p value	
Flagellin (IgA + IgG)										
Placebo	64	3.29	0.10	59	3.27	0.10				
Calcium 1 g	62	3.50	0.10	58	3.52	0.10	0.04	0.06	0.54	1.01
Calcium 2 g	63	3.26	0.10	57	3.31	0.10	0.06	0.06	0.28	1.02
LPS (IgA + IgG)										
Placebo	64	2.20	0.09	59	2.21	0.10				
Calcium 1 g	62	2.23	0.10	58	2.27	0.10	0.03	0.06	0.58	1.01
Calcium 2 g	63	2.30	0.10	57	2.31	0.10	0.01	0.06	0.91	1.00
Permeability score[3]										
Placebo	64	5.49	0.17	59	5.48	0.17				
Calcium 1 g	62	5.74	0.17	58	5.79	0.17	0.07	0.10	0.51	1.01
Calcium 2 g	63	5.56	0.17	57	5.62	0.18	0.07	0.10	0.49	1.01

Abbreviations: Ig, immunoglobulin; LPS, lipopolysaccharide; SE, standard error

Note: The unit for any permeability biomarker alone or in combination is optical density (OD).

[1] Absolute treatment effect = ([treatment group follow-up - treatment group baseline] - [placebo group follow-up - placebo group baseline]); actual calculations of mean, SE and p value from the linear mixed model. Covariates included random intercept, follow-up visit, treatment group, and treatment group by follow-up visit interaction.

[2] Relative effect = [(treatment group follow-up/treatment group baseline) / (placebo follow-up/placebo baseline)].

[3] Defined as the sum of the optical densities of all permeability biomarkers

CHAPTER 3. EFFECTS OF CALCIUM SUPPLEMENTATION ON CIRCULATING BIOMARKERS OF INFLAMMATION AND OXIDATIVE STRESS IN COLORECTAL ADENOMA PATIENTS: A RANDOMIZED CONTROLLED TRIAL

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Abstract

Inflammation and oxidative stress play important roles in colorectal carcinogenesis. There is strong evidence that calcium reduces risk for colorectal neoplasms, possibly through its ability to bind bile acids and prevent their colonic toxicity (which occurs via an oxidative mechanism and results in an inflammatory response). In a previously reported pilot, randomized, controlled trial among sporadic colorectal adenoma patients we found that those on 2.0 g/d of calcium, relative to those on placebo, had an estimated drop in a combined cytokine z-score by 48% ($p = 0.18$) over six months. To follow-up these promising preliminary findings, we tested the efficacy of two doses of supplemental calcium (1.0 or 2.0 g/d) relative to placebo on modulating circulating biomarkers of inflammation (C-reactive protein [CRP] and 10 cytokines) and oxidative stress (F_2-isoprostanes) over a 4-month treatment period among 193 patients with previous sporadic, colorectal adenoma in a randomized, double-blinded, placebo-controlled clinical trial. The inflammation markers were measured in plasma using electrochemiluminescence detection-based immunoassays, and F_2-isoprostanes were measured in plasma using gas chromatography-mass spectrometry. Over a 4-month treatment period, we found no appreciable effects of calcium on CRP, cytokines, or F_2-isoprostanes ($p > 0.4$), overall or within strata of several major risk factors for colorectal carcinogenesis, such as body mass index and regular use of nonsteroidal anti-inflammatory drugs. Overall, our results provide no evidence that calcium supplementation favorably modulates concentrations of circulating biomarkers of inflammation or oxidative stress over four months among patients with a previous colorectal adenoma.

Introduction

Colorectal cancer, a disease highly correlated with Western lifestyles,[2,74] is the second leading cause of cancer deaths in the US.[1] Calcium is a plausible and evidently well-supported dietary chemopreventive agent against colorectal neoplasms.[31] A recent meta-analysis of fifteen prospective observational studies reported that every 300 mg/day increase of total calcium intake was associated with a statistically significant 8% lower risk of colorectal cancer.[155] In addition, a major randomized controlled trial found statistically significantly reduced recurrence of colorectal adenoma (a well-accepted precursor of colorectal cancer) with calcium supplementation.[8]

Multiple mechanisms have been proposed for calcium's chemopreventive properties against colorectal carcinogenesis.[164] The earliest and probably the most prominent hypothesis was that calcium can bind bile and fatty acids in the colon lumen by forming insoluble soaps and thus prevent their colonic toxicity, which occurs via an oxidative mechanism and results in an inflammatory response and increased proliferation.[4,9,10] It is well accepted that inflammation is causally linked to colorectal carcinogenesis, and reducing inflammation reduces risk for colorectal neoplasms.[73,75,79,145,256] Evidence that oxidative stress (which is intimately linked with inflammation[257]) is modifiable and associated with risk for colorectal neoplasms is growing.[93,258-260] We hypothesized that calcium may reduce oxidative damage and inflammation in the colon, which could be reflected in the circulation and unlikely be due to systemic actions of calcium, because circulating levels of calcium are maintained in a very narrow range. Our group previously conducted a pilot clinical trial among colorectal adenoma patients and found that calcium supplementation over 6 months reduced plasma levels of several pro-inflammatory biomarkers (individually and combined as an inflammation z-score),[23] as well as colon tissue 8-hydroxy-2′-deoxyguanosine (8-OH-dG) as a biomarker of oxidative DNA damage.[19] Other than our pilot study, only a few clinical trials previously reported the effect of calcium or dairy (a rich source of calcium) on inflammation or oxidative stress markers among humans,[85-90] but some of

these studies had relatively small sample sizes,[85,86,89,90] or restricted the study population to generally healthy adults[86-88] for whom the levels of inflammation and oxidative stress markers may be relatively low and perhaps not amenable to subsequent change.

To address these gaps in the literature, we tested the effects of two doses of calcium supplementation on panels of circulating biomarkers of inflammation (C-reactive protein [CRP], tumor necrosis factor [TNF]-α, interleukin [IL]-1β, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-17, vascular endothelial growth factor [VEGF], and interferon [IFN]-γ) and oxidative stress (F_2-isoprostanes) in a randomized, clinical trial among 193 patients with previous sporadic colorectal adenomas ("the Calcium Trial"). The biomarkers in the inflammation panel were chosen to represent different aspects of the inflammatory response/immunomodulation in order to provide a more complete summary of the overall effect of calcium on inflammation. Categories of markers represented included mediators of natural and adaptive immunity (*e.g.*, TNF-α and IL-4, respectively); inflammation promotion and inhibition (*e.g.*, IL-6 and IL-10, respectively); cytokines originating from different cell sources, such as T, B, NK, Th1, and Th2 cells, macrophages, fibroblasts, epithelial cells, and others; cytokines with different cell targets; and cytokines with different primary effects.

Patients and Methods

This study was an adjunct investigation using data and blood samples from a chemoprevention trial conducted from 1990 – 1994 in the Minneapolis, MN metropolitan area.[17] The parent trial was approved by the Committee on Use of Human Subjects in Research of the University of Minnesota. Each study participant provided written informed consent.

Participant Population

Details on the eligibility criteria and recruitment protocol of the parent trial were described previously.[17] Briefly, adults aged 30 – 74 years and in general good health were

eligible if they had a history of pathology-confirmed adenomatous polyps within the previous 5 years. Subjects were recruited from the patient population of a major private-practice gastroenterology group in Minneapolis-St. Paul, MN. Subjects were excluded if any of the following criteria were met: having contraindications to calcium supplementation or rectal biopsies; having clinical conditions, dietary habits, or medication that would otherwise affect the safety, adherence, or interpretation of the study results; or failure to take > 80% of their pills in a 4-week placebo run-in trial.

Clinical Trial Protocol

As previously described,[17] individuals who passed the initial eligibility screening were invited for an eligibility visit, during which they were interviewed and their medical/pathology records were reviewed. Those eligible then entered a 4-week placebo run-in trial. Only individuals without substantial perceived side effects and who had taken > 80% of their pills in the run-in trial were ultimately considered eligible (n = 193). Eligible participants then underwent a baseline visit and were randomly assigned (stratified by sex) to one of three groups: a placebo control group (n = 66) and 1.0 g (n = 64) and 2.0 g (n = 63) elemental calcium supplementation groups. Randomization was blinded to all participants and all study personnel and laboratory staff. The calcium tablets (prepared by SmithKline Beecham, Pittsburgh, PA) contained calcium carbonate and were taken twice daily with meals. The placebo pills contained no calcium, magnesium, vitamin D, and chelating agents; they were otherwise identical to calcium tablets in size, appearance, and taste.

At the baseline visit, we collected information on demographic and lifestyle factors as well as medical history and medication use for each participant viaa self-administered questionnaire, and additionally collected dietary data using a Willett semi-quantitative food-frequency questionnaire. The treatment period for the parent trial was 6 months, and participants

were instructed to maintain their usual diets during the study. After random assignment, all participants attended follow-up visits at 1, 2, 4, and 6 months. Pill-taking adherence was evaluated at each follow-up visit by questionnaire, interview, and pill count. Blood samples were collected at the baseline and 4-month follow-up visits. Participants sat comfortably in a chair for five minutes with both of their feet on the floor before venipuncture. Blood was drawn into pre-chilled Vacutainers, and immediately placed on ice and shielded from light. Tubes were immediately processed, plasma and serum were aliquotted into separate cryopreservation tubes, the air was displaced with nitrogen, and the aliquots were immediately shipped to the laboratory for storage in a -80^0C freezer.

Laboratory Protocol

Concentrations of inflammation biomarkers were measured at the Emory Multiplexed Immunoassay Core (EMIC), using electrochemiluminescence detection-based immunoassays based on the Meso-Scale Discovery Sector 2400 instrument. We conducted an individual assay for CRP, and a 10-plex assay for IFN-γ, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-17, TNF-α, and VEGF. All biomarkers were measured in duplicate, according to the manufacturer's protocol, and technicians were blinded to treatment assignment. The average intra-assay coefficient of variation (CV) for CRP was 4.59%, for IFN-γ 16.71%, for IL-10 5.66%, for IL-12 6.89%, for IL-17 21.26%, for IL-1β 13.01%, for IL-4 17.61%, for IL-6 6.99%, for IL-8 3.48%, for TNF-α 4.29%, and for VEGF 4.49%. The results for biomarkers with CVs ≥ 15 (IFN-γ, IL-17, and IL-4) were excluded from further analyses because they were considered insufficiently reliable.

F_2-isoprostanes were measured at the University of Minnesota Molecular Epidemiology Biomarker Research Laboratory by gas chromatography-mass spectrometry (GC-MS) using an Agilent 6890 Series GC and an Agilent 5973N Mass Selective Detector. For quality control, we

included two control samples, measured in duplicate, for each batch; the average intra-assay CV was 11.5% and 12.5%, respectively, for these two control samples.

Statistical Analysis

Treatment groups were assessed for comparability of baseline characteristics using analysis of covariance (ANCOVA) for continuous variables, and the chi-square or Fisher's exact test for categorical variables, adjusting for sex as appropriate. Among the 193 participants, blood samples were available for measuring inflammation biomarkers on 190 participants and for F_2-isoprostane on 188 participants at baseline; blood samples were available for all biomarkers at follow-up among 176 participants. For one biomarker (IL-1β), the biomarker levels for 4% (n = 13) of the samples were below the detection limit, and were assigned a value equal to half of the detection limit.

Primary analysis was based on original assignment of treatment group at randomization regardless of adherence (intent-to-treat). Because the biomarker values were not normally distributed, they were log-transformed before statistical testing. Treatment effects on the biomarkers from baseline to 4-months follow-up for the 1 g/d and 2 g/d calcium groups relative to the placebo group were estimated using a mixed linear models procedure for repeated measures data as implemented in SAS Institute's Mixed Procedure (SAS version 9.4; SAS Institute, Cary, NC). Predictors in the model included visit, treatment groups, and a treatment by visit interaction term. Since it was necessary to apply natural log transformation to the biomarker values, the main effect for each individual biomarker was estimated on a multiplicative scale based on geometric means. Accordingly, a relative effect, defined as [(treatment group follow-up mean) / (treatment group baseline mean)] / [(placebo follow-up mean) / (placebo baseline mean)], was obtained from the Mixed model. Its interpretation is somewhat analogous to that of an odds ratio (e.g., a relative effect of 1.10 means that the proportional change in the treatment group is 10%

higher than that in the placebo group). In addition, we also manually calculated an absolute treatment effect defined as [(treatment group follow-up mean) - (treatment group baseline mean)] - [(placebo follow-up mean) - (placebo baseline mean)], directly using the geometric means for each group at baseline and follow-up.

We considered that no single marker of inflammation could represent all of the complex aspects of inflammation / immunomodulation, and thus calculated a cytokine summary z-score. Briefly, an individual z-score was calculated for each cytokine as $z = (x - \mu)/\delta$, where x is the natural log-transformed values for each individual marker at a given visit, and μ and δ are the sex-specific mean and standard deviation of the log-transformed biomarker value at baseline, respectively. Each individual z-score at baseline fits a standard normal distribution with a mean of 0 and a standard deviation of 1. Then a combined z-score was calculated by summing the individual z-scores (we included the z-score for IL-10 with a negative sign considering its anti-inflammatory properties).[239] Since the z-score was normally distributed, it was not log-transformed in the modeling process, and its main effect was estimated using the Mixed model on an additive scale as an absolute treatment effect (defined above), based on arithmetic means.

We also conducted baseline analyses using analysis of covariance (ANCOVA), to investigate whether baseline levels of CRP, the cytokine summary z-score, or F_2-isoprostanes were associated with sex, body measurements, smoking status, and a comprehensive Oxidative Balance Score (OBS, which reflects combined contributions of anti-oxidant and pro-oxidant exposures, with a higher score indicating lower oxidative stress),[240,241] with adjustment for sex and BMI as appropriate.

Results

The mean age of study subjects was 59 years, 63% were men, 99% were White, and 28% had a family history of colorectal cancer in a first-degree relative. The treatment groups were balanced on major demographic, diet, and lifestyle factors at baseline (Table 3.1).

Adherence to visit attendance averaged 95.3%, and did not differ among the three groups. In each group, the mean percentage of pills taken was 97%, and > 98% of all participants took > 80% of their pills. Table 3.2 shows the geometric mean concentrations of each biomarker at baseline and follow-up, as well as the relative and absolute treatment effects by calcium supplementation. Overall, we did not observe an effect of calcium supplementation (1 g/d or 2 g/d) on individual biomarkers of inflammation and oxidative stress. Opposite to our hypothesis, we noted statistically significant increases of 12% and 8% in the concentrations of IL-12p40 and TNF-α, respectively for those treated with 1 g/d but not 2 g/d of calcium. The effect of calcium on a cytokine summary z-score is presented in Table 3.3. From baseline to follow-up, the z-score decreased by 0.39, 0.13, and 0.26 in the placebo group and the 1 g/d and 2 g/d calcium groups, respectively, suggesting relative increases of the cytokine levels in both treatment groups compared to the placebo; however, none of these estimates were statistically significant. The results were also null within strata of age at enrollment, sex, smoking status, family history of colorectal cancer in a first degree relative, body mass index, regular use of nonsteroidal anti-inflammatory drugs (NSAIDs), total fat intake, dietary fiber intake, and the OBS, or limiting the analysis to those with good adherence (data not shown).

To provide possible insight into whether the null results for the calcium intervention were valid or likely due to the age of the blood samples, we analyzed baseline associations of CRP, the cytokine summary z-score, and F_2-isoprostanes with selected participant characteristics previously reported to be associated with inflammation and oxidative stress (Table 3.4). Overall, mean concentrations of these biomarkers were higher among women, those with a larger BMI or

waist-hip ratio, current smokers, or those with higher oxidative stress as indicated by a lower OBS (overall, diet-specific, or lifestyle-specific).

Discussion

The results from this first full-scale, dose-response trial of calcium and biomarkers of inflammation and oxidative stress indicate that supplementation with 1 or 2 g/d of elemental calcium has no effects on circulating biomarkers of inflammation and oxidative stress in sporadic colorectal adenoma patients over a 4-month treatment period.

Chronic inflammation is an important hallmark of cancer,[261] including colorectal cancer.[79] Several biomarkers of inflammation have been previously linked to colorectal cancer risk in population studies. For example, in a meta-analysis of prospective studies (including 1,159 colorectal cancer cases and 37,986 controls), CRP was statistically significantly associated with higher risk for colorectal cancer (RR per unit increase of log-transformed CRP 1.12, 95% CI 1.01, 1.25).[76] Also, serum levels of several pro-inflammatory cytokines, including VEGF, TNF-α, IL-6, and IL-8, were found to be higher in colorectal cancer cases than in controls.[80] Oxidative stress, intimately linked with inflammation,[257] primarily acts through reactive oxygen and nitrogen species (RONS); RONS can induce damage in almost all cellular components, including oxidizing cellular lipids (lipid peroxidation),[94] which is believed to be a major determinant of oxidative stress-related colorectal carcinogenesis,[95,96] and F_2-isoprostanes has been recognized as the most reliable marker of lipid peroxidation *in vivo*.[94,97] Therefore, the selection of CRP, cytokines, and F_2-isoprostanes as the endpoints in our calcium intervention trial is well supported, and modulation of these biomarkers by calcium could have implications for further modulation of risk for colorectal neoplasms.

Previous animal studies have reported that treating mice with calcium (with or without vitamin D) reduced inflammation (IL-1β, TNF-α, IL-6)[83-85] or oxidative stress (ROS production,

NADPH oxidase mRNA, and plasma malondialdehyde).[85] Among humans, results from our previous pilot clinical trial suggested that calcium may reduce plasma IL-6, IL-1β, and an inflammation z-score[23] as well as oxidative DNA damage in the normal colorectal mucosa among colorectal adenoma patients.[19] Apart from our pilot study, three other human clinical trials tested the effect of calcium on inflammation biomarkers. Gannagé-Yared *et al.* reported no effect of 1.0 g/d calcium plus 800 IU/d vitamin D_3 on serum CRP, IL-6, and TNF-α among 47 healthy post-menopausal women over 12 weeks.[86] Similarly, Grey *et al.* reported no effect of 1 g/d of calcium on serum CRP level among 116 healthy post-menopausal women over 12 months,[87] and Pittas *et al.* reported no effect of 500 mg/d calcium plus 700 IU/d vitamin D_3 supplementation on CRP and IL-6 among non-diabetic adults over 3 years.[88] However, the null results in the above three studies may be partially explained by the relatively low levels of cytokines in healthy participants, as opposed to those likely with higher levels of gut or systemic inflammation, such as colorectal adenoma patients.[102] In addition, three studies reported that diets high in dairy reduced the levels of CRP, TNF-α, IL-6, monocyte chemoattractant protein-1, and oxidative stress biomarkers in overweight or obese adults [85,89,90] who may have higher levels of systemic inflammation than individuals with normal weight,[262] but whether the effects were due to calcium or other dairy components could not be ascertained.

In the current study, we observed no effect of calcium on circulating biomarkers of inflammation and oxidative stress, which is inconsistent with the preliminary findings from our pilot trial. This discrepancy could be due to several reasons. The original blood samples for the current study were collected back in the early 1990s, which raised concerns that some analytes in the samples could have deteriorated over the years. However, the blood samples were immediately processed and appropriately stored in a -80 ^{0}C freezer since the original collection, and no additional freeze-thaw cycles were introduced before we aliquotted the samples for the current study. Concentrations of inflammation markers were comparable to those in the pilot

trial, which had a much shorter gap between sample collection and laboratory measurement.[23] Most importantly, associations of these biomarkers at baseline with sex, body measurements, and an oxidative balance score were consistent with previous findings from our group[102,263,264] and other groups (e.g.,[242,265,266]), supporting the validity of our biomarker measurements. Another possibility is that although the current study was originally designed to have a 6-month treatment period (same as the pilot trial), blood samples were only collected at baseline and at a 4-month follow-up visit since the blood biomarkers were not the pre-specified primary trial outcome; thus, this shorter treatment period may have been insufficient to allow an effect of calcium to become detectable. Finally, it is possible that calcium truly has no effect on systemic inflammation and oxidative stress, whether or not it has effects on inflammation in the colorectal mucosa, and our previously reported preliminary findings were due to chance.

A major strength of the study is that it is the first full-scale dose-response trial to test the effect of calcium on systemic indicators of inflammation and oxidative stress. We had 99% power to detect effect estimates equal to those estimated in the preliminary studies (e.g., an absolute effect of -0.65 for the inflammation z-score), and 80% power to detect effect estimates that are half the size or less than those found in our preliminary studies. For the inflammation biomarkers, we chose a panel of markers to represent different aspects of the inflammatory response/immunomodulation in order to provide a more complete summary of the overall effect of calcium on inflammation. There are also several limitations of this study, including the above-mentioned long storage period of blood samples and relatively short treatment period. Also, because this study was based on a clinical trial population, the findings may not be generalizable to a general population; however, the population may reflect a typical clinic population with adenoma removal. Although not all adenomas become cancerous, most sporadic cancers form from adenomas, and it is important to understand mechanisms and intervene preventively at earlier points in the carcinogenic process.

In summary, taken together with previous literature, the results from this study do not support the hypothesis that calcium supplementation favorably modulates circulating biomarkers of inflammation and oxidative stress among patients with previous colorectal adenoma over a 4-month treatment period. Future full-scale studies, especially those with a longer follow-up period, and that include biomarkers of inflammation and oxidative stress in the normal appearing colorectal mucosa, are needed to provide additional insights into the effects of calcium and further clarify its role as a chemopreventive agent against colorectal neoplasia.

Tables and Figures

Table 3.1. Selected baseline characteristics of the study participants in the Calcium Trial [1]

Characteristics	Treatment group			P-value[2]
	Placebo (n = 66)	Calcium 1 g (n = 64)	Calcium 2 g (n = 63)	
Age, yrs.	60 (9)	60 (9)	58 (10)	0.37
Men (%)	64	63	62	0.98
White (%)	98	100	100	> 0.99
College graduate (%)	35	19	33	0.08
Employed (%)	52	45	56	0.48
Family history (%)	26	25	30	0.78
Take aspirin[3] (%)	21	27	16	0.34
Take non-aspirin NSAID[3] (%)	9	11	10	0.92
Currently smoke (%)	20	16	24	0.53
Alcohol intake, g/d	11 (19)	13 (20)	8 (13)	0.20
Body mass index, kg/m^2				
Men	28.0 (3.8)	29.0 (3.1)	28.8 (4.5)	0.47
Women	30.1 (5.2)	28.1 (8.4)	26.3 (4.4)	0.12
Vigorous/moderate physical activity, MET-hours/d	33 (21)	30 (22)	28 (21)	0.47
Dietary intakes				
Total energy, kcal/d	2,097 (753)	2,000 (627)	2,102 (633)	0.63
Total fat, g/d	64 (27)	62 (24)	70 (24)	0.19
Dietary fiber, g/d	24 (10)	22 (7)	22 (9)	0.33
Total vitamin D, IU/d	345 (251)	294 (268)	314 (207)	0.48
Total calcium, mg/d	884 (339)	787 (364)	855 (416)	0.33
Phosphorous, mg/d	1,359 (435)	1,248 (441)	1,327 (418)	0.34
Omega-3 fatty acids, g/d	0.2 (0.2)	0.2 (0.2)	0.2 (0.2)	0.41
Take any vitamin supplement(s) (%)	38	38	33	0.82

Abbreviations: MET, metabolic equivalents of task; NSAID, nonsteroidal anti-inflammatory drug

[1] Unless otherwise specified, values presented are mean (standard deviation).

[2] P values calculated from ANCOVA for continuous variables, and chi-square or Fisher's exact test for categorical variables. Sex was included as a covariate when appropriate.

[3] Regularly take once or more a week

Table 3.2. Changes in plasma concentrations of biomarkers of inflammation and oxidative stress among colorectal adenoma patients in response to calcium supplementation in the Calcium Trial

Biomarker	Baseline			4-month follow-up			Relative treatment effect[2]			Absolute effect[3]
	n	Mean[1]	95% CI	n	Mean[1]	95% CI	Mean	95% CI	P value	
Inflammation										
CRP (μg/ml)										
Placebo	65	1.62	1.20, 2.18	60	1.68	1.24, 2.28	—	—	—	—
1 g calcium	62	2.81	2.08, 3.82	58	2.65	1.94, 3.61	0.91	0.66, 1.25	0.55	-0.22
2 g calcium	63	1.66	1.23, 2.26	58	1.96	1.44, 2.68	1.14	0.83, 1.56	0.43	0.24
IL-6 (pg/ml)										
Placebo	65	2.05	1.77, 2.38	60	2.10	1.80, 2.44	—	—	—	—
1 g calcium	62	2.75	2.36, 3.20	58	2.52	2.16, 2.94	0.89	0.72, 1.10	0.30	-0.28
2 g calcium	63	1.95	1.67, 2.26	58	2.32	1.98, 2.71	1.16	0.94, 1.43	0.16	0.32
IL-8 (pg/ml)										
Placebo	65	5.59	5.03, 6.21	60	5.39	4.84, 6.00	—	—	—	—
1 g calcium	62	5.72	5.14, 6.37	58	5.58	5.00, 6.22	1.01	0.91, 1.13	0.84	0.06
2 g calcium	63	5.70	5.12, 6.35	58	5.20	4.66, 5.81	0.95	0.85, 1.05	0.32	-0.30
IL-10 (pg/ml)										
Placebo	65	1.86	1.40, 2.49	60	2.07	1.55, 2.76	—	—	—	—
1 g calcium	62	2.17	1.62, 2.90	58	2.29	1.71, 3.07	0.95	0.81, 1.12	0.55	-0.09
2 g calcium	63	2.21	1.65, 2.96	58	2.51	1.87, 3.36	1.02	0.87, 1.20	0.77	0.09
IL-12p40 (pg/ml)										
Placebo	65	18.73	16.05, 21.86	60	18.71	16.02, 21.86	—	—	—	—
1 g calcium	62	18.75	16.05, 21.92	58	20.94	17.90, 24.50	1.12	1.01, 1.23	0.03	2.21
2 g calcium	63	17.42	14.89, 20.38	58	18.23	15.56, 21.35	1.05	0.95, 1.16	0.36	0.83
TNF-α (pg/ml)										
Placebo	65	1.41	1.28, 1.55	60	1.41	1.28, 1.55	—	—	—	—
1 g calcium	62	1.32	1.20, 1.45	58	1.42	1.29, 1.56	1.08	1.01, 1.15	0.04	0.10

(Table continues on the next page)

Biomarker	Baseline			4-month follow-up			Relative treatment effect[2]			Absolute effect[3]
	n	Mean[1]	95% CI	n	Mean[1]	95% CI	Mean	95% CI	P value	
2 g calcium	63	1.42	1.29, 1.56	58	1.45	1.32, 1.60	1.02	0.96, 1.10	0.48	0.03
VEGF (pg/ml)										
Placebo	65	76.39	63.95, 91.26	60	76.17	63.62, 91.20	—	—	—	—
1 g calcium	62	85.02	71.01, 101.8	58	79.56	66.32, 95.44	0.94	0.81, 1.09	0.42	-5.24
2 g calcium	63	80.10	66.87, 95.96	58	73.00	60.80, 87.66	0.91	0.79, 1.06	0.25	-6.88
IL-1β (pg/ml)										
Placebo	65	0.16	0.13, 0.20	60	0.13	0.10, 0.16	—	—	—	—
1 g calcium	62	0.17	0.14, 0.22	58	0.16	0.12, 0.20	1.12	0.72, 1.72	0.62	0.02
2 g calcium	63	0.16	0.13, 0.21	58	0.14	0.11, 0.17	1.03	0.67, 1.59	0.90	0.01
Oxidative stress										
F_2-isoprostane (pg/ml)										
Placebo	64	86.82	77.01, 97.87	60	85.94	76.02, 97.15	—	—	—	—
1 g calcium	61	80.15	70.93, 90.57	58	81.81	72.24, 92.65	1.03	0.87, 1.22	0.72	2.54
2 g calcium	63	84.61	74.96, 95.49	58	85.38	75.37, 96.72	1.02	0.86, 1.21	0.82	1.65

Abbreviations: CI, confidence interval; CRP, C-reactive protein; IL, interleukin; TNF, tumor necrosis factor; VEGF: vascular endothelial growth factor

[1] Geometric means

[2] Calculated as (treatment group geometric mean at follow-up / treatment group geometric mean at baseline) / (placebo group geometric mean at follow-up) / (placebo group geometric mean at baseline); mean, 95% CI, and p-value obtained from the repeated measures mixed linear model

[3] Calculated as (treatment group geometric mean at follow-up - treatment group geometric mean at baseline) – (placebo group geometric mean at follow-up - placebo group geometric mean at baseline)

Table 3.3. Changes in plasma cytokine summary z-score[1] among colorectal adenoma patients in response to calcium supplementation in the Calcium Trial

	Baseline			4-month follow-up			Change from baseline to follow-up	Absolute treatment effect[3]		
	n	Mean[2]	95% CI	n	Mean[2]	95% CI		Mean	95% CI	P value
Placebo	65	-0.11	-0.92, 0.70	60	-0.50	-1.33, 0.32	-0.39	—	—	—
1 g calcium	62	0.40	-0.42, 1.22	58	0.27	-0.57, 1.10	-0.13	0.26	-0.64, 1.17	0.57
2 g calcium	63	-0.25	-1.07, 0.57	58	-0.51	-1.35, 0.33	-0.26	0.13	-0.77, 1.03	0.78

Abbreviations: CI, confidence interval

[1] Summary z-score of pro- and anti-inflammatory cytokines (IL-6, IL-1β, TNF-α, IL-8, IL-12p40, VEGF, and IL-10) calculated as the summation of the z-value for each cytokine [$z = (x - \mu)/\delta$, where x is the natural log-transformed values for each individual marker, and μ and δ are the sex-specific mean and standard deviation of the natural log-transformed biomarker value, respectively, at baseline]. The z-value for IL-10 was included with a negative sign.

[2] Arithmetic means

[3] Calculated as (treatment group arithmetic mean at follow-up - treatment group arithmetic mean at baseline) - (placebo group arithmetic mean at follow-up - placebo group arithmetic mean at baseline); mean, 95% CI, and p-value obtained from the repeated measures mixed linear model

Table 3.4. Mean levels of inflammation and oxidative stress biomarkers by demographic and lifestyle factors in the Calcium Trial

	CRP (μg/ml)				Cytokine z-score				F_2-isoprostanes (pg/ml)			
	N	Mean[1]	95% CI[1]	*P* value[1]	N	Mean[1]	95% CI[1]	*P* value[1]	N	Mean[1]	95% CI[1]	*P* value[1]
Sex												
Male	119	1.91	1.54, 2.36		119	-0.02	-0.61, 0.57		118	74.99	68.06, 82.63	
Female	71	2.05	1.56, 2.70	0.69	71	0.03	-0.73, 0.80	0.91	70	101.72	89.67, 115.38	< 0.01
BMI, kg/m^2												
< 25	49	1.31	0.94, 1.82		49	-1.07	-1.99, -0.14		49	82.52	70.95, 95.98	
25 - 27.49	34	1.24	0.83, 1.87		34	-0.68	-1.82, 0.47		32	73.97	60.95, 89.76	
27.50 - 29.99	41	2.30	1.59, 3.33		41	0.75	-0.29, 1.78		41	92.64	78.23, 109.71	
30 - 34.99	51	3.29	2.37, 4.56		51	0.86	-0.05, 1.78		51	90.81	78.14, 105.53	
≥ 35	15	2.70	1.49, 4.88	< 0.01	15	0.42	-1.25, 2.08	< 0.01	15	108.22	82.42, 142.09	0.05
Waist-hip ratio[2]												
Tertile 1	63	1.34	1.00, 1.81		63	-1.06	-1.87, -0.24		62	74.43	65.11, 85.09	
Tertile 2	64	2.00	1.48, 2.69		64	-0.06	-0.87, 0.75		63	95.70	83.72, 109.39	
Tertile 3	63	2.86	2.12, 3.86	< 0.01	63	1.12	0.31, 1.93	< 0.01	63	93.28	81.67, 106.53	0.02
Current cigarette smoker												
Yes	38	3.46	2.37, 5.05		38	1.01	-0.05, 2.07		38	97.55	81.91, 116.18	
No	149	1.70	1.40, 2.06	< 0.01	149	-0.28	-0.82, 0.26	0.03	147	85.45	78.13, 93.46	0.19
OBS[b]												
Tertile 1	70	2.48	1.88, 3.28		70	0.09	-0.70, 0.88		69	94.13	82.71, 107.14	
Tertile 2	57	2.29	1.69, 3.12		57	0.28	-0.59, 1.16		57	88.62	76.88, 102.16	
tertile 3	60	1.39	1.04, 1.87	0.01	60	-0.24	-1.08, 0.60	0.59	59	80.34	69.97, 92.23	0.10
OBS-diet[2]												
Tertile 1	61	2.32	1.72, 3.13		61	0.60	-0.23, 1.43		60	103.12	90.11, 118.02	
Tertile 2	66	1.95	1.45, 2.61		66	-0.52	-1.33, 0.29		66	84.95	74.57, 96.77	
Tertile 3	60	1.78	1.31, 2.40	0.22	60	0.06	-0.77, 0.9	0.37	59	77.19	67.40, 88.40	< 0.01
OBS-lifestyle[2]												
Tertile 1	45	2.79	1.97, 3.94		45	0.65	-0.33, 1.64		45	95.79	81.55, 112.53	
Tertile 2	78	2.31	1.78, 2.99		78	-0.04	-0.78, 0.69		77	84.80	75.09, 95.77	
Tertile 3	67	1.31	0.98, 1.74	< 0.01	67	-0.37	-1.19, 0.45	0.13	66	84.91	74.24, 97.13	0.30

Abbreviations: BMI, body mass index; CI, confidence interval; CRP, C-reactive protein; OBS, oxidative balance score

[1] Mean, standard error, and p value were calculated using analysis of covariance (ANCOVA). Models for all variables except sex were adjusted for sex (men/women). Models for all variables except BMI and waist-hip ratio also adjusted for BMI (continuous). P value is for trend if explanatory variable has > two categories. Geometric means presented for CRP and F_2-isoprostane because of the non-normality of the original observations.

[2] Tertiles are sex-specific.

CHAPTER 4. CALCIUM, VITAMIN D, DAIRY PRODUCTS, AND MORTALITY AMONG COLORECTAL CANCER SURVIVORS: THE CANCER PREVENTION STUDY-II NUTRITION COHORT

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Abstract

Purpose: Higher calcium, vitamin D, and dairy product intakes are associated with lower colorectal cancer incidence, but their impacts on colorectal cancer survival are unclear. We evaluated associations of calcium, vitamin D, and dairy product intakes before and after colorectal cancer diagnosis with all-cause and colorectal cancer-specific mortality among colorectal cancer patients.

Patients and Methods: This analysis included 2,284 participants in a prospective cohort who were diagnosed with invasive, non-metastatic colorectal cancer after baseline (1992 or 1993) and up to 2009. Mortality follow-up was through 2010. Pre-diagnosis risk factor information was collected on the baseline questionnaire. Post-diagnosis information was collected viaquestionnaires in 1999 and 2003 and was available for 1,111 patients.

Results: A total of 949 participants with colorectal cancer died during follow-up, including 408 from colorectal cancer. In multivariable-adjusted Cox proportional hazards regression models, post-diagnosis total calcium intake was inversely associated with all-cause mortality (relative risk [RR] for those in the highest relative to the lowest quartiles, 0.72; 95% confidence interval [CI], 0.53-0.98; $p_{trend} = 0.02$) and associated with marginally statistically significant reduced colorectal cancer-specific mortality (RR,0.59; 95% CI, 0.33-1.05; $p_{trend} = 0.01$). An inverse association with all-cause mortality was also observed for post-diagnosis milk intake (RR, 0.72; 95% CI, 0.55-0.94; $p_{trend} = 0.02$), but not vitamin D intake. Pre-diagnosis calcium, vitamin D, and dairy product intakes were not associated with any mortality outcomes.

Conclusion: Higher post-diagnosis intakes of total calcium and milk may be associated with lower risk of death among patients with non-metastatic colorectal cancer.

Introduction

The overall five-year relative survival for colorectal cancer is 64% in the U.S. but decreases to 12% for distant metastatic disease.[267] The associations of dietary factors with colorectal cancer incidence have been extensively reported,[74] but their roles for colorectal cancer survival are largely unknown.[183] Current dietary guidelines for cancer survivors are primarily based on incidence studies.[184] Empirical knowledge of modifiable prognostic factors, including diet, for colorectal cancer patients is needed for the over 3.5 million colorectal cancer survivors worldwide.[25]

Higher intakes of calcium, vitamin D, and dairy products are generally associated with lower risk of colorectal cancer incidence in observational studies.[6,268] In addition, a major randomized, clinical trial of 1,200 mg of supplemental calcium versus placebo among 930 colorectal adenoma patients reported a 19% reduced risk of adenoma recurrence.[8] In contrast, the Women's Health Initiative clinical trial reported no effect of calcium plus vitamin D supplementation on colorectal cancer incidence,[37,159,160] but suggestive benefits were observed among those not taking personal calcium or vitamin D supplements[162] and those not concurrently randomized to estrogen therapies.[161] Two studies reported null associations of pre-diagnostic calcium intake with colorectal cancer survival.[26,27] The main circulating biomarker of vitamin D, 25(OH) D, was associated with lower risk of mortality among colorectal cancer patients.[213-216] To our knowledge, no study has examined whether total dairy or milk are associated with survival among colorectal cancer patients. We investigated associations of pre- and post-diagnosis calcium (total, dietary, and supplemental), vitamin D (total and dietary), and dairy product (total and milk only) intakes with all-cause and colorectal cancer-specific mortality in a prospective study of men and women diagnosed with invasive, non-metastatic colorectal cancer.

Patients and Methods

Study Cohort

Men and women in this study were selected from among the 184,000 participants in the Cancer Prevention Study II (CPS-II) Nutrition Cohort, a prospective study of cancer incidence that began in 1992.[269] A 10-page, self-administered questionnaire was used to collect information at baseline regarding demographics, medical history, physical activity, body size, cancer screening and early detection, diet and other factors. Follow-up questionnaires were sent to participants biennially, beginning in 1997, to update exposure information and to learn of new cancer diagnoses. The CPS-II Nutrition Cohort is approved by the institutional review board of Emory University.

By the end of incidence follow-up on June 30, 2009, 3,832 of the 181,293 participants who had no personal history of the disease at baseline had been diagnosed with invasive colon or rectal cancer. Of these 3,832 colorectal cancer patients, 2,188 were first self-reported on a follow-up questionnaire and then verified by review of medical records, while 865 patients had their diagnoses confirmed after self-report via linkage with state cancer registries. An additional 779 patients were initially identified as cancer deaths through linkage to the National Death Index (NDI);[270] among those 779 patients, 531 colorectal cancer diagnoses were confirmed, either through linkage with state cancer registries (n = 529) or by examination of medical records (n = 2).

Among the 3,832 colorectal cancer patients, the following exclusions were applied: deaths determined through NDI that were not verified through medical records or cancer registries (n = 248), prevalent cancers (except for non-melanoma skin cancer) at baseline (n = 387), implausible diagnosis date (n = 11), missing or unknown stage at diagnosis (n = 136), TNM summary stage IV or distant SEER stage at diagnosis (n = 421), non-adenocarcinoma histology (n = 50), implausible death date (n = 2), and poor-quality dietary data at baseline (n = 293). We

decided, *a priori*, to exclude patients with distant metastatic disease, consistent with previous studies from this cohort,[186,194,271,272] because the 5-year relative survival in this group is so poor that it is unlikely that diet would substantially affect long-term mortality.

After exclusions, 2,284 participants (1,274 men and 1,010 women) were included in this analysis. Among them, 1,682 were diagnosed with colon cancer (International Classification of Diseases for Oncology [ICD-O]: C18.0, C18.2-C18.9) and 602 with rectal cancer (ICD-O: C19.9, C20.9). By SEER summary stage, 1,154 participants were diagnosed with localized disease (malignant tumors limited to the colon or rectum) while 1,130 participants had regional disease (tumors that spread to adjacent tissue or regional lymph nodes through the bowel wall).

Study Outcomes

All participants were followed through December 31, 2010 to ascertain their vital status and cause of death (if applicable) through linkage to the NDI. Cause of death was obtained for 99.3% of all known deaths in the cohort. The primary outcome in this study was all-cause mortality. The secondary outcome was mortality specifically due to colorectal cancer (ICD Ninth Revision [ICD-9]: 153, 154; ICD Tenth Revision [ICD-10]: C18, C19, C20), defined from the singular underlying cause of death from NDI records. Other major causes of death in this cohort include cardiovascular diseases (CVD), neurodegenerative disease, other types of cancer (primarily lung and pancreas cancer), and respiratory system diseases.

Pre- and Post-diagnosis Diet

Pre-diagnosis diet was assessed at baseline (1992 or 1993) using a modified brief Block Food Frequency Questionnaire (FFQ).[269,273,274] Post-diagnosis diet, where available, was assessed in 1999 and 2003, using a modified Willett FFQ.[269,275-277] Both FFQs used similar questions on usual intake of dairy foods (major sources of dietary calcium and vitamin D, calculated by summing up total servings of milk, yogurt, ice cream, and cheese), and on calcium supplements

and multivitamins (the major source of supplemental vitamin D during this time period) (Table 4.1). For patients diagnosed after baseline and before the date of the 1999 survey completion, the 1999 survey was used for post-diagnosis diet. For patients diagnosed after 1999 and before the date of the 2003 survey completion, the 2003 survey was used for post-diagnosis diet. No post-diagnosis diet data are available from participants who did not return an eligible 1999 or 2003 post-diagnosis survey or from participants who were diagnosed after 2003. Of the 2,284 patients included in the pre-diagnosis analysis, 1,111 (48.6%) reported post-diagnosis diet.

Statistical Analysis

Sex- and questionnaire-specific quartiles were created for total calcium (i.e., diet plus supplements), dietary calcium, total vitamin D (i.e., diet plus supplements), dietary vitamin D, dairy, and milk. Questionnaire-specific categories were created for supplemental calcium (3 levels) among men and women combined based on visually inspecting the distribution and selecting interpretable cut-off points.

We used multivariable Cox proportional hazards models to calculate relative risks (RRs) and 95% confidence intervals (CIs). The underlying time axis for all Cox models was time since diagnosis. For pre-diagnosis models, person-time began on the date of diagnosis. For post-diagnosis models, we used delayed entry Cox models wherein person-time started on the date they returned their post-diagnosis FFQ. In all analyses, person-time ended on the date of death or the end of follow-up (December 31, 2010), whichever came first. The proportional hazards assumption was evaluated for the main exposures with a likelihood ratio test by comparing models with and without an interaction term between an exposure and time; no violations were detected.

All analyses were adjusted for age at diagnosis and tumor stage at diagnosis by stratifying within models. For pre-diagnosis models, we chose *a priori* to adjust for sex and baseline energy

intake, and we additionally adjusted for baseline total folate intake because it changed the RR estimates by approximately 10%. Other demographic, lifestyle and clinical covariates were evaluated but none changed the RR estimates by more than 10%. Covariates in the basic post-diagnosis models also included sex and post-diagnosis energy intake, and additionally included post-diagnostic total folate in the multivariate model, to be consistent with the pre-diagnosis models. Baseline dietary intakes were evaluated as covariates in corresponding post-diagnosis models but did not materially change the RRs, so they were excluded. For each model the linear trend between exposure and mortality risk was assessed using the Wald test and modeling exposure as a continuous variable.

In sensitivity analyses, we excluded participants with a history of diabetes, myocardial infarction, and stroke at baseline, and death within two years of diagnosis. In addition, because treatment or serious illness may influence diet, we conducted sensitivity analyses excluding: 1) participants who completed FFQs within 1 year of diagnosis (one year before diagnosis for pre-diagnostic models and one year after diagnosis for post-diagnosis models); and 2) deaths within two years of the post-diagnosis questionnaire for post-diagnosis models. We tested for statistical interaction of each diet variable with age at diagnosis, sex, tumor stage, tumor sub-site, pre- or post-diagnosis BMI, physical activity, total energy and total folate intakes, using likelihood ratio tests. All analyses were conducted using SAS version 9.3 (SAS Institute, Cary, NC).

Results

Participants were, on average, aged 64 years at baseline and 73 years at diagnosis. Fifty-six percent of participants were men, and most reported their race as white. There were no differences across quartiles of pre-diagnostic total calcium intake in the distributions of year of diagnosis, sex, tumor stage, grade, sub-site, treatment, and history of hypertension, myocardial infarction, diabetes, and stroke (Table 4.2). High calcium consumers were slightly older, better

educated, more physically active, leaner, more likely to use NSAIDs and postmenopausal hormones (women only), less likely to smoke, and more likely to have a healthier overall diet.

Among the 2,284 patients included in the pre-diagnosis analyses, 949 deaths occurred (408 from colon or rectal cancer) during a mean follow-up of 7.5 years (standard deviation, 4.6 years; range, 2 days to 18.1 years). No statistically significant associations were observed for any of the pre-diagnosis diet variables with any of the mortality outcomes (Tables 4.3 and 4.4). The results were not meaningfully different after further adjusting for other covariates or after additional sensitivity analyses (data not shown). The results were also null after we included patients with metastatic or unknown tumor stage (Table 4.5). In analyses restricted to the 1,111 participants who were included in the post-diagnosis analyses, pre-diagnosis use of supplemental calcium ≥250 mg/d was statistically significantly associated with higher risk of all-cause mortality (RR, 1.65; 95% CI, 1.16 to 2.35) (Table 4.6); this risk was primarily due to an increased RR for CVD mortality (RR, 1.83; 95% CI, 0.82 to 4.09).

Among the 1,111 patients included in the post-diagnosis analyses, 429 deaths occurred (143 from colon or rectal cancer) during a mean follow-up of 7.6 years (standard deviation, 3.4 years; range, 20 days to 11.3 years). The mean time between diagnosis and completing the post-diagnosis questionnaire was 2.6 years. As shown in Table 4.7, comparing the highest to the lowest quartiles, total calcium (RR, 0.72; 95% CI, 0.53 to 0.98; $p_{trend} = 0.02$) and milk (RR, 0.72; 95% CI, 0.55 to 0.94; $p_{trend} = 0.02$) intakes were associated with lower all-cause mortality. Further adjustment for pre-diagnostic total calcium and milk had no discernible effect on the results. A marginally statistically significant inverse association with all-cause mortality was observed for total dairy (RR, 0.75; 95% CI, 0.56 to 1.01; $p_{trend} = 0.05$). Total calcium was also inversely associated with colorectal cancer-specific mortality (highest vs. lowest quartile RR, 0.59; 95% CI, 0.33 to 1.05; $p_{trend} = 0.01$) (Table 4.8).

Because post-diagnosis diet and supplement use may be influenced by serious illness preceding death (reverse causation), we conducted a sensitivity analysis excluding deaths within the first two years of follow-up after completion of the post-diagnosis questionnaire. The results after this exclusion appeared similar to the original results. The RRs for the highest compared to the lowest quartile of total calcium and milk, respectively, were 0.69 (95% CI, 0.48 to 0.98; p_{trend} = 0.03) and 0.68 (95% CI, 0.50 to 0.93; p_{trend} = 0.02) for all-cause mortality; and 0.53 (95% CI, 0.24 to 1.19; p_{trend} = 0.02) for total calcium and colorectal-cancer specific mortality.

There was no evidence that the inverse associations of post-diagnosis total calcium and milk intakes with all-cause mortality were modified by age at diagnosis (< 70 years vs. ≥ 70 years), sex, tumor stage (localized vs. regional), tumor sub-site (colon vs. rectum), post-diagnosis BMI (obese vs. not-obese), physical activity (< median vs. ≥ median), total energy (< median vs. ≥ median) or total folate (< median vs. ≥ median) intakes (results stratified by stage shown in Table 4.9, other data not shown).

Discussion

This study suggests that higher intakes of total calcium and milk after colorectal cancer diagnosis are associated with lower risk of mortality. These associations persisted after adjusting for important covariates, such as sex and tumor stage, and after several sensitivity analyses. We found no evidence that calcium, vitamin D, or dairy product intakes before colorectal cancer diagnosis were associated with mortality. To our knowledge, this is the first study to report associations of dairy and milk (both pre- and post-diagnosis) with colorectal cancer survival, and also the first to assess the role of post-diagnosis calcium and vitamin D intakes.

Calcium, 25(OH)D (the major circulating form of vitamin D), and dairy products are associated with lower risk of incident colorectal cancer based on several meta-analyses.[6,156,268] An earlier CPS-II Nutrition Cohort study reported inverse associations of colorectal cancer

incidence with total calcium and total vitamin D intakes.[154] The World Cancer Research Fund and American Institute for Cancer Research Continuous Update Project in 2011 concluded that calcium and milk were both "probable" factors associated with lower colorectal cancer risk.[31]

In contrast to the substantial evidence of a role for calcium, vitamin D, and dairy products in colorectal cancer primary prevention, the role of these factors in colorectal cancer survival is less studied.[183] In two cohort studies, pre-diagnosis dietary calcium intake was not associated with all-cause mortality among colorectal cancer patients, consistent with our findings.[26,27] 25(OH) D, either pre- or post-diagnosis, was associated with longer colorectal cancer survival in four previous studies;[213-216] in the current study, we observed no association with dietary vitamin D intake, which may not optimally reflect serum vitamin D status. In a large, pooled analysis, vitamin D intake was positively associated with serum 25(OH)D level, but the associations were relatively weak (Spearman correlation was 0.22 for dietary vitamin D and 0.29 for total vitamin D).[278]

In the current study, we found a statistically significant lower risk of death among patients with higher post-diagnostic intakes of total calcium and milk. Though not completely understood, several possible biological mechanisms might underlie these associations. Clinical trials conducted among patients with a previous colorectal adenoma indicated that daily treatment with calcium, compared to placebo, was associated with lower risk of colorectal adenoma recurrence,[8,157,158] Potential mechanisms include: calcium's ability to bind to bile and fatty acids and prevent or lower toxicity;[4,9,10] direct effects on colonocyte proliferation,[17,279] differentiation,[11] and apoptosis;[12] and, alterations in K-ras mutations.[163] Although these mechanisms were originally proposed in the primary prevention context, it is reasonable to hypothesize that calcium may also act through these mechanisms after diagnosis to reduce the risk of cancer recurrence, thus ultimately improving chance of survival. Direct clinical or epidemiological evidence of calcium in colorectal cancer progression is limited, but *in vitro* evidence suggests that calcium

may promote E-cadherin expression and suppress β-catenin/TCF activation through the calcium sensing receptor (CaSR), and restrain their malignant behaviors.[18] Thus, calcium may be capable of limiting growth and distant metastasis from cancer cells that escaped the colon at the time of treatment. In our data, the strong inverse association of post-diagnosis total calcium intake with colorectal cancer specific mortality was consistent with these mechanisms.

While post-diagnosis calcium intake was associated with lower risk of all-cause and colorectal cancer-specific mortality, there were no such associations with pre-diagnosis diet. Reasons for these discrepant findings are unclear. It is possible that calcium may have short-term, rather than long-term, effects on colorectal cancer progression and survival, and therefore only post-diagnosis diet is relevant in this context. It is also important to note that different FFQs were used to assess pre- and post-diagnosis diet. The correlation coefficient (Pearson) between pre- and post-diagnosis total calcium intake was 0.37: this moderate correlation might suggest that participants changed their calcium intake after cancer diagnosis or, alternatively, this could reflect differences in the dietary assessment instruments. The FFQs used in this study included the major food and beverage sources of calcium and vitamin D and validation studies have shown good agreements between estimates from diet recall and these FFQs (e.g., Pearson correlation coefficients ranged from 0.57 to 0.66 for calcium and from 0.52 to 0.88 for dairy products).[274,276,277] Therefore, we believe that the low correlations between pre- and post-diagnosis diet are more likely due to real changes in diet after cancer diagnosis.

We observed a potential higher risk of all-cause mortality from pre-diagnosis supplemental calcium intake, especially when restricting the analysis to the 1,111 participants who were included in the post-diagnosis analyses. Further research should address if this is a real potential harm to colorectal cancer patients.

Milk may be associated with improved survival among colorectal cancer patients because it is a rich source of dietary calcium and vitamin D. In addition, milk is a primary dietary source

of conjugated linoleic acid, which was found to inhibit colorectal cancer cell growth *in vitro*.[280,281] Other potentially beneficial components in dairy products include butyric acid, lactoferrin, and fermentation products.[280]

The strengths of our study include its large sample size, prospective design, and detailed pre- and post-diagnosis questionnaire information. We were also able to examine cause-specific mortality. Limitations include the lack of information on adverse effects from treatment and tumor recurrence. FFQs may underestimate diet-disease associations compared to more objective biomarker measurements due to non-differential misclassification. For large cohort studies, however, FFQs offer a feasible method to detect potential associations (especially when using energy-adjusted nutrients) in the absence of biomarker measurements.[282] As in most studies of this type, estimates of the effects of pre-diagnosis exposures are potentially biased due to selecting patients who survived until the occurrence of colorectal cancer or the first post-diagnosis questionnaire.[237,283,284]

In conclusion, higher intakes of total calcium and milk after, but not before, colorectal cancer diagnosis may be associated with lower overall mortality. Our findings, if replicated in future observational studies and randomized trials, will provide important guidance for cancer survivors who are actively seeking diet and lifestyle changes to improve their prognosis.

Tables and Figures

Table 4.1. Comparison of questions on each food frequency questionnaire on usual intake of dairy foods, calcium supplements and multivitamins, Cancer Prevention Study II Nutrition Cohort

	1992[1]	1999[2]	2003[2]
Milk	Whole milk & beverages with whole milk; 2% milk & beverages with 2% milk; Skim milk, 1% or buttermilk	Whole milk; 2% milk; Skim or 1% milk	Whole milk; 2% milk; Skim or 1% milk
Other dairy products	Cheeses and cheese spreads (regular and low-fat); Ice cream (regular and low-fat); Yogurt (regular and low-fat, including frozen); Restaurant pizza	Cheese (cottage or ricotta, and other); Ice cream (regular and non-fat/sherbet); Yogurt (plain or artificially sweetened, frozen, and other); Pizza	Cheese (cottage or ricotta, and other); Ice cream (regular and non-fat/sherbet); Yogurt (plain or artificially sweetened, frozen, and other); Pizza
Calcium supplements	Calcium or Dolomite 1. Frequency per week or per day 2. Amount in each tablet (250mg, 500mg, 600mg, or 750mg)	Calcium 1. Regular use: Yes/no 2. Amount per day (≤ 900mg [calculated as 500mg], ≥901mg [calculated as 1,000mg], unknown)	Calcium 1. Regular use: Yes/no 2. Pills per week 3. Amount in each pill (≤350mg [calculated as 250mg], ≥400mg [calculated as 500mg], unknown)
Multivitamins	Multivitamin 1. Use at least once per week yes/no 2. Type • Stress-tabs type; • Therapeutic, Theragran type; • One-a-day type, or Centrum 3. Number of tablets per day or per week	Multivitamin 1. Currently yes/no 2. Frequency per week 3. Brand (write-in)	Multivitamin 1. Currently yes/no 2. Frequency per week 3. Brand (write-in)

[1]Dairy in 1992 calculated as: all types of milk (8 ounce glass serving) + cheese and cheese spreads (2 ounce serving) + ice cream (1 ½ cup serving) + yogurt (1 cup serving) + cheese on pizza (1 ½ ounce serving)

[2]Dairy in 1999 and 2003 calculated as: all types of milk (8 ounce glass serving) + ice cream (1 ½ cup serving) + yogurt (1 cup serving) + cottage cheese (2 cup serving) + processed cheese (2 ounce serving) + hard cheese (1½ ounce serving) + cheese on pizza (1½ ounce serving)

Table 4.2. Baseline Characteristics of Colorectal Cancer Patients by Quartiles of Pre-diagnostic Total Calcium Intake in the CPS-II Nutrition Cohort

Characteristic	Quartile of Total Calcium Intake (mg/day)[1] Q1 (n=570) No. (%)	Q2 (n=572) No. (%)	Q3 (n=570) No. (%)	Q4 (n=572) No. (%)	p-value[2]
Age at colorectal cancer diagnosis (yrs.)					
< 65	93 (16.3)	69 (12.1)	61 (10.7)	47 (8.2)	< 0.01
65 - < 70	141 (24.7)	117 (20.5)	112 (19.6)	97 (17.0)	
70 - < 75	134 (23.5)	169 (29.5)	148 (26.0)	172 (30.1)	
75 - < 80	131 (23.0)	141 (24.7)	151 (26.5)	147 (25.7)	
80+	71 (12.5)	76 (13.3)	98 (17.2)	109 (19.1)	
Year of colorectal cancer diagnosis					
1992 - 1996	121 (21.2)	142 (24.8)	129 (22.6)	134 (23.4)	0.95
1997 - 2000	173 (30.4)	165 (28.8)	167 (29.3)	175 (30.6)	
2001 - 2004	148 (26.0)	151 (26.4)	151 (26.5)	144 (25.2)	
2005 - 2009	128 (22.5)	114 (19.9)	123 (21.6)	119 (20.8)	
Sex					
Male	318 (55.8)	319 (55.8)	318 (55.8)	319 (55.8)	1.00
Female	252 (44.2)	253 (44.2)	252 (44.2)	253 (44.2)	
Race/ethnicity					
White/White-Hispanic	551 (96.7)	563 (98.4)	561 (98.4)	562 (98.3)	0.08
Black/Black-Hispanic	11 (1.9)	6 (1.0)	6 (1.1)	2 (0.3)	
Other/missing	8 (1.4)	3 (0.5)	3 (0.5)	8 (1.4)	
Education					
Less than high school	60 (10.5)	35 (6.1)	29 (5.1)	34 (5.9)	< 0.01
High school degree	189 (33.2)	173 (30.2)	149 (26.1)	131 (22.9)	
Some college/trade school	153 (26.8)	168 (29.4)	176 (30.9)	176 (30.8)	
College graduate	164 (28.8)	193 (33.7)	215 (37.7)	228 (39.9)	
SEER summary stage					
Localized	294 (51.6)	302 (52.8)	270 (47.4)	288 (50.3)	0.29
Regional	276 (48.4)	270 (47.2)	300 (52.6)	284 (49.7)	
Tumor grade at diagnosis					
Well differentiated	68 (11.9)	68 (11.9)	73 (12.8)	69 (12.1)	0.43
Moderately differentiated	364 (63.9)	338 (59.1)	353 (61.9)	340 (59.4)	
Poorly differentiated	74 (13.0)	108 (18.9)	92 (16.1)	107 (18.7)	
Undifferentiated	7 (1.2)	7 (1.2)	9 (1.6)	6 (1.0)	

(Table continues on the next page)

Characteristic	Quartile of Total Calcium Intake (mg/day)[1] Q1 (n=570) No. (%)	Q2 (n=572) No. (%)	Q3 (n=570) No. (%)	Q4 (n=572) No. (%)	p-value[2]
Colorectal Cancer diagnosis site					
Colon	407 (71.4)	431 (75.3)	421 (73.9)	423 (74.0)	0.50
Rectum	163 (28.6)	141 (24.7)	149 (26.1)	149 (26.0)	
First course of cancer treatment					
Surgery					
No	12 (2.1)	12 (2.1)	13 (2.3)	11 (1.9)	0.97
Yes	415 (72.8)	411 (71.9)	409 (71.8)	425 (74.3)	
Chemotherapy					
No	258 (45.3)	239 (41.8)	243 (42.6)	261 (45.6)	0.83
Yes	169 (29.6)	184 (32.2)	179 (31.4)	175 (30.6)	
Radiation					
No	386 (67.7)	386 (67.5)	373 (65.4)	395 (69.1)	0.79
Yes	41 (7.2)	37 (6.5)	49 (8.6)	41 (7.2)	
Family history of colorectal cancer in 1982					
No	533 (93.5)	536 (93.7)	537 (94.2)	538 (94.1)	0.96
Yes	37 (6.5)	36 (6.3)	33 (5.8)	34 (5.9)	
History of diabetes					
No	520 (91.2)	513 (89.7)	517 (90.7)	519 (90.7)	0.84
Yes	50 (8.8)	59 (10.3)	53 (9.3)	53 (9.3)	
History of stroke					
No	558 (97.9)	557 (97.4)	561 (98.4)	557 (97.4)	0.58
Yes	12 (2.1)	15 (2.6)	9 (1.6)	15 (2.6)	
History of myocardial infarction					
No	529 (92.8)	525 (91.8)	526 (92.3)	529 (92.5)	0.93
Yes	41 (7.2)	47 (8.2)	44 (7.7)	43 (7.5)	
History of hypertension					
No	342 (60.0)	345 (60.3)	366 (64.2)	336 (58.7)	0.26
Yes	228 (40.0)	227 (39.7)	204 (35.8)	236 (41.3)	
Physical activity (MET-hrs./wk.)					
Q1	90 (15.8)	61 (10.7)	60 (10.5)	49 (8.6)	<0.01
Q2	204 (35.8)	192 (33.6)	180 (31.6)	180 (31.5)	
Q3	144 (25.3)	173 (30.2)	172 (30.2)	172 (30.1)	
Q4	123 (21.6)	138 (24.1)	148 (26.0)	166 (29.0)	
BMI (kg/m^2)					
< 18.5	6 (1.1)	5 (0.9)	3 (0.5)	9 (1.6)	<0.01

(Table continues on the next page)

Characteristic	Quartile of Total Calcium Intake (mg/day)[1] Q1 (n=570) No. (%)	Q2 (n=572) No. (%)	Q3 (n=570) No. (%)	Q4 (n=572) No. (%)	p-value[2]
18.5 - < 25	187 (32.8)	190 (33.2)	218 (38.2)	253 (44.2)	
25 - < 30	259 (45.4)	258 (45.1)	237 (41.6)	218 (38.1)	
30+	112 (19.6)	108 (18.9)	103 (18.1)	84 (14.7)	
Cigarette smoking status					
Never	228 (40)	210 (36.7)	222 (38.9)	230 (40.2)	< 0.01
Current	74 (13)	43 (7.5)	45 (7.9)	26 (4.5)	
Former	268 (47)	315 (55.1)	302 (53.0)	309 (54.0)	
NSAID use (No. pills/mon.)					
0	279 (48.9)	258 (45.1)	241 (42.3)	246 (43.0)	<0.01
1 - < 15	96 (16.8)	92 (16.1)	79 (13.9)	61 (10.7)	
15 - < 30	32 (5.6)	47 (8.2)	67 (11.8)	69 (12.1)	
30 - < 60	96 (16.8)	99 (17.3)	112 (19.6)	127 (22.2)	
≥ 60	46 (8.1)	54 (9.4)	50 (8.8)	51 (8.9)	
HRT use among post-menopausal women					
None	119 (49.0)	124 (50.4)	110 (44.5)	94 (38.5)	0.03
Current	50 (20.6)	64 (26.0)	78 (31.6)	78 (32.0)	
Former	65 (26.8)	52 (21.1)	50 (20.2)	58 (23.8)	
Dietary characteristics					
Alcohol intake, drinks/day					
Non-Drinker	207 (36.3)	229 (40.0)	227 (39.8)	255 (44.6)	< 0.01
< 1	178 (31.2)	212 (37.1)	234 (41.1)	209 (36.5)	
≥ 1	173 (30.4)	124 (21.7)	98 (17.2)	99 (17.3)	
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	
Energy intake (kcal/day)	1,543.2 (605.6)	1,617.8 (606.5)	1,611.1 (583.0)	1,577.6 (594.3)	0.01
Dietary folate intake (µg/day)	214.8 (81.3)	254.5 (90.4)	268.0 (88.1)	288.0 (95.4)	< 0.01
Total folate intake (µg/day)	257.2 (161.2)	361.1 (210.4)	424.7 (238.9)	563.7 (389.3)	< 0.01
Fruit/vegetable intake (servings/day)	2.8 (1.5)	3.2 (1.6)	3.5 (1.8)	3.5 (1.7)	< 0.01
Red/processed meat intake (servings/week)	6.4 (4.1)	5.7 (3.6)	5.2 (3.9)	4.4 (3.4)	< 0.01
Whole grain intake (g/day)	44.7 (52.6)	59.8 (63.2)	65.7 (63.8)	69.4 (60.5)	< 0.01

Note: some percentages do not add up to 100% due to missing data or rounding

Abbreviations: CPS, Cancer Prevention Study; Q, quartile; No., number; SD, standard deviation; SEER, Surveillance Epidemiology and End Results; MET, metabolic equivalent; BMI, body mass index; NSAID, non-steroidal anti-inflammatory drug; HRT, hormone replacement therapy

[1]Quartiles in men: < 578, 578 - <776, 776 - <1,044, ≥ 1,044; quartiles in women: < 553, 553 - <776, 776 - <1,156, ≥ 1,156

[2]P values derived from Chi-square test for differences in frequencies across total calcium strata for categorical predictors, and t test for continuous predictors with continuous total calcium intake

Table 4.3. Associations of 1992 Pre-diagnostic Calcium, Vitamin D, and Dairy Intakes with All-Cause Mortality among Non-Metastatic Colorectal Cancer Patients in the CPS-II Nutrition Cohort

Exposure	Range[1] (men; women)	Total No. Deaths	Person-Years	Base model[2]		MV model[3]	
				RR	95% CI	RR	95% CI
Total calcium, mg/day							
Q1[4]	< 578; < 553	227	4,288	1.00	—	1.00	—
Q2	578 - < 776; 553 - < 776	227	4,460	0.91	0.75, 1.10	0.93	0.77, 1.13
Q3	776 - < 1,044; 776 - < 1,156	234	4,255	0.85	0.70, 1.03	0.88	0.72, 1.08
Q4	≥ 1,044; ≥ 1,156	261	4,235	0.96	0.80, 1.15	0.99	0.81, 1.21
P_{trend}[5]					0.99		0.68
Dietary calcium, mg/day							
Q1[4]	< 548; < 486	243	4,146	1.00	—	1.00	—
Q2	548 - < 729; 486 - < 629	222	4,496	0.84	0.70, 1.02	0.86	0.71, 1.04
Q3	729 - < 949; 629 - < 849	237	4,272	0.84	0.69, 1.01	0.86	0.71, 1.04
Q4	≥ 949; ≥ 849	247	4,325	0.85	0.70, 1.02	0.86	0.71, 1.04
P_{trend}[5]					0.13		0.21
Supplemental calcium, mg/day							
C1[4,6]	0	575	10,733	1.00	—	1.00	—
C2	0.1 - < 250	229	3,891	1.01	0.86, 1.19	1.13	0.90, 1.42
C3	≥ 250	145	2,615	1.12	0.92, 1.36	1.22	0.96, 1.54
P_{trend}[5]					0.34		0.10
Total vitamin D, IU/day							
Q1[4]	< 122; < 111	229	4,284	1.00	—	1.00	—
Q2	122 - < 191; 111 - < 201	218	4,370	0.87	0.71, 1.05	0.90	0.74, 1.10
Q3	191 - < 425; 201 - < 467	244	4,278	0.92	0.76, 1.11	0.97	0.78, 1.19
Q4	≥ 425; ≥ 467	258	4,306	0.95	0.79, 1.14	1.09	0.82, 1.47
P_{trend}[5]					0.92		0.32
Dietary vitamin D, IU/day							
Q1[4]	< 105; < 90	232	4,283	1.00	—	1.00	—

(Table continues on the next page)

Exposure	Range[1] (men; women)	Total No. Deaths	Person-Years	Base model[2]		MV model[3]	
				RR	95% CI	RR	95% CI
Q2	105 - < 157; 90 - < 136	238	4,347	0.97	0.81, 1.18	1.00	0.82, 1.21
Q3	157 - < 226; 136 - < 202	225	4,295	0.89	0.73, 1.07	0.91	0.75, 1.11
Q4	≥ 226; ≥ 202	254	4,313	0.94	0.78, 1.13	0.97	0.80, 1.17
P_{trend}[5]					0.43		0.63
Total dairy, servings/week							
Q1[4]	< 5.5; < 5.0	243	4,044	1.00	—	1.00	—
Q2	5.5 - < 9.6; 5.0 - < 8.9	213	4,610	0.78	0.64, 0.95	0.79	0.65, 0.96
Q3	9.6 - < 14.5; 8.9 - < 13.4	244	4,351	0.85	0.70, 1.03	0.87	0.72, 1.05
Q4	≥ 14.5; ≥ 13.4	249	4,233	0.86	0.71, 1.06	0.88	0.72, 1.09
P_{trend}[5]					0.47		0.62
Milk, servings/week							
Q1[4]	0; 0	262	4,895	1.00	—	1.00	—
Q2	0.1 - < 5.7; 0.1 - < 5.1	204	3,771	1.01	0.84, 1.23	1.01	0.84, 1.23
Q3	5.7 - < 10.5; 5.1 - < 10.1	237	4,293	0.97	0.81, 1.17	0.99	0.82, 1.19
Q4	≥ 10.5; ≥ 10.1	246	4,280	0.94	0.78, 1.13	0.95	0.79, 1.15
P_{trend}[5]					0.36		0.46

Abbreviations: Q, quartile; C, category; RR, relative risk; CI, confidence interval; MV, multivariable

[1]Range obtained from each sex-specific quartile of all exposures, except for supplemental calcium, which was obtained from each category for both sexes combined

[2]Base model adjusted for age at diagnosis, sex, tumor stage, and 1992 total energy intake

[3]Multivariable model adjusted for age at diagnosis, sex, tumor stage, and 1992 total energy and total folate intakes

[4]Reference group

[5]P_{trend} calculated by using the median exposure in each quartile, specific to sex, for all exposures except for supplement calcium, which was calculated using the actual categories, i.e. 1, 2, and 3, for both sexes

[6]Supplemental calcium was categorized based on visually inspecting the distribution of the variable

Table 4.4. Associations of 1992 Prediagnostic Calcium, Vitamin D, and Dairy Intakes with Colorectal Cancer Mortality among Non-Metastatic Colorectal Cancer Patients in the CPS-II Nutrition Cohort

Exposure	Range[1] (men; women)	Total No. Deaths	Person-Years	Base model[2]		MV model[3]	
				RR	95% CI	RR	95% CI
Total calcium, mg/day							
Q1[4]	< 578; < 553	101	4,288	1.00	—	1.00	—
Q2	578 - < 776; 553 - < 776	103	4,460	0.99	0.75, 1.32	1.04	0.77, 1.39
Q3	776 - < 1,044; 776 - < 1,156	102	4,255	0.88	0.66, 1.17	0.94	0.69, 1.26
Q4	≥ 1,044; ≥ 1,156	102	4,235	0.95	0.72, 1.26	1.01	0.74, 1.38
P_{trend}[5]					0.78		0.91
Dietary calcium, mg/day							
Q1[4]	< 548; < 486	106	4,146	1.00	—	1.00	—
Q2	548 - < 729; 486 - < 629	99	4,496	0.93	0.70, 1.24	0.96	0.72, 1.28
Q3	729 - < 949; 629 - < 849	107	4,272	0.95	0.72, 1.25	0.99	0.74, 1.31
Q4	≥ 949; ≥ 849	96	4,325	0.82	0.62, 1.10	0.86	0.64, 1.16
P_{trend}[5]					0.18		0.30
Supplemental calcium, mg/day							
C1[4,6]	0	250	10,733	1.00	—	1.00	—
C2	0.1 - < 250	95	3,891	0.98	0.77, 1.25	1.11	0.79, 1.57
C3	≥ 250	63	2,615	1.07	0.79, 1.43	1.18	0.83, 1.66
P_{trend}[5]					0.76		0.36
Total vitamin D, IU/day							
Q1[4]	< 122; < 111	96	4,284	1.00	—	1.00	—
Q2	122 - < 191; 111 - < 201	108	4,370	1.06	0.80, 1.40	1.12	0.83, 1.49
Q3	191 - < 425; 201 - < 467	99	4,278	0.90	0.67, 1.21	1.00	0.72, 1.38
Q4	≥ 425; ≥ 467	105	4,306	0.98	0.73, 1.30	1.14	0.73, 1.78
P_{trend}[5]					0.77		0.61
Dietary vitamin D, IU/day							
Q1[4]	< 105; < 90	103	4,283	1.00	—	1.00	—

(Table continues on the next page)

Exposure	Range[1] (men; women)	Total No. Deaths	Person-Years	Base model[2]		MV model[3]	
				RR	95% CI	RR	95% CI
Q2	105 - < 157; 90 - < 136	109	4,347	1.01	0.76, 1.33	1.03	0.78, 1.36
Q3	157 - < 226; 136 - < 202	93	4,295	0.87	0.65, 1.16	0.90	0.67, 1.22
Q4	≥ 226; ≥ 202	103	4,313	0.91	0.69, 1.21	0.96	0.72, 1.28
P_{trend}[5]					0.39		0.61
Total dairy, servings/week							
Q1[4]	< 5.5; < 5.0	110	4,044	1.00	—	1.00	—
Q2	5.5 - < 9.6; 5.0 - < 8.9	91	4,610	0.83	0.62, 1.10	0.84	0.63, 1.13
Q3	9.6 - < 14.5; 8.9 - < 13.4	107	4,351	0.89	0.67, 1.18	0.92	0.69, 1.23
Q4	≥ 14.5; ≥ 13.4	100	4,233	0.86	0.63, 1.17	0.89	0.65, 1.22
P_{trend}[5]					0.55		0.73
Milk, servings/week							
Q1[4]	0; 0	110	4,895	1.00	—	1.00	—
Q2	0.1 - < 5.7; 0.1 - < 5.1	88	3,771	1.07	0.80, 1.43	1.06	0.79, 1.42
Q3	5.7 - < 10.5; 5.1 - < 10.1	114	4,293	1.07	0.81, 1.40	1.08	0.82, 1.42
Q4	≥ 10.5; ≥ 10.1	96	4,280	0.95	0.71, 1.28	0.98	0.73, 1.32
P_{trend}[5]					0.62		0.80

Abbreviations: Q, quartile; C, category; RR, relative risk; CI, confidence interval; MV, multivariable

[1]Range obtained from each sex-specific quartile of all exposures, except for supplemental calcium, which was obtained from each category for both sexes combined

[2]Base model adjusted for age at diagnosis, sex, tumor stage, and 1992 total energy intake

[3]Multivariable model adjusted for age at diagnosis, sex, tumor stage, and 1992 total energy and total folate intakes

[4]Reference group

[5]P_{trend} calculated by using the median exposure in each quartile, specific to sex, for all exposures except for supplement calcium, which was calculated using the actual categories, i.e. 1, 2, and 3, for both sexes

[6]Supplemental calcium was categorized based on visually inspecting the distribution of the variable

Table 4.5. Associations of 1992 Pre-diagnostic Calcium, Vitamin D, and Dairy Intakes with All-Cause Mortality among Colorectal Cancer Patients of All Stages in the CPS-II Nutrition Cohort

Exposure	Range[1] (men; women)	Total No. Deaths	Person-Years	Base model[2]		MV model[3]	
				RR	95% CI	RR	95% CI
Total calcium, mg/day							
Q1[4]	< 579; < 545	337	4,562	1.00	—	1.00	—
Q2	579 - < 775; 545 - < 773	335	4,874	0.93	0.79, 1.09	0.96	0.81, 1.13
Q3	775 - < 1,033; 773 - < 1,152	344	4,563	0.89	0.76, 1.05	0.94	0.79, 1.11
Q4	≥ 1,033; ≥ 1,152	366	4,628	0.96	0.82, 1.12	1.00	0.84, 1.19
P_{trend}[5]					0.85		0.75
Dietary calcium, mg/day							
Q1[4]	< 548; < 481	350	4,427	1.00	—	1.00	—
Q2	548 - < 724; 481 - < 620	337	4,859	0.86	0.73, 1.01	0.88	0.75, 1.04
Q3	724 - < 944; 620 - < 848	342	4,640	0.92	0.78, 1.07	0.95	0.81, 1.12
Q4	≥ 944; ≥ 848	353	4,701	0.87	0.75, 1.03	0.91	0.77, 1.07
P_{trend}[5]					0.22		0.45
Supplemental calcium, mg/day							
C1[4,6]	0	851	11,618	1.00	—	1.00	—
C2	0.1 - < 250	321	4,219	1.01	0.88, 1.16	1.11	0.91, 1.35
C3	≥ 250	210	2,790	1.06	0.90, 1.26	1.14	0.94, 1.40
P_{trend}[5]					0.52		0.19
Total vitamin D, IU/day							
Q1[4]	< 123; < 108	343	4,534	1.00	—	1.00	—
Q2	123 - < 191; 108 - < 193	324	4,728	0.87	0.74, 1.03	0.92	0.78, 1.09
Q3	191 - < 420; 193 - < 461	350	4,660	0.94	0.80, 1.11	1.02	0.85, 1.22
Q4	≥ 420; ≥ 461	365	4,704	0.95	0.81, 1.11	1.08	0.84, 1.40
P_{trend}[5]					0.99		0.33
Dietary vitamin D, IU/day							
Q1[4]	< 106; < 90	345	4,562	1.00	—	1.00	—
Q2	106 - < 158; 90 - < 135	338	4,758	0.88	0.75, 1.03	0.90	0.77, 1.06
Q3	158 - < 227; 135 - < 203	331	4,737	0.88	0.75, 1.03	0.92	0.78, 1.09

(Table continues on the next page)

Exposure	Range[1] (men; women)	Total No. Deaths	Person-Years	Base model[2]		MV model[3]	
				RR	95% CI	RR	95% CI
Q4	≥ 227; ≥ 203	368	4,571	0.97	0.83, 1.14	1.02	0.86, 1.20
P_{trend}[5]					0.98		0.52
Total dairy, servings/week							
Q1[4]	< 5.5; < 4.9	350	4,394	1.00	—	1.00	—
Q2	5.5 - < 9.6; 4.9 - < 8.8	321	4,886	0.81	0.68, 0.95	0.82	0.70, 0.97
Q3	9.6 - < 14.6; 8.8 - < 13.3	352	4,761	0.89	0.76, 1.05	0.92	0.78, 1.09
Q4	≥ 14.6; ≥ 13.3	359	4,585	0.92	0.77, 1.09	0.95	0.80, 1.14
P_{trend}[5]					0.88		0.79
Milk, servings/week							
Q1[4]	0 ; 0	440	5,365	1.00	—	1.00	—
Q2	0.1 - < 5.5; 0.1 - < 4.8	286	3,847	0.97	0.82, 1.14	0.96	0.82, 1.14
Q3	5.5 - < 10.4 ; 4.8 - < 10.0	344	4,859	0.92	0.78, 1.08	0.93	0.80, 1.09
Q4	≥ 10.4; ≥ 10.0	368	4,677	0.95	0.81, 1.12	0.98	0.83, 1.15
P_{trend}[5]					0.48		0.72

Abbreviations: Q, quartile; C, category; RR, relative risk; CI, confidence interval; MV, multivariable

[1]Range obtained from each sex-specific quartile of all exposures, except for supplemental calcium, which was obtained from each category for both sexes combined

[2]Base model adjusted for age at diagnosis, sex, tumor stage, and 1992 total energy intake

[3]Multivariable model adjusted for age at diagnosis, sex, tumor stage, and 1992 total energy and total folate intakes

[4]Reference group

[5]P_{trend} calculated by using the median exposure in each quartile, specific to sex, for all exposures except for supplement calcium, which was calculated using the actual categories, i.e. 1, 2, and 3, for both sexes

[6]Supplemental calcium was categorized based on visually inspecting the distribution of the variable

Table 4.6. Associations of 1992 Pre-diagnostic Calcium, Vitamin D, and Dairy Intakes with All-Cause Mortality among Colorectal Cancer Patients with Postdiagnosis Data (n = 1,111) in the CPS-II Nutrition Cohort

Exposure	Range[1] (men; women)	Total No. Deaths	Person-Years	Base model[2]		MV model[3]	
				RR	95% CI	RR	95% CI
Total calcium, mg/day							
Q1[4]	< 578; < 553	100	2,733	1.00	—	1.00	—
Q2	578 - < 776; 553 - < 776	101	2,909	0.84	0.63, 1.12	0.87	0.64, 1.17
Q3	776 - < 1,044; 776 - < 1,156	107	2,765	0.88	0.65, 1.17	0.91	0.67, 1.23
Q4	≥ 1,044; ≥ 1,156	121	2,913	0.91	0.68, 1.20	0.96	0.70, 1.30
P_{trend}[5]					0.87		0.79
Dietary calcium, mg/day							
Q1[4]	< 548; < 486	109	2,556	1.00	—	1.00	—
Q2	548 - < 729; 486 - < 629	98	3,031	0.69	0.52, 0.92	0.69	0.51, 0.92
Q3	729 - < 949; 629 - < 849	106	2,676	0.81	0.61, 1.08	0.82	0.61, 1.10
Q4	≥ 949; ≥ 849	116	3,058	0.74	0.56, 0.97	0.75	0.56, 1.00
P_{trend}[5]					0.13		0.19
Supplemental calcium, mg/day							
C1[4,6]	0	251	6,987	1.00	—	1.00	—
C2	0.1 - < 250	110	2,682	1.00	0.79, 1.27	1.31	0.94, 1.83
C3	≥ 250	68	1,652	1.34	0.99, 1.80	1.65	1.16, 2.35
P_{trend}[5]					0.12		0.01
Total vitamin D, IU/day							
Q1[4]	< 122; < 111	109	2,718	1.00	—	1.00	—
Q2	122 - < 191; 111 - < 201	88	2,843	0.69	0.51, 0.93	0.70	0.52, 0.95
Q3	191 - < 425; 201 - < 467	114	2,792	0.84	0.64, 1.12	0.85	0.63, 1.16
Q4	≥ 425; ≥ 467	118	2,968	0.79	0.60, 1.05	0.91	0.58, 1.43
P_{trend}[5]					0.49		0.85
Dietary vitamin D, IU/day							
Q1[4]	< 105; < 90	105	2,679	1.00	—	1.00	—
Q2	105 - < 157; 90 - < 136	102	2,826	0.88	0.66, 1.18	0.89	0.66, 1.19
Q3	157 - < 226; 136 - < 202	107	2,901	0.85	0.64, 1.12	0.86	0.65, 1.15

(Table continues on the next page)

Exposure	Range[1] (men; women)	Total No. Deaths	Person-Years	Base model[2]		MV model[3]	
				RR	95% CI	RR	95% CI
Q4	≥ 226; ≥ 202	115	2,915	0.84	0.63, 1.11	0.85	0.63, 1.13
P_{trend}[5]					0.22		0.27
Total dairy, servings/week							
Q1[4]	< 5.5; < 5.0	105	2,490	1.00	—	1.00	—
Q2	5.5 - < 9.6; 5.0 - < 8.9	92	3,028	0.72	0.54, 0.98	0.73	0.54, 0.99
Q3	9.6 - < 14.5; 8.9 - < 13.4	116	2,941	0.83	0.63, 1.10	0.84	0.63, 1.12
Q4	≥ 14.5; ≥ 13.4	116	2,862	0.80	0.59, 1.09	0.82	0.60, 1.13
P_{trend}[5]					0.42		0.52
Milk, servings/week							
Q1[4]	0; 0	118	3,259	1.00	—	1.00	—
Q2	0.1 - < 5.7; 0.1 - < 5.1	99	2,304	1.23	0.93, 1.64	1.23	0.93, 1.64
Q3	5.7 - < 10.5; 5.1 - < 10.1	100	2,883	0.89	0.67, 1.19	0.91	0.69, 1.21
Q4	≥ 10.5; ≥ 10.1	112	2,874	0.89	0.67, 1.19	0.90	0.68, 1.20
P_{trend}[5]					0.14		0.17

Abbreviations: Q, quartile; C, category; RR, relative risk; CI, confidence interval; MV, multivariable

[1]Range obtained from each sex-specific quartile of all exposures, except for supplemental calcium, which was obtained from each category for both sexes combined

[2]Base model adjusted for age at diagnosis, sex, tumor stage, and 1992 total energy intake

[3]Multivariable model adjusted for age at diagnosis, sex, tumor stage, and 1992 total energy and total folate intakes

[4]Reference group

[5]P_{trend} calculated by using the median exposure in each quartile, specific to sex, for all exposures except for supplement calcium, which was calculated using the actual categories, i.e., 1, 2, and 3, for both sexes

[6]Supplemental calcium was categorized based on visually inspecting the distribution of the variable

Table 4.7. Associations of Post-diagnosis Calcium, Vitamin D, and Dairy Intakes with All-Cause Mortality among Non-Metastatic Colorectal Cancer Patients in the CPS-II Nutrition Cohort

Exposure	1999 Range[1] (men; women)	2003 Range[1] (men; women)	Total No. Deaths	Person-Years	Base model[2]		MV model[3]	
					RR	95% CI	RR	95% CI
Total calcium, mg/day								
Q1[4]	< 581; < 713	< 683; < 773	112	1,903	1.00	—	1.00	—
Q2	581 - < 775; 713 - < 1,170	683 - < 882; 773- < 1,131	118	2,098	0.89	0.67, 1.18	0.89	0.67, 1.18
Q3	775 - <1,105; 1,170 - < 1,598	882 - < 1,162; 1,131 - < 1,591	100	2,078	0.72	0.54, 0.96	0.72	0.53, 0.98
Q4	≥ 1,105; ≥ 1,598	≥ 1,162; ≥ 1,591	99	2,325	0.72	0.54, 0.97	0.72	0.53, 0.98
P_{trend}[5]						0.01		0.02
Dietary calcium, mg/day								
Q1[4]	< 532; < 525	< 613; < 609	105	1,804	1.00	—	1.00	—
Q2	532 - < 683; 525 - < 671	613 - < 765; 609 - < 766	118	2,277	0.84	0.63, 1.12	0.84	0.63, 1.11
Q3	683 - < 885; 671 - < 892	765 - < 968; 766 - < 990	90	2,100	0.69	0.51, 0.92	0.69	0.51, 0.93
Q4	≥ 885; ≥ 892	≥ 968; ≥ 990	116	2,223	0.85	0.64, 1.12	0.86	0.65, 1.14
P_{trend}[5]						0.17		0.21
Supplemental calcium, mg/day								
C1[4,6]	0	0	221	3,966	1.00	—	1.00	—
C2	0.1 - < 500	0.1 - < 500	108	2,001	0.90	0.70, 1.16	0.95	0.72, 1.27
C3	≥ 500	≥ 500	100	2,437	0.94	0.72, 1.23	0.98	0.73, 1.31
P_{trend}[5]						0.55		0.88
Total vitamin D, IU/day								
Q1[4]	< 164; < 151	< 194; < 219	105	1,894	1.00	—	1.00	—
Q2	164 - < 302; 151 - < 379	194 - < 389; 219 - < 509	101	2,087	0.79	0.59, 1.07	0.81	0.59, 1.10
Q3	302 - < 559; 379 - < 588	389 - < 603; 509 - < 685	108	2,167	0.90	0.67, 1.21	0.97	0.67, 1.40
Q4	≥ 559; ≥ 588	≥ 603; ≥ 685	115	2,256	0.80	0.60, 1.07	0.88	0.57, 1.35
P_{trend}[5]						0.16		0.35
Dietary vitamin D, IU/day								
Q1[4]	< 122; < 100	< 132; < 103	103	2,065	1.00	—	1.00	—

(Table continues on the next page)

Exposure	1999 Range[1] (men; women)	2003 Range[1] (men; women)	Total No. Deaths	Person-Years	Base model[2]		MV model[3]	
					RR	95% CI	RR	95% CI
Q2	122 - < 178; 100 - < 155	132 - < 188; 103 - < 178	115	2,144	0.99	0.75, 1.31	0.99	0.75, 1.31
Q3	178 - < 245; 155 - < 229	188 - < 267; 178 - < 257	105	2,109	0.94	0.70, 1.25	0.95	0.71, 1.27
Q4	≥ 245; ≥ 229	≥ 267; ≥ 257	106	2,085	0.89	0.67, 1.19	0.90	0.67, 1.21
P_{trend}[5]						0.29		0.33
Total dairy, servings/week								
Q1[4]	< 4.7; < 3.9	< 5.1; < 4.8	115	1,931	1.00	—	1.00	—
Q2	4.7 - < 8.2; 3.9 - < 7.7	5.1 - < 8.8; 4.8 - < 8.1	109	2,106	0.91	0.69, 1.20	0.91	0.69, 1.21
Q3	8.2 - < 11.7; 7.7 - < 11.6	8.8 - < 12.3; 8.1 - < 12.4	98	2,068	0.73	0.54, 0.98	0.73	0.54, 0.98
Q4	≥ 11.7; ≥ 11.6	≥ 12.3; ≥ 12.4	107	2,299	0.75	0.55, 1.00	0.75	0.56, 1.01
P_{trend}[5]						0.05		0.05
Milk, servings/week								
Q1[4]	< 1.1; < 1.0	< 1.0; < 1.0	106	1,844	1.00	—	1.00	—
Q2	1.1 - < 5.6; 1.0 - < 3.5	1.0 - < 5.6; 1.0 - < 3.3	109	2,135	0.84	0.64, 1.12	0.85	0.64, 1.13
Q3	5.6 - < 7.0; 3.5 - < 7.0	5.6 - < 7; 3.3 - < 7.0	41	881	0.76	0.52, 1.11	0.76	0.52, 1.12
Q4	≥ 7.0; ≥ 7.0	≥ 7.0; ≥ 7.0	173	3,543	0.71	0.55, 0.93	0.72	0.55, 0.94
P_{trend}[5]						0.01		0.02

Abbreviations: Q, quartile; C, category; RR, relative risk; CI, confidence interval; MV, multivariable

[1]Range obtained from each questionnaire- and sex-specific quartile of all exposures, except for supplemental calcium, which was obtained from each category for both sexes combined

[2]Base model adjusted for age at diagnosis, sex, tumor stage, and post-diagnosis total energy intake

[3]Multivariable model adjusted for age at diagnosis, sex, tumor stage, and post-diagnosis total energy and total folate intakes

[4]Reference group

[5]P_{trend} calculated by using the median exposure in each quartile, specific to sex, for all exposures except for supplement calcium, which was calculated using the actual categories, i.e., 1, 2, and 3, for both sexes

[6]Supplemental calcium was categorized based on visually inspecting the distribution of the variable

Table 4.8. Associations of Postdiagnosis Calcium, Vitamin D, and Dairy Intakes with Colorectal Cancer Mortality among Non-Metastatic Colorectal Cancer Patients in the CPS-II Nutrition Cohort

Exposure	1999 Range[1] (men; women)	2003 Range[1] (men; women)	Total No. Deaths	Person-Years	Base model[2] RR	Base model[2] 95% CI	MV model[3] RR	MV model[3] 95% CI
Total calcium, mg/day								
Q1[4]	< 581; < 713	< 683; < 773	37	1,903	1.00	—	1.00	—
Q2	581 - < 775; 713 - < 1,170	683 - < 882; 773 - < 1,131	49	2,098	1.11	0.69, 1.78	1.15	0.71, 1.86
Q3	775 - <1,105; 1,170 - < 1,598	882 - < 1,162; 1,131 - < 1,591	33	2,078	0.75	0.46, 1.24	0.81	0.48, 1.38
Q4	≥ 1,105; ≥ 1,598	≥ 1,162; ≥ 1,591	24	2,325	0.54	0.31, 0.94	0.59	0.33, 1.05
P_{trend}[5]						<0.01		0.01
Dietary calcium, mg/day								
Q1[4]	< 532; < 525	< 613; < 609	35	1,804	1.00	—	1.00	—
Q2	532 - < 683; 525 - < 671	613 - < 765; 609 - < 766	36	2,277	0.86	0.52, 1.42	0.85	0.51, 1.41
Q3	683 - < 885; 671 - < 892	765 - < 968; 766 - < 990	35	2,100	0.90	0.55, 1.48	0.98	0.59, 1.62
Q4	≥ 885; ≥ 892	≥ 968; ≥ 990	37	2,223	0.91	0.56, 1.47	1.00	0.61, 1.63
P_{trend}[5]						0.53		0.83
Supplemental calcium, mg/day								
C1[4,6]	0	0	74	3,966	1.00	—	1.00	—
C2	0.1 - < 500	0.1 - < 500	42	2,001	0.92	0.61, 1.38	1.04	0.65, 1.69
C3	≥ 500	≥ 500	27	2,437	0.58	0.35, 0.95	0.65	0.38, 1.11
P_{trend}[5]						0.04		0.13
Total vitamin D, IU/day								
Q1[4]	< 164; < 151	< 194; < 219	33	1,894	1.00	—	1.00	—
Q2	164 - < 302; 151 - < 379	194 - < 389; 219 - < 509	38	2,087	0.84	0.51, 1.38	0.99	0.59, 1.66
Q3	302 - < 559; 379 - < 588	389 - < 603; 509 - < 685	34	2,167	0.85	0.51, 1.42	1.31	0.66, 2.58
Q4	≥ 559; ≥ 588	≥ 603; ≥ 685	38	2,256	0.90	0.54, 1.49	1.74	0.80, 3.77
P_{trend}[5]						0.45		0.52
Dietary vitamin D, IU/day								
Q1[4]	< 122; < 100	< 132; < 103	32	2,065	1.00	—	1.00	—

(Table continues on the next page)

Exposure	1999 Range[1] (men; women)	2003 Range[1] (men; women)	Total No. Deaths	Person-Years	Base model[2]		MV model[3]	
					RR	95% CI	RR	95% CI
Q2	122 - < 178; 100 - < 155	132 - < 188; 103 - < 178	34	2,144	0.76	0.45, 1.28	0.78	0.46, 1.32
Q3	178 - < 245; 155 - < 229	188 - < 267; 178 - < 257	37	2,109	1.01	0.61, 1.68	1.11	0.67, 1.85
Q4	≥ 245; ≥ 229	≥ 267; ≥ 257	40	2,085	1.18	0.72, 1.93	1.28	0.77, 2.10
P_{trend}[5]						0.31		0.19
Total dairy, servings/week								
Q1[4]	< 4.7; < 3.9	< 5.1; < 4.8	37	1,931	1.00	—	1.00	—
Q2	4.7 - < 8.2; 3.9 - < 7.7	5.1 - < 8.8; 4.8 - < 8.1	31	2,106	0.73	0.44, 1.22	0.73	0.44, 1.23
Q3	8.2 - < 11.7; 7.7 - < 11.6	8.8 - < 12.3; 8.1 - < 12.4	41	2,068	0.87	0.53, 1.44	0.92	0.56, 1.52
Q4	≥ 11.7; ≥ 11.6	≥ 12.3; ≥ 12.4	34	2,299	0.71	0.42, 1.19	0.73	0.44, 1.23
P_{trend}[5]						0.26		0.32
Milk, servings/week								
Q1[4]	< 1.1; < 1.0	< 1.0; < 1.0	33	1,844	1.00	—	1.00	—
Q2	1.1 - < 5.6; 1.0 - < 3.5	1.0 - < 5.6; 1.0 - < 3.3	33	2,135	0.88	0.53, 1.45	0.90	0.54, 1.49
Q3	5.6 - < 7.0; 3.5 - < 7.0	5.6 - < 7; 3.3 - < 7.0	14	881	0.85	0.43, 1.65	0.85	0.44, 1.67
Q4	≥ 7.0; ≥ 7.0	≥ 7.0; ≥ 7.0	63	3,543	0.87	0.55, 1.38	0.93	0.59, 1.49
P_{trend}[5]						0.61		0.81

Abbreviations: Q, quartile; C, category; RR, relative risk; CI, confidence interval; MV, multivariable

[1]Range obtained from each questionnaire- and sex-specific quartile of all exposures, except for supplemental calcium, which was obtained from each category for both sexes combined

[2]Base model adjusted for age at diagnosis, sex, tumor stage, and post-diagnosis total energy intake

[3]Multivariable model adjusted for age at diagnosis, sex, tumor stage, and post-diagnosis total energy and total folate intakes

[4]Reference group

[5]P_{trend} calculated by using the median exposure in each quartile, specific to sex, for all exposures except for supplement calcium, which was calculated using the actual categories, i.e. 1, 2, and 3, for both sexes

[6]Supplemental calcium was categorized based on visually inspecting the distribution of the variable

Table 4.9. Associations of Post-diagnosis Calcium, Vitamin D, and Dairy Intakes with All-Cause Mortality among Colorectal Cancer Patients in the CPS-II Nutrition Cohort Stratified by Tumor Stage at Diagnosis[1]

Exposure	Localized Stage				Regional Stage				$p_{interaction}$
	Total No. Deaths	Person-Years	RR	95% CI	Total No. Deaths	Person-Years	RR	95% CI	
Total calcium, mg/day									
Q1[2]	55	1,150	1.00	—	57	753	1.00	—	
Q2	60	1,237	1.00	0.67, 1.49	58	861	0.79	0.52, 1.20	
Q3	60	1,190	0.97	0.64, 1.46	40	888	0.52	0.33, 0.82	
Q4	46	1,332	0.69	0.45, 1.07	53	993	0.74	0.47, 1.16	
P_{trend}[3]				0.12				0.09	0.08
Dietary calcium, mg/day									
Q1[2]	50	1,031	1.00	—	55	773	1.00	—	
Q2	65	1,351	0.91	0.61, 1.35	53	926	0.75	0.50, 1.14	
Q3	47	1,211	0.72	0.47, 1.11	43	889	0.67	0.43, 1.04	
Q4	59	1,316	0.83	0.55, 1.25	57	907	0.90	0.60, 1.34	
P_{trend}[3]				0.30				0.49	0.75
Supplemental calcium, mg/day									
C1[2,4]	122	2,404	1.00	—	99	1,562	1.00	—	
C2	48	1,179	0.87	0.59, 1.29	60	822	1.05	0.69, 1.61	
C3	51	1,326	1.00	0.67, 1.48	49	1,111	0.99	0.64, 1.54	
P_{trend}[3]				0.92				0.96	0.68
Total vitamin D, IU/day									
Q1[2]	57	1,186	1.00	—	48	708	1.00	—	
Q2	52	1,096	0.88	0.57, 1.34	49	991	0.76	0.48, 1.19	
Q3	60	1,270	0.98	0.60, 1.60	48	897	0.99	0.55, 1.77	
Q4	52	1,358	0.64	0.36, 1.13	63	897	1.33	0.68, 2.59	
P_{trend}[3]				0.13				0.79	0.23
Dietary vitamin D, IU/day									
Q1[2]	61	1,254	1.00	—	42	811	1.00	—	

(Table continues on the next page)

Exposure	Localized Stage				Regional Stage				$p_{interaction}$
	Total No. Deaths	Person-Years	RR	95% CI	Total No. Deaths	Person-Years	RR	95% CI	
Q2	58	1,241	0.93	0.64, 1.36	57	903	1.13	0.73, 1.74	
Q3	53	1,149	0.86	0.58, 1.27	52	960	1.07	0.69, 1.67	
Q4	49	1,266	0.71	0.47, 1.08	57	820	1.18	0.77, 1.82	
P_{trend}[3]				0.07				0.57	0.53
Total dairy, servings/week									
Q1[2]	62	1,196	1.00	—	53	735	1.00	—	
Q2	54	1,208	0.93	0.63, 1.38	55	898	0.91	0.61, 1.38	
Q3	49	1,096	0.85	0.57, 1.28	49	971	0.63	0.40, 0.98	
Q4	56	1,409	0.69	0.46, 1.05	51	889	0.82	0.53, 1.27	
P_{trend}[3]				0.06				0.37	0.42
Milk, servings/week									
Q1[2]	58	1,100	1.00	—	48	744	1.00	—	
Q2	55	1,282	0.76	0.51, 1.13	54	853	1.00	0.66, 1.52	
Q3	21	464	0.75	0.43, 1.28	20	417	0.75	0.43, 1.31	
Q4	87	2,063	0.67	0.46, 0.98	86	1,480	0.80	0.54, 1.18	
P_{trend}[3]				0.05				0.17	0.84

Abbreviations: Q, quartile; C, category; RR, relative risk; CI, confidence interval; MV, multivariable

[1]Only showing multivariate model results adjusted for age at diagnosis, sex, tumor stage, and post-diagnosis total energy and total folate intakes

[2]Reference group

[3]P_{trend} calculated by using the median exposure in each quartile, specific to sex, for all exposures except for supplement calcium, which was calculated using the actual categories, i.e., 1, 2, and 3, for both sexes

[4]Supplemental calcium was categorized based on visually inspecting the distribution of the variable

CHAPTER 5. CALCIUM INTAKE AND MORTALITY FROM ALL CAUSES, CANCER, AND CARDIOVASCULAR DISEASE: THE CANCER PREVENTION STUDY II NUTRITION COHORT

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Abstract

Calcium has an established role in promoting bone health, but its effects on other outcomes remain unclear. Especially, the potential adverse influence of supplemental calcium on cardiovascular diseases (CVD) has raised concerns. We investigated associations of total, dietary, and supplemental calcium with all-cause, CVD-, and cancer-specific mortality among 132,823 participants in the Cancer Prevention Study II Nutrition Cohort, using multivariable-adjusted Cox proportional hazards regression models with cumulative updating of exposures. During a mean follow-up of 17.5 years, 43,186 deaths occurred. For men, supplemental calcium intake ≥1,000 mg/d was associated with higher all-cause mortality (relative risk [RR]: 1.17; 95% confidence interval [CI]: 1.03, 1.33) and marginally statistically significantly higher CVD-specific mortality (RR: 1.22; 95% CI: 0.99, 1.51), but was not associated with cancer-specific mortality (RR: 1.03; 95% CI: 0.79, 1.33). For women, supplemental calcium was inversely associated with mortality from all causes and CVD, but not cancer. Dietary and total calcium intakes were not associated with all-cause mortality in men and were inversely associated with mortality in women. In conclusion, calcium intake is generally not associated with higher mortality in this cohort, but for men, supplemental calcium intake ≥ 1,000 mg/d may be associated with increased all-cause and CVD-specific mortality.

Introduction

In the United States, recommendations to consume foods rich in calcium and vitamin D are included in dietary guidelines.[151] In addition, dietary supplements containing calcium are used by 43% of the overall population and 62% of individuals 71 years of age or older.[285] Adequate calcium intake is important for bone health and several major physiologic functions.[30] However, beyond its benefits for bone health, the effects of calcium on other health outcomes are largely unclear.

The relationship between calcium and cardiovascular disease (CVD) is complex and may depend on the source of calcium.[219] Dietary calcium is generally weakly associated with lower risk of incident or fatal CVD.[219] In contrast, studies on supplemental calcium have reported conflicting results: while positive associations with CVD risk and mortality were reported in several epidemiological studies,[32-34] especially in men, null or inverse associations were reported in others.[35-38,286,287] A meta-analysis of several randomized clinical trials, primarily among older women, reported that calcium supplementation increased myocardial infarction (MI) risk by 24%, and the risk of a composite of MI or stroke by 15%.[39] Although the trials included in the meta-analysis were not primarily designed to assess the effect of calcium supplementation on cardiovascular events, these results raised concerns about the potential harms of supplemental calcium on the cardiovascular system.

With regard to cancer, there is generally consistent observational evidence that calcium intake is inversely associated with colorectal cancer,[6,7,155] and a major clinical trial found statistically significantly reduced colorectal adenoma recurrence with calcium supplementation.[8] In addition, some evidence suggests that total or dietary calcium may be associated with lower risk of breast cancer,[40,41] and total calcium or dairy intake may be positively associated with risk of prostate cancer,[42] but the World Cancer Research Fund considers the level of evidence "limited" for both types of cancer.[43,44]

As reported herein, we comprehensively examined the associations of supplemental calcium intake with mortality from all causes and specifically due to CVD and cancer, and secondarily examined the associations of total and dietary calcium intake with mortality outcomes, in a large US prospective cohort study of men and women.

Methods

Study Population

The analytic cohort for this analysis consisted of men and women participating in the Cancer Prevention Study (CPS)-II Nutrition Cohort, a prospective study of cancer incidence and mortality among US adults,[269] which is a subset of the original CPS-II mortality cohort.[288] At baseline enrollment (1992 – 1993), approximately 184,000 participants completed a mailed 10-page self-administered questionnaire regarding demographics, body size, medical history, diet, and other major lifestyle factors. Follow-up questionnaires were sent to participants in 1997 and biennially thereafter to update exposure information and to learn of new cancer diagnoses (response rate ≥ 89% for each follow-up questionnaire). The CPS-II Nutrition Cohort is approved by the institutional review board of Emory University.

For this analysis, we excluded participants with history of cancer at baseline (n = 21,785), previous history of MI (n = 11,559), and stroke (n = 2,513) because these individuals may have changed their diets as a result of their diagnosis. We also excluded those with poorly completed dietary assessment instruments at baseline (n = 14,136) or uninterpretable calcium supplement use (n = 1,369). After exclusions, 132,823 subjects remained eligible for this analysis, including 59,744 men and 73,079 women.

Dietary Assessment

We first assessed calcium intake of participants at baseline (1992 – 1993) using a 68-item modified Block Food Frequency Questionnaire (FFQ),[269,273,274] and updated this information in 1999 and 2003 using a 152-item modified Willett FFQ.[269,275-277] Both FFQs included similar questions on the major food and beverage sources of calcium (Table 5.1).[289] FFQ estimates for calcium as well as dairy products (major source of dietary calcium) were in good agreement with estimates from dietary recalls or food records in validation studies (Pearson correlation

coefficients ranged from 0.57 to 0.66 for calcium and from 0.52 to 0.88 for dairy products).[274,276,277] Dietary calcium was adjusted for energy intake using the residual method,[152] and total calcium was calculated as energy-adjusted dietary calcium plus raw supplemental calcium intake.

Outcome Ascertainment

Vital status and cause of death were ascertained through linkage to the National Death Index up to December 31, 2012. The primary outcomes of this study were death from all causes, all cancers (International Classification of Diseases [ICD]-9 140-208, ICD-10 C00-C97), and all CVD (ICD-9 390-459, ICD-10 I00-I99). Secondary outcomes included mortality specifically from colorectal cancer (ICD-9 153-154, ICD-10 C18-C20), lung cancer (ICD-9 162, ICD-10 C33-C34), female breast cancer (ICD-9 174-175, ICD-10 C50), prostate cancer (ICD-9 185, ICD-10 C61), coronary heart disease (ICD-9 410-414, ICD-10 I20-I25), and stroke (ICD-9 430-438, ICD-10 I60-I69).

Statistical Analysis

Supplemental calcium intake was categorized into four levels based on interpretable cut-points for both sexes (0, 0.1- < 500, 500 - < 1,000, and ≥ 1,000 mg/d). Total calcium and dietary calcium were categorized according to sex- and questionnaire-specific quintiles. To best estimate long-term calcium intake, we cumulatively updated calcium intake at 1999 and 2003 by taking the mean of all reported intakes up to that year, to predict outcomes that occurred during the subsequent period. Values for missing data were carried forward from the previous questionnaire.

We used Cox proportional hazards models to estimate relative risks (RRs) and 95% confidence intervals (CIs). The underlying time axis for all models was time since baseline enrollment. Person-time began on the date of baseline enrollment, and ended on the date of death

or the end of mortality follow-up (December 31, 2012), whichever came first. We assessed the proportional hazards assumption using a likelihood ratio test by comparing models with and without an interaction term between a main exposure (in categories) and time.

We chose *a priori* to conduct all analyses separately for men and women because of previously reported heterogeneity by sex,[34] and to adjust for total energy intake (quintiles) and age at enrollment (year) in all models. We also adjusted for quintile intakes of whole grain, red and processed meat, and total folate, and for cigarette smoking (never smokers; former smokers who quit ≥ 30 years ago, 20 - < 30 years ago, or < 20 years ago; and current smokers who smoke < 15 cigarettes/d, or ≥ 15 cigarettes/d), alcohol consumption (0, 0.1 - <1 and ≥1 drinks/d), body mass index(BMI; <18.5, 18.5 – 24.9, 25 – 29.9, and ≥30 kg/m^2), education (less than high school, high school graduate, some college/trade school, and at least college graduate), and hormone replacement therapy (HRT) for women (none, former, or current use), because these variables either changed the RR by over 10% or were established predictors of the outcome. Other covariates considered included race, marital status, physical activity, use of aspirin/other nonsteroidal anti-inflammatory drugs (NSAIDs), multivitamin use, history of diabetes, high cholesterol, hypertension, and osteoporosis, and intakes of vegetables, fruit, vitamin D, and (for supplemental calcium models only) dairy products. Dietary calcium was included in supplemental calcium models, and *vice versa*. We additionally adjusted for recent colonoscopy or sigmoidoscopy in models for colorectal cancer mortality, and for recent mammography in models for female breast cancer mortality. The Wald test was used to assess linear trends for the association between calcium and mortality by assigning the sex-specific median value for the quintile (for total and dietary calcium) or an ordinal variable for supplemental calcium category, and modeling it as a continuous variable.

Several sensitivity analyses were conducted to assess the robustness of our findings. We excluded the first two years of follow-up, and used age as the alternative time scale to account for

the increased failure potential for an older participant over a younger participant at study entry. Because development of comorbidities may affect participants' subsequent calcium intake, we stopped cumulative updating of calcium if incident cancer was diagnosed or a diagnosis of CVD was reported by that time point. We excluded participants with a history of diabetes or chronic obstructive pulmonary disease at baseline. We also repeated the analyses using baseline calcium data with no updating.

We tested for effect modification of supplemental calcium use and all-cause mortality by age at enrollment (< 65y vs. ≥ 65y), BMI (< 30 vs. ≥ 30 kg/m^2), physical activity (< median metabolic equivalent-hours per week vs. ≥ median), smoking status (never vs. former vs. current), aspirin use (non-user vs. user), other NSAID use (non-user vs. user), and HRT use (non- or former-user vs. current user) using likelihood ratio tests. All analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC).

Results

The mean age of participants at baseline was 62.6 years (standard deviation, 6.3 years), and 45% of participants were men. Most self-reported their race as white. Baseline characteristics of men and women by supplemental calcium intake are shown in Table 5.2. Participants with higher supplemental calcium use were generally older, better educated, more physically active, leaner, less likely to smoke, more likely to use NSAIDs and currently use HRT (women), and to have an overall healthier diet. Participants with 0.1 - <500 mg/d of supplemental calcium intake were more likely to take multivitamins, whereas those with ≥500 mg/d of intake were more likely to take individual calcium supplements; this is because multivitamins were counted as a source of low levels of calcium, while individual calcium supplements are necessary to reach higher intake levels.

Among the 132,823 participants, 43,186 deaths (24,413 in men; 18,773 in women) occurred during a mean follow-up of 17.5 years (standard deviation, 4.5 years; range, 1 day to 20.2 years), including 13,157 from cancer and 13,916 from CVD. As shown in Table 5.3, among men, supplemental calcium intake was directly associated with higher all-cause mortality when daily consumption was 1,000 mg or more, compared to none (RR: 1.17; 95% CI: 1.03, 1.33) but not when daily consumption was < 1,000 mg (P_{trend} = 0.18). A similar pattern in men was observed between supplemental calcium and CVD-specific mortality (RR: 1.22; 95% CI: 0.99, 1.51 for supplemental calcium use ≥ 1,000 mg/d), whereas no association was observed with cancer-specific mortality. Men with ≥1,000 mg/d of supplemental calcium intake were also at higher risk of death from all other causes (RR: 1.22; 95% CI: 0.99, 1.51), particularly death from respiratory system diseases (data not shown). Among women, there were inverse associations between supplemental calcium use and mortality from all causes and CVD, but not cancer (Table 5.3). When we repeated the above analyses using baseline supplemental calcium intake with no cumulative updating, the results were largely unchanged in men, whereas in women, intakes ≥ 1,000 mg/d was no longer associated with lower all-cause mortality (Table 5.4), opposite to our main results. None of the other sensitivity analyses, such as excluding the first two years of follow-up, changed the results materially.

The associations of supplemental calcium with the four leading types of cancer (lung cancer, colorectal cancer, female breast cancer and prostate cancer) and two major types of CVD (coronary heart disease [CHD] and stroke) are shown in Table 5.5. Supplemental calcium was associated with marginally statistically significantly higher CHD mortality in men when daily consumption exceeded 1,000 mg/d. In women, supplemental calcium was inversely associated with mortality from colorectal cancer, breast cancer and CHD, and had a U-shaped association with stroke mortality.

We observed effect modification by the use of aspirin and HRT in the association between supplemental calcium use and all-cause mortality (Figure 1). For men, taking ≥ 1,000 mg/d of supplemental calcium was more strongly associated with higher all-cause mortality among aspirin non-users compared to regular users ($P_{interaction} < 0.01$). For women, taking ≥ 1,000 mg/d of supplemental calcium was only associated with lower all-cause mortality among regular aspirin users or HRT never/former users ($P_{interaction} = 0.01$).

Total or dietary calcium intakes were not associated with all-cause mortality in men but were inversely associated with all-cause mortality in women, in cumulatively updated models; in addition, total calcium was inversely associated with mortality specifically due to total cancer or CVD in women, but not men; dietary calcium was generally not associated with cancer or CVD in either men or women (Tables 5.6 and 5.7).

Discussion

The results from this large prospective cohort study suggest that supplemental calcium intake ≥ 1,000 mg/d may be associated with higher mortality (especially from CVD) in men, but not in women. Total and dietary calcium intakes were generally not associated with all-cause mortality in men, but were inversely associated with all-cause mortality in women. Our results add to the previous evidence of a potential adverse effect of higher levels of supplemental calcium on cardiovascular health in men.

We are not aware of any randomized clinical trials that were specifically designed to test the effect of calcium supplements on CVD events as the primary outcome; however, meta-analyses of several trials that included CVD as a secondary outcome reported higher risks of MI and a composite outcome (MI or stroke) among subjects randomized to calcium (typically 0.6 - 2 g/d elemental calcium) compared to placebo.[39,290] Several large observational studies also reported positive associations of supplemental calcium use and CVD risk or mortality: The

EPIC-Heidelberg cohort found that regular use of supplemental calcium was associated with higher risk of incident MI in both sexes,[33] a study in Finland observed a 24% statistically significant higher risk of incident CHD among women who took any calcium/calcium + vitamin D supplements,[32] and the NIH-AARP study reported higher risk of CVD death among men who consumed > 1,000 mg/d of supplemental calcium, but not among women.[34] Conversely, several other large observational studies reported null or inverse associations between supplemental calcium use and CVD risk or mortality.[35-37,286,287] Reasons for the conflicting results in the observational studies are unclear, but may relate to the different doses examined: our study and the NIH-AARP study found direct associations of supplemental calcium with cardiovascular events in men only at very high doses, whereas several previous studies with null results compared lower doses of supplemental calcium intake with none, in men or women.[36,286,287]

In our cohort, we observed marginally statistically significantly higher risk of CVD mortality in men only. One of the mechanisms underlying the potential adverse cardiovascular effect of supplemental calcium may be vascular calcification. There is evidence that ingesting calcium supplements, but not calcium-rich foods, may lead to an acute increase in serum calcium.[219,221,222] This increase may be sustained long-term, as evidenced by a persistently lower level of serum parathyroid hormone (which acts to increase the level of calcium when the concentration is low) during two years of calcium supplementation among 323 healthy men.[235] High serum calcium may contribute to vascular calcification, a complex process that resembles osteogenesis,[291] as shown in several studies among dialysis patients that consistently reported positive associations between arterial calcification and calcium supplement use or higher serum calcium level.[223-225] Vascular calcification has been linked to higher CVD risk or mortality across several ethnic groups.[226-230] This adverse effect may counteract several protective effects of calcium on the cardiovascular system (e.g., favorable regulation of cholesterol levels, blood pressure, and insulin sensitivity).[219]

The observed heterogeneity by sex in our cohort was consistent with results from the NIH-AARP cohort, which also primarily consists of older white participants and was initiated in the 1990s.[34] Reasons for observing no excess mortality associated with calcium supplementation in women are unclear, especially considering that in previous calcium trials that reported elevated CVD risks, the participants were primarily older women not concurrently taking vitamin D supplements (i.e., in many, the primary trial outcome was bone health). Notably, the Women's Health Initiative Calcium/Vitamin D trial found higher risk of cardiovascular events with calcium and vitamin D treatment only if the participant was not taking personal calcium supplements, thus raising an interesting hypothesis that an abrupt change in serum calcium may account for the adverse effects.[39] Based on this hypothesis, Xiao *et al.* proposed that in the NIH-AARP cohort, women on average started taking supplemental calcium at younger ages than men, and the serum calcium level had acutely increased and was sustained thereafter long before the study baseline, precluding the observation of adverse effects during the study.[34] If this hypothesis is true, then the results from our study and the NIH-AARP study may not necessarily contradict the two European studies (EPIC-Heidelberg and the Finland study), which reported an elevated CVD risk with regular supplemental calcium intake for women or both sexes combined, because supplemental calcium consumption is relatively uncommon in Europe even among women.[32,33] Our study did not collect information on the duration of supplemental calcium use; however, when we did a sensitivity analysis using 1999 as the baseline and restricted the analysis to non-users inf 1992, we still observed lower mortality in women taking ≥ 1,000 mg/d of supplemental calcium (data not shown), providing no support for this hypothesis. Future studies with detailed duration data may provide more insights to evaluate this hypothesis.

An alternative explanation for the observed heterogeneity by sex is different patterns of bias for men and women. It is possible that for women, calcium supplement use may be associated with better health care or health-seeking behaviors (which in turn may be associated

with lower mortality), because of the known benefit of calcium in preventing or treating osteoporosis primarily for women. In contrast, for men, using high doses of supplemental calcium (especially ≥ 1,000 mg/d) may be associated with medical conditions that may be associated with higher mortality, thus possibly resulting in confounding by indication. However, our study did not comprehensively collect information on the above-mentioned potential confounding factors throughout all questionnaires; therefore, whether the heterogeneity by sex is due to potential biases warrants investigation in future studies.

That calcium supplement doses greater than 1,000 mg were associated with higher mortality risk in men, and yet the highest quintile consumption level of total calcium (diet plus supplements) was not associated with increased risk could be partially explained by the fact that dietary intakes drive total intakes in men: the mean supplemental calcium intake among men in the top quintile of total calcium intake was 242, 318, and 337 mg/day in 1992, 1999, and 2003, respectively.

We observed effect modification by aspirin in the association between supplemental calcium and all-cause mortality: despite the differences in the overall RR in men and women, both RR estimates were statistically significantly lower among aspirin users, compared to among non-users. The interaction between calcium and aspirin was previously reported in the context of colorectal adenoma prevention, with calcium and NSAIDs/aspirin acting synergistically to reduce risk of advanced colorectal adenoma.[292] We are not aware of previous reports on such interactions in relation to overall mortality, but it was previously reported that calcium enhanced anti-inflammatory properties of aspirin in rats,[293] and inflammation plays a key role in the development of major causes of death such as cancer and CVD.[294,295] Therefore, an interaction between calcium and aspirin in lowering all-cause mortality is plausible. We also observed an interaction with HRT in women, such that the inverse association between supplemental calcium and all-cause mortality was only observed among HRT never or former users, but not current

users. An interaction between calcium and estrogen use was also reported in the Women's Health Initiative, in which calcium and vitamin D treatment marginally reduced colorectal cancer risk only among subjects not concurrently randomized to estrogen therapy.[161] The authors proposed several mechanisms, some of which may be relevant to CVD or other causes of death, including the ability of estrogen therapy to increase calcium binding protein levels and induce bone mineralization, both leading to reduced systemic bioavailability of calcium.[161] This may mask an inverse association between supplemental calcium and mortality in women. These potential interactions deserve further investigation. It is also possible that the observed interactions were due to chance.

The strengths of this study include its prospective design, large sample size, and detailed information on covariates. The large sample size enabled us to study very high levels of supplemental calcium intake (e.g., ≥ 1000 mg/d, which is uncommon in men) at which adverse effects on CVD risk may occur. Repeated assessments captured long-term calcium intake, which was not always available in other studies. There are also several limitations. We used different FFQs at the baseline and follow-up dietary assessments; however, both FFQs were well-validated and asked similar questions, especially concerning the major sources of calcium intake in the United States. Although we adjusted for a variety of covariates, there may still be confounding by unmeasured confounders (e.g., access to health care).

In conclusion, we found that calcium intake generally was not associated with higher risk of mortality, however a high intake of supplemental calcium (≥ 1000 mg/d) was associated with increased all-cause and CVD mortality in men only. The potential adverse effect of high supplemental calcium consumption on the cardiovascular system warrants further study.

Tables and Figures

Table 5.1. Comparison of Questions on Each Food Frequency Questionnaire on Usual Intake of Dairy Foods, Calcium Supplements and Multivitamins, Cancer Prevention Study II Nutrition Cohort (1992 – 2012)

	1992 [1]	1999 [2]	2003 [2]
Milk	Whole milk & beverages with whole milk; 2% milk & beverages with 2% milk; Skim milk, 1% or buttermilk	Whole milk; 2% milk; Skim or 1% milk	Whole milk; 2% milk; Skim or 1% milk
Other dairy products	Cheeses and cheese spreads (regular and low-fat); Ice cream (regular and low-fat); Yogurt (regular and low-fat, including frozen); Restaurant pizza	Cheese (cottage or ricotta, and other [e.g. American, cheddar, etc.]); Ice cream (regular and non-fat/sherbet); Yogurt (plain or artificially sweetened, frozen, and other); Pizza	Cheese (cottage or ricotta, and other [e.g. American, cheddar, etc.]); Ice cream (regular and non-fat/sherbet); Yogurt (plain or artificially sweetened, frozen, and other); Pizza
Calcium supplements	Calcium or Dolomite 3. Frequency per week or per day 4. Amount in each tablet (250mg, 500mg, 600mg, or 750mg)	Calcium 3. Regular use: Yes/no 4. Amount per day (≤ 900mg [calculated as 500mg], ≥901mg [calculated as 1,000mg], unknown)	Calcium 4. Regular use: Yes/no 5. Pills per week 6. Amount in each pill (≤350mg [calculated as 250mg], ≥400mg [calculated as 500mg], unknown)
Multivitamins	Multivitamin 4. Use at least once per week yes/no 5. Type • Stress-tabs type; • Therapeutic, Theragran type; • One-a-day type, or Centrum 6. Number of tablets per day or per week	Multivitamin 4. Currently yes/no 5. Frequency per week 6. Brand (write-in)	Multivitamin 4. Currently yes/no 5. Frequency per week 6. Brand (write-in)

[1] Dairy in 1992 calculated as: all types of milk (8 ounce glass serving) + cheese and cheese spreads (2 ounce serving) + ice cream (1 ½ cup serving) + yogurt (1 cup serving) + cheese on pizza (1 ½ ounce serving).

[2] Dairy in 1999 and 2003 calculated as: all types of milk (8 ounce glass serving) + ice cream (1 ½ cup serving) + yogurt (1 cup serving) + cottage cheese (2 cup serving) + processed cheese (2 ounce serving) + hard cheese (1½ ounce serving) + cheese on pizza (1½ ounce serving).

Table 5.2. Baseline Characteristics of Participants by Categories of Supplemental Calcium, Cancer Prevention Study II Nutrition Cohort (1992-2012) [1]

	Supplemental calcium intake (mg/d)							
	Men				Women			
	0	0.1 – <500	500 – < 1,000	≥ 1,000	0	0.1 – <500	500 – < 1,000	≥ 1,000
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Age at enrollment (years)								
<60	11,450 (28.7)	4,364 (25.7)	450 (20.1)	162 (23.5)	13,918 (39.5)	7,797 (37.3)	3,663 (35.3)	2,273 (35.0)
60 - <65	12,041 (30.2)	5,008 (29.5)	648 (28.9)	188 (27.2)	9,690 (27.5)	5,670 (27.1)	2,881 (27.7)	1,780 (27.4)
65 - <70	11,047 (27.7)	4,789 (28.3)	638 (28.4)	193 (28.0)	7,230 (20.5)	4,461 (21.3)	2,383 (22.9)	1,422 (21.9)
70+	5,323 (13.4)	2,788 (16.4)	508 (22.6)	147 (21.3)	4,439 (12.6)	2,990 (14.3)	1,460 (14.1)	1,022 (15.7)
Race								
White/White-Hispanic	38,858 (97.5)	16,539 (97.6)	2,192 (97.7)	678 (98.3)	34,349 (97.4)	20,336 (97.2)	10,162 (97.8)	6,402 (98.5)
Black/Black-Hispanic	463 (1.2)	169 (1.0)	17 (0.8)	3 (0.4)	538 (1.5)	323 (1.5)	75 (0.7)	24 (0.4)
Other/Missing	540 (1.4)	241 (1.4)	35 (1.6)	9 (1.3)	390 (1.1)	259 (1.2)	150 (1.4)	71 (1.1)
Education								
Less than high school	3,151 (7.9)	1,020 (6.0)	131 (5.8)	33 (4.8)	1,765 (5.0)	923 (4.4)	345 (3.3)	151 (2.3)
High school degree	7,905 (19.8)	2,703 (15.9)	337 (15.0)	115 (16.7)	12,087 (34.3)	6,361 (30.4)	2,850 (27.4)	1,634 (25.2)

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	Supplemental calcium intake (mg/d)							
	Men				Women			
	0 N (%)	0.1 – <500 N (%)	500 – < 1,000 N (%)	≥ 1,000 N (%)	0 N (%)	0.1 – <500 N (%)	500 – < 1,000 N (%)	≥ 1,000 N (%)
Some college/trade school	9,995 (25.1)	4,299 (25.4)	553 (24.6)	163 (23.6)	10,688 (30.3)	6,779 (32.4)	3,315 (31.9)	2,210 (34.0)
College graduate	18,563 (46.6)	8,831 (52.1)	1,210 (53.9)	378 (54.8)	10,503 (29.8)	6,717 (32.1)	3,812 (36.7)	2,472 (38.0)
Physical activity (MET-hrs./wk.) [2]								
Q1	5,323 (13.4)	1,701 (10.0)	207 (9.2)	70 (10.1)	3,809 (10.8)	1,733 (8.3)	667 (6.4)	425 (6.5)
Q2	11,153 (28.0)	4,378 (25.8)	536 (23.9)	184 (26.7)	11,676 (33.1)	6,101 (29.2)	2,877 (27.7)	1,709 (26.3)
Q3	5,873 (14.7)	2,830 (16.7)	391 (17.4)	116 (16.8)	6,264 (17.8)	4,380 (20.9)	2,275 (21.9)	1,371 (21.1)
Q4	8,854 (22.2)	4,057 (23.9)	532 (23.7)	159 (23.0)	6,797 (19.3)	4,267 (20.4)	2,178 (21.0)	1,441 (22.2)
Q5	8,232 (20.7)	3,827 (22.6)	560 (25.0)	156 (22.6)	6,350 (18.0)	4,236 (20.3)	2,304 (22.2)	1,509 (23.2)
BMI (kg/m^2)								
< 18.5	124 (0.3)	82 (0.5)	12 (0.5)	8 (1.2)	509 (1.4)	336 (1.6)	219 (2.1)	209 (3.2)
18.5 - < 25	13,174 (33.0)	6,638 (39.2)	996 (44.4)	278 (40.3)	16,372 (46.4)	10,599 (50.7)	5,967 (57.4)	3,942 (60.7)
25 - < 30	19,952 (50.1)	7,980 (47.1)	975 (43.4)	308 (44.6)	11,475 (32.5)	6,598 (31.5)	2,934 (28.2)	1,670 (25.7)
≥ 30	6,017 (15.1)	1,992 (11.8)	229 (10.2)	81 (11.7)	6,266 (17.8)	3,041 (14.5)	1,145 (11.0)	595 (9.2)

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	Supplemental calcium intake (mg/d)							
	Men				Women			
	0 N (%)	0.1 – <500 N (%)	500 – < 1,000 N (%)	≥ 1,000 N (%)	0 N (%)	0.1 – <500 N (%)	500 – < 1,000 N (%)	≥ 1,000 N (%)
History of diabetes								
No	36,926 (92.6)	15,763 (93.0)	2,115 (94.3)	645 (93.5)	33,352 (94.5)	19,941 (95.3)	9,990 (96.2)	6,279 (96.6)
Yes	2,935 (7.4)	1,186 (7.0)	129 (5.7)	45 (6.5)	1,925 (5.5)	977 (4.7)	397 (3.8)	218 (3.4)
History of hypertension								
No	25,487 (63.9)	10,888 (64.2)	1,490 (66.4)	460 (66.7)	23,610 (66.9)	14,402 (68.8)	7,361 (70.9)	4,705 (72.4)
Yes	14,374 (36.1)	6,061 (35.8)	754 (33.6)	230 (33.3)	11,667 (33.1)	6,516 (31.2)	3,026 (29.1)	1,792 (27.6)
History of high cholesterol								
No	15,744 (39.5)	7,089 (41.8)	921 (41.0)	276 (40.0)	15,900 (45.1)	9,801 (46.9)	4,900 (47.2)	3,014 (46.4)
Yes	24,117 (60.5)	9,860 (58.2)	1,323 (59.0)	414 (60.0)	19,377 (54.9)	11,117 (53.1)	5,487 (52.8)	3,483 (53.6)
NSAID use								
None	18,402 (46.2)	6,135 (36.2)	788 (35.1)	258 (37.4)	16,748 (47.5)	8,414 (40.2)	4,109 (39.6)	2,535 (39.0)
1 - <15 Pills/Mo	5,840 (14.7)	2,422 (14.3)	289 (12.9)	98 (14.2)	5,858 (16.6)	3,409 (16.3)	1,543 (14.9)	869 (13.4)
15 - <30 Pills/Mo	3,875 (9.7)	2,077 (12.3)	243 (10.8)	82 (11.9)	3,014 (8.5)	2,157 (10.3)	1,055 (10.2)	593 (9.1)
30 - <60 Pills/Mo	7,052 (17.7)	3,863 (22.8)	550 (24.5)	128 (18.6)	4,341 (12.3)	3,210 (15.3)	1,760 (16.9)	1,039 (16.0)

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	Supplemental calcium intake (mg/d)							
	Men				Women			
	0 N (%)	0.1 – <500 N (%)	500 – < 1,000 N (%)	≥ 1,000 N (%)	0 N (%)	0.1 – <500 N (%)	500 – < 1,000 N (%)	≥ 1,000 N (%)
≥60 Pills/Mo	3,471 (8.7)	1,894 (11.2)	297 (13.2)	107 (15.5)	3,996 (11.3)	2,847 (13.6)	1,497 (14.4)	1,165 (17.9)
Smoking status								
Never Smoker	13,471 (33.8)	5,666 (33.4)	857 (38.2)	242 (35.1)	19,383 (54.9)	11,374 (54.4)	5,721 (55.1)	3,517 (54.1)
Current Smoker	3,849 (9.7)	1,407 (8.3)	109 (4.9)	34 (4.9)	3,469 (9.8)	1,772 (8.5)	641 (6.2)	369 (5.7)
Former Smoker	22,273 (55.9)	9,764 (57.6)	1,262 (56.2)	413 (59.9)	12,003 (34.0)	7,513 (35.9)	3,909 (37.6)	2,557 (39.4)
Alcohol consumption								
Non-Drinker	12,964 (32.5)	5,536 (32.7)	810 (36.1)	283 (41.0)	16,899 (47.9)	9,582 (45.8)	4,466 (43.0)	2,889 (44.5)
< 1 Drink/Day	15,786 (39.6)	6,773 (40.0)	865 (38.5)	224 (32.5)	13,320 (37.8)	8,339 (39.9)	4,343 (41.8)	2,668 (41.1)
≥ 1 Drink/Day	10,366 (26.0)	4,393 (25.9)	542 (24.2)	176 (25.5)	4,356 (12.3)	2,733 (13.1)	1,461 (14.1)	883 (13.6)
Hormone replacement therapy								
None					17,282 (49.0)	8,612 (41.2)	3,508 (33.8)	1,851 (28.5)
Current user					9,607 (27.2)	7,028 (33.6)	4,439 (42.7)	3,102 (47.7)
Former user					6,585 (18.7)	4,156 (19.9)	1,877 (18.1)	1,206 (18.6)

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	Supplemental calcium intake (mg/d)							
	Men				Women			
	0 N (%)	0.1 – <500 N (%)	500 – < 1,000 N (%)	≥ 1,000 N (%)	0 N (%)	0.1 – <500 N (%)	500 – < 1,000 N (%)	≥ 1,000 N (%)
Multivitamin use								
No Current Use	37,924 (95.1)	754 (4.4)	619 (27.6)	242 (35.1)	33,008 (93.6)	2,541 (12.1)	3,494 (33.6)	2,504 (38.5)
Current Use	1,218 (3.1)	16,177 (95.4)	1,609 (71.7)	442 (64.1)	1,311 (3.7)	18,290 (87.4)	6,778 (65.3)	3,919 (60.3)
Use of individual calcium supplements								
No	39,061 (98.0)	14,143 (83.4)	57 (2.5)	0 (0)	34,265 (97.1)	12,973 (62.0)	67 (0.6)	0 (0)
Yes	0 (0)	2,245 (13.2)	2,183 (97.3)	690 (100.0)	0 (0)	7,396 (35.4)	10,319 (99.3)	6,497 (100.0)
Dietary composition	Mean (SD)				Mean (SD)			
Total energy intake (kcal/day)	1,828.1 (628.5)	1,803.0 (603.3)	1,769.6 (599.5)	1,759.2 (581.5)	1,366.7 (490.1)	1,371.8 (479.0)	1,354.2 (450.8)	1,338.0 (446.8)
Red/processed meat intake (servings/wk)	6.1 (4.0)	5.5 (3.8)	5.0 (3.9)	4.8 (3.9)	4.6 (3.2)	4.1 (3.0)	3.8 (3.0)	3.6 (2.9)
Total folate intake (µg/d)	283.3 (118.9)	663.8 (219.8)	685.9 (429.5)	774.8 (684.6)	251.3 (132.3)	584.4 (249.1)	555.2 (323.0)	573.8 (420.7)
Fruit/vegetable intake (servings /d)	3.0 (1.6)	3.3 (1.6)	3.7 (1.7)	3.6 (1.7)	3.4 (1.6)	3.6 (1.7)	3.9 (1.7)	4.0 (1.8)
Whole grain intake (g/d)	60.4 (63.7)	71.2 (71.2)	85.4 (81.9)	88.8 (90.8)	52.3 (47.5)	60.1 (50.5)	63.9 (51.4)	66.1 (55.0)
Total vitamin D intake (IU/d)	179.3 (104.5)	552.2 (213.4)	523.3 (425.1)	565.9 (556.0)	155.0 (95.0)	480.1 (239.4)	421.8 (304.3)	431.3 (382.5)

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	Supplemental calcium intake (mg/d)							
	Men				Women			
	0 N (%)	0.1 – <500 N (%)	500 – < 1,000 N (%)	≥ 1,000 N (%)	0 N (%)	0.1 – <500 N (%)	500 – < 1,000 N (%)	≥ 1,000 N (%)
Dairy intake (servings/d)	1.7 (1.3)	1.8 (1.3)	1.7 (1.3)	1.6 (1.3)	1.5 (1.2)	1.7 (1.2)	1.6 (1.2)	1.6 (1.2)

Abbreviations: BMI, body mass index; MET, metabolic equivalent; NSAID, non-steroidal anti-inflammatory drug; Q, quintile; SD, standard deviation.

[1] Some percentages do not add up to 100% because of missing data or rounding.

[2] Quintiles in men: < 3.5, 3.5 – < 6, 6 – < 14, 14 – < 24.5, and ≥ 24.5; quintiles in women: < 3.5, 3.5 – < 4, 4 – <14, 14 – < 18.5, and ≥ 18.5.

Table 5.3. Associations of Supplemental Calcium with Mortality from All Causes, Cancer, and Cardiovascular Disease, Cancer Prevention Study II Nutrition Cohort (1992 - 2012) [1]

Supplemental calcium (mg/d) [2]	Men					Women				
	# of deaths	Person-years	RR	95% CI	P_{trend}	# of deaths	Person-years	RR	95% CI	P_{trend}
All-cause mortality										
0	12,103	544,192	Ref		0.18	5,639	427,737	Ref		<0.01
0.1 - < 500	10,736	401,700	1.01	0.97, 1.04		8,032	510,804	0.90	0.87, 0.94	
500 - < 1,000	1,328	49,750	1.01	0.95, 1.08		3,857	285,534	0.84	0.80, 0.88	
≥ 1,000	246	9,461	1.17	1.03, 1.33		1,245	99,439	0.93	0.87, 0.99	
Cancer mortality										
0	3,723	544,192	Ref		0.19	1,783	427,737	Ref		< 0.01
0.1 - < 500	3,175	401,700	1.05	0.99, 1.11		2,497	510,804	0.99	0.92, 1.06	
500 - < 1,000	380	49,750	1.05	0.94, 1.18		1,159	285,534	0.86	0.79, 0.94	
≥ 1,000	60	9,461	1.03	0.79, 1.33		380	99,439	0.94	0.83, 1.06	
Cardiovascular disease mortality										
0	4,129	544,192	Ref		0.39	1,760	427,737	Ref		<0.01
0.1 - < 500	3,564	401,700	0.96	0.91, 1.02		2,413	510,804	0.83	0.78, 0.90	
500 - < 1,000	418	49,750	0.91	0.82, 1.01		1,184	285,534	0.81	0.74, 0.88	
≥ 1,000	93	9,461	1.22	0.99, 1.51		355	99,439	0.84	0.74, 0.94	
Mortality from all other causes										
0	4,251	544,192	Ref		0.06	2,096	427,737	Ref		0.02
0.1 - < 500	3,997	401,700	1.01	0.96, 1.07		3,122	510,804	0.89	0.84, 0.95	
500 - < 1,000	530	49,750	1.09	0.99, 1.20		1,514	285,534	0.84	0.78, 0.91	
≥ 1,000	93	9,461	1.22	0.99, 1.51		510	99,439	0.99	0.89, 1.10	

Abbreviations: CI, confidence interval; RR, relative risk.

[1] Supplemental calcium models adjusted for age at enrollment, intakes of total energy, whole grain, red/processed meat, and total folate, smoking and alcohol drinking doses, education level, body mass index, dietary calcium intake, and hormone replacement therapy (for women).

[2] Supplemental calcium intake was cumulatively updated in 1999 and 2003.

Table 5.4. Associations of Baseline Supplemental Calcium Use with Mortality from All-Causes, Cancer, and Cardiovascular Disease, Cancer Prevention Study II Nutrition Cohort (1992 – 2012) [1]

Supplemental calcium (mg/d) [2]	Men					Women				
	# of deaths	Person-years	RR	95% CI	P_{trend}	# of deaths	Person-years	RR	95% CI	P_{trend}
All-cause mortality										
0	15,937	674,226	Ref		0.04	9,080	639,012	Ref		0.27
0.1- < 500	7,159	282,580	1.02	0.97, 1.06		5,379	377,999	0.97	0.93, 1.01	
500 -< 1,000	996	37,156	1.04	0.97, 1.11		2,590	189,117	0.97	0.92, 1.02	
≥ 1,000	321	11,140	1.14	1.02, 1.28		1,724	117,385	1.05	0.99, 1.11	
Cancer mortality										
0	4,875	674,226	Ref		0.79	2,875	639,012	Ref		0.58
0.1- < 500	2,091	282,580	0.97	0.90, 1.05		1,682	377,999	1.00	0.92, 1.08	
500 -< 1,000	293	37,156	1.06	0.93, 1.21		760	189,117	0.93	0.85, 1.02	
≥ 1,000	79	11,140	0.98	0.78, 1.23		502	117,385	1.01	0.91, 1.12	
Cardiovascular disease mortality										
0	5,350	674,226	Ref		0.40	2,757	639,012	Ref		0.60
0.1- < 500	2,426	282,580	1.05	0.97, 1.13		1,656	377,999	0.95	0.87, 1.03	
500 -< 1,000	315	37,156	0.96	0.85, 1.09		811	189,117	0.98	0.90, 1.07	
≥ 1,000	113	11,140	1.17	0.96, 1.41		488	117,385	0.96	0.86, 1.07	
Mortality from all other causes										
0	5,712	674,226	Ref		0.02	3,448	639,012	Ref		0.01
0.1- < 500	2,642	282,580	1.03	0.96, 1.11		2,041	377,999	0.96	0.89, 1.03	
500 -< 1,000	388	37,156	1.08	0.97, 1.21		1,019	189,117	0.99	0.91, 1.07	
≥ 1,000	129	11,140	1.24	1.03, 1.48		734	117,385	1.16	1.06, 1.26	

Abbreviations: CI, confidence interval; RR, relative risk.

[1] Supplemental calcium models adjusted for age at enrollment, intakes of total energy, whole grain, red/processed meat, dietary calcium, and total folate, smoking and alcohol drinking doses, education level, body mass index, and hormone replacement therapy (for women).

[2] Supplemental calcium intake was obtained from the baseline questionnaire with no cumulative updating.

Table 5.5. Associations of Supplemental Calcium with Mortality from Specific Types of Cancer and Cardiovascular Disease, Cancer Prevention Study II Nutrition Cohort (1992 - 2012) [1]

Men						Women					
Supplemental calcium mg/d [2]	# of deaths	Person-years	RR	95% CI	P_{trend}	Supplemental calcium mg/d [2]	# of deaths	Person-years	RR	95% CI	P_{trend}
Colorectal cancer						**Colorectal cancer**					
0	346	544,192	Ref		0.80	0	199	427,737	Ref		0.05
0.1 - < 500	222	401,700	0.89	0.72, 1.11		0.1 - < 500	240	510,804	1.00	0.80, 1.24	
≥ 500 [3]	41	59,211	1.15	0.81, 1.63		500 - < 1,000	94	285,534	0.76	0.58, 1.00	
						≥ 1,000	30	99,439	0.81	0.54, 1.22	
Prostate cancer						**Breast cancer**					
0	369	544,192	Ref		0.23	0	173	427,737	Ref		0.02
0.1 - < 500	381	401,700	1.16	0.97, 1.38		0.1 - < 500	283	510,804	0.96	0.77, 1.20	
≥ 500 [3]	52	59,211	1.12	0.82, 1.53		500 - < 1,000	114	285,534	0.73	0.56, 0.95	
						≥ 1,000	35	99,439	0.78	0.53, 1.14	
Lung cancer						**Lung cancer**					
0	1016	544,192	Ref		0.20	0	416	427,737	Ref		0.62
0.1 - < 500	801	401,700	1.18	1.04, 1.33		0.1 - < 500	520	510,804	1.07	0.92, 1.24	
≥ 500 [3]	88	59,211	1.03	0.81, 1.30		500 - < 1,000	260	285,534	1.06	0.89, 1.26	
						≥ 1,000	77	99,439	1.05	0.81, 1.36	
Coronary heart disease						**Coronary heart disease**					
0	2184	544,192	Ref		0.50	0	742	427,737	Ref		<0.01
0.1 - < 500	1869	401,700	0.96	0.89, 1.04		0.1 - < 500	988	510,804	0.86	0.77, 0.96	
500 - < 1,000	216	49,750	0.89	0.77, 1.03		500 - < 1,000	460	285,534	0.80	0.70, 0.91	
≥ 1,000	52	9,461	1.28	0.97, 1.70		≥ 1,000	129	99,439	0.76	0.63, 0.93	

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Men						Women					
Supplemental calcium mg/d [2]	# of deaths	Person-years	RR	95% CI	P_{trend}	Supplemental calcium mg/d [2]	# of deaths	Person-years	RR	95% CI	P_{trend}
Stroke						**Stroke**					
0	674	544,192	Ref		0.12	0	397	427,737	Ref		0.14
0.1 - < 500	600	401,700	0.92	0.80, 1.05		0.1 - < 500	606	510,804	0.86	0.74, 0.99	
≥ 500 [3]	82	59,211	0.84	0.66, 1.08		500 - < 1,000	298	285,534	0.80	0.67, 0.94	
						≥ 1,000	105	99,439	0.96	0.76, 1.21	

Abbreviations: CI, confidence interval; RR, relative risk.

[1] Models adjusted for age at enrollment, intakes of total energy, whole grain, red/processed meat, and total folate, smoking and alcohol drinking doses, education level, body mass index, dietary calcium intake, and hormone replacement therapy (for women). We additionally adjusted for colonoscopy/sigmoidoscopy in colorectal cancer models, and mammography in female breast cancer models.

[2] Supplemental calcium intake was cumulatively updated in 1999 and 2003.

[3] Results combined for 500 - <1,000 and ≥ 1,000 categories because of the small numbers of deaths.

Table 5.6. Associations of Total Calcium with Mortality from All Causes, Cancer, and Cardiovascular Disease, Cancer Prevention Study II Nutrition Cohort (1992 – 2012) [1]

Men						Women					
Quintile [2]	# of deaths	Person-years	RR	95% CI	P_{trend}	Quintile [2]	# of deaths	Person-years	RR	95% CI	P_{trend}
Primary mortality outcomes											
All-cause						**All-cause**					
Q1	4,984	200,063	Ref		0.58	Q1	4,429	260,673	Ref		< 0.01
Q2	4,829	201,393	0.97	0.93, 1.01		Q2	3,866	264,523	0.92	0.88, 0.96	
Q3	4,750	201,850	0.96	0.92, 1.00		Q3	3,582	265,962	0.87	0.83, 0.91	
Q4	4,797	201,724	0.98	0.94, 1.02		Q4	3,470	266,414	0.86	0.82, 0.90	
Q5	5,053	200,072	1.00	0.95, 1.04		Q5	3,426	265,940	0.85	0.81, 0.90	
Cancer						**Cancer**					
Q1	1,552	200,063	Ref		0.69	Q1	1,336	260,673	Ref		< 0.01
Q2	1,530	201,393	1.03	0.96, 1.11		Q2	1,241	264,523	0.98	0.90, 1.06	
Q3	1,429	201,850	0.99	0.92, 1.06		Q3	1,139	265,962	0.92	0.85, 1.00	
Q4	1,434	201,724	1.02	0.94, 1.10		Q4	1,089	266,414	0.90	0.82, 0.98	
Q5	1,393	200,072	0.99	0.92, 1.07		Q5	1,014	265,940	0.85	0.78, 0.93	
CVD						**CVD**					
Q1	1,634	200,063	Ref		0.77	Q1	1,341	260,673	Ref		< 0.01
Q2	1,598	201,393	0.96	0.89, 1.03		Q2	1,207	264,523	0.93	0.86, 1.00	
Q3	1,638	201,850	0.97	0.90, 1.04		Q3	1,076	265,962	0.85	0.78, 0.92	
Q4	1,609	201,724	0.95	0.88, 1.02		Q4	1,064	266,414	0.85	0.78, 0.93	
Q5	1,725	200,072	0.97	0.91, 1.05		Q5	1,024	265,940	0.81	0.74, 0.89	

(Table continues on the next page)

Men						Women					
Quintile [2]	# of deaths	Person-years	RR	95% CI	P_{trend}	Quintile [2]	# of deaths	Person-years	RR	95% CI	P_{trend}
Secondary mortality outcomes											
Colorectal cancer						**Colorectal cancer**					
Q1	142	200,063	Ref		0.10	Q1	170	260,673	Ref		< 0.01
Q2	137	201,393	1.04	0.82, 1.32		Q2	112	264,523	0.70	0.55, 0.90	
Q3	113	201,850	0.88	0.68, 1.14		Q3	102	265,962	0.67	0.52, 0.87	
Q4	120	201,724	0.96	0.74, 1.24		Q4	103	266,414	0.71	0.54, 0.92	
Q5	97	200,072	0.81	0.61, 1.07		Q5	76	265,940	0.56	0.41, 0.75	
Prostate cancer						**Breast cancer**					
Q1	147	200,063	Ref		0.74	Q1	125	260,673	Ref		0.03
Q2	163	201,393	1.08	0.86, 1.35		Q2	145	264,523	1.11	0.87, 1.42	
Q3	167	201,850	1.09	0.87, 1.37		Q3	126	265,962	0.95	0.73, 1.23	
Q4	169	201,724	1.11	0.88, 1.40		Q4	100	266,414	0.77	0.58, 1.01	
Q5	156	200,072	0.99	0.77, 1.26		Q5	109	265,940	0.83	0.63, 1.11	
Lung cancer						**Lung cancer**					
Q1	460	200,063	Ref		0.43	Q1	335	260,673	Ref		0.89
Q2	428	201,393	1.09	0.95, 1.25		Q2	278	264,523	1.06	0.90, 1.25	
Q3	364	201,850	1.01	0.88, 1.16		Q3	226	265,962	0.95	0.79, 1.13	
Q4	354	201,724	1.06	0.92, 1.23		Q4	236	266,414	1.09	0.91, 1.30	
Q5	299	200,072	0.95	0.81, 1.12		Q5	198	265,940	1.01	0.83, 1.22	
Coronary heart disease						**Coronary heart disease**					
Q1	886	200,063	Ref		0.34	Q1	581	260,673	Ref		< 0.01
Q2	851	201,393	0.94	0.85, 1.03		Q2	508	264,523	0.90	0.80, 1.02	
Q3	864	201,850	0.93	0.84, 1.03		Q3	444	265,962	0.82	0.72, 0.93	

(Table continues on the next page)

Men						Women					
Quintile [2]	# of deaths	Person-years	RR	95% CI	P_{trend}	Quintile [2]	# of deaths	Person-years	RR	95% CI	P_{trend}
Q4	806	201,724	0.87	0.78, 0.96		Q4	396	266,414	0.74	0.65, 0.85	
Q5	914	200,072	0.95	0.86, 1.05		Q5	390	265,940	0.73	0.64, 0.84	
Stroke						**Stroke**					
Q1	264	200,063	Ref		0.96	Q1	322	260,673	Ref		0.02
Q2	240	201,393	0.88	0.73, 1.05		Q2	277	264,523	0.85	0.72, 1.00	
Q3	278	201,850	0.99	0.83, 1.18		Q3	260	265,962	0.79	0.66, 0.93	
Q4	284	201,724	1.00	0.84, 1.19		Q4	279	266,414	0.84	0.71, 1.00	
Q5	290	200,072	0.95	0.79, 1.14		Q5	268	265,940	0.77	0.65, 0.92	

Abbreviations: CI, confidence interval; RR, relative risk.

[1] Total calcium models adjusted for age at enrollment, intakes of total energy, whole grain, red/processed meat, and total folate, smoking and alcohol drinking doses, education level, body mass index, and hormone replacement therapy (for women).

[2] Total calcium intake was cumulatively updated in 1999 and 2003. The quintile cut-off points for total calcium intake (mg/d) in 1992 were 571, 720, 884, and 1,136 for men; and 542, 729, 962, and 1,341 for women. The quintile cut-off points for cumulatively updated total calcium intake (mg/d) in 1999 were 601, 745, 906, and 1,148 for men; and 652, 890, 1,140, and 1,454 for women. The quintile cut-off points for cumulatively updated total calcium intake (mg/d) in 2003 were 630, 774, 931, and 1,162 for men; and 706, 948, 1,190, and 1,492 for women.

Table 5.7. Associations of Dietary Calcium with Mortality from All Causes, Cancer, and Cardiovascular Disease, Cancer Prevention Study II Nutrition Cohort (1992 – 2012) [1]

	Men						Women				
Quintile [2]	# of deaths	Person-years	RR	95% CI	P_{trend}	Quintile [2]	# of deaths	Person-years	RR	95% CI	P_{trend}
Primary mortality outcomes											
All-cause						**All-cause**					
Q1	5,010	200,054	Ref		0.89	Q1	4,115	262,221	Ref		0.06
Q2	4,750	201,737	0.94	0.90, 0.98		Q2	3,742	264,971	0.94	0.90, 0.98	
Q3	4,750	201,829	0.95	0.91, 0.98		Q3	3,557	265,968	0.92	0.88, 0.96	
Q4	4,862	201,333	0.97	0.93, 1.01		Q4	3,559	265,901	0.91	0.87, 0.96	
Q5	5,041	200,150	0.98	0.94, 1.02		Q5	3,800	264,452	0.95	0.90, 0.99	
Cancer						**Cancer**					
Q1	1,544	200,054	Ref		0.19	Q1	1,256	262,221	Ref		0.21
Q2	1,532	201,737	1.02	0.95, 1.10		Q2	1,239	264,971	1.04	0.96, 1.12	
Q3	1,488	201,829	1.03	0.95, 1.11		Q3	1,085	265,968	0.93	0.86, 1.01	
Q4	1,392	201,333	0.98	0.91, 1.06		Q4	1,129	265,901	0.97	0.90, 1.06	
Q5	1,382	200,150	0.97	0.90, 1.05		Q5	1,110	264,452	0.96	0.88, 1.05	
CVD						**CVD**					
Q1	1,630	200,054	Ref		0.27	Q1	1,201	262,221	Ref		0.42
Q2	1,548	201,737	0.92	0.86, 0.98		Q2	1,122	264,971	0.94	0.87, 1.02	
Q3	1,550	201,829	0.92	0.85, 0.98		Q3	1,119	265,968	0.96	0.88, 1.05	
Q4	1,724	201,333	1.00	0.94, 1.08		Q4	1,067	265,901	0.90	0.83, 0.98	
Q5	1,752	200,150	0.99	0.92, 1.06		Q5	1,203	264,452	0.96	0.88, 1.05	

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Men						Women					
Quintile [2]	# of deaths	Person-years	RR	95% CI	P_{trend}	Quintile [2]	# of deaths	Person-years	RR	95% CI	P_{trend}
Secondary mortality outcomes											
Colorectal cancer						**Colorectal cancer**					
Q1	137	200,054	Ref		0.06	Q1	129	262,221	Ref		0.16
Q2	133	201,737	1.02	0.80, 1.29		Q2	130	264,971	1.08	0.84, 1.38	
Q3	110	201,829	0.87	0.67, 1.13		Q3	103	265,968	0.88	0.67, 1.15	
Q4	136	201,333	1.09	0.85, 1.40		Q4	99	265,901	0.85	0.65, 1.11	
Q5	93	200,150	0.75	0.57, 0.99		Q5	102	264,452	0.88	0.67, 1.16	
Prostate cancer						**Breast cancer**					
Q1	155	200,054	Ref		0.13	Q1	107	262,221	Ref		0.79
Q2	172	201,737	1.04	0.83, 1.29		Q2	138	264,971	1.29	1.00, 1.66	
Q3	170	201,829	1.02	0.82, 1.28		Q3	107	265,968	1.00	0.76, 1.31	
Q4	153	201,333	0.91	0.72, 1.14		Q4	134	265,901	1.23	0.94, 1.60	
Q5	152	200,150	0.88	0.70, 1.12		Q5	119	264,452	1.09	0.83, 1.44	
Lung cancer						**Lung cancer**					
Q1	459	200,054	Ref		0.59	Q1	349	262,221	Ref		0.72
Q2	416	201,737	1.04	0.91, 1.19		Q2	273	264,971	0.96	0.82, 1.13	
Q3	364	201,829	1.01	0.88, 1.16		Q3	221	265,968	0.87	0.73, 1.04	
Q4	343	201,333	1.01	0.88, 1.17		Q4	215	265,901	0.89	0.75, 1.07	
Q5	323	200,150	0.98	0.84, 1.13		Q5	215	264,452	0.98	0.82, 1.17	
Coronary heart disease						**Coronary heart disease**					
Q1	883	200,054	Ref		0.93	Q1	522	262,221	Ref		0.04
Q2	810	201,737	0.89	0.81, 0.98		Q2	452	264,971	0.88	0.77, 1.00	
Q3	819	201,829	0.89	0.81, 0.98		Q3	459	265,968	0.91	0.80, 1.03	

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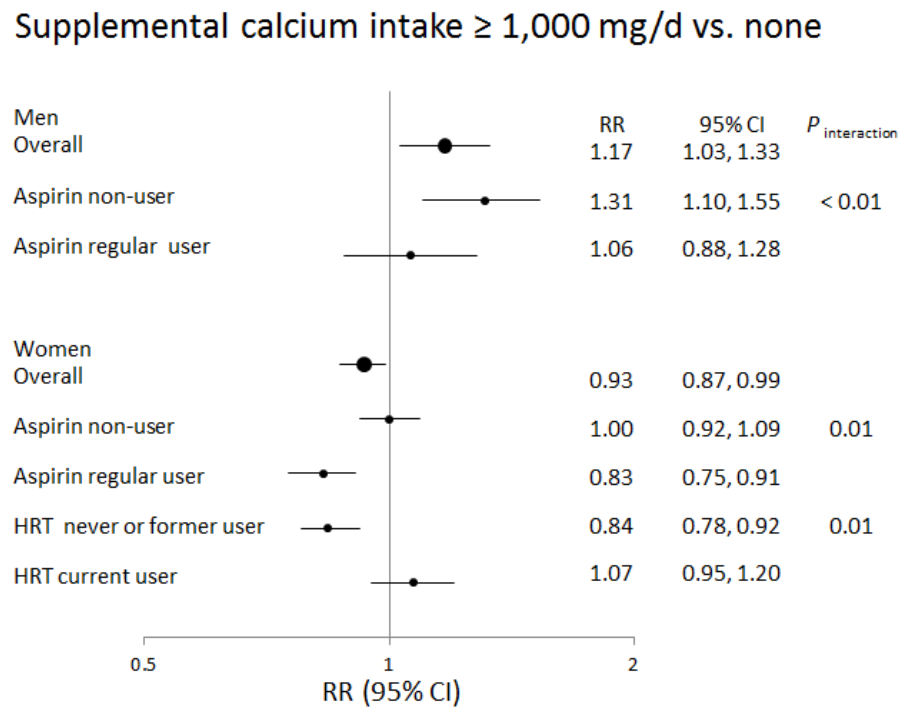
	Men					Women					
Quintile [2]	# of deaths	Person-years	RR	95% CI	P_{trend}	Quintile [2]	# of deaths	Person-years	RR	95% CI	P_{trend}
Q4	896	201,333	0.97	0.88, 1.06		Q4	416	265,901	0.82	0.71, 0.93	
Q5	913	200,150	0.95	0.86, 1.05		Q5	470	264,452	0.87	0.76, 1.00	
Stroke						**Stroke**					
Q1	263	200,054	Ref		0.15	Q1	283	262,221	Ref		0.62
Q2	235	201,737	0.84	0.70, 1.00		Q2	273	264,971	0.94	0.79, 1.11	
Q3	246	201,829	0.88	0.73, 1.05		Q3	281	265,968	0.98	0.83, 1.16	
Q4	304	201,333	1.05	0.89, 1.25		Q4	269	265,901	0.90	0.76, 1.08	
Q5	308	200,150	1.03	0.87, 1.23		Q5	300	264,452	0.95	0.80, 1.13	

Abbreviations: CI, confidence interval; RR, relative risk.

[1] Dietary calcium models adjusted for age at enrollment, intakes of total energy, whole grain, red/processed meat, supplemental calcium, and total folate, smoking and alcohol drinking doses, education level, body mass index, and hormone replacement therapy (for women).

[2] Dietary calcium intake was cumulatively updated in 1999 and 2003. The quintile cut-off points for dietary calcium intake (mg/d) in 1992 were 538, 672, 816, and 1,028 for men; and 465, 592, 730, and 930 for women. The quintile cut-off points for cumulatively updated dietary calcium intake (mg/d) in 1999 were 552, 672, 795, and 981 for men; and 506, 626, 751, and 932 for women. The quintile cut-off points for cumulatively updated dietary calcium intake (mg/d) in 2003 were 572, 688, 806, and 983 for men; and 538, 657, 778, and 952 for women.

Figure 1. Supplemental calcium and all-cause mortality in the Cancer Prevention Study II Nutrition Cohort (1992-2012), stratified by use of aspirin and hormone replacement therapy (for women only).



Abbreviations for Figure 1: CI, confidence interval; HRT, hormone replacement therapy; RR, relative risk.

CONCLUSIONS AND PUBLIC HEALTH RELEVANCE

For this dissertation I investigated the role of calcium consumption in relation to biomarkers of risk for colorectal cancer and other chronic diseases, survival from colorectal cancer, and risk for all-cause and cause-specific mortality. My investigations entailed a full-scale randomized clinical trial and a prospective cohort study.

In the first study, using data from a large, randomized clinical trial, we tested the effect of calcium supplementation over a 4-month treatment period on plasma biomarkers of inflammation, oxidative stress, and gut permeability among patients with previous colorectal adenoma, but did not observe appreciable effects of either dose of calcium (1 g/d or 2 g/d) on these biomarkers, overall or stratified by major colorectal cancer risk factors. As a secondary analysis, we found that men or those with greater adiposity may have greater gut permeability, and that markers of gut permeability and systemic inflammation may be directly associated with one another. These findings from the secondary analysis may provide important information for future epidemiological studies for evaluating the role of gut permeability biomarkers in the etiology of cancer and other diseases, including which participant characteristics and risk factors should be considered as potential confounders or effect modifiers.

In the second study, we evaluated the associations of pre- and post-diagnosis intakes of calcium, vitamin D, and dairy products with colorectal cancer-specific and overall survival among colorectal cancer survivors. We found that post-diagnosis higher intakes of total calcium or milk may be associated with lower overall survival, and higher intakes of total calcium may also be associated with lower colorectal cancer-specific survival, but pre-diagnosis intakes of calcium, vitamin D, or dairy products were not associated survival. Our findings, if confirmed in future studies (especially in randomized clinical trials), may inform the development of dietary guidelines specifically for colorectal cancer patients, and benefit the more than 3.5 million

colorectal cancer survivors worldwide who may be actively seeking diet and lifestyle changes to improve their prognosis.

In the third study, among a large cohort of participants without a history of cancer or CVD at baseline, over 17.5 years of follow-up, we observed that men taking ≥ 1,000 mg/d of supplemental calcium were at higher risk of all-cause mortality whereas women were at lower risk. The results were also suggestive of higher risk of mortality from CVD among men. These findings in men are consistent with the previously reported potential direct associations of supplemental calcium with CVD events in several large prospective cohorts and found in secondary analyses from clinical trials (the latter predominantly among women), and warrants further investigation. In contrast to the findings for supplemental calcium, total or dietary calcium was not associated with higher mortality among men and women. Overall, our findings contribute to building a better understanding of whether calcium consumption, on average, would be of public health benefit. Furthermore, our findings may contribute to the eventual development of personalized recommendations for calcium intake in clinical and public health practice.

FUTURE DIRECTIONS

In my first dissertation project, we found no effect of calcium on circulating biomarkers of inflammation, oxidative stress, and gut permeability. Since the blood samples were originally collected in the 1990s, we cannot rule out the possibility that the blood samples may have deteriorated over the years, although we found no evidence to support this possibility. I propose to investigate these effects again in another large, full-scale, randomized clinical trial, with more recently collected blood samples. I also propose to evaluate colon tissue biomarkers of inflammation, oxidative stress, and gut permeability that could more directly reflect the potential

role of calcium in preventing the colonic toxicity of bile and fatty acids to the gut mucosa. Also, because the parent trial for the first dissertation study has available data on colon tissue proliferation at baseline and follow-up, I also propose to evaluate whether there are correlations of tissue proliferation with circulating biomarkers of inflammation, oxidative stress, and gut permeability. If data on colorectal adenoma recurrence become available in this or future studies, it would also be of interest to evaluate whether these circulating or tissue biomarkers of risk for colorectal carcinogenesis are associated with colorectal adenoma recurrence.

In my second dissertation project, we observed that higher post-diagnosis intakes of total calcium and milk were associated with lower mortality among colorectal cancer survivors. I propose three additional analyses, if the data become available. The first analysis is to evaluate whether post-diagnosis intakes of total calcium and milk are also associated with less colorectal cancer recurrence (i.e., more favorable recurrence-free survival), which has not been evaluated previously. The second analysis is to evaluate adverse treatment effects as potential confounders in the diet-colorectal cancer survival association, because it is possible that those with stronger treatment side effects (such as nausea) consume lower levels of calcium or dairy products, and they may also be at higher risk of mortality after diagnosis. The third analysis is to evaluate the diet-colorectal cancer survival associations according to molecular phenotypes of cancer, such as microsatellite instability (MSI) status or *KRAS* mutation, because colorectal cancer is not a single disease entity, and the impact of diet on survival outcomes could be different according to the molecular characteristics of the tumor.

In my third dissertation project, we reported that a high level of supplemental calcium use was associated with higher mortality in men, but lower mortality in women. I propose to evaluate in a future study whether the heterogeneity by sex may be partially due to different confounding patterns among men and women. Men taking a high level of supplemental calcium may have indications such as certain medical histories, and thus may be at a higher risk of mortality; for

women, because the benefits of calcium in both health outcomes are well-recognized, those with sufficient supplemental calcium use may be more health conscious or have better health care, and could be at lower risk of mortality. A future study with detailed information on these potential confounders is warranted to clarify whether confounding contributes to the heterogeneity by sex. Also, it has been hypothesized that the adverse effect of supplemental calcium on CVD could be due to a sudden change of serum calcium levels, and an adverse effect was observed only in men because women generally had been taking calcium supplements long before the study began, and the serum calcium level was sustained from before the study baseline. I propose to evaluate the association of supplemental calcium with mortality stratified by the duration of supplemental calcium use, if the data on duration of use are available in a future study. In addition, in my third there may have been time-varying confounding by the incidence of chronic diseases, if calcium intake at an earlier time point is associated with risk of certain chronic diseases, which could subsequently affect one's calcium intake at a later time point, and those chronic diseases could also be associated with mortality outcomes. I propose to explore this issue using G-computation to account for potential time-varying confounding from the development of chronic diseases during the follow-up period.

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