# Calcium and Magnesium in Relation to Colorectal Neoplasia, Lipid Profile and Uric Acid

By

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ASR | Age standardized rate |
| ASU | Annual study update |
| ATP | Adenosine triphosphate |
| BMI | Body mass index |
| BQ | Baseline questionnaire |
| Ca:Mg | Calcium: magnesium |
| CaD | Calcium plus vitamin D |
| CaSR | Calcium-sensing receptor |
| CI | Confidence interval |
| CRC | Colorectal cancer |
| CRP | C-reactive protein |
| CSFII | Continuing survey of food intakes by individuals |
| CV | Clinic visit |
| DHQ | Dietary history questionnaire |
| DM | Diabetes mellitus |
| DNA | Deoxyribonucleic acid |
| DQX | Dietary questionnaire |
| DRI | Dietary reference intake |
| DRP | Death review process |
| FSG | Flexible 60-cm sigmoidoscopy |
| HDL-c | High-density lipoprotein cholesterol |
| HMG-CoA | 2-hydroxy-3-methyl-glutaryl-coenzyme A |
| HR | Hazards ratios |
| IGF-1 | Insulin-like growth factor 1 |

| | |
|---|---|
| IU | International unit |
| *KCNJ1* | Potassium channel, inwardly rectifying subfamily J, member 1 |
| LCAT | Lecithin cholesterol acyltransferase |
| LDL-c | Low-density lipoprotein cholesterol |
| LPL | Lipoprotein lipase |
| MRA | Medical record abstraction |
| NDI | National death index |
| NDS | Nutrient Data System |
| NHANES | National Health and Nutrition Examination Survey |
| NSAIDs | Non-steroidal anti-inflammatory drugs |
| OR | Odds ratio |
| PLCO | Prostate, Lung, Colorectal and Ovarian |
| PPCCT | Personalized Prevention of Colorectal Cancer Trial |
| SCU | Study of Colonoscopy Utilization |
| *SLC2A9* | Solute carrier family 2, member 9 |
| *SLC12A1* | Solute carrier family 12, member 1 |
| SRSR | Survey Research Shared Resource |
| $T_0$ | Entry year |
| $T_3$ | The 3-year point |
| $T_5$ | The 5-year point |
| TCPS | Tennessee Colorectal Polyp Study |
| TIARS | Tennessee-Indiana Metachronous adenoma Study |
| *TRPM7* | Transient receptor potential melastatin 7 |
| U.S. | United States |
| USDA | United States department of agriculture |

| | |
|---|---|
| VLDL | Very low-density lipoprotein |
| WHI | Women’s Health Initiative |
| $1,25(OH)_2D3$ | 1,25-dihydroxyvitamin D3 |

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# Chapter 1. Introduction

## A. Background and significance

Colorectal cancer (CRC) is the third most common incident cancer and the fifth leading cancer killer in 2012 in the world [1]. In the Unites States (U.S.), the estimated age-standardized rate (ASR) of CRC incidence is more than twice of that in the world (42.4 per 100,000 based on data from 2008-2012 vs 17.2 per 100,000 for both sexes) [1,2], despite the slightly decreasing trend over time [3]. Therefore, understanding the etiology of CRC and developing effective preventive strategies are still the most attractive public health goals.

CRC arises from the malignant transformation of adenomas and polyps [4]. The development of CRC is complex and multi-factorial. Both external factors and/or inherited genetic factors play an essential role in the initiation and progression of the adenoma-carcinoma sequence [5]. Epidemiological studies have reported inverse [6–14] as well as null associations [15,16] between calcium intake and the risk of colorectal neoplasia. Although inconsistent, calcium, one of nutrients with chemopreventive effects, was found to reduce 20-30% of risk for colorectal adenoma [17–20] and cancer [21,22]. Clinical trials also found that calcium supplementation reduced about 20% risk of metachronous colorectal adenoma [23–25]. In addition, recent studies indicate that calcium: magnesium (Ca:Mg) intake ratio modified the associations between intakes of calcium and magnesium and risk of gastrointestinal neoplasia [17,26,27], and total, cardiovascular disease and cancer mortalities [28]. These studies also indicated that a ratio between 1.7 and 2.6 is needed for calcium intake to be protective against risk of colorectal neoplasia,

and total, cardiovascular disease and cancer mortalities [17,26–28]. It is possible that lack of consideration in Ca:Mg balance may explain some of the inconsistency in previous studies on calcium intake.

The removal of polyps within an endoscopy screening is the most effective prevention strategy to reduce the morbidity and mortality of CRC [29]. However, recent evidence suggested that colonoscopy is more related to the reduction of left-side colon deaths, but not right-side [30–32]. Furthermore, annual recurrence rates in patients with small and advanced adenomas were as high as 19.3% and 22.9%, respectively [33]. Therefore, additional preventive strategies are necessary.

Studies have shown that individuals with type 2 diabetes [34–37] and metabolic syndrome [38–40] have an elevated risk of CRC . Magnesium deficiency is a contributor to age-related diseases including cancer and cardiovascular disease. [41–44]. Calcium and magnesium may share the same homeostatic regulation system [45] primarily through absorption in the intestine and reabsorption in the kidneys to maintain the balance of Ca:Mg [45,46]. Nevertheless, calcium and magnesium compete with each other in (re)absorption and transport [47–51]. Experimental evidence in animals showed that calcium-adequate and magnesium-deficient diets may increase heart lipid peroxidation, plasma levels of triglycerides, and inflammatory markers; whereas both calcium-deficient and magnesium-deficient diets were associated with significant reductions in inflammatory markers, lipid peroxidation and a normal plasma triglyceride level [52,53]. However, no previous human study has examined the potential modifying effect of the

Ca:Mg intake ratio in relation to the lipid panel and uric acid, which may have a role in the development of colorectal neoplasia. This is supported by findings from recent studies which indicated that Ca:Mg intake ratio modified the associations between intakes of calcium and magnesium and risk of total, cardiovascular disease and cancer mortalities [28].

**B. Study objectives**

This thesis is composed of three projects and has three well-connected purposes:

Project A. Calcium: magnesium intake ratio and colorectal carcinogenesis, results from the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial.

1A. To determine the modifying effect of Ca:Mg intake ratio on the association between calcium intake and the risk of colorectal neoplasia.

1B. To measure the association between calcium intake and risks of incident adenoma, metachronous adenoma, and CRC.

1C. To assess whether calcium intake provides additional protection against incident colorectal neoplasia in individuals receiving endoscopic screenings

Project B. Ca:Mg Ratio and Lipid Profile, a Randomized Clinical Trial.

1A. To examine the effect of magnesium supplementation may have in the change of lipid biomarkers in the Personalized Prevention of CRC Trial (PPCCT)

1B. To investigate whether reducing the Ca:Mg intake ratio under 2.6 among participants with high ratios by magnesium supplementation changes the serum lipid level in the PPCCT.

Project C. Ca:Mg Ratio and Uric acid, a Randomized Clinical Trial.

1A. To examine whether the magnesium supplementation may have a role in uric acid metabolism

1B. To investigate whether reducing the Ca:Mg intake ratio under 2.6 among participants with high ratios by magnesium supplementation changes the serum uric acid level in the PPCCT

**C. Organization of the thesis**

This thesis is divided into five chapters. *Chapter 1* is an overall introduction of the studies including the introduction of background and significance of the studies, the objectives of the thesis and the structure of the thesis. *Chapter 2* reviews the topic of main exposures as well as outcomes in details. *Chapter 3* presents the research methods applied in studies including descriptions of populations, the data collection, power analyses, and the statistical analysis. Chapter 4 is a result section. Chapter 5 discusses and summaries key findings, significance and implications of the study results.

# Chapter 2. Literature review

## A. Colorectal neoplasia

### 1. The worldwide and United States burden

CRC is the third most common incident cancer with an estimated worldwide age-standardized rate (ASR) of 17.2 per 100,000 for both sexes in 2012 [1]. Men usually have slightly higher incidence rates than women (overall sex ratio 1.44:1) [1]. Based on cancer statistics from the Surveillance, Epidemiology, and End results program, CRC incidence rates have great geographical variations [54]. The majority (55%) of the cases occur in more developed regions [54]. Australia/New Zealand has the highest estimated incidence rates with ASRs of 44.8 and 32.2 per 100,000 in men and women, respectively. On the other hand, the lowest incidence occurs in Western Africa (ASR: 4.5 and 3.8 per 100,000 for men and women respectively) [54]. However, global trends are changing over time: certain countries such as Japan and Korea with historically low incidence are currently increasing; while historically high-risk countries are variably increasing (Finland, Norway), remaining steady (in most Western and Northern European countries) or decreasing (the U.S.) [3].

The worldwide estimated age-standardized mortality rate is 8.5 per 100,000 for both sexes in 2012, which ranked CRC as the fifth leading cancer related death in the world [1]. There is less geographical variation in mortality rates; however, more deaths (52%) happen in the less developed regions of the world [54]. The estimated mortality rates are the highest in Central and Eastern Europe (ASR: 20.3 per 100,000 for men, 11.7 per

100,000 for women), and the lowest in Western Africa (ASR: 3.5 and 3.0 for men and women per 100,000, respectively) [54].

CRC is the third most common incident cancer in the U.S. [55], with an incidence rate of 43.7 per 100,000 men and women per year based on 2007-2011 statistics [56]. CRC is also the third leading cause of cancer-related death [55] with 15.9 deaths per 100,000 for both sexes per year [56]. It appears that there is an average 3% reduction per year for CRC incidence (**Figure 2.1**) and mortality (**Figure 2.2**) rates from the years 2002 to 2011 [57].

CRC incidence and mortality rates vary widely by race and ethnicity within the U.S. [55]. Generally, both incidence and mortality rates are highest in blacks and lowest in Asians/Pacific Islanders. Based on data from 2006 to 2010, incidence rates in blacks are nearly 50% higher than those in Asians/Pacific Islanders; death rates in blacks (29.4 per 100,000 population) are almost twice as high as those in Asians/Pacific Islanders (13.1) [55].

The stage of CRC is determined by “size and extent of the primary tumor, absence or presence of regional lymph node involvement, and absence or presence of distant metastases” [58]. The stage of cancer at diagnosis is essential in determining the type of therapy and estimating prognosis outcome [3]. The earlier CRC is discovered and treated, the better survival rates appear. The five-year survival rates are 90.3%, 70.4% and 12.5% for local, regional and distant stages, respectively [55]. Forty percent of patients with CRC

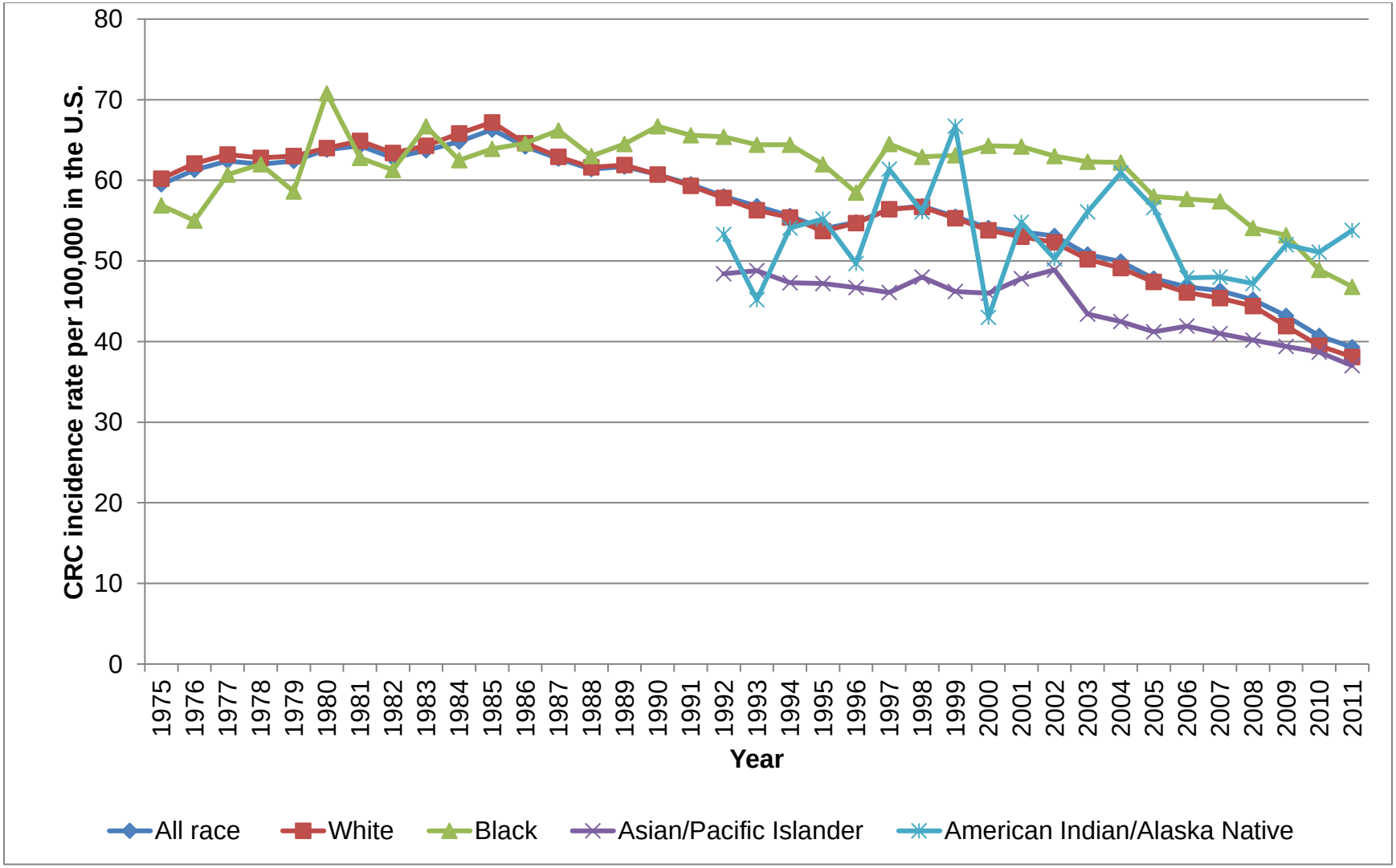


**Figure 2.1 The age-standardized CRC incidence rate by ethnicities in the U.S.**

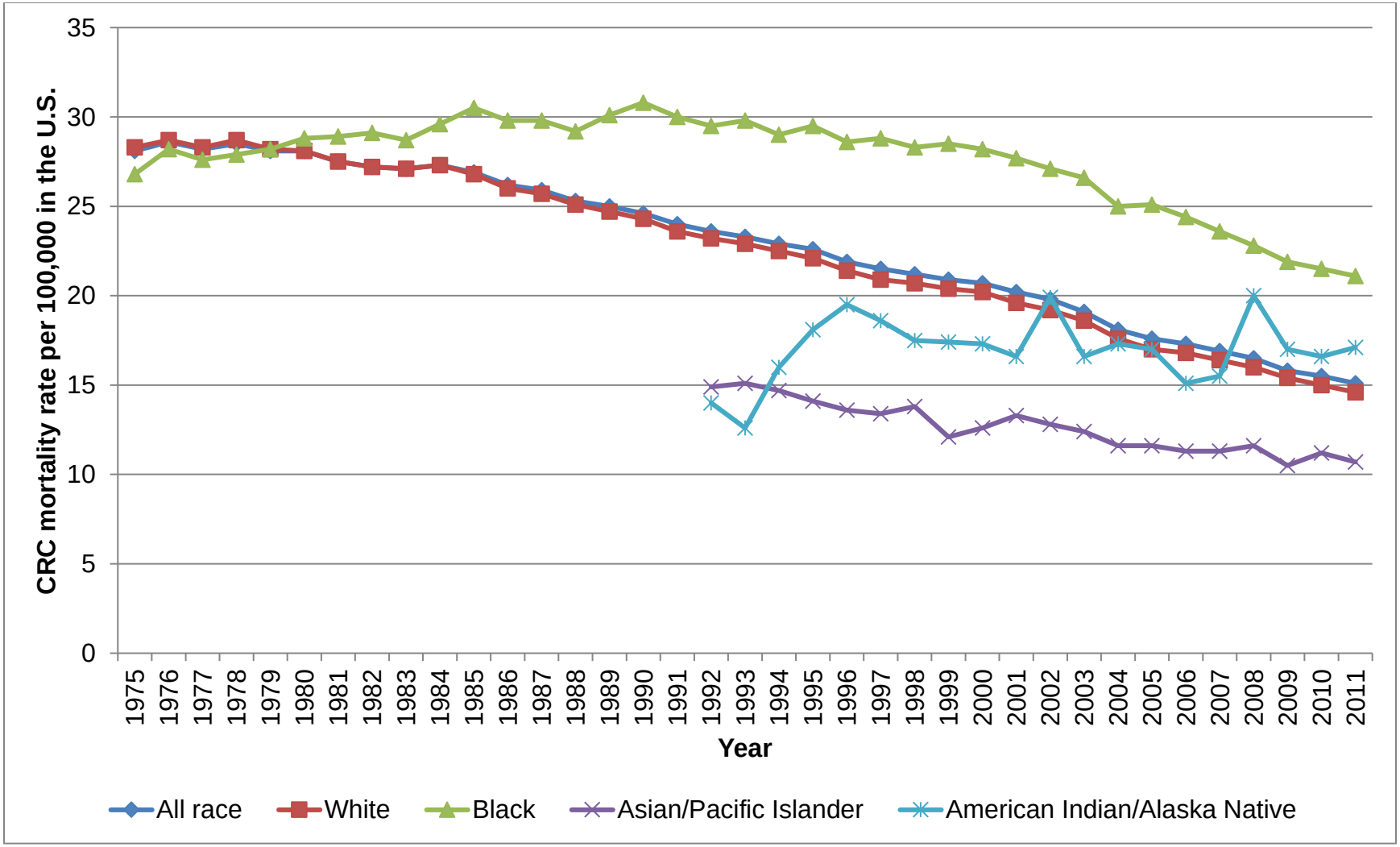


**Figure 2.2 The age-standardized CRC mortality rate by ethnicities in the U.S.**

are diagnosed with localized disease and 36% and 20% of CRC s are diagnosed with regional and distant disease, respectively [55]. In an early stage, CRC is usually asymptomatic while symptoms such as a change in bowel habits, rectal bleeding, and blood in stool may appear in a later stage [3]. Both the absence of symptoms until later stages and the availability/use of screening affect the stage of diagnosis.

CRC is most commonly diagnosed in the proximal colon (42%) followed by the rectum (28%) and distal colon (23%) [55]. The etiologies and carcinogenesis of CRC varies substantially by site of diagnosis [55,59–61]. The frequency of CRC by subsites varies with age and sex. In the population with age over 65 years, women have higher rates of CRC incidence and mortality, compared with men [62]. Women, especially aged 80 years and older, have a higher proportion of proximal colon cancer, while men are more likely to be at risk of rectal cancer [63–65]. It was hypothesized that oral contraceptive and hormone related-factors may explain potential differences in CRC risk by subsite and sex [63,66]. However, the mechanism of sex differences in subsite-specific CRC has not been fully understood.

## 2. The development of colorectal neoplasia

### 2.1 Anatomic features and physical function of the colon and rectum

An integral part of the digestive system, the colon is the first part of the large bowel, is connected to the small bowel at the lower right abdomen and is about 5 feet long [67]. The four sections of the colon include ascending, transverse, descending, and sigmoid regions. The colon absorbs mineral nutrients and water from the food passed through

this tube, and retains the waste matter which is eventually expelled it through the rectum and anus, which is the end of the large intestine that is about 6-8 inches long [67].

The wall of colon and rectum is made up of several layers. From the inner to the outer, its layers include mucosa, submucosa, thick muscle layers, subserosa and serosa (only for colon but not for rectum) [68]. The mucosa is the inner lining of colon and rectum and contains three layers (epithelium, connective tissue, and thin muscle layer). The submucosa is a layer of fibrous tissue under the mucosa [68]. A thick muscle layer beneath submucosa is vital in expelling fecal matter [68].

### 2.2 The natural history of adenoma-carcinoma sequence

A polyp is a small cluster of nonmalignant cells and can form in the lining of colon or rectum [69]. Adenomas and hyperplastic polyps are two broad types of polyps that may develop. The colorectal adenoma is a benign glandular tumor of the colon and rectum [70,71]. Previous studies have identified the potential precursor role of adenomas in CRC development, thus a hypothesis of adenoma to carcinoma sequence was first reported in 1951 [72]. Although it is still debatable, geographical, pathological as well as epidemiological evidence indicate the possibility of the adenoma-carcinoma sequence. The adenoma prevalence in geographical areas was correlated with CRC incidence in those same regions [73]. The evidence of reduction in CRC incidence after endoscopic removal of adenomas supports the theory of the adenoma-cancer sequence because removal of adenomas by polypectomy has been shown to reduce the incidence and mortality of CRC [74,75]. Moreover, colorectal carcinomas were observed to arise at the

site of polyps left *in situ* and [70]. In addition, there is evidence of a temporal relationship between adenoma and CRC development: the average age of diagnosis with an adenoma is, on average, about 4 or 5 years younger than the average age of cancer diagnosis [70]. The time length of the adenoma-carcinoma sequence varies among individuals [70]. The whole period may range from 10 to 15 years [67].

The removal of polyps within an endoscopy screening is the most effective prevention strategy to reduce the morbidity and mortality of CRC [29]. However, polypectomy has been shown to be ineffective in inhibiting the progression of CRC carcinogenesis in around 24% of adenoma cases [76]. Recent evidence also suggests that colonoscopy is more effective in reduction of colorectal cancer deaths associated with the distal colon, but not proximal colon [30–32].

Adenomas are very common and the prevalence varies from 7.4 to 52.5% [77]. A meta-analysis of 18 studies reported that the pooled prevalence of adenomas was 30.2% in an average at-risk adult population [78]. Approximately one third to one half of people will develop one or more adenomas in their lifetime [79]. However, the risk of transformation from adenoma to carcinoma in an individual is low. Colonoscopy screening is used to locate and remove adenomas. Follow-up studies among participants with negative colonoscopy findings at baseline suggested the incidence rate of subsequent CRC within 10 years was 0.006% [80] while the CRC incidence rate after four years of follow up increased to around 0.6% for participants with advanced adenoma at the baseline [4].

Even though the malignancy rate for an adenoma is low, adenomas, compared with hyperplastic polyps, are most likely to develop into CRC [67].

The characteristics of adenomas are important predictors for subsequent carcinogenesis. Adenoma size was one of main predictors in the progression to malignancy [70,71]. Recent reviews indicated that the proportion of malignancy for adenomas under 1 cm is around 1.3%, the malignant potential for adenomas with a diameter of 1-2 cm increases to 9.7%, and nearly one third to half of large-size adenomas with a diameter over 2 cm develop to invasive cancers [70]. In addition, villous adenoma was more likely to progress to carcinoma compared to tubular adenoma [70,71]. Other known factors that predisposed to CRC were adenomas with advanced features, familial polyposis and ulcerative colitis [70]. Advanced features include one of the following: >25% villous histology, size larger than 1 cm, and/or presence of high-grade dysplasia [81,82]. The coexistence of two or more adenomas diagnosed at the same time or within 6 months is defined as synchronous adenomas [83]. The incidence of advanced adenomas is higher in individuals who have synchronous adenomas [84]. The U.S. population undergoing endoscopy screening has a prevalence of 3.4 to 7.6% for advanced adenomas [4]. Sessile serrated polyps are saw-toothed and differ from traditional adenomas [4]. The accurate prevalence for sessile serrated polyps is unknown but current best estimate was approximately 6 to 12% [4]. Metachronous adenomas are adenomas detected half to one year after the previous polypectomy [83]. Thus, it is possible that individuals with synchronous, large, advanced adenomas and sessile serrated polyps are at a higher risk of subsequent metachronous and advanced

adenomas as well as CRC [4]. A cohort study based on private practice settings reported that the number of polyps at baseline was the only significant predictor for metachronous adenoma [85]. Additionally, another cohort study in the Netherlands has reported that three or more adenomas identified at initial colonoscopy was a significant risk factor for further lesions at subsequent surveillance [81].

CRC includes malignancies originating from the epithelial cells of the gastrointestinal tract, which includes the colon, rectum and appendix [82]. The carcinogenesis progress is usually accumulated defects in tumor suppressor genes, oncogenes, and deoxyribonucleic acid (DNA) repair genes, which control cell growth, division, and apoptosis [86]. However, the majority of genetic abnormalities that is involved in the formation of carcinoma is believed to start with somatic genetic mutations rather than heritable mutations [3]. The genetic abnormalities can be caused by endogenous factors such as hormones and cellular nutrient metabolism or exogenous factors such as radiations, toxins and chemicals [3]. Genetic components such as aforementioned accumulated defects may influence an individual's propensity to develop polyps or CRC. Lifestyle or environmental factors could also initiate and promote the formation of polyps and subsequent CRC [87].

## 3. Risk factors for colorectal neoplasia

There are several unmodifiable risk factors for colorectal neoplasia such as age over 50 years [55], male gender, a family history of CRC including multiple first-degree relatives with CRC or relatives with CRC diagnosed at a young age, and personal history of

chronic inflammatory bowel disease [84]. A large number of studies have investigated modifiable risk factors associated with the development of and/or death due to CRC. Although it is inconsistent, behavioral factors which seem to decrease risk include being physically active, eating healthy, keeping an optimal body mass index (18.5 to 25), abstinence from smoking, no to light alcohol consumption, hormonal replacement therapy for postmenopausal women, regular endoscopy screening to remove polyps, and use of non- steroidal anti-inflammatory drugs (NSAIDs) [67]. Oral intake of NSAIDs is considered to be one of the most promising prevention strategies for colorectal carcinogenesis chemoprevention [88]. However, the long-term use of NSAIDs also carries risks for side effects.

Over the past few decades, a large number of studies have investigated the role diet has on the risk of CRC. Nutrients such as fiber [89], vegetables [90], vitamin C and E [91], carotenoids and other antioxidants [90,92–94] have been extensively studied and the results are inconsistent. Recent large epidemiologic studies and/or clinical trials regarding the chemopreventive effects of these nutrients did not provide further evidence to support the potential benefits of these nutrients. Contrary to the original hypothesis, folic acid supplementation appeared to increase rather than reduce the risk of colorectal neoplasia [95]. Similarly, although debatable, calcium alone or along with vitamin D probably protects against cancer [96–99].

## 4. Calcium and carcinogenesis

### 4.1 Calcium intake and ionized calcium

Calcium is a vital mineral in human metabolism and can be obtained from the diet [100]. Ionized calcium balance is a comprehensive function of oral intake, intestinal absorption, renal reabsorption and skeletal modeling [101]. Ionized calcium is vital for cellular functions and initiation of numerous physiological metabolic pathways [102]. Intracellular calcium functions as a secondary messenger in pathways that involve secretion of many hormones and neurotransmitters [102].

### 4.2 Mechanisms of calcium in relation to cancer

The homeostasis of cancer is regulated by molecular-calcium pathways including: active energy-dependent calcium transporters, calcium-permeable ion channels, calcium-binding and storage proteins, and calcium-dependent effectors [103]. Despite inconsistencies, calcium may also function by reducing cell proliferation, stimulating differentiation, inducing apoptosis and regulating the cell-cycle of normal and tumor colorectal cells [104–107]. Therefore, aberrant calcium signaling may be associated with uncontrolled proliferation, unregulated apoptosis, and metastasis of cancer cells. Although it may be assertive to state that aberrant calcium signaling is a cause of molecular and tissue homeostasis change and subsequent carcinogenesis; it would be better to consider aberrant calcium signaling as a dynamic consequence of genome, epigenetics, environmental factors, adaptation and compensation that triggers cancer initiation or progression[103]. In other words, mutations, over/under expression,

regulations of calcium handling toolkits of proteins are potential causes of aberrant calcium signaling in cancer [103].

Newmark *et al.* first reported that calcium may have a chemopreventive effect on CRC in 1984 [108]. Calcium is known to bind secondary bile acids and ionized fatty acids in the colon lumen to form insoluble calcium soaps, preventing bile acids and fatty acids from damaging the mucosa of the intestinal lumen by inhibiting their proliferative effects [108]. Several studies [108–110] have supported that dietary calcium may decrease cytotoxicity, nitroso compounds, and lipoperoxidation in feces of dimethylhydrazine-initiated rats and human volunteers, thereby subsequently reducing CRC risk attributed to processed meat intake.

### 4.3 Epidemiological studies on calcium and colorectal neoplasia

A few studies have reported a significant protective effect of calcium on colorectal neoplasia [18,110,11,8] while a few studies have not detected such beneficial effect [125,126]. A pooled-analysis of 10 cohort studies suggested that the highest quintile of total calcium consumption from both dietary and supplemental sources may confer a 14% reduction of risk in CRC versus the lowest quintile, with the association only being significant among people with high intake of vitamin D [22]. Epidemiologic studies found high calcium consumption was associated with approximate 20-30% reduction in risk of colorectal adenoma [17–20], metachronous colorectal adenoma [23–25], and overall CRC risk [21,22]. The protective effect of calcium supplementation may extend up to 5 years after cessation of

active treatment [127]. **Figure 2.3** is a hypothesized diagram of calcium intake in relation to adenoma-carcinoma sequence.

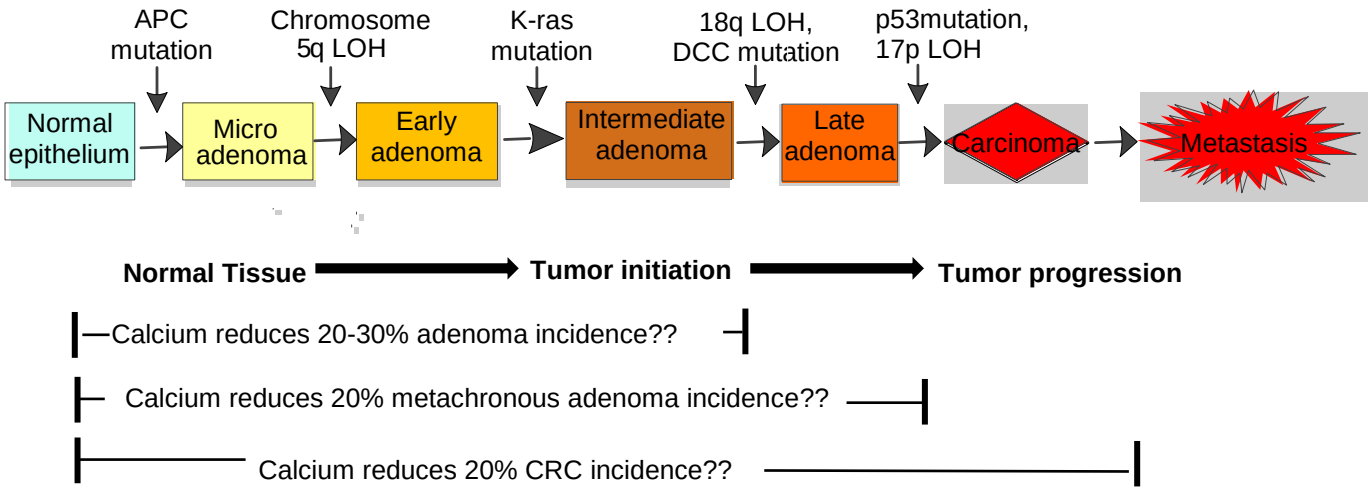


**Figure 2.3 Calcium intake on adenoma-carcinoma sequence derived from Morán A et al. 2010**

Consistent with the results from these epidemiologic studies, two large intervention trials observed that calcium supplementation moderately reduced the risk of metachronous colorectal adenoma [23,24], while one trial found that this protective effect may only be present in those with a high blood level of vitamin D [25]. Baron *et al.* [127] found in the observational phase of a clinical trial that the protective effect of calcium supplementation on metachronous colorectal adenoma rate extends up to 5 years after cessation of active treatment. However, results from a very recent large-scale randomized clinical trial (Women's Health Initiative, WHI) do not support an effect of calcium plus vitamin D (CaD) supplementation for 7 years on the incidence of CRC [15]. An updated analysis of WHI study showed that among participants with no personal calcium or vitamin D supplements, CaD was significantly associated with decrease the risk of total cancers and non-significantly reduces the risk of CRC ; however, CaD did

not alter cancer risk in women who took personal calcium or vitamin D supplements [128]. Clinical trials regarding calcium supplementation for at least 6 months on risk of colorectal neoplasia is summarized in **Table 2.1**.

Calcium sensing receptor (*CaSR*), mainly expressed in the parathyroid gland and the renal tubules of the kidney, controls calcium homeostasis by regulating the release of parathyroid hormone and by inhibiting the reabsorption of calcium in the kidney [111,112]. Polymorphisms of the *CaSR* gene were reported to affect serum calcium concentrations [111,113,114]. However, genetic epidemiological studies have reported both significant [115–119] and null association [120–123] between variant alleles of the *CaSR* gene and risk of colorectal tumors. A meta-analysis [124] of the *CaSR* gene found that polymorphisms of rs1042636 were associated with reduced risk of proximal and distal CRC; variant alleles of rs1801726 were associated with increased risk of distal but not proximal CRC; and rs1801725 polymorphisms were not associated with risk of CRC.

**5. Magnesium and carcinogenesis**

Magnesium, involved in more than 300 metabolic functions [129], is an essential element for the body. Similar to the distribution of calcium in humans, approximately 60% of the magnesium is reserved in the bones and teeth, 20% in the muscle tissue, 19% in soft tissues such as heart, brain, liver and kidney, and less than 1% in the extracellular fluid including erythrocytes and serum [130]. An analysis of data from the National Health and Nutrition Examination Survey (NHANES) of 2005-2006 found that a majority of

Table 2.1 Randomized clinical trials of calcium supplementation and the risk of colorectal neoplasia

| Authors | Study characteristics | Interventions | Outcomes | Baseline calcium (mg/day) | Main findings Highest vs lowest category of calcium OR (95%CI) |
|---|---|---|---|---|---|
| Baron J, et al. 2015 | N=2259, Multicenter, Patients aged 45-75 and with recently diagnosed adenomas and no known colorectal polyps remaining after complete colonoscopy | Daily vitamin D3 (1000 IU) N=420, calcium as carbonate (1200 mg) N=419, both N=421, or neither N=415, for a mean duration of 4 years | Colorectal metachronous adenoma | -- | Ca vs no Ca: 0.95 (0.85-1.06)<br><br>Ca by baseline Ca≤597 mg/day: 0.90 (0.78-1.05)<br>Ca by baseline Ca>597 mg/day: 0.97 (0.82-1.15)<br><br>Ca by Ca:Mg ≤ median [a]: 0.68 (0.52, 0.90)<br>Ca by Ca:Mg > median [a]: 0.98 (0.75, 1.28) |
| Bonithon Kopp, 2000 | 665 patients with a history colorectal adenomas | 2 g elemental calcium, or 3.5 g fibre, or placebo daily for 3 years | Colorectal metachronous adenoma | 944±364 | Calcium /placebo: 0.66 (0.38,1.17) |

[a]: results among 930 subjects were previously published by Dai Q[11], 2012.

Americans at all ages have less magnesium intake than recommended daily intakes [131]. Adult men aged 71 years and older and adolescent females are most likely to have low magnesium intakes [131]. A low magnesium diet in the long term is associated with reduced serum magnesium and intracellular free magnesium in red blood cells [132].

Magnesium is a necessary nutrient needed to provide energy supply to cells. It plays a vital role in the active transport of calcium and potassium ions across cell membranes [129]. Magnesium is also inversely associated with anti-inflammatory biomarkers [133]. Larsson *et al*. [134] proposed evidence that magnesium may prevent colon cancer by reducing oxidative stress, improving insulin sensitivity, or by other means of decreasing colonic epithelial cell proliferation.

Fewer studies have investigated possible associations between magnesium and colorectal neoplasia than have been conducted for association with calcium. A case-control study found that magnesium level in drinking water was not associated with death from cancers of the colon [135] and rectum [136]. Two prospective studies observed an inverse association between total magnesium intake and incident CRC [134,137], although one study reported that the inverse association was only limited to colon cancer [137]. A cohort study in Japanese has indicated the inverse association was only significant for men but not for women [138]. However, two prospective studies have reported null results [139,140].

## 6. Calcium to magnesium ratio

Micronutrients of calcium, magnesium, phosphorus, and vitamin D share common food sources and are considered to be “metabolically interactive” [141]. Magnesium has an active role in adenosine triphosphatase, which is the energy source for ion exchange pump [142]. Intracellular magnesium deficiency may result in an increase in intracellular calcium [143]. Examining the effect of a single micronutrient without taking into account the influence of complementary or opposing nutrients may lead to residual confounding and incomplete risk assessment [144].

Calcium and magnesium share the same homeostatic regulatory system [45] primarily through absorption in intestine and reabsorption in kidney to keep the balance of Ca:Mg [45,46]. Nevertheless, magnesium and calcium compete with each other in (re)absorption and transport [47–51]. High calcium intake reduces absorption of both magnesium and calcium [145] whereas moderate magnesium deprivation results in negative magnesium balance while increased calcium retention [146]. Extracellular concentration of magnesium has been shown to competitively inhibit the transportation of calcium [147]. In addition, magnesium supplementation has been associated with increased urinary calcium excretion when calcium intake was less than 800 mg/day [148], suggesting that magnesium may suppress calcium reabsorption when Ca:Mg intake ratio is very low [28]. Therefore, the Ca:Mg intake ratio may take an important role in regulating calcium and magnesium (re)absorption. Several recent studies have postulated and preliminarily confirmed that the dietary ratio of calcium to magnesium modifies the effects of calcium and magnesium intakes on carcinogenesis [17,28,149]. In addition, one animal study has

indicated that the severity of inflammation caused by magnesium deficiency could be reduced by calcium deficiency [52]. Growing evidence strongly suggestes that the balance of calcium and magnesium plays an essential role in many diseases [52]. Considering that more than 45**%** of the U.S. population aged 40 or older are users of calcium supplements, an investigation into the balance of calcium and magnesium (Ca:Mg ratio) in colorectal carcinogenesis is particularly important.

### 6.1 Calcium to magnesium ratio and chronic diseases

Magnesium and calcium also potentially antagonize each other in many physiologic activities [47,48], including insulin resistance [150], oxidative stress [151], systematic inflammation [134], cell proliferation, differentiation, angiogenesis, and apoptosis [47,49,52,108,152,153]. These activities may subsequently result in carcinogenesis. Dai *et al.* first reported that dietary intake ratio of Ca:Mg modified the association between intakes of calcium and magnesium in relation to colorectal adenoma [17]. Ca:Mg ratio also interacted with a functional common polymorphism in the gene encoding *TRPM7*, a channel regulating $Ca^{2+}$ and $Mg^{2+}$ [154], in relation to both colorectal adenoma and hyperplastic polyps [17]. A subsequent randomized clinical trial reported that calcium supplementation only reduced risk of colorectal metachronous adenoma when the baseline Ca:Mg was less than or equal to 2.63 [26]. In addition, a high serum Ca:Mg was significantly associated with an increased risk of high-grade prostate cancer even after adjusting for serum levels of calcium and magnesium [149]. In support of these findings, one case-control study in Belgians has reported a high calcium but low magnesium was associated with increased risk of bladder cancer [144]. East Asian populations have a

similar intake level of magnesium as the U.S. population [155,156]. A high magnesium intake at U.S. recommended dietary allowance level was associated with increased risk of total mortality, cardiovascular diseases and cancer mortality in East-Asian populations with a very low Ca:Mg ratio (1.7(median)) [28,156]; whereas in the U.S. population with a high Ca:Mg ratio (median=3.0) [155], high magnesium intake was significantly linked to reduced risk of these diseases [134,139]. In the same cohort study conducted in east Asians, the association between intakes of calcium and magnesium with the risk of total, cardiovascular disease and/or cancer mortalities were modified by Ca:Mg ratio, but not by calcium or magnesium intake alone [28]. These findings were consistent with that from the Nurses' Health Study which reported that the interaction between total calcium and magnesium intakes was not statistically significant in relation to the risk of CRC incidence [139]. The direct associations between Ca:Mg ratio and chronic diseases are summarized in **Table 2.2** and the modification effect of Ca:Mg ratio on associations between calcium intake or serum calcium levels and the risk of common chronic diseases are presented in **Table 2.3**.

## 7. Hypothesized explanations for inconsistent associations between calcium and colorectal neoplasia

Factors such as the dose and duration of calcium and vitamin D treatment have been proposed to explain the inconsistency in findings across studies [99,157]. Prospective studies of CRC and calcium intake suggested that 600-1000mg of calcium per day may be ideal for CRC risk reduction, with no further protection beyond this range [158]. The Nurses' Health Study and the Health Professionals Follow-up Study also indicated a

Table 2.2 The direct effect of Ca:Mg ratio on the risk of common chronic diseases

| Authors | Population | Exposures Components | | Outcomes | Study Design | Main findings Highest vs lowest category of calcium OR (95%CI) |
|---|---|---|---|---|---|---|
| | | Dietary Ca/Mg | Serum Ca/Mg | | | |
| Dai Q, et al[1]. 2010 | 494 NMHS participants including 331 cases and 163 controls | -- | Yes | Prostate cancer cases | Case-control | Higher VS lower Ca:Mg ratio: 2.81 (1.24, 6.36) |
| Takata Y, et al. 2013 | 71, 267 females From SWHS in China | Yes | -- | Lung cancer | Cohort | Higher VS lower Ca:Mg ratio: 0.62 (0.47, 0.82) |
| Sun, Y, et al[18]. 2013 | -- | -- | Yes | Proliferation of prostate cancer cells | In-vitro study | An increase in the serum ratio of $Ca^{2+}/Mg^{2+}$ promoted proliferation for the initiation/progression of prostate cancer cells by activating TRPM7 channel |
| Ziniewicz HK, 2015 | 48 neonates | -- | Yes | Cardiovascular disease markers, insulin sensitivity/resistance markers (e.g. glucose, insulin, HOMA) | Cross-sectional study | The Ca:Mg ratio is negatively related to arylesterase (AE) ($p<0.01$), AE/HDL-c ($p<0.05$), and low levels of insulin<br><br>Serum calcium negative correlated with HDL-c ($p<0.05$), arylesterase (AE) ($p<0.01$), the Apo A1/Apo B ($p<0.05$) and AE/HDL-c ($p<0.05$) ratios<br><br>Neonates within the highest quartile for Mg displayed significantly higher levels of LDL-c and homocysteine ($p<0.05$) |

Table 2.3 Studies of the modification effect of Ca:Mg ratio on associations between calcium and the risk of diseases

| Authors | Population | Exposures Components | | Outcomes | Study Design | Main findings Highest vs lowest category of calcium OR (95%CI) |
|---|---|---|---|---|---|---|
| | | Diet | Supplement | | | |
| Dai Q, et al[2]. 2007 | 898 cases and 1306 controls from the TCPS | Yes | Yes | Colorectal adenoma cases , hyperplastic polyp cases | Case-control | Diet Ca: 1.12 (0.76, 1.65)<br>Total Ca: 0.98 (0.68, 1.40)<br>Total Ca by Ca:Mg ≤ 2.78: 0.72 (0.30, 1.69)<br>Total Ca by Ca:Mg > 2.78: 1.32 (0.69, 2.53) |
| Dai Q[11]. 2012 | 930 subjects | -- | Yes (Treatment) | Colorectal metachronous adenoma | Randomized clinical trials | Ca by Ca:Mg ≤ median: 0.68 (0.52, 0.90)<br>Ca by Ca:Mg > median: 0.98 (0.75, 1.28) |
| Dai et al, 2013 | Over 130,000 participants. From SWHS and SMHS in China | Yes | Yes | All-cause mortality and disease-specific mortality | Cohort | **Men**<br>Total mortality by Ca:Mg ≤1.7: 0.95 (0.66, 1.36)<br>Total mortality by Ca:Mg >1.7: 0.59 (0.44, 0.80)<br>CHD mortality by Ca:Mg ≤1.7: 1.64 (0.73, 3.70)<br>CHD mortality by Ca/Mg>1.7: 0.48 (0.21, 1.09)<br>**Women**<br>Total mortality by Ca:Mg ≤1.7: 1.03 (0.78, 1.36)<br>Total mortality by Ca:Mg >1.7: 1.06 (0.81, 1.39)<br>CHD mortality by Ca:Mg ≤1.7: 1.92 (1.00, 3.70)<br>CHD mortality by Ca/Mg>1.7: 0.85 (0.41, 1.76) |

daily intake of 700-800mg calcium is beneficial for CRC prevention [90]. These findings may explain the null effects found in the WHI, in which women had a total calcium intake of approximately 2150 mg/day (1000 mg of Ca carbonate plus a baseline mean intake of 1151 mg) [158], which is far from the beneficial range summarized from prospective studies. However, this does not explain why another clinical trial using similar or even higher levels of calcium supplementation (1200 mg/day) and total calcium intake (>2100 mg/day) found calcium intake was associated with reduced risk of metachronous adenoma [23]. It is plausible that there is a population difference. Participants recruited in these metachronous adenoma trials have a high risk of colorectal polyps, whereas participants included in the WHI are generally post-menopausal female subjects.

Furthermore, evidence from earlier studies suggested that there is a substantial variation of the ability to (re)absorb calcium in healthy people, which is mostly attributed to genetic variation[159]. Although not consistent, several previous studies found the association between calcium intake and CRC risk may be modified by genetic polymorphisms in *vitamin D receptor* [160,161] and *calcium-sensing receptor* [162,163]. Dai *et al.* also reported the association of calcium intake and risk of colorectal adenoma may differ by polymorphism in the *transient receptor potential melastatin 7* (*TRPM7*) gene [17]. Moreover, high calcium intake was significantly associated with reduced adenoma risk among carriers with variant alleles in genes of potassium channel, inwardly rectifying subfamily J, member 1 (*KCNJ1*) and solute carrier family 12, member 1 (*SLC12A1*) [164]. Future research on adequate nutrients intake and targeting for subpopulations with high

risk such as certain genetic profiles (gene-nutrient interactions) is going to advance the field of cancer chemoprevention and prevention of other common chronic diseases.

It is also reasonable to investigate whether the balance of calcium and other nutrients could help to explain the discrepancy. The beneficial effect of nutrient supplementation was reported in a large clinical trial conducted in a population in China, at high risk of nutritional deficiency [165]. This indicated that supplementation of nutrients may only be effective among high-risk populations with nutritional deficiency. Furthermore, from these trials, we learned that excessive supplementation of nutrients also could lead to adverse effects [166]. High levels of calcium consumption or serum levels were associated with a reduced risk of CRC or adenoma[22] but an increased risk of advanced/fatal prostate cancer [167–174]. Growing evidence showed that some nutrients or metals, including calcium, showed protective effects on cancer at a normal range while harmful effects at a high dose [165]. These evidences further support the hypothesis of nutrients balance. The Ca:Mg intake ratio, a hypothesized marker for calcium and magnesium balance, has not been considered in most of previous studies, except for some recent publications [17,26], in which the inverse association between calcium intake and adenoma prevalence and recurrence only appeared when the Ca:Mg intake ratio is low. Thus, studies on Ca:Mg ratio [17,26,149] provide further evidence for the theory of nutrients balance.

**8. Chronic diseases related to colorectal neoplasia**

Epidemiological studies have shown that diabetes [34–37] and metabolic syndrome [38–40] are strongly associated with risk for CRC development. The metabolic syndrome is a

cluster of metabolic abnormalities including dyslipidemia (hypertriglyceridemia and decreased fasting serum high-density lipoprotein cholesterols (HDL-c)), glucose intolerance, hypertension, insulin resistance, and central obesity. Higher blood pressure, total cholesterol, triglyceride, and lower HDL-c levels have been associated with increased risk of colorectal adenoma [175]. Hypertension, one of main components in metabolic syndrome, is a risk factor for recurrent colorectal adenoma [176]. A recent meta-analysis has reported that metabolic syndrome was associated with 34% increase in the risk of colorectal neoplasia [177]. Diabetes was associated with an elevated risk for both proximal and distal colon adenomas, but was more strongly associated with proximal colon adenoma and with large adenomas (≥5 mm in diameter) [178].The mechanism underlying these relations is not completely understood.

**B. The lipid profile**

**1. The composition and physical function of lipid profile**

The lipid profile or lipid panel is a pattern of lipids in the blood, which is also a group of blood tests used to assess the risk of developing cardiovascular disease or to monitor treatment effect. Typically, the lipid profile includes low-density lipoprotein cholesterol (LDL-c), HDL-c, triglycerides, and total cholesterol [179]. Some lipid measurements also include very low-density lipoprotein (VLDL) if the LDL-c is very high.

LDL-c mainly facilitates the transportation of fat and a small amount protein from liver to other peripheral tissues [179]. VLDL is produced in the liver primarily from dietary triglycerides [179]. It is usually estimated by a percentage of the triglyceride. The main

function of VLDL is to distribute the triglyceride produced in the liver. HDL-c is called "good" cholesterol [179]. It was postulated that HDL-c helps transport cholesterol from peripheral tissues back to the liver, where the majority of cholesterol were catabolized and excreted [180–182]. Higher levels of HDL-c is associated with lower risk for cardiovascular disease [179]. Triglycerides are the fats absorbed in the blood through diet or made by the liver in response to diets rich in sugars, refined carbohydrates, or fats [179]. The body utilizes triglycerides to store energy and provide a source energy to muscles. Only small amounts are found in the blood. Cholesterol is necessary for constructing the lipid layers in cells and for hormone production [179]. Excessive blood cholesterol accelerates blockages of inside arteries, which can increase risk for cardiovascular disease.

**2. The development of cardiovascular disease**

Initially, cholesterol deposits and accumulates in the arteries that supply to the heart [183]. With gradual accumulation over time, the lumen of the artery begin to narrow. This process is called atherosclerosis. Atherosclerosis in the coronary artery is known as coronary artery disease, which is the most common type of cardiovascular disease [184].

**3. Contributing factors of cardiovascular heart disease**

Heart disease is the leading cause of death in the U.S. [197]. Multiple factors (i.e. diabetes, smoking, high blood pressure, obesity, physical inactivity, and genetics) could affect lipid panel and are deemed as contributing factors of cardiovascular disease [198]. Greater control of risk factors and improved treatment are considered main contributors

to the reduction of mortality [199,200]. The administration of statin treatment is associated with a 20% reduction in total cholesterol, 28% reduction in LDL-c, 13% reduction in triglycerides, and 5% increase in HDL-c [188]. Combinations of fibrate, niacin (North and South America), omega-3 fatty acids or ezetimibe with a statin further reduced cardiovascular risk [201]. However, a large residual risk of cardiovascular disease remains unexplained [187,201].

**4. Magnesium and lipids**

Kobayashi first reported in an ecological study that the hardness of drinking water was inversely associated with mortality rate due to stroke [305]. Since then, numerous studies have investigated the associations of calcium and magnesium content in drinking water [306] or dietary intake [213,220,236,307–309] on lipid metabolism and the risk of subsequent cardiovascular disease. Early observational studies reported positive [227], inverse [228], and null [229] associations between blood magnesium and cholesterol.

Early experimental data from animal studies have shown that magnesium plays a role in the pathogenesis and treatment of vascular disease [202–204]. In several species, magnesium deficiency was associated with marked hypertriglyceridemia, hypercholesterolemia, increased LDL-c, and a decrease in the percentage of cholesterol transported by HDL-c [202–204]. Increased magnesium intake has been shown to improve insulin sensitivity, hyperglycemia, hyperlipidemia, and oxidative stress and has been shown to reduce lipid peroxidation in fructose-fed rats [205].These findings indicate that magnesium deficiency worsens several parameters of lipids.

A number of small-scale clinical trials have also generated inconsistent results on the effects of magnesium supplementation on lipid profile. Magnesium supplementation significantly improved lipid profile, including a reduction in total cholesterol, LDL-c and triglycerides in a few trials [206,207], but not in others [208,209] [210,211]. A meta-analysis of nine clinical trials found magnesium treatment significantly, but weakly, increased HDL-c, and had no effect on LDL-c and triglycerides [212]. A clinical trial among patients with type 2 diabetes mellitus has observed a significant fall in serum total cholesterol, LDL-c and triglycerides as well as a rise in HDL-c levels in 4 to 8 weeks after the initiation of magnesium supplementation. This effect continued to be observed till the end of the study 12 weeks [213]. However, none of these previous studies have considered the Ca:Mg balance and this may explain a part of the inconsistency in previous studies.

**5. Calcium and magnesium with lipid**

High calcium supplementation was linked to increased risk of myocardial infarction [232,233]. Conversely, some studies found that high calcium from dietary sources were associated with a reduced risk of cardiovascular disease [234,235]. Two grams of calcium carbonate supplementation was associated with deceased triglycerides and possibly total cholesterol in a small clinical trial with 92 colorectal adenoma patient [236]. Previous intervention trials also generated inconsistent results on the effect of calcium supplementation on lipid profile [307,313,314]. It was hypothesized that dietary sources of calcium usually goes together with reasonably high magnesium dietary intake, which leads to a reasonable Ca:Mg ratio. In contrast, calcium supplements alone substantially increase the Ca:Mg ratio. This may explain the differential effects of calcium from

dietary [234,235] and supplemental sources [232,233] on cardiovascular disease risk and mortality.

Experimental studies have suggested that the balance between calcium and magnesium may modify markers of lipid profile. Evidence from animal studies reported that calcium-adequate and magnesium-deficient diets lead to increases in heart lipid peroxidation, plasma levels of triglycerides and inflammatory markers; whereas a combination of calcium-deficient and magnesium-deficient diets cause significant reductions in inflammatory markers, lipid peroxidation and a normalization of plasma triglyceride concentration [52,53]. These findings highlight the critical role of the dietary Ca:Mg balance in the development of dyslipidemia and subsequent cardiovascular disease.

Patients with cardiovascular disease such as variant angina [237] and myocardial infarction [238] usually have magnesium deficiency [239]. Calcium channel-blocker therapy was proved to increase serum or fluid magnesium [237,238]. Dai *et al.* proposed that calcium and/or magnesium intake may be associated with an increased risk of cardiovascular disease mortality if the Ca:Mg ratio is either ≥2.6 or ≤1.7, but associated with a reduced risk or no risk when the Ca:Mg ratio is between 1.7 and 2.6 [28]. The modifying effect of the Ca:Mg ratio may explain the inconsistent results of previous studies pertaining to calcium and magnesium intakes and cardiovascular disease risk [216,220] or between magnesium supplementation and lipid profile change [206–209,211,212].

Previous trials of magnesium supplementation enrolled subjects with both high and low Ca:Mg ratios; the high doses of magnesium generally used in these trials may have resulted in a very low Ca:Mg ratio (<1.7), particularly for those with a low ratio at baseline [212]. These findings indicate the critical role of the dietary Ca:Mg balance in cardiovascular disease development. In other words, the effects of dietary magnesium and calcium on cardiovascular disease may differ by Ca:Mg intake ratio levels in the diet.

**6. Potential mechanisms for magnesium efficacy on cardiovascular disease**

The enzyme activity of lecithin cholesterol acyltransferase (LCAT) [240] and lipoprotein lipase (LPL) [202], which lowers triglycerides levels and raises HDL-c levels, requires the involvement of magnesium [241]. The lipid lowering effect of magnesium could be attributed to improved activity of LPL or insulin, or decreased actions of lipogenic liver enzymes [242,243]. Moreover, magnesium$^{2+}$- adenosine triphosphate (ATP) determines the rate-limiting enzyme in the cholesterol biosynthesis, which is associated with cholesterol levels [241]. In the pathway of cholesterol biosynthesis, the conversion of 2-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) to mevalonate requires the enzyme, HMG-CoA reductase, which is the rate-limiting enzyme [244]. The statins and magnesium inhibit that enzyme [244].

The role of Ca:Mg intake ratio in lipid metabolism is less frequently studied. Thus, there are no reports of whether the change of Ca:Mg intake ratio by magnesium supplementation may have a role in lipid metabolism. No previous studies investigated

the hypothesis that lipid profile is an potential intermediate biomarker between Ca:Mg ratio and risk of colorectal neoplasia and cardiovascular disease.

## C. Uric acid

### 1. Known factors that affect uric acid

Uric acid is a metabolic product of purine nucleotides. The serum uric acid reflects the balance between production and excretion [245]. Both exogenous (dietary) and endogenous purines are the major sources of uric acid for humans. Approximately 70% of daily excretion of uric acid is through the kidney [246].

### 1.1 Increase of uric acid

Diets rich in purines, high-fructose corn syrup, and table sugar are a risk factor of elevated uric acid [247,248]. Liver and kidney [249] have a high amount of purines. A moderate amount of purines is found in meats such as beef, lamb, pork, poultry and seafood [249]. Certain vegetables such as asparagus, beans, cauliflower, green peas, mushrooms, oatmeal, spinach, etc., contain a moderate level of purines [249]. In addition, hereditary factors, rapid weight loss, and diuretic medications could cause elevation of uric acid [250]. Certain drugs including thiazide diuretics and inosine can increase uric acid levels in the blood by interfering with renal clearance [251]. Behavioral factors such as fasting and rapid weight loss can temporarily elevate uric acid levels. Correcting low or deficient zinc levels can help elevate serum uric acid in Wilson's disease by ameliorating liver function and uric acid synthesis [252]. A higher serum uric acid level was associated with diabetes mellitus, hypertension, and increased triglycerides level [43].

### 1.2 Decrease of uric acid

A purine-free diet was associated with approximately 35% decline in plasma urate for healthy males [253]. A cohort study with hemodialysis patients has indicated that factors such as cardiovascular diseases, increased age, creatinine, fasting blood glucose, corrected calcium, phosphate, corrected calcium plus phosphate product, and LDL-c levels were associated with lower serum uric acid levels [43].

## 2. Uric acid and disease

Abnormal concentrations of uric acid may be an indicator of certain diseases [245]. Hyperuricemia is associated with a variety of diseases such as all-site cancer incidence and mortality [321], hypertension [254–256], cardiovascular disease [257–260], diabetes [261], gout, kidney disease [262–264], and obesity [265]. Hypouricemia was linked with multiple sclerosis [266,267]. Although epidemiologic studies have indicated rich evidence that elevated serum uric acid is independently associated with metabolic abnormalities, the mechanisms behind these associations have not been fully understood and are not well-established [268].

### 2.1 Magnesium, uric acid and type 2 diabetes

Whether elevated serum uric acid is a risk factor for diabetes has long been a matter of debate. Even since the early 1980s, uric acid has been associated with the development of diabetes although these associations were not adjusted for potential confounders [269]. Moreover, this report showed that serum uric acid levels were higher in prediabetics than in nondiabetics [269,270]. A prospective follow-up study showed high

serum uric acid measured at the baseline is associated with higher risk of type 2 diabetes, independent of obesity, dyslipidemia, and hypertension [271]. For each 1 mg/dl increment in uric acid levels, approximately 60% increased risk of incident type 2 diabetes was observed [272]. Although statistically significant associations have been extensively reported, one study in Japanese men reported a null association [273]. Another study has found a negative association between serum uric acid and diabetes [274]. Changes in serum urate (salt of uric acid) were associated with changes in serum magnesium in women with type 2 diabetes and non-diabetic men [275].

### 2.2 Magnesium, uric acid and metabolic syndrome

Hyperuricemia, a relatively new risk factor of metabolic syndrome, has been associated with a higher prevalence of metabolic syndrome [261]. A study has suggested fructose-induced hyperuricemia may play a pathogenic role in metabolic syndrome [276]. This is consistent with the increased consumption of fructose-containing beverages (such as fruit juices and soft drinks sweetened with sugar and high-fructose corn syrup) and the epidemic of diabetes and obesity in recent decades [277]. Serum uric acid is closely associated with metabolic syndrome in females than males [278]. One study has demonstrated elevated uric acid and lower serum magnesium were significantly associated with insulin resistance [279].

### 2.3 Calcium, magnesium, uric acid and cardiovascular disease

Viazzi *et al.* found that serum uric acid was associated with a positive history of cardiovascular disease and high Framingham risk score, which is independent of

metabolic syndrome [280]. A meta-analysis reported that 1 mg/dl increase in uric acid level was associated with 13% increased risk of incident hypertension [281]. Elevated serum uric acid level a is positively associated with cardiovascular mortality [257]. In addition, elevated levels of serum uric acid was associated with the presence of coronary artery calcium [42], a marker of atherosclerosis. A number of studies adjusted for multiple risk factors suggested that uric acid may be an independent risk factor for cardiovascular disease [257,282–284]. Another set of studies have indicated that uric acid is not independent of other established risk factors, especially hypertension, for the development of cardiovascular disease [285–288]. The role of uric acid in cardiovascular disease is still debated [265].

However, the majority of studies evaluating uric acid are observational studies. Moreover, our previous findings suggest a modifying effect of the Ca:Mg ratio on associations between calcium or magnesium intake and risk of colorectal [17,26] and esophagenal neoplasia [27], total, cardiovascular disease and cancer mortalities [28]. To our knowledge, no previous randomized trial has evaluated the effect of magnesium on uric acid status. Further, no previous study has examined whether the Ca:Mg intake ratio plays a role in uric acid metabolism and whether uric acid is an intermediate biomarker between Ca:Mg ratio and risk of colorectal neoplasia and cardiovascular disease.

## Chapter 3. Research strategies

### A. The Prostate, Lung, Colorectal, and Ovarian (PLCO) screening trial (project A)

#### 1. Study design: overall description

The PLCO is a ten-study-center, randomized, controlled trial comparing cancer screening tests and usual care to determine the effects of screening on cancer-related mortality. The details of the study design were described elsewhere [289,290]. Nine of ten study centers (Denver, CO; Detroit, MI; Honolulu, HI; Marshfield, WI; Minneapolis, MN; Pittsburgh, PA; Salt Lake City, UT; St. Louis, MO; and Washington, DC) began enrollment of eligible participants in November 1993. The University of Alabama began enrollment in January 1998. About 155,000 subjects were recruited between November 1993 and July 2001. After stratification by age and gender within each study center, participants were individually randomized to the intervention arm or control arm in equal proportions (block randomization). The date of enrollment is the date of randomization. Participants assigned to the intervention arm were invited to receive screening exams for prostate, lung, colorectal and ovarian cancers, whereas participants assigned to the control arm received routine medical care. The screening methods were digital rectal examination plus serum prostate-specific antigen (for prostate), chest X-ray (for lung), flexible 60-cm sigmoidoscopy (FSG) (for colorectum), cancer antigen 125 and transvaginal ultrasound (for ovary). The data analyses for this proposed project were primarily within the screening arm of the PLCO trial (n=77,444). The control arm of the PLCO initially had 77,453 participants.

## 2. Study population

According the original PLCO study design [289,290], the exclusion criteria used to select eligible subjects were listed as follows:

1) Aged < 60 or >74 at the time of randomization; Beginning in January 1996, participants aged < 55 or >74 at the time of randomization were excluded;
2) Under cancer treatment (excluding basal and squamous cell skin cancer) at the entry of the trial;
3) With known diagnosis of colorectal, lung, ovarian or prostate cancer;
4) With previous surgical removal of the entire prostate, one lung or the entire colon;
5) With prior surgical removal of both ovaries. Starting in October 1995, this criteria was deleted;
6) Participating in another cancer screening or cancer primary prevention trial;
7) Males who have taken Proscar in the past 6 months;
8) Starting in April 1995, males who had more than one prostate specific antigen (PSA) test in the three years prior to enrollment;
9) Starting in April 1995, participants who had a colonoscopy, sigmoidoscopy, or barium enema in the three years prior to enrollment;
10) Unable or unwilling to provide informed consent.

## 3. Data collection

### 3.1 Baseline Questionnaire (BQ)

At the entry of the trial, participants were asked to complete a BQ. The BQ collected information on demography (e.g., age, sex, race, education, and marital status), family cancer history, personal medical history (including the history of colorectal polyp, nonsteroidal anti-inflammatory drugs use, cancer screening history within 3 years), anthropometry (height, weight), lifestyle factors including smoking history and physical activities. In the intervention arm, 75,611 (97.6%) of participants finished the BQ. In the control arm, 74,366 (96.0%) of subjects has a valid BQ.

### 3.2 Dietary Information

#### 3.2.1 Dietary Questionnaire (DQX)

The DQX which included 137 food items at baseline was administered among intervention arm participants to obtain dietary intake over the 12 months before enrollment, and 14 questions about supplement intake. In the intervention arm, 60,358 (77.9%) of participants completed the DQX and 1,721 of subjects were further excluded because of invalid DQX. A questionnaire was invalid for any of the following four criteria [291]: 1) Missing date of completion; 2) Death before the completion of the DQX; 3) Eight or more missing items in food frequency questionnaire; and 4) Extreme (highest or lowest 1% for each gender) caloric intake.

#### 3.2.2 Dietary History Questionnaire (DHQ)

In December 1998, a second dietary questionnaire, the DHQ with 114 food items, was implemented. The DHQ was administered to participants of the control arm at $T_0$ (time

of year 0) and intervention arm at $T_3$ (time of year 3). Participants who had already passed these windows received the DHQ at their randomization anniversary in either 1999 or 2000. Among enrolled participants, 111, 477 (72.0%) individuals have completed the DHQ. Four criteria were used to confirm the validation of questionnaires: 1) A date of completion must exist; 2) The date of completion must not be later than the date of death; 3) Fewer than 8 missing frequencies; 4) Caloric intake is within normal range (excluding top and bottom 1% for each gender). Only valid food frequency questionnaires (N=106,522) were used for dietary analyses, of which 58,637 were in the intervention arm and 47,885 were in the control arm.

## 4. Nutrients

### 4.1 Dietary nutrients

Usual frequency of intake (less than once a month, once a month, twice to thrice a month, once a week, twice a week, three to four times a week, five to six times a week, once a day, two or more times a day) and portion size (small, medium, large) were queried. The usual dietary intakes of nutrients, including calcium, magnesium, and vitamin D, were derived from frequency and portion-size responses from DHQ and DQX, in which nutrient values per portion were multiplied by the daily frequency of intake and summed across all relevant food items. Nutrient composition databases which were constructed based on U.S. Department of Agriculture (USDA) food composition tables (USDA's 1994-96 Continuing Survey of Food Intakes by Individuals [CSFII]) [292]. Cut points between small and medium portions and between medium and large portions corresponded to the 25$^{th}$ and 75$^{th}$ percentiles, respectively, for portion sizes reported by

participants in the CSFII, 51 years or older [292]. Dietary calcium or dietary vitamin D intakes were calculated from all dietary sources, without supplements, respectively.

### 4.2 Nutrients from supplement use

Supplement use was calculated from single and/or multi-vitamins based on the responses to the supplement questions on the DQX or DHQ. The food frequency questionnaire also collected information on multivitamin, single vitamin, and mineral supplement use with questions about current use, past use (2 and 5 years ago), dosage, and years of intake [293]. Values for multivitamins were derived from the third National Health and Nutrition Examination Survey (NHANES III) database. Supplemental calcium, magnesium, or vitamin D intake were calculated by summing the intake from the single vitamin supplement calcium (the specific doses for calcium, dolomite, Tums, and so forth.) or vitamin D and multivitamins (162 magnesium calcium and 400 international unit (IU) of vitamin D per one-a-day multivitamin pill; 400 IU of vitamin D per therapeutic or high-dose types), as defined by the generic multivitamins most frequently reported by participants ages 55 to 74 years in the NHANES III cohort [294]. In this dissertation project, analysis of calcium intake from the supplements is mainly based on current intake (0-2 years before enrollment). Total calcium and vitamin D intake is defined as dietary plus supplemental intake.

## 5. Screening and diagnostics

FSG was performed at entry ($T_0$) and then at the 3 ($T_3$) or 5 ($T_5$)-year point. Participants randomized before Oct 1994 underwent a second screen at $T_3$. For participants who

were randomized between Oct 1994 and Nov 1995, a $T_3$ FSG were scheduled; however, $T_5$ FSG were offered if $T_3$ were missed [295]. Intervention arm participants randomized on/after Dec 1995 were scheduled for FSG at $T_5$. In the intervention arm, 65,000 subjects underwent baseline FSG and 42,000 received $T_3$ or $T_5$ FSG. There were 39,000 participants had FSGs both at baseline and at the $T_3$ or $T_5$ FSG. The screening results were divided into three mutually exclusive result categories. An FSG was defined as “positive FSG” if one or more of the following were found: rectal nodule, rectal and/or colon mass, and/or colon polyp. An FSG was defined as “inadequate” if a <50-cm depth of insertion was achieved or visual inspection was limited to <90% of the mucosal surface due to inadequate bowel preparation. Otherwise, an FSG was defined “negative” group if there was no detection of a polyp or mass. Subjects with inadequate screening could return for a second examination. Among 7,522 inadequate cases, 632 returned and 7,099 continued to be defined as inadequate after the second screening.

Participants with a positive screening were referred to their health care providers for further diagnostic evaluation and follow-up. Within 12 months after a positive screening, the PLCO abstracted medical records pertaining to diagnostics. Information on date of diagnosis, the anatomic location, the size and histology of polyps and masses were collected. In addition, classifications of malignant tumors (TNM) clinical and pathologic stages were abstracted for participants with CRC.

**6. Follow-up**

Study centers actively tracked subjects to collect, assemble, organize, and abstract medical record information related to diagnosis during the follow-up. Participants would

be followed until death or termination of the study (13 years from randomization) to ascertain all cancers of the prostate, lung, colorectal, and ovary, as well as deaths from all causes. The main sources by which study personnel learned of participants' diagnosis/death were (1) abnormal FSG screening, (2) the administration of the Annual Study Update (ASU) questionnaires, (3) reports from relatives, friends, or physicians, and (4) National Death Index (NDI) plus searches. Study centers also attempted to obtain a death certificate for each death that occurred on or before December 31, 2009. Information from the death certificate was abstracted and coded in the trial database. The underlying cause of death from death certificate was derived according to rules established by the National Center for Health Statistics. In order to ensure a more accurate assessment of the trial endpoints, a Death Review Process (DRP) was conducted and medical records were reviewed for all deaths that might have been due to prostate, lung, colorectal and ovarian cancers. The DRP cause of death was considered authoritative and will be used in statistical analyses of the primary endpoints.

In addition, cancer incidence, stages, and case survival data were also collected using the ASU questionnaire. The ASU was followed by linkage to medical records to confirm the diagnosis. The diagnosis of colorectal neoplasia was through medical record abstraction (MRA).

## 7. Outcomes in the PLCO trial

### 7.1 Incidence of colorectal adenoma

Participants (n=22,521) with a negative $T_0$ FSG in the intervention arm of the PLCO trial were eligible to be included in the sub-cohort for evaluation of incident colorectal

adenoma risk. In addition, an adequate $T_{3/5}$ screen was required to further classify an incident adenoma case or control. Cases must have a positive $T_{3/5}$ screen which led to the discovery of a left-sided adenoma [296]. Controls must have a negative $T_{3/5}$ FSG. Filters including a valid BQ (n=22,500), no cancer history before BQ (n=21,693), complete DQX (n=19,503), a valid DQX (n=19,088), no cancer history before DQX (n=19,076) were further applied. Finally, 1,147 incident colorectal adenoma cases and 17,929 control participants were identified. An adenoma with ≥1 cm in size, high-grade dysplasia, or villous component was defined as an advanced adenoma (**Figure 3.1**).

### 7.2 Metachronous colorectal adenoma (recurrent adenoma)

The Study of Colonoscopy Utilization (SCU) was designed as an ancillary study nested within the PLCO trial, which aimed to investigate the utilization of subsequent colonoscopy in the PLCO screening arm. Participants with a positive baseline FSG screening, diagnostic endoscopy within 6 months from baseline, and no cancer findings were invited to complete interviewer-administered telephone-based SCU questionnaire [296]. A baseline adenoma was defined as an adenoma found within the first 18 months following a positive $T_0$ FSG screen, on the first endoscopy that followed the screen, or on an endoscopy within six months of the first endoscopy following the screen. A questionnaire collected information on all known colonoscopy after randomization.

Medical record abstraction was performed to verify the collected questionnaire information. Individuals who were diagnosed with an adenoma in the second endoscopy were defined as recurrent colorectal adenoma cases. Participants not in SCU but with a

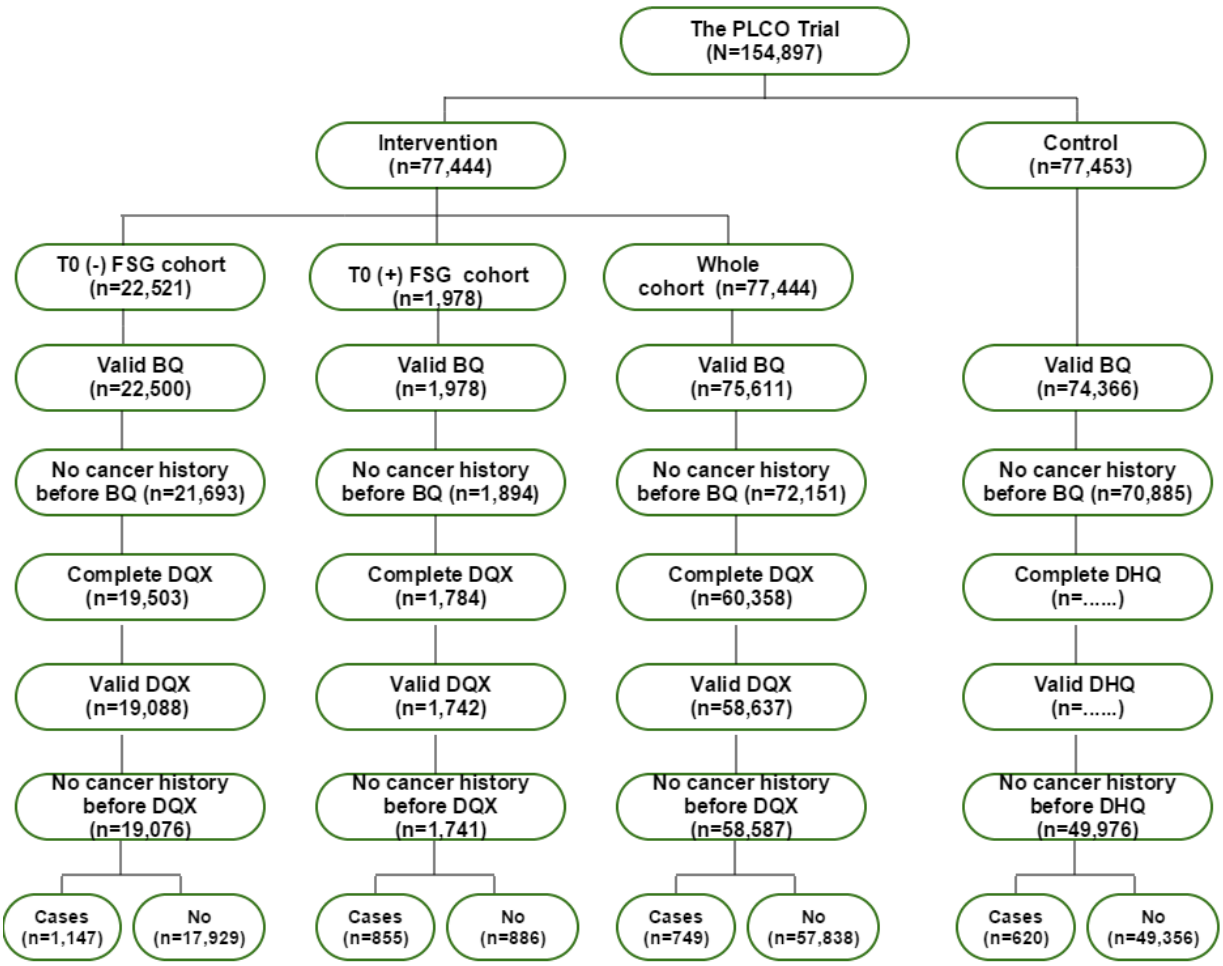


**Figure 3.1 The flow chart of the population sample size in the PLCO**

positive $T_{3/5}$ screen which resulted in an endoscopy that discovered recurrence were also included.

We further restricted the population to those who were diagnosed with adenoma at baseline endoscopy, had a second endoscopy, completed a valid BQ (n=1,978), no cancer history before BQ (n=1,894), complete DQX (n=1,784), a valid DQX (n=1,742), and who had no cancer history before DQX (n=1,741). The final analysis included 855 colorectal metachronous adenoma cases and 886 controls (**Figure 3.1**).

### 7.3 Incident CRC

Medical records related to diagnostic follow-up, a diagnosis of CRC, and cancer complications were collected. The intervention arm of the PLCO trial was further restricted to a valid BQ (n=75,611), no history of any cancer prior to BQ (n=72,151), completed a DQX (n=60,358), has valid DQX (n=58,637), and no history of any cancer prior to DQX (n=58,587). The control arm of the PLCO trial was further restricted to a valid BQ (n=74,366), no history of any cancer prior to BQ (n=70,885), and no history of any cancer prior to DQX (n=49,976). The final analysis included 58,587 subjects in the intervention arm, of which 749 were incident CRC cases and 57,838 CRC free controls. The control arm was reduced to 49,976 participants with valid BQ, DQX and no cancer history, of whom 620 were incident CRC cases and 49,356 were CRC free participants (**Figure 3.1**).

## 8. Statistical Analysis

The incidence of colorectal adenoma and metachronous adenoma were only followed up within the intervention arm of the PLCO. The information on CRC incidence was abstracted within both intervention and control arms. Thus, participants included in this study were from both intervention and control arms of the PLCO trial.

### 8.1 Power analysis

The main purpose of the project A was to investigate the association between calcium intake and the risk of various stages of colorectal neoplasia (incident and metachronous adenoma, and incident CRC). Among these three longitudinal outcomes, the number of

events is lowest for CRC incidence (n=749). Since the number of events is a major determinant of power in the survival analysis, the power analysis was conducted on the uses the CRC incidence. We expect power will be larger for other outcomes such as incidence and recurrence of adenoma with more events for similar HR's.

This power analysis was based on the following parameters: 29,288 experimental subjects (whose calcium intake is higher than 1135 magnesium per day (median)), 29,288 control subjects (whose calcium intake is no more than 1135 magnesium per day), an accrual interval of 1,095.75 days, and additional follow-up after the accrual interval of 4,748 days. An estimated median survival time of the control group is 230,115.5 days. If the true hazard ratio of control subjects relative to experiment subjects is 1.25, we will be able to reject the null hypothesis that the experimental and control survival curves are equal with a power of 0.893. The type I error probability associated with this test of the null hypothesis is 0.05. The power analysis was done using the PS software. **Table 3.1** shows the detailed alternatives to this power analysis.

Table 3.1 Study power for main effect of Ca intake in relationship to CRC in Aim 1

| Median survival | Hazard Ratio (Relative Risk) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| time (days)* | 1.05 | 1.10 | 1.15 | 1.20 | 1.21 | 1.22 | 1.23 | 1.24 | 1.25 | 1.30 |
| 230115.5 | 0.113 | 0.294 | 0.537 | 0.754 | 0.788 | 0.820 | 0.847 | 0.872 | 0.893 | 0.961 |
| 226463 | 0.115 | 0.297 | 0.543 | 0.761 | 0.795 | 0.826 | 0.853 | 0.877 | 0.898 | 0.964 |
| 175328 | 0.134 | 0.367 | 0.651 | 0.857 | 0.885 | 0.908 | 0.927 | 0.943 | 0.956 | 0.989 |
| 138803 | 0.156 | 0.444 | 0.751 | 0.925 | 0.944 | 0.958 | 0.970 | 0.978 | 0.984 | 0.998 |
| 102270 | 0.195 | 0.561 | 0.865 | 0.977 | 0.985 | 0.990 | 0.994 | 0.996 | 0.998 | 1.000 |
| 65745 | 0.274 | 0.747 | 0.967 | 0.998 | 0.999 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| 29220 | 0.518 | 0.972 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

*: This median survival time for controls is estimated using a formula of t × log(1/2) / log(p); *i.e.*: t is the observed maximal follow-up time of 4,748 days at which p = 0.98864 is the probably that a control subject survived.

### 8.2 The list of potential co-variables

Continuous variables included age at randomization, body mass index (BMI), total intakes of calcium, calories, copper, folic acid, iron, magnesium, phosphorous, vitamins A, $B_6$, $B_{12}$, C, D, and E, zinc. Categorical variables contained aspirin use (yes, no), cigarette smoking status (never, current cigarette smoker, and former cigarette smoker), drinker (yes, no), education (<8 years, 8-11 years, 12 years or completed high school, post-high school training other than college, some college, college graduate, and postgraduate), family history of CRC (yes, no), race (white, black, Hispanic, Asian, pacific islander, American Indian), sex (female, male), vigorous physical activity (none, <1, 1, 2, 3, $\geqslant$4 h/week). Due to the small count in certain groups, education status was reorganized to less than high school, 12 years high school, post-high school or some college, and college graduate or more; race was categorized into white, black, Asian and others. Other necessary data transformation of variables followed the rule of non-violation of assumptions of proportional hazards. Potential confounding factors was selected based on biological plausibility, literature reports and ≥10% change in relative risks [297].

### 8.3 Effect modifiers

It was hypothesized that the association between calcium intake and the risk of colorectal neoplasia differs by sex, race and the levels of Ca:Mg ratio, magnesium and vitamin D. Thus, these factors were considered as possible effect modifiers. Likelihood ratio tests, comparing the differences in log-likelihoods for interaction terms in the full model and no interaction terms in the reduced model, were used to assess the

significance (<0.05) of interactions. For the outcome of CRC incidence, Wald test was conducted to assess interactions.

### 8.4 Analytical methods

Summary statistics for both continuous (mean ± standard deviation) and categorical variables (count and percent) were used to describe study populations. Chi-square tests for categorical variables, t-tests and generalized linear models for continuous variables were conducted to evaluate differences in the distribution of potential confounding factors between different categories of calcium intake. Person-years for CRC incidence were calculated from the date of randomization to the date of the most recently completed end point follow-up questionnaire, the date of CRC diagnosis, death, or end of follow-up, whichever came first [31].

For incident and metachronous adenoma, the occurrence of disease as a case or control rather than time to event was considered; thus, unconditional logistic regression models were used to calculate odds ratios (OR) and 95% confidence intervals (95%CIs) to estimate the associations after adjusting for covariates. Hazard ratios (HR) and 95%CIs for incident CRC were estimated using Cox proportional hazard models. Potential confounding factors were selected based on biological plausibility, literature reports and/or ≥10% change in relative risks [297]. Confounding factors evaluated included age, sex, race, education, recruitment site, family history of CRC, body mass index, smoking status, alcohol consumption, exercise and daily intakes of total energy, vitamin D and magnesium. Tests for trend across categories were performed in

regression models by assigning the score j to the jth level of the variable selected. An *a priori* stratified analysis by Ca:Mg intake ratio was conducted. For metachronous adenoma and CRC incidence in the intervention arm, stratified analyses on baseline adenoma characteristics (e.g. advanced and/or synchronous adenoma) and subsites were performed. All tests were 2-sided and statistical significance level was set at 0.05. Statistical analyses were performed using SAS statistical software (version 9.4; SAS Institute, Cary, NC).

**B. The Personalized Prevention of CRC Trial (PPCCT, project B&C)**

**1. Study Population**

Adenoma and/or hyperplastic polyp cases aged from 40 to 85 years were recruited from two major sources: 1) Two existing large adenoma studies (Tennessee Colorectal Polyp Study (TCPS) and Tennessee-Indiana Adenoma Recurrence Study (TIARS)) in which participants were diagnosed between 2003 to 2010 and agreed to be contacted for future studies; and 2) Patients with colorectal adenoma and/or hyperplastic polyp diagnosed from 2008 to 2012 at Vanderbilt University Hospital. Participants had a calcium intake ≥ 700 mg/day and < 2000 mg/day measured with two 24-hour dietary recalls. In addition, the Ca:Mg intake ratio of participants was greater than 2.6. A list of exclusion criteria was applied: a history of colectomy, inflammatory bowel disease, any organ transplantation, cancer other than non-melanoma skin cancer, gastric bypass, chronic renal diseases and hepatic cirrhosis, chronic ischemic heart disease, diarrhea, type I diabetes mellitus, pituitary dwarfism; current use of lithium carbonate therapy,

blood anticoagulant drugs, digoxin and licorice; without contact information and informed consent.

**2. Enrollment**

Trained research staff reviewed the medical record of potential participants. If participants were eligible, an introductory letter signed by the parent study principal investigator and another introductory letter signed by the principal investigator and the gastroenterologist described the study and invited them to participate. A few days after the letter was mailed, the trained research staff of the Survey Research Shared Resource called the potential participant to provide more detailed information about the study, answered questions about the study, and to see if they may be interested in participating. During the telephone conversation, a series of yes/no screening questions were administered to determine eligibility based on the exclusion criteria. If the person was eligible, implied verbal consent was obtained to conduct the baseline interview survey and 24-hour dietary recalls prior to the first in-person clinic visit (CV#1). For eligible participants from Vanderbilt University Hospital and other sources, implied verbal consent was obtained to collect a mouthwash sample for genotype assay and then to conduct two 24 hour dietary recalls and a baseline interview survey. Eligible participants were scheduled for three clinic visits.

**3. Randomization and study design**

The project of 2&3 are nested in the parent PPCCT study. The PPCCT is a 2×2 factorial design (magnesium treatment and *TRPM*7 genotype) which enrolled 250 participants, of which 239 completely finished the study and 11 subjects finished part of the study

before withdrawing. The primary endpoints of the PPCCT were the expression of carcinogenesis biomarkers in colorectal mucosa. Within each group of individuals with different *TRPM7* genotypes (GG vs GA/AA), a permuted-block randomization algorithm was used to allocate the subjects into either the treatment or placebo arm.

**4. Dietary Ca:Mg intake assessment, intervention and clinic visit**

Two 24-hour dietary recall assessments were used to estimate the baseline Ca:Mg intake ratio. The recalls were based on the Minnesota Nutrient Data System (NDS) and were administered by the Survey Research Shared Resource. Information on the participant's use of medications, nutritional supplements, and other conditions was collected. Based on a participant's baseline Ca:Mg intake ratio, the participant was assigned to a customized dose that would reduce the Ca:Mg intake ratio to less than 2.6 but above 2.0. Identical-appearing placebos were made to match magnesium capsules.

The intervention period was designed to be 12 weeks. Eligible participants were scheduled for three clinic visits (week 1, 6 and 12). Before each clinic visit, participants received a study package, which included an instruction booklet, a Food Amounts booklet, clinic visit questionnaire forms, and diet, health, and probiotics questionnaires. Information on the participant's use of medications, nutritional supplements, and other conditions were collected. Rectal biopsy, blood, and urine biospecimens were collected and processed in each clinic visit. Anthropometric measurements (blood pressures,

weight, height, waist and hip circumstance) were measured at least twice in each clinic visit.

**5. Biomarkers assay**

As of April, 2014, 150 participants had finished the trial and their samples were assayed for a lipid profile and uric acid. Blood was collected from a forearm vein at each clinic visit after participants had fasted for at least 8 hours. The blood was clotted and immediately centrifuged to separate serum, which was rapidly cooled and frozen at -80$^{o}$C before biochemistry analysis. The 150 pairs of pre- and post-treatment serum samples of participants who finished the trial were sent to the Vanderbilt Lipid Laboratory for assay.

The four lipid biomarkers (LDL-c, HDL-c, total cholesterol, and triglycerides) as well as uric acid were assayed by the Vanderbilt Lipid Laboratory, which is standardized by the Centers for Disease Control and Prevention for lipid analysis [298]. Total cholesterol, HDL-c, triglycerides, and uric acid were measured using the Reagents of ACE® Cholesterol (#SA1010), HDL-c (#SA1038), triglycerides (#SA1023), and uric acid (#SA1025) respectively [298]. The detailed assay procedure can be referred to the appropriate Alfa Wassermann Diagnostic Technologies, LLC Clinical Chemistry Systems Operator's Manual. When triglycerides was less than or equal to 400 mg/dl, the LDL-c was a calculated by subtracting HDL-c and one-fifth of triglycerides from the total cholesterol. Otherwise, the direct LDL-c was directly measured using the ACE LDL-c Reagent (#SA1040) [298]. Based on values measured from quality control samples, intra-assay and

inter-assay variation coefficients were 1.28% and 4.03% for LDL-c; 0.63% and 2.19% for HDL-c; 0.67% and 5.12% for triglycerides; 0.75% and 2.06% for total cholesterol, 1.72% and 7.64% for uric acid.

### 6. Statistical power

The estimate of power is focused on the primary endpoint of LDL-c and uric acid. The conservative estimate of power is based on McNemar's test. The paired fourfold table of the McNemar's test was presented in **Table 3.2**. Our pilot data shows that 38% at baseline had abnormal LDL-c, and after 12 weeks of treatment 25% had abnormal LDL-c. The same pattern was found for uric acid in the pilot data. One third with abnormal LDL-c at baseline lowered their LDL-c to normal range, and none of those with normal LDL-c at baseline became abnormal after 12 weeks of treatment. In the biomarker assay, we have measured 150 pairs (150 subjects before and after treatment) of serum samples. We assumed that the balance of treatment and placebo was 1:1. Thus, we had 75 pairs of LDL-c and uric acid measures in the treatment group. With a 0.05 two-sided significance level, a sample size of 75 will have 95% power to detect a difference in proportions of 0.125 ($p_{10} - p_{01}$) when the proportion of discordant pairs is expected to be 0.135 ($p_{10} + p_{01}$). **Table 3.3** contains estimated power for McNemar's test of equality of paired proportions. We do not expect a significant change of biomarkers in the placebo arm, thus we have an equal number (n=75) of subjects in the placebo arm.

Table 3.2 The paired fourfold table of the McNemar's test

| | | Post-treatment | |
|---|---|---|---|
| | | Normal | Abnormal |
| Pre-treatment | Normal | $p_{00}$ | $p_{01}$ |
| | Abnormal | $p_{10}$ | $p_{11}$ |

| Table 3.3 Study power for lipid panels (project 2) and uric acid (project 3) in the PPCCT | | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| Test significance level, α | 0.050 | 0.050 | 0.050 | 0.050 | 0.050 | 0.050 |
| Difference in proportions, $p_{10} - p_{01}$ | 0.125 | 0.115 | 0.105 | 0.095 | 0.085 | 0.075 |
| Proportions of discordant pairs, $p_{10} + p_{01}$ | 0.135 | 0.125 | 0.115 | 0.105 | 0.095 | 0.085 |
| Power | 0.95 | 0.92 | 0.88 | 0.83 | 0.75 | 0.66 |

## 7. Analytic methods

The parent PPCCT is still blinded for primary outcomes; thus, an independent statistician outside of the study team conducted the statistical analyses for the current study. Summary statistics for lipid biomarkers as continuous variables (mean ± standard deviation, median, and inter quartile range) was derived for each randomization group. The distributions of continuous outcomes were assessed for normality by using the Kolmogorov-Smirnov test. Both within-group change and between-group comparisons were evaluated. Paired t-test (normal) or the Wilcoxon-signed rank test (non-normal) was conducted to evaluate whether pre- and post-treatment values were different within each group (treatment or placebo). Either the parametric two-sample t-test or the non-parametric Wilcoxon Rank Sum test was performed to evaluate between-group differences. Generalized linear regression analysis was performed to examine the effect of magnesium treatment on lipid profile while adjusting for baseline lipid biomarkers. All participants had a baseline Ca:Mg ratio ≥ 2.6. Participants were thus classified into three groups based on the concordance between the baseline Ca:Mg ratio and the Ca:Mg ratio measured from the FFQ (the median interval to baseline was around 5.0 years): long-term high ratio (FFQ Ca:Mg ratio ≥ 2.6), recent high ratio (FFQ Ca:Mg ratio < 2.6), and unknown ratio (Ca:Mg ratio from the FFQ is unmeasured). Stratified analyses were conducted by the categories of the change in the Ca:Mg ratio for both long-term and recent high. The statistical significance was 0.05 using two-sided test.

The data analysis used software R and SAS® (version 9.4; SAS Institute, Cary, North Carolina).

# Chapter 4. Results

## A. Calcium: magnesium intake ratio and colorectal carcinogenesis, results from the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial

### Characteristics of the selected population

The distribution of selected demographic characteristics and potential confounders in cases and controls for incident and metachronous adenoma are shown in **Table 4.1**. Compared to controls, adenoma cases were more likely to be male, smokers, physically inactive, and had higher body mass index, higher intake of energy, but lower intakes of calcium, magnesium and vitamin D. Compared to participants without metachronous adenoma, metachronous adenoma cases were also more likely to be male and obese, to have family history of CRC, higher intake of total energy but lower intake of calcium. Metachronous adenoma cases were less likely to be white.

The selected basic characteristics in the intervention and control arms for the study of incident CRC are compared in **Table 4.2**. For both intervention and control arms, compared to the lower calcium intake (<600mg/day), participants with higher calcium intake were less likely to be male, but more likely to be white, never smokers, aspirin users, physically active, and had lower BMI, higher education achievement and family history of CRC , and higher intakes of energy, magnesium and vitamin D.

### Incident colorectal adenoma

There was no significant association between calcium intake and risk of incident colorectal adenoma (**Table 4.3**). However, compared to the lowest category of calcium

intake, higher intakes of calcium were associated with a suggestive reduced risk of advanced and/or synchronous adenoma (*P* for trend, 0.05). In the stratified analysis Ca:Mg ratios, the inverse association between calcium intake and advanced and/or synchronous adenoma primarily appeared in participants with Ca:Mg ratios ranging from 1.7 to 2.5 (*P* for trend, 0.05). No significant association was observed when the Ca:Mg ratio was above 2.5.

**Metachronous (recurrent) adenoma**

We found calcium intake was not related to risk of metachronous adenoma (**Table 4.4**). We also did not find significant associations in the stratified analysis by Ca:Mg intake ratio (**Table 4.4**), location of adenoma (i.e. distal or proximal, data not shown), and baseline adenoma characteristics (i.e. advanced/synchronous adenoma) (**Supplement Table 4.7**).This null association was similar for advanced and/or metachronous adenoma (**Table 4.4**). The Ca:Mg ratio stratified analysis showed null results as well.

**CRC incidence**

We found calcium intake was significantly related to a reduced risk of CRC (*P* for trend, 0.03) (**Table 4.5**). This significant inverse association was primarily present for distal CRC (*P* for trend, <0.01) but not for proximal colon cancer. In the analysis stratified by the Ca:Mg intake ratio, we found the inverse association was only statistically significant in participants whose Ca:Mg ratio ranged from 1.7 to 2.5 (*P* for trend, 0.04). There was a significant interaction between calcium intake and the Ca:Mg ratio in relation to distal CRC (*P* for interaction, <0.01).

**Stratified analysis by treatment**

We found, in the control arm without regular endoscopy, the significant association between calcium intake and risk of incident distal CRC remained (*P* for trend, <0.01, **Table 4.6**) and this inverse association appeared to be primarily in those with Ca:Mg intake ratios between 1.7 and 2.5, although this was not statistically significant. However, in the intervention arm with endoscopy screening, the inverse association between calcium intake and risk of incident distal CRC was of borderline significance (*P* for trend, 0.06, **Table 4.6**). In further analysis stratified by feature of the baseline adenomas, we found among participants with advanced/synchronous adenoma at baseline, higher calcium intake was inversely associated with risk of incident CRC (*P* for trend, 0.04) in the intervention arm (**Supplement Table 4.7**). Comparable data on were not available in the control arm.

Table 4.1 Selected descriptive characteristics of adenoma cases and controls

| Characteristics | Adenoma | | | Metachronous adenoma | | |
|---|---|---|---|---|---|---|
| | Cases (N=1147) | Controls (N=17929) | *P* value | Cases (N=855) | Controls (N=886) | *P* value |
| Age (years), Mean ± SD | 62.1±5.3 | 62.3±5.2 | 0.13 | 62.9±4.8 | 62.7±5.2 | 0.41 |
| Sex, % | | | | | | |
| Men | 67.5 | 55.9 | <.0001 | 72.4 | 60.0 | <.0001 |
| Race, % | | | | | | |
| White | 90.6 | 88.9 | 0.07 | 93.0 | 96.1 | 0.009 |
| Education, % | | | | | | |
| College or higher | 38.7 | 39.5 | 0.38 | 38.0 | 35.7 | 0.68 |
| Smoking status, % | | | | | | |
| Never smoker | 42.6 | 53.4 | | 34.5 | 37.5 | |
| Former smoker | 47.3 | 41.4 | | 52.1 | 49.3 | |
| Current smoker | 10.1 | 5.2 | <.0001 | 13.4 | 13.2 | 0.42 |
| Alcohol consumption, % | 81.1 | 79.1 | 0.10 | 84.0 | 83.1 | 0.61 |
| Family History of CRC , % | 10.5 | 9.1 | 0.13 | 13.2 | 13.7 | 0.79 |
| Aspirin use, % | 48.4 | 46.8 | 0.30 | 44.7 | 43.9 | 0.74 |
| Physically inactive, % | 16.6 | 12.0 | <.0001 | 15.1 | 15.2 | 0.93 |
| Body mass index≥ 30, % | 25.3 | 21.9 | 0.003 | 27.9 | 23.7 | 0.02 |
| Daily nutrients intake, Mean ± SE | | | | | | |
| Total energy (kcal)[1] | 2187.0±23.6 | 2086.5±6.0 | <.0001 | 2185.5±27.6 | 2088.9±27.1 | 0.01 |
| Total calcium (mg)[2] | 1160.5±15.8 | 1255.1±4.0 | <.0001 | 1119.0±16.6 | 1196.8±16.3 | 0.0008 |
| Total magnesium (mg) [2] | 431.6±3.1 | 444.4±0.8 | <.0001 | 431.4±3.7 | 439.4±3.6 | 0.12 |
| Vitamin D (mcg/day) [2] | 10.8±0.3 | 12.1±0.1 | <.0001 | 10.4±0.3 | 11.0±0.3 | 0.09 |

[1] Least squares mean value, SE

[2] Least squares mean value, SE, adjusting for total energy

Table 4.2 Selected descriptive characteristics of participants by calcium intake (mg/day)

| Characteristics | Intervention arm | | | | | Control arm | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | < 600 | 600-1200 | *1200-1600* | *≥1600* | *P* | < 600 | 600-1200 | *1200-1600* | *≥1600* | *P* |
| N | 7323 | 24475 | 12354 | 14383 | | 12678 | 21185 | 9584 | 6487 | |
| No. of CRC cases | 100 | 315 | 128 | 154 | | 174 | 242 | 99 | 63 | |
| Person-years | 80222 | 273937 | 138958 | 161286 | | 142777 | 241744 | 110113 | 74392 | |
| Age (years) | | | | | | | | | | |
| Mean[1] | 62.6 | 62.7 | 62.6 | 62.6 | | 62.6 | 62.5 | 62.5 | 62.5 | |
| SE | 0.06 | 0.03 | 0.05 | 0.04 | 0.12 | 0.05 | 0.04 | 0.05 | 0.07 | <.0001 |
| Sex (men), % | 53.7 | 58.6 | 50.2 | 39.2 | <.0001 | 55.9 | 52.2 | 36.1 | 34.0 | <.0001 |
| Race (white), % | 81.3 | 90.2 | 93.1 | 94.7 | <.0001 | 85.2 | 91.9 | 93.4 | 95.7 | <.0001 |
| Education, % | | | | | | | | | | |
| College or higher | 48.5 | 56.9 | 59.3 | 61.1 | <.0001 | 54.0 | 57.9 | 59.3 | 61.1 | <.0001 |
| Smoking status, % | | | | | | | | | | |
| Never smoker | 42.4 | 44.5 | 47.9 | 52.2 | | 44.0 | 47.1 | 50.6 | 51.6 | |
| Former smoker | 44.0 | 44.8 | 43.3 | 40.5 | | 44.4 | 43.9 | 41.3 | 40.9 | |
| Current smoker | 13.6 | 10.7 | 8.8 | 7.3 | <.0001 | 11.6 | 9.0 | 8.1 | 7.5 | <.0001 |
| Alcohol consumption, % | 75.6 | 78.7 | 77.8 | 76.2 | <.0001 | 72.8 | 73.8 | 72.5 | 69.7 | <.0001 |
| Family history of CRC , % | 10.3 | 10.9 | 10.5 | 11.0 | 0.0038 | 9.9 | 10.3 | 10.2 | 10.4 | 0.61 |
| Aspirin use, % | 43.0 | 46.4 | 49.1 | 49.3 | <.0001 | 45.2 | 47.7 | 47.5 | 48.5 | <.0001 |
| Body mass index ≥ 30, % | 25.1 | 24.1 | 24.3 | 22.7 | <.0001 | 24.5 | 23.8 | 21.2 | 22.3 | <.0001 |
| Total energy (kcal)[2] | | | | | | | | | | |
| Mean[1] | 1340.8 | 1913.4 | 2236.7 | 2477.5 | | 1296.0 | 1781.0 | 1867.9 | 2217.1 | |
| SE | 8.4 | 4.6 | 6.5 | 6.0 | <.0001 | 6.0 | 4.6 | 6.9 | 8.4 | <.0001 |
| Total magnesium (mg)[2] | | | | | | | | | | |
| Mean[3] | 351.4 | 410.3 | 454.3 | 500.7 | | 324.3 | 364.9 | 391.1 | 432.3 | |
| SE | 1.2 | 0.6 | 0.9 | 0.8 | <.0001 | 0.8 | 0.6 | 0.9 | 1.1 | <.0001 |
| Vitamin D (mcg/day) | | | | | | | | | | |
| Mean[2] | 4.0 | 9.1 | 13.8 | 19.3 | | 6.6 | 9.0 | 10.9 | 13.8 | |
| SE | 0.09 | 0.07 | 0.05 | 0.07 | <.0001 | 0.05 | 0.03 | 0.05 | 0.06 | <.0001 |

[1] Least squares mean value, SE

[2] Least squares mean value, SE, adjusting for total energy

Table 4.3 ORs and 95% CIs[1] for colorectal adenoma incidence by calcium intake

| Ca Intake (mg/day) | Any adenoma | | Advanced and/or synchronous adenoma | |
|---|---|---|---|---|
| | Cases | OR (95% CI) | Cases | OR (95% CI) |
| **All** | | | | |
| <600 | 139 | 0.89 (0.72-1.12) | 55 | 1.17 (0.82-1.68) |
| 600-1200 | 545 | 1.00 (Ref) | 192 | 1.00 (Ref) |
| 1200-1600 | 219 | 0.82 (0.68-0.97) | 70 | 0.71 (0.52-0.96) |
| ≥1600 | 244 | 0.84 (0.69-1.03) | 83 | 0.80 (0.58-1.11) |
| $P_{trend}$[2] | | 0.17 | | 0.05 |
| **Ca:Mg ratio is between 1.7 to 2.5** | | | | |
| <600 | 64 | 0.91 (0.66-1.26) | 31 | 1.50 (0.92-2.47) |
| 600-1200 | 284 | 1.00 (Ref) | 93 | 1.00 (Ref) |
| 1200-1600 | 62 | 0.72 (0.51-1.02) | 19 | 0.60 (0.33-1.08) |
| ≥1600 | 35 | 0.91 (0.55-1.51) | 11 | 0.64 (0.27-1.54) |
| $P_{trend}$[2] | | 0.61 | | 0.05 |
| **Ca:Mg ratio is above 2.5** | | | | |
| <600 | 13 | 0.86 (0.47-1.60) | 2 | 0.45 (0.11-1.88) |
| 600-1200 | 190 | 1.00 (Ref) | 63 | 1.00 (Ref) |
| 1200-1600 | 154 | 0.81 (0.64-1.03) | 50 | 0.74 (0.50-1.12) |
| ≥1600 | 207 | 0.81 (0.62-1.06) | 72 | 0.77 (0.49-1.22) |
| $P_{trend}$[2] | | 0.16 | | 0.43 |
| $P_{interaction}$[3] | | 0.94 | | 0.11 |

[1] Adjusted for age (continuous), sex, BMI (<25, 25-30, ≥30), education (less than high school, 12 years or completed high school, post high school training other than college, some college, college graduate, postgraduate), race (white, black, asian or others), family history of CRC (yes or no), cigarette (never smoked cigarettes, current or former), hours spent in vigorous activities (less than1 hour/week, 1 hour/week, 2 hours/week, 3 hours/week, 4+ hours/week), and total energy and vitamin D intake

[2] Assigned the score j to the $j^{th}$ level of calcium intake and evaluated the significance of Wald test

[3] Estimated the full model with interaction term of calcium intake and Ca:Mg ratio and without this term in reduce model using likelihood ratio test

Table 4.4 ORs and 95% CIs[1] for metachronous colorectal adenoma incidence by calcium intake

| Calcium Intake (mg/day) | Any metachronous adenoma | | Advanced and/or metachronous adenoma | |
|---|---|---|---|---|
| | Cases | OR (95% CI) | Cases | OR (95% CI) |
| **All** | | | | |
| <600 | 124 | 1.23 (0.87-1.73) | 65 | 1.45 (0.96-2.19) |
| 600-1200 | 393 | 1.00 (Ref) | 198 | 1.00 (Ref) |
| 1200-1600 | 190 | 1.02 (0.77-1.34) | 96 | 0.97 (0.69-1.38) |
| ≥1600 | 148 | 0.83 (0.60-1.15) | 81 | 0.92 (0.61-1.37) |
| $P_{trend}$[2] | | 0.15 | | 0.21 |
| **Ca:Mg ratio is between 1.7 to 2.5** | | | | |
| <600 | 63 | 1.33 (0.82-2.17) | 32 | 1.44 (0.80-2.62) |
| 600-1200 | 194 | 1.00 (Ref) | 91 | 1.00 (Ref) |
| 1200-1600 | 53 | 0.90 (0.52-1.56) | 24 | 1.03 (0.51-2.07) |
| ≥1600 | 22 | 2.34 (0.82-6.68) | 11 | 3.32 (0.95-11.64) |
| $P_{trend}$[2] | | 0.89 | | 0.90 |
| **Ca:Mg ratio is above 2.5** | | | | |
| <600 | 9 | 0.85 (0.32-2.27) | 4 | 0.76 (0.22-2.59) |
| 600-1200 | 137 | 1.00 (Ref) | 74 | 1.00 (Ref) |
| 1200-1600 | 133 | 1.02 (0.69-1.50) | 70 | 0.86 (0.53-1.38) |
| ≥1600 | 124 | 0.73 (0.47-1.13) | 68 | 0.65 (0.37-1.13) |
| $P_{trend}$[2] | | 0.21 | | 0.18 |
| $P_{interaction}$[3] | | 0.53 | | 0.40 |

[1] Adjusted for age (continuous), sex, BMI (<25, 25-30, ≥30), education (less than high school, 12 years or completed high school, post high school training other than college, some college, college graduate, postgraduate), race (white, black, asian or others), family history of CRC (yes or no), cigarette (never smoked cigarettes, current or former), hours spent in vigorous activities (less than1 hour/week, 1 hour/week, 2 hours/week, 3 hours/week, 4+ hours/week), and total energy, magnesium and vitamin D intake

[2] Assigned the score j to the $j^{th}$ level of calcium intake and evaluated the significance of Wald test

[3] Estimated the full model with interaction term of calcium intake and Ca:Mg ratio and without this term in reduce model using likelihood ratio test

Table 4.5 HRs and 95% CIs[1] for CRC incidence by calcium intake

| Calcium Intake (mg/day) | CRC incidence | | Proximal CRC | | Distal CRC | |
|---|---|---|---|---|---|---|
| | Cases | HR (95% CI) | Cases | HR (95% CI) | Cases | HR (95% CI) |
| **All** | | | | | | |
| <600 | 274 | 1.12 (0.95-1.33) | 133 | 0.94 (0.74-1.19) | 141 | 1.38 (1.08-1.76) |
| 600-1200 | 557 | 1.00 (Ref) | 302 | 1.00 (Ref) | 254 | 1.00 (Ref) |
| 1200-1600 | 227 | 0.85 (0.72-1.01) | 134 | 0.94 (0.75-1.17) | 93 | 0.75 (0.58-0.97) |
| ≥1600 | 217 | 0.89 (0.73-1.07) | 121 | 0.97 (0.75-1.26) | 92 | 0.75 (0.56-1.01) |
| $P_{trend}$[2] | | 0.03 | | 0.99 | | <0.01 |
| **Ca:Mg ratio is between 1.7 to 2.5** | | | | | | |
| <600 | 101 | 1.12 (0.85-1.47) | 50 | 0.95 (0.65-1.39) | 51 | 1.34 (0.90-1.99) |
| 600-1200 | 269 | 1.00 (Ref) | 144 | 1.00 (Ref) | 124 | 1.00 (Ref) |
| 1200-1600 | 51 | 0.70 (0.49-1.01) | 28 | 0.80 (0.48-1.33) | 23 | 0.62 (0.37-1.06) |
| ≥1600 | 21 | 0.89 (0.51-1.55) | 11 | 1.19 (0.56-2.54) | 9 | 0.59 (0.25-1.39) |
| $P_{trend}$[2] | | 0.17 | | 0.94 | | 0.04 |
| **Ca:Mg ratio is above 2.5** | | | | | | |
| <600 | 25 | 1.16 (0.75-1.79) | 10 | 0.78 (0.41-1.51) | 15 | 1.76 (0.98-3.14) |
| 600-1200 | 213 | 1.00 (Ref) | 124 | 1.00 (Ref) | 89 | 1.00 (Ref) |
| 1200-1600 | 174 | 0.91 (0.73-1.13) | 105 | 0.97 (0.73-1.29) | 69 | 0.83 (0.59-1.17) |
| ≥1600 | 195 | 0.93 (0.73-1.20) | 109 | 0.97 (0.70-1.35) | 83 | 0.84 (0.57-1.24) |
| $P_{trend}$[2] | | 0.41 | | 0.93 | | 0.12 |
| $P_{interaction}$[3] | | 0.08 | | 0.64 | | <0.01 |

[1] Adjusted for arm, age (continuous), sex, BMI (<25, 25-30, ≥30), education (less than high school, 12 years or completed high school, post high school training other than college, some college, college graduate, postgraduate), race (white, black, asian or others), family history of CRC (yes or no), cigarette (never smoked cigarettes, current or former), hours spent in vigorous activities (less than1 hour/week, 1 hour/week, 2 hours/week, 3 hours/week, 4+ hours/week), and total energy and vitamin D intake

[2] Assigned the score j to the $j^{th}$ level of calcium intake and evaluated the significance of Wald test

[3] Estimated the full model with interaction term of calcium intake and Ca:Mg ratio and without this term in reduce model using likelihood ratio test

Table 4.6 HRs and 95% CIs[1] for CRC incidence by arms

| Calcium Intake (mg/day) | Distal CRC in the intervention arm | | Distal CRC in the control arm | |
|---|---|---|---|---|
| | Cases | HR (95% CI) | Cases | HR (95% CI) |
| **All** | | | | |
| <600 | 52 | 1.42 (0.97-2.07) | 89 | 1.43 (1.03-1.98) |
| 600-1200 | 135 | 1.00 (Ref) | 119 | 1.00 (Ref) |
| 1200-1600 | 51 | 0.74 (0.52-1.06) | 42 | 0.78 (0.54-1.13) |
| ≥1600 | 62 | 0.85 (0.58-1.25) | 30 | 0.71 (0.45-1.12) |
| $P_{trend}$ | | 0.06 | | <0.01 |
| **Ca:Mg ratio is between 1.7 to 2.5** | | | | |
| <600 | 26 | 1.31 (0.77-2.25) | 25 | 1.42 (0.80-2.53) |
| 600-1200 | 71 | 1.00 (Ref) | 53 | 1.00 (Ref) |
| 1200-1600 | 15 | 0.68 (0.35-1.32) | 8 | 0.58 (0.24-1.41) |
| ≥1600 | 7 | 0.71 (0.25-1.99) | 2 | 0.55 (0.11-2.65) |
| $P_{trend}$ | | 0.19 | | 0.11 |
| **Ca:Mg ratio is above 2.5** | | | | |
| <600 | 7 | 1.63 (0.72-3.69) | 8 | 2.03 (0.88-4.65) |
| 600-1200 | 51 | 1.00 (Ref) | 38 | 1.00 (Ref) |
| 1200-1600 | 36 | 0.74 (0.46-1.18) | 33 | 0.96 (0.58-1.57) |
| ≥1600 | 55 | 0.82 (0.49-1.36) | 28 | 0.86 (0.47-1.60) |
| $P_{trend}$ | | 0.25 | | 0.29 |
| $P_{interaction}$ | | 0.03 | | 0.07 |

[1] Adjusted for age (continuous), sex, BMI (<25, 25-30, ≥30), education (less than high school, 12 years or completed high school, post high school training other than college, some college, college graduate, postgraduate), race (white, black, asian or others), family history of CRC (yes or no), cigarette (never smoked cigarettes, current or former), and total energy and vitamin D intake

[2] Assigned the score j to the $j^{th}$ level of calcium intake and evaluated the significance of Wald test

[3] Estimated interaction term of calcium intake and Ca:Mg ratio in Wald test

Supplemental Table 4.7 ORs[1] and 95% CIs for additional outcomes by calcium intake

| Ca Intake | Metachronous adenoma | | CRC in intervention arm | |
|---|---|---|---|---|
| (mg/day) | Cases | OR (95% CI) | Cases | HR (95% CI) |
| **No advanced/synchronous adenoma at baseline** | | | | |
| <600 | 37 | 1.92 (1.02-3.59) | 73 | 0.98 (0.74-1.30) |
| 600 -1200 | 98 | 1.00 (Ref) | 245 | 1.00 (Ref) |
| 1200 -1600 | 46 | 0.97 (0.57-1.66) | 107 | 0.86 (0.67-1.10) |
| ≥1600 | 41 | 0.97 (0.52-1.81) | 133 | 0.99 (0.76-1.28) |
| $P_{trend}$[2] | | 0.26 | | 0.80 |
| **Has advanced/synchronous adenoma at baseline** | | | | |
| <600 | 87 | 1.03 (0.70-1.52) | 21 | 1.46 (0.83-2.59) |
| 600 -1200 | 295 | 1.00 (Ref) | 51 | 1.00 (Ref) |
| 1200 -1600 | 144 | 1.01 (0.73-1.40) | 14 | 0.52 (0.26-1.00) |
| ≥1600 | 107 | 0.78 (0.54-1.14) | 17 | 0.66 (0.34-1.30) |
| $P_{trend}$[2] | | 0.28 | | 0.04 |
| $P_{interaction}$[3] | | 0.55[3] | | 0.09[4] |

[1] Adjusted for age (continuous), sex, BMI (<25, 25-30, ≥30), education (less than high school, 12 years or completed high school, post high school training other than college, some college, college graduate, postgraduate), race (white, black, asian or others), family history of CRC (yes or no), cigarette (never smoked cigarettes, current or former), hours spent in vigorous activities (less than1 hour/week, 1 hour/week, 2 hours/week, 3 hours/week, 4+ hours/week), and total energy and vitamin D intake

[2] Assigned the score j to the $j^{th}$ level of calcium intake and evaluated the significance of Wald test

[3] Estimated the full model with interaction term of calcium intake and Ca:Mg ratio and without this term in reduce model using likelihood ratio test

**B. Ca:Mg Ratio and lipid profile, a randomized clinical trial**

The participants from the magnesium supplementation arm did not significantly differ from those from the placebo arm on age, sex, body mass index, smoking, drinking, physical activity, education, anti-hypertensive and oral hypoglycemic medication use. A higher percentage of participants used lipid-modulating drugs in the placebo arm compared to the magnesium treatment arm (**Table 4.8**). The Kolmogorov-Smirnov test showed that the distribution of four lipid biomarkers was not normal (data not shown).

We found magnesium treatment did not significantly affect the lipid profile, including LDL-c, HDL-c, triglycerides, and total cholesterol (**Table 4.9**). Further, compared to the placebo arm, the treatment arm also did not significantly affect lipid profile. We further conducted analysis among participants with long-term high Ca:Mg ratios measured by two 24-hour dietary recalls and the previously administered FFQ (**Table 4.10**). We found magnesium treatment led to a significant increase of HDL-c level by 5 mg/dl (*P* value, <0.001), but no changes were found for LDL-c, triglycerides, and total cholesterol within the magnesium treatment group. In the placebo arm, LDL-c significantly decreased (*P* value, 0.044). Compared to the placebo arm, magnesium treatment significantly increased HDL-c (*P* value, 0.004) and total cholesterol (*P* value, 0.017) even after controlling for controlling for baseline lipid profile. After Bonferroni multiple comparison correction, only the effect on HDL-c remained statistically significant.

Finally, we conducted analysis among participants with recent high Ca:Mg ratio measures (i.e. >2.6 from two 24-hour dietary recalls, but <2.6 from FFQ conducted

earlier) **(Table 4.11).** We found within the magnesium treatment arm, magnesium treatment significantly reduced HDL-c by 3 mg/dl (*P* value, 0.025), but not others. In the placebo arm, levels of triglycerides significantly reduced (*P* value, 0.043). Compared to the placebo arm, magnesium treatment reduced HDL-c (*P* value, 0.099), but increased triglycerides (*P* value, 0.054) at borderline significance. After adjusting for baseline lipid profile, the effect on HDL-c still remained of borderline significance (*P* value, 0.055), although the significance disappeared after Bonferroni correction.

Table 4.8 Descriptive characteristics of the included population

| | Magnesium (N=76) | Placebo (N=74) | *P* value |
|---|---|---|---|
| Age | 60.4±8.2 | 62.0±7.8 | 0.303[1] |
| Sex - male (%) | 47 (62%) | 41 (55%) | 0.423[2] |
| Body mass index (BMI) | 29.5±6.2 | 29.9±6.1 | 0.533[1] |
| Waist to hip ratio | 0.97±0.06 | 0.97±0.05 | 0.5[1] |
| *TRPM* 7 Genotype - GG (%) | 61 (80%) | 56 (76%) | 0.519[2] |
| Smoking status (%) | | | 0.293[2] |
| Never | 35 (47%) | 44 (59%) | |
| Ever | 33 (44%) | 25 (34%) | |
| Current | 7 (9%) | 5 (7%) | |
| Drinking status (%) | | | 0.078[2] |
| Never | 21 (28%) | 32 (43%) | |
| Ever | 20 (26%) | 11 (15%) | |
| Current | 35 (46%) | 31 (42%) | |
| Physically active in ≥ 2 days per week (%) | 57 (75%) | 60 (81%) | 0.369[2] |
| Education under college (%) | 7 (9%) | 8 (11%) | 0.744[2] |
| Lipid-modulating medication use - yes (%) | 18 (24%) | 32 (43%) | 0.011[2] |
| Oral hypoglycemic drug use - yes (%) | 3 (4%) | 6 (8%) | 0.283[2] |
| Anti-hypertensive drug use - yes (%) | 33 (43%) | 35 (47%) | 0.634[2] |
| Ca:Mg ratio measured by FFQ | | | 0.813[2] |
| High (≥2.6) | 35 (46%) | 33 (45%) | |
| Low (<2.6) | 21 (28%) | 18 (24%) | |
| Not available | 20 (26%) | 23 (31%) | |
| Baseline Ca:Mg ratio | 3.5 (3.0-4.0) | 3.6 (3.0-4.1) | 0.875[1] |

For continuous variables: X±SD, median (the lower quartile-the upper quartile)
Tests used:[1]Wilcoxon test; [2]Pearson test

Table 4.9 Within and between group comparison for lipid biomarkers among all participants

| | N | Magnesium (N=76) | | | | Placebo (N=74) | | | | Between group | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Week 0 | Week 12 | Differences | $P^1$ | Week 0 | Week 12 | Differences | $P^1$ | $P^2$ | $P^3$ |
| LDL-c | 150 | 115 (95-153)<br>126±51 | 117 (92-150)<br>124±45 | -5 (-15-10.2)<br>-1.8±23.6 | 0.320 | 118 (94-140)<br>120±37 | 108 (88-142)<br>117±40 | -4.5 (-17.2-13.5)<br>-2.7±28 | 0.197 | 0.73 | 0.58 |
| HDL-c | 150 | 54 (44-71)<br>62±27 | 56 (44-72)<br>62±24 | 1.5 (-4-6)<br>0.1±10.1 | 0.403 | 56 (47-73)<br>61±20 | 55 (46-71)<br>60±19 | -1 (-5-5)<br>-0.7±9.2 | 0.627 | 0.38 | 0.53 |
| TG | 150 | 113 (82-168)<br>135±81 | 114 (83-164)<br>142±96 | 5 (-17-42.8)<br>6.6±57.4 | 0.238 | 119 (77-156)<br>133±75 | 110 (79-157)<br>133±78 | -3.5 (-28.8-15.3)<br>-0.9±50.3 | 0.575 | 0.23 | 0.38 |
| TC | 150 | 199 (164-255)<br>214±73 | 201 (168-250)<br>213±62 | 0.5 (-14.8-16)<br>-0.9±33 | 0.943 | 199 (173-234)<br>207±50 | 196 (169-228)<br>203±52 | -5.5 (-18.8-16.3)<br>-4.0±37.2 | 0.278 | 0.40 | 0.37 |

a (b-c) represent the median a, the lower quartile b, and the upper quartile c for continuous variables

$x \pm s$ represents $\bar{X} \pm SD$

LDL-c: low-density lipoprotein cholesterol, HDL-c: high-density lipoprotein cholesterol, TG: triglycerides, TC: total cholesterol

N is the number of non-missing values

$P^1$: the P value for within group differences using Wilcoxon signed rank test

$P^2$: the P value for between group differences using Wilcoxon rank-sum test

$P^3$: the P value for between group comparison while adjusting for baseline level for each biomarker in the generalized linear model

Table 4.10 Within and between group comparison for lipid biomarkers among long-term high ratio participants

| | N | Magnesium (N=35) | | | | Placebo (N=33) | | | | Between group | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Week 0 | Week 12 | Differences | $P^1$ | Week 0 | Week 12 | Differences | $P^1$ | $P^2$ | $P^3$ |
| LDL-c | 68 | 108 (86-140)<br>112±48 | 112 (90-139)<br>115±41 | 1 (-12-21)<br>2.5±27.3 | 0.473 | 120 (87-135)<br>117±37 | 104 (86-128)<br>108±35 | -11 (-23-8)<br>-8.8±21.8 | 0.044 | 0.035 | 0.066 |
| HDL-c | 68 | 53 (43-63)<br>54±19 | 56 (43-69)<br>58±20 | 5 (-0.5-8.5)<br>4.4±6.7 | <0.001 | 49 (47-60)<br>54±12 | 50 (46-59)<br>53±12 | -2 (-5-5)<br>-0.8±7.6 | 0.555 | 0.004 | 0.004 |
| TG | 68 | 107 (68-192)<br>142±99 | 114 (84-154)<br>151±118 | 8 (-12.5-39)<br>9±59.8 | 0.207 | 124 (84-162)<br>143±83 | 100 (77-201)<br>144±91 | -5 (-33-16)<br>0.7±58.1 | 0.668 | 0.270 | 0.569 |
| TC | 68 | 181 (152-236)<br>195±67 | 190 (165-244)<br>202±55 | 10 (-7.5-28)<br>7.5±33.1 | 0.118 | 195 (172-216)<br>200±49 | 186 (165-206)<br>189±45 | -12 (-37-10)<br>-10.2±30.7 | 0.072 | 0.019 | 0.017 |

a (b-c) represent the median a, the lower quartile b, and the upper quartile c for continuous variables

$x \pm s$ represents $\bar{X} \pm SD$

LDL-c: low-density lipoprotein cholesterol, HDL-c: high-density lipoprotein cholesterol, TG: triglycerides, TC: total cholesterol

N is the number of non-missing values

$P^1$: the P value for within group differences using Wilcoxon signed rank test

$P^2$: the P value for between group differences using Wilcoxon rank-sum test

$P^3$: the P value for between group comparison while adjusting for baseline level for each biomarker in the generalized linear model

Table 4.11 Within and between group comparison for lipid biomarkers among recent high ratio participants

| | N | Magnesium (N=21) | | | | Placebo (N=19) | | | | Between group | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Week 0 | Week 12 | Differences | *P*[1] | Week 0 | Week 12 | Differences | *P*[1] | *P*[2] | *P*[3] |
| LDL-c | 39 | 110 (96-131)<br>112±32 | 107 (85-133)<br>110±32 | -7 (-11-5)<br>-1.9±17.2 | 0.244 | 110 (88-137)<br>114±39 | 102 (81-139)<br>110±38 | -4.5 (-10.5-3)<br>-4.4±16.6 | 0.246 | 0.98 | 0.675 |
| HDL-c | 39 | 52 (44-64)<br>55±14 | 50 (44-60)<br>53±12 | -3 (-6-2)<br>-2.7±4.8 | 0.025 | 48 (44-64)<br>56±17 | 50 (42-70)<br>57±19 | -1 (-3.5-5.8)<br>1.3±7.7 | 0.861 | 0.099 | 0.055 |
| TG | 39 | 110 (81-154)<br>116±50 | 116 (77-163)<br>122±58 | 5 (-8-45)<br>6±43.5 | 0.444 | 131 (98-149)<br>132±52 | 109 (77-122)<br>111±48 | -13.5 (-39.5-2.2)<br>-20.3±43.1 | 0.043 | 0.054 | 0.115 |
| TC | 39 | 188 (162-227)<br>190±42 | 184 (164-217)<br>187±39 | 0 (-14-8)<br>-3.3±19 | 0.432 | 195 (169-217)<br>196±47 | 188(157-211)<br>189±44 | -4.5 (-17-2)<br>-7.2±24.8 | 0.133 | 0.47 | 0.685 |

a (b-c) represent the median a, the lower quartile b, and the upper quartile c for continuous variables

$\bar{x} \pm s$ represents $\bar{X} \pm 1SD$

LDL-c: low-density lipoprotein cholesterol, HDL-c: high-density lipoprotein cholesterol, TG: triglycerides, TC: total cholesterol

N is the number of non-missing values

P[1]: the P value for within group differences using Wilcoxon signed rank test

P[2]: the P value for between group differences using Wilcoxon rank-sum test

P[3]: the P value for between group comparison while adjusting for baseline level for each biomarker in the generalized linear model

### C. Ca:Mg ratio and uric acid, a randomized clinical trial

Overall, no significant difference in the change of uric acid from baseline to week 12 was observed in the magnesium treatment arm (**Table 4.12**). Among subjects with a long-term high Ca:Mg ratio, uric acid was lowered by 0.4 mg/dl within the placebo arm (*P* value, 0.011), which further resulted in a significant difference between magnesium treatment arm and placebo arm (*P* value, 0.026). However, this reduction is not significant after we adjusted for baseline uric acid (*P* value, 0.057).

Table 4.12 Within and between group comparison for uric acid levels

| Magnesium | | | | | Placebo | | | | | Between group | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| N | Week 0 | Week 12 | Differences | $P^1$ | N | Week 0 | Week 12 | Differences | $P^1$ | $P^2$ | $P^3$ |
| **All participants** | | | | | | | | | | | |
| 76 | 6.1 (4.6-6.5)<br>6.0±1.6 | 5.8 (4.9-6.8)<br>6.0±1.6 | 0 (-0.6-0.5)<br>0±0.9 | 0.886 | 74 | 5.9 (4.9-7.1)<br>6.2±1.6 | 5.7 (4.9-6.8)<br>6.0±1.6 | -0.1 (-0.8-0.5)<br>-0.1±1.0 | 0.29 | 0.44 | 0.457 |
| **Long-term high ratio participants** | | | | | | | | | | | |
| 35 | 5.5 (4.3-6.4)<br>5.5±1.3 | 5.5 (4.7-6.5)<br>5.6±1.4 | 0.1 (-0.6-0.6)<br>0.1±0.9 | 0.568 | 33 | 5.8 (4.8-7.3)<br>6.1±1.5 | 5.7 (4.9-6.2)<br>5.7±1.4 | -0.4 (-1.0-0.2)<br>-0.4±0.8 | 0.011 | 0.026 | 0.057 |
| **Recent high ratio participants** | | | | | | | | | | | |
| 21 | 6.3 (5.1-6.5)<br>6.1±1.3 | 6.0 (4.8-6.8)<br>6.0±1.4 | 0 (-0.4-0.3)<br>-0.1± 0.7 | 0.711 | 18 | 6.0 (5.0-6.7)<br>6.1±2.0 | 5.5 (4.8-6.6)<br>5.9±1.5 | -0.1 (-0.4-0.5)<br>-0.2±1.2 | 0.896 | 0.99 | 0.739 |

a (b-c) represent the median a, the lower quartile b, and the upper quartile c for continuous variables
$x \pm s$ represents $\bar{X} \pm SD$
N is the number of non-missing values
$P^1$: the *P* value for within group differences using Wilcoxon signed rank test
$P^2$: the *P* value for between group differences using Wilcoxon rank-sum test
$P^3$: the *P* value for between group comparison while adjusting for baseline level for each biomarker in the generalized linear model

# Chapter 5. Discussion and summary

## A. Project A-discussion

In the current study, we found higher intakes of calcium, compared to low calcium intake (<600 magnesium/day), were not related to a reduced risk of incident adenoma, but were associated with a suggestive reduced risk for advanced/synchronous adenomas. The inverse association primarily appeared in subjects with Ca:Mg intake ratios between 1.7 and 2.5. Although calcium intake was not related to risk of metachronous adenoma regardless of initial adenoma characteristic and Ca:Mg intake ratio, we found higher intakes of calcium were associated with a reduced risk of incident distal CRC. Further, the inverse association with incident distal CRC was also primarily present in those with Ca:Mg intake ratios ranging from 1.7 to 2.5. Moreover, we found the inverse association was only significant among control arm without regular endoscopy, and became weaker in the intervention arm with regular endoscopy. We found the inverse association was only significant for those with baseline advanced/synchronous adenomas. To our knowledge, this is the first study to examine whether regular endoscopy use modifies the association between calcium and incident CRC risk.

Both cohort studies and earlier intervention trials relatively consistently found high calcium intake or calcium supplementation was related to moderately reduced risks of adenoma, metachronous adenoma and CRC [18–20,22,23,25,299,300]. However, results from a very recent large-scale randomized clinical trial (WHI) do not support an effect of calcium plus vitamin D supplementation on the incidence of CRC after 7 years of follow-

up [15]. Very recently, Baron et al reported from a large scale randomized trial that calcium supplementation did not reduce risk of colorectal metachronous adenoma [16].

It is possible the follow-up time in the WHI was not sufficiently long to observe an effect. CRC is believed to arise, in the overwhelming majority of cases, from adenomas via the well-established adenoma-carcinoma sequence [301]. Our findings from the current study may provide possible explanations for the inconsistent results [125,126] in the previous studies on the effect of calcium on the colorectal carcinogenesis [17–20]. Earlier studies hypothesized that the chemoprevention effect of calcium intake on CRC may primarily exert in its early stages (adenoma) [15]. Our findings are consistent with previous epidemiologic data [21,22] indicating higher calcium intake may only inhibit early colorectal carcinogenesis at the stage of incident adenoma [17–20,300] and the association may be stronger for prevention of advanced adenoma, a premalignant lesion for CRC [4], than other types of adenoma or polyps [302]. The possibility is consistent with the observation that the magnitude of reduction in overall CRC risk associated with high calcium intake is similar to the reduction in adenoma risk.

We did not find calcium intake was related to risk of metachronous colorectal adenoma in the current study. However, the sample size, and thus statistical power, is smaller for this outcome than for the analyses of incident adenoma and incident cancer. Although earlier randomized trials found calcium supplementation reduced risk of colorectal metachronous adenoma [23], a recent trial did not find calcium supplementation reduced risk of metachronous colorectal adenoma [16]. As such, other underlying factors may

account for the inconsistency between these randomized trials such as the change of the Ca:Mg intake ratio over the time. The Ca:Mg intake ratio has increased from about 2.6 [303] during the period the earlier trials [23,299] were conducted to over 3 when the recent randomized trial was conducted [16].

We found in the current study that the Ca:Mg intake ratio modifies the association between calcium intake and development of adenoma and CRC . We also found the significant inverse associations between calcium intake and advanced/synchronous adenomas, or distal CRC may primarily appear in those with Ca:Mg intake ratios between 1.7 and 2.5. We reported earlier that the dietary intake ratio of Ca:Mg modified the association between intakes of calcium and magnesium in relation to prevalent colorectal adenoma [154]. In a subsequent randomized clinical trial, calcium supplementation only reduced risk of metachronous colorectal adenoma when the baseline Ca:Mg was below 2.63 [26]. Very recently, we found that Ca:Mg ratio modified the associations between intakes of calcium and magnesium and risk of esophageal neoplasia [27]. Consistent with our finding, one case-control study conducted in Belgians reported that a high calcium intake with a low magnesium intake was associated with increased risk of bladder cancer [144]. In studies conducted in East Asian populations with a low Ca:Mg intake ratio (a median around 1.7), we found the association between intakes of calcium and magnesium with the risk of total, cardiovascular and/or cancer mortalities were modified by Ca:Mg ratio, but not by calcium or magnesium intake alone [28]. Thus, the balance between calcium and magnesium intake is an important factor to

consider in the investigation of associations between intakes of calcium and magnesium and cancer development.

In addition to the Ca:Mg intake ratio, we found the inverse association between calcium intake and risk of incident distal CRC was stronger in the control arm compared to the intervention arm with multiple flexible sigmoidoscopies. These findings suggested the association between calcium intake and colorectal carcinogenesis may be attenuated by endoscopy screening and the removal of polyps with an endoscopy, which is the most effective prevention strategy to reduce the morbidity and mortality of CRC [29].

The protective association with higher calcium intake was mainly in distal CRC, with approximately 30-50% reduced risk. This finding is consistent with a meta-analysis of 17 cohort studies, in which the protective effect of calcium intake was more evident for distal colon cancer compared with proximal colon tumors (33% vs 25% of risk reduction) [12]. Previous etiologic studies indicated proximal and distal sections differ in physiological functions, fecal composition and transit, as well as suggested that environmental factors such as diet mainly affect distal colon carcinogenesis [304].

No previous study has prospectively evaluated the associations of calcium intake with incident adenoma, metachronous adenoma, and incident cancer in the same cohort using the same food frequency questionnaire, which minimizes misclassification that can occur when combining dietary estimates from multiple studies with different food

frequency questionnaire instruments. The PLCO screening trial provides a unique and unparalleled opportunity to prospectively and concurrently evaluate these associations. One weakness is that the PLCO is a sigmoidoscopy-based randomized trial, which only screened distal colorectum. Thus, incident adenoma cases mainly included adenomas from the left side of the bowel although metachronous adenoma and incident CRC cases included cases from both proximal and distal colorectum. However, it is interesting that recent reports found that colonoscopy was linked to reduced deaths from distal colorectum, but not the right [30,32] while similarly a recent report from the PLCO observed sigmoidoscopic screenings only reduced distal, but not proximal CRC mortality [31]. Further, sigmoidoscopic screenings also significantly reduced both distal and proximal CRC incidence in the PLCO [31]. Thus, the PLCO provides a unique opportunity to test whether calcium intake confers additional protection against proximal and distal CRC among those receiving sigmoidoscopic screening(s). Similar to other nutritional epidemiological studies using food frequency questionnaire, there are possibly non-differential measurement errors which may bias the results to the null.

In conclusion, higher calcium intake may primarily be related to reduced risks of incident advanced/synchronous adenoma and incident distal CRC among subjects with Ca:Mg intake ratios between 1.7 and 2.5, or participants without regular endoscopy and those with advanced/multiple adenomas at baseline.

**B. Project B-discussion**

In this study, we found overall magnesium supplementation did not affect lipid profile. However, among individuals whose Ca:Mg intake ratios were consistently higher than 2.6, reducing the Ca:Mg ratio through magnesium treatment significantly led to a beneficial effect on HDL-c (i.e. an increase by 5 mg/dl). This change remained significant after adjusting for baseline HDL-c and multiple comparisons. Conversely, among subjects with a Ca:Mg intake ratio which varied over time (i.e. <2.6 from FFQ, but >2.6 in two 24-hour dietary recalls), reducing the Ca:Mg ratio through magnesium treatment may have reduced HDL-c, although the change was not significant after correcting for multiple comparison.

Although not consistent [315,316], earlier clinical trials indicated an increase of HDL-c by 3 to 4 mg/dl with oral magnesium supplementation among diabetic patients [311]. Similarly, a more recent meta-analysis of clinical trials among participants with diabetes found magnesium significantly increased HDL-c by around 3 mg/dl, and had no effect on LDL-c, triglycerides, and total cholesterol [212]. Consistent with these studies, our results also suggested null effects of magnesium treatment on LDL-c, triglycerides, and total cholesterol, although previous clinical trials [310,311] generated inconsistent results. These findings are biologically plausible since both patients with type 2 diabetes [312] and individuals with high Ca:Mg intakes ratio (i.e. ratios >2.6) [17,26,27] are at high risk of magnesium deficiency. On the other hand, we previously found among those with very low Ca:Mg intake ratios (i.e. ratio<1.7), high intakes of magnesium were related to an increased risk of total mortality or mortality due to cardiovascular disease [28]. This may

explain in our study further reducing the Ca:Mg ratio via magnesium supplementation may even decrease HDL-c among those with inconsistent ratios measured by FFQ (i.e. ratio<2.6).

It is possible that previous trials of magnesium supplementation enrolled subjects with both high and low Ca:Mg ratios; the high doses of magnesium treatment generally used in these trials may have led to a very low Ca:Mg ratio (<1.7), particularly for those with a low ratio at baseline [212]. The modifying effect of the Ca:Mg ratio may explain the inconsistent results in previous clinical trials of magnesium supplementations. Likewise, the findings for dietary and supplement calcium [232,233] in relation to cardiovascular disease have also been inconsistent. It is likely that dietary sources of calcium are usually accompanied by high dietary magnesium intake, and this, in turn, leads to a moderate Ca:Mg ratio from dietary sources. In contrast, calcium supplementation alone substantially increases the Ca:Mg ratio. This may explain the differential effects of calcium from dietary [234,235] and supplemental sources [232,233] on cardiovascular disease risk and mortality. Some recent randomized trials found high calcium supplementation led to increased risk of myocardial infarction [308]. Therefore, instead of considering absolute calcium and magnesium alone, the Ca:Mg ratio is another critical factor in lipid metabolism as well as other physiologic activities.

The body's calcium and magnesium status is an integrated function of oral intake, intestinal absorption, renal reabsorption and skeletal remodeling [101]. Calcium and magnesium have common food sources and are considered to be "metabolically

interactive" as well [141]. Calcium and magnesium may share the same homeostatic regulation system [45] primarily through absorption in the intestine and reabsorption in the kidneys to maintain the balance of Ca:Mg [45,46]. Nevertheless, calcium and magnesium compete with each other in (re)absorption and transport [47–51]. A high calcium intake reduces absorption of both magnesium and calcium [145] whereas moderate magnesium deprivation results in negative magnesium balance but increased calcium retention [146]. In addition, magnesium supplementation increased urinary calcium excretion if calcium intake was less than 800 mg/day [148], suggesting that magnesium may suppress calcium reabsorption when Ca:Mg intake ratio is very low [28]. Therefore, the Ca:Mg intake ratio may have an important role in regulating calcium and magnesium (re)absorption. A high Ca:Mg intake ratio implies relatively high calcium but low magnesium intake; thus, magnesium supplementation is essential for lowering the already-high calcium status among participants with a high Ca:Mg ratio.

Although potential mechanisms of magnesium deficiency on the development of cardiovascular disease is not completely understood, animal studies have suggested that magnesium intake is highly related to atherogenic process [317]. Specifically, magnesium affects apolipoproteins and enzymes involved in lipid metabolism [317]. The enzyme activity of lecithin cholesterol acyltransferase (LCAT) [240], lipoprotein lipase (LPL) [202], and 2-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase [244], requires the involvement of magnesium [241]. In the pathway of cholesterol biosynthesis, HMG-CoA reductase is a rate-limiting enzyme to transform HMG-CoA to mevalonate [244]. This conversion process could be affected by statins and regulated by magnesium [244].

Magnesium supplementation among participants with a long-term high Ca:Mg ratio may their improve magnesium deficiency or insufficiency and subsequently change the overall cholesterol biosynthesis process. Therefore, we observed a significant increase of HDL-c.

Participants in this study are otherwise healthy volunteers with previous diagnoses of hyperplastic polyps or colorectal adenomas, which is different from those reported in previous studies [318–320]. The baseline levels of lipid profile for the participants are close to the normal range. In this study, the average of HDL-c is around 55 mg/dl and LDL-c is around 115 mg/dl. Thus, a 5 mg/dl increase in HDL-c may be more beneficial than a decrease in LDL-c among this group of participants, which has very significant implications for the prevention of cardiovascular disease. It is possible that the "regression to the mean" effect exists. Thus, we further adjusted for baseline level of lipid profile in final models. Serum calcium and magnesium is strictly regulated by the homeostasis system [231]. Thus, serum calcium and magnesium are not sensitive markers of total body calcium and magnesium status. In our study, dietary intake and supplemental use of calcium and magnesium were used as a surrogate of total body calcium and magnesium status. However, these measurements have not taken into account gastrointestinal absorption.

In conclusion, reducing the high Ca:Mg ratio through magnesium supplementation may increase HDL-c in participants with long-term high Ca:Mg intake ratios, but may have a detrimental effect among subjects with Ca:Mg ratios <2.6 or not remaining stable. If this

finding is confirmed, it provides a novel and inexpensive approach for the prevention of cardiovascular disease.

**C. Project C-discussion**

In this randomized trial, reducing Ca:Mg ratios through magnesium supplementation did not affect concentrations of serum uric acid. Previously, we found the Ca:Mg intake ratio modified the associations between intakes of calcium and magnesium and risk of gastrointestinal neoplasia [17,26,27], total, cardiovascular disease and cancer mortalities [28]. No previous studies have investigated the effect of the Ca:Mg ratio on uric acid. To our knowledge, this is the first clinical trial to investigate whether reducing the Ca:Mg intake ratio through magnesium supplementation affected uric acid. Our findings indicated the Ca:Mg ratio may not affect risk of cancer or cardiovascular disease by modifying uric acid. Two previous studies found that magnesium status was correlated with uric acid status [275,322]. However, these are cross-sectional studies which are difficult to determine their temporal relationships. It is possible that uric acid is correlated with lipid profiles, inflammation biomarkers and other pathways. Future studies should evaluate whether the Ca:Mg ratio may affect pathways such as lipid profiles [323] as well as inflammatory responses [324,325]. We also cannot exclude the possibility that the null result in the current study is due to a small sample size so future larger studies may be needed.

**D. Summary, conclusions and future directions**

CRC, a worldwide health burden, is the 3rd most common incident cancer and the 3rd leading cause of cancer death in the United States [55]. A few modifiable risk factors were

associated with colorectal carcinogenesis. Although it is inconsistent, good behavioral factors which seem to decrease risk include being physically active, eating healthy, keeping an optimal body mass index (18.5 to 25), abstinence from smoking, no to light alcohol consumption, hormonal replacement therapy for postmenopausal women, regular endoscopy screening to remove polyps, aspirin [326,327] and other non-steroidal anti-inflammatory drugs [88] would potentially prevent against CRC [67].

Calcium, a chemopreventive nutrient, was suggested to reduce the risk of incident adenoma [17–20], metachronous adenoma [23–25] and cancer [21,22]. The Ca:Mg intake ratio was suggested to modify the associations between intakes of calcium and magnesium and risk of gastrointestinal neoplasia [17,26,27], and total, cardiovascular disease and cancer mortalities [28]. Previous studies also indicated that a ratio between 1.7 and 2.6 is potentially the range needed for calcium intake to be protective against these outcomes [17,26–28]. It is plausible that calcium intake and the Ca:Mg intake ratio play an essential role in the development of CRC .

We evaluated the associations between calcium intake and the risks of incident colorectal adenoma (1,147 cases), metachronous adenoma (855 cases) and incident CRC (697 and 578 cases in intervention and control arms, respectively) among 108,563 PLCO participants aged 55 to 74 years. Compared to low calcium intake (<600 mg/day), higher intakes of calcium were not related to a reduced risk of incident or metachronous colorectal adenoma, but were related to a suggestive reduced risk of advanced and/or synchronous adenomas and to a significantly reduced risk of CRC, especially for distal

CRC. The inverse association was primarily apparent in participants whose Ca:Mg ratios ranged from 1.7 to 2.5. In addition, the statistically significant association between calcium intake and risk of incident distal CRC appeared to be primarily present in the control arm without regular endoscopy.

The 150 pairs of pre- and post-treatment serum samples of participants in the PPCCT study who finished the trial were sent to the Vanderbilt Lipid Laboratory assaying of lipid panel and uric acid. Overall, magnesium treatment did not significantly affect the levels of the lipid profile including LDL-c, HDL-c, triglycerides, and total cholesterol. Among participants with a long-term high Ca:Mg ratio, magnesium supplementation led to a statically significant increase of HDL-c level by 5 mg/dl. No significant difference in the change of uric acid from baseline to week 12 was observed in the magnesium treatment arm.

This dissertation suggests higher calcium intake may primarily be related to reduced risks of incident advanced and/or synchronous adenoma as well as incident distal CRC among subjects with Ca:Mg intake ratios between 1.7 and 2.5, or participants without regular endoscopy and those with advanced and/or synchronous adenomas at baseline. Thus, this study adds additional evidence to understand associations between calcium intake and risk of CRC development at various stages. Previous studies suggested that the association between intakes of calcium and magnesium with the risk of total, cardiovascular and/or cancer mortalities were modified by Ca:Mg ratio, but not by calcium or magnesium intake alone [28]. In this study, the potential modifying effect of

Ca:Mg intake ratios was further suggested. It is possible that lack of considering Ca:Mg balance may explain some of the inconsistency in previous studies on either calcium or magnesium intake. Moreover, the association between calcium intake and risk of CRC appears to be stronger in individuals without endoscopic screenings. To our best knowledge, this is the first study to examine whether regular endoscopy use modifies the association between calcium intake and incident CRC risk.

Recent studies indicate that Ca:Mg intake ratio may modify the associations between intakes of calcium and magnesium and risk of gastrointestinal neoplasia [17,26,27], and total, cardiovascular disease and cancer mortalities [28]. This study found that reducing a high Ca:Mg ratio through magnesium supplementation may increase HDL-c in participants with long-term high Ca:Mg intake ratios. A meta-analysis of nine randomized trials conducted among type 2 diabetic patients, individuals at high risk of magnesium deficiency [312], showed that oral magnesium supplementation significantly, but weakly, increased HDL-c by 0.08 mmol/l, and had no effect on LDL-c and triglycerides [212]. Our study is consistent with this meta-analysis and found a stronger effect of magnesium supplement when the Ca:Mg ratio is considered. These human findings are consistent with the evidence from animal studies indicating that calcium-adequate and magnesium-deficient diets lead to increases in heart lipid peroxidation, plasma levels of triglycerides, as well as inflammatory markers; whereas both calcium-deficient and magnesium-deficient diets normalize the levels of inflammatory markers, lipid peroxidation and plasma triglycerides concentration [52,53].

Both metabolic syndrome and deficits of calcium or magnesium are potential risk factors of colorectal neoplasia. However, no studies have examined whether the Ca:Mg ratio may have a role in uric acid metabolism nor whether uric acid is an intermediate between the association of the Ca:Mg ratio and colorectal neoplasia. Our findings indicated the Ca:Mg ratio may not affect risk of cancer or cardiovascular disease by modifying uric acid. Future studies should evaluate whether the Ca:Mg ratio may affect biomarkers such as lipid profiles [323] and uric acid. We also cannot exclude the possibility that the null result for uric acid in the current study is due to a small sample size so future larger studies may be needed.

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