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Circulating insulin-like growth factor-related biomarkers: Correlates and responses to calcium supplementation in colorectal adenoma patients

Caroline Y. Um, PhD¹, Veronika Fedirko, PhD^{1,2,3}, W. Dana Flanders, MD, DSc^{2,3}, Christine Höflich, PhD⁴, Elisa Wirthgen, PhD^{4,5}, and Roberd M. Bostick, MD, MPH^{1,2,3}

¹Nutrition and Health Sciences Program, Emory University, Atlanta, GA

²Department of Epidemiology, Emory University, Atlanta, GA

³Winship Cancer Institute, Emory University, Atlanta, GA

⁴Ligandis GbR, Gülzow, Germany

⁵Institute of Genome Biology, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany

Abstract

Circulating insulin-like growth factor 1 (IGF-1) may be directly associated with colorectal cancer risk, and IGF binding protein 3 (IGFBP-3) is one of the most abundantly expressed binding proteins in various cancers. Calcium intakes, primarily from food, have been directly associated with circulating IGF-1, but whether supplemental calcium affects IGF-1 and IGFBP-3 is unknown. We tested the effects of 1.0 and 2.0 grams of supplemental elemental calcium daily on circulating IGF-1 and IGFBP-3 concentrations in colorectal adenoma patients in a randomized, double-blinded, placebo-controlled clinical trial (n=193). IGF-1 and IGFBP-3 were quantified using enzyme-linked immunoassay and quantitative Western Ligand blot, respectively. We also assessed cross-sectional associations of these biomarkers with participants' baseline characteristics. We found no appreciable effect of calcium relative to placebo on circulating IGF-1, IGFBP-3, or the IGF-1:IGFBP-3 molar ratio. Mean IGF-1 concentrations were 11.1% higher in those with greater milk intakes ($P=0.05$). Mean IGF-1 and IGFBP-3 concentrations were, respectively, 18.0% ($P=0.003$) and 16.5% ($P=0.01$) higher in men and were monotonically lower with increasing age (both $P=0.01$). IGFBP-3 was 17.7% higher among those with higher relative to no alcohol consumption ($P=0.04$). While these results support previous findings that IGF-1 concentrations are higher with greater milk intakes, and IGF-1 and IGFBP-3 concentrations differ according to sex and age, they provide no evidence to suggest that supplemental calcium appreciably affects circulating IGF-1, IGFBP-3, or the IGF-1:IGFBP-3 molar ratio in sporadic colorectal adenoma patients.

Corresponding author and reprint requests: Dr. Roberd M. Bostick, 1518 Clifton Road NE, Atlanta, GA, 30322, Work phone: 404-727-2671, Fax: 404-727-8737, rmbosti@emory.edu.

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Keywords

insulin-like growth factor 1; colorectal neoplasms; milk; clinical trial

INTRODUCTION

Circulating insulin-like growth factor 1 (IGF-1) concentrations were directly associated with risk for colorectal, breast, and prostate cancers in previous observational studies.^{1–4} IGF-1 is an essential growth factor, critical for normal growth and maintenance of homeostasis, but may potentially act as a potent mitogen, anti-apoptotic peptide, and promoter of angiogenesis when overexpressed.⁵ Circulating IGF-1 is predominantly bound to insulin-like growth factor binding protein 3 (IGFBP-3) to increase stability and regulate distribution to tissues.⁶ There is no consistent evidence that IGFBP-3 levels are associated with risk of colorectal cancer but may be an indicator of bioavailable IGF-1.

Colorectal cancer is the second leading cause of cancer deaths among men and women combined in the United States.⁷ Calcium has been consistently associated with lower risk of colorectal neoplasms,^{8–11} and supplemental calcium reduced sporadic colorectal adenoma recurrence in two^{12,13} of three^{12–14} large randomized controlled trials. The hypothesized mechanisms of calcium's anti-neoplastic action are through its binding of secondary bile acids and free fatty acids in the colon, which reduces epithelial cell exposure to their damaging effects,^{15,16} and by directly inhibiting proliferation and inducing differentiation of colonic epithelial cells.^{17,18}

Milk products are a common source of dietary calcium in the average American diet, and tend to be inversely associated with risk of colorectal cancer, similar to calcium.^{8,19} However, clinical trials suggest that milk product consumption may increase circulating IGF-1 levels, and cross-sectional studies reported positive associations of milk consumption with IGF-1 concentrations.^{20,21} This may be attributable to higher levels of IGF-1 measured in milk produced from cows administered bovine somatotropin (bST) hormone (the most commonly consumed milk in the US), in comparison to bST-free or organically produced milks.²² However, calcium may also be obtained from supplemental sources, and to our knowledge, whether supplemental calcium affects circulating IGF-1 and IGFBP-3 concentrations is unknown, and no clinical trial tested this. Thus, we tested the effects of 1.0 and 2.0 g of supplemental elemental calcium on IGF-1 and IGFBP-3 concentrations in colorectal adenoma patients in a randomized, double-blinded, placebo-controlled trial.

MATERIALS AND METHODS**Participant Population**

For this investigation, we used data and stored blood samples from a randomized, double-blinded, placebo-controlled, three-arm, parallel-group clinical trial of calcium supplementation conducted in Minneapolis-St. Paul, Minnesota from 1990 – 1993. The recruitment methods and study protocol of this trial were previously described.^{23,24} Briefly, patients 30–74 years of age with a history of a pathology confirmed colorectal adenomatous

polyp within the previous 5 years were recruited from a large, private gastroenterology practice. Eligible participants were in good health and must have consumed a western-style diet that included meat and no more than 1,100 mg of calcium daily. Additional exclusion criteria included current use of calcium supplements; contraindications to calcium supplementation or rectal biopsies; clinical conditions, dietary habits, or medication that would otherwise affect safety, adherence, or interpretation of the study results; or less than 80% adherence to a study pill-taking in a 4-week placebo run-in trial.

Clinical Trial Protocol

As previously described,²³ of the patients who were invited for an eligibility visit and subsequently completed the placebo run-trial, 193 were randomized. Participants underwent a baseline visit and were randomly assigned to one of three treatment groups: a placebo control group (n = 66) and 1.0 g (n = 64) and 2.0 g (n = 63) elemental calcium supplementation groups. Randomized group assignment was blinded to all participants, study personnel, and laboratory staff. The calcium carbonate and placebo tablets (prepared by SmithKline Beecham, Pittsburgh, PA) were administered twice daily with meals, and all pills were identical in size, appearance, and taste.

Medical history, medication use, and information on demographic, dietary, and lifestyle factors were collected at the eligibility and baseline visits via self-administered questionnaires. Usual dietary intakes over the previous 12 months were collected using a 153-item Willett semi-quantitative food-frequency questionnaire (FFQ). The treatment period was 6 months with follow-up visits at 1, 2, 4, and 6 months. Participants were instructed to follow their usual diets, and pill-taking adherence was assessed at each follow-up visit by interview, questionnaire, and pill count. Non-fasting venous blood samples were collected only at the baseline and 4-month follow-up visits. Participants were comfortably seated in a chair for 5 minutes with both feet on the floor before venipuncture. Blood was drawn into pre-chilled Vacutainer tubes and immediately placed on ice and shielded from light. The blood was immediately processed, plasma and serum were aliquoted into separate cryopreservation tubes, air was displaced with nitrogen, and the aliquots were immediately placed in a -80°C freezer for storage. During the course of the trial, duplicate blood samples were collected on 35 participant visits as blinded quality control samples.

Laboratory Protocol

IGF-1 was measured at Ligandis laboratory (Ligandis GbR, Gülzow, Germany) in October 2015 using the solid-phase sandwich enzyme-linked immunoassay (Mediagnost). Briefly, serum samples and recombinant human standards were added to wells pre-coated with human IGF-1 monoclonal antibody and incubated. Unbound material was removed by washing, and streptavidin-horseradish peroxidase (HRP) conjugated anti-human IGF-1 antibody was added. Wells were washed again to remove unbound streptavidin-HRP, and a substrate solution reactive with HRP was added. After incubation, stop solution was added, and absorbance was measured using an automated ELISA reader (Dynex technologies OPSYSMR).

Insulin-like growth factor binding protein 2 (IGFBP-2) and IGFBP-3 concentrations were also measured at Ligandis laboratory in April 2014, using quantitative Western Ligand blot to ensure detection of intact IGFBPs. For this analysis, serum samples and serial dilutions of recombinant human IGFBP standards (R&D Systems, Wiesbaden, Germany) in artificial serum matrix were diluted 1:20 and boiled in sample buffer [312.5 mM Tris (pH 6.8), 50% (w/v) glycerol, 5 mM EDTA (pH 8), 1% (w/v) SDS and 0.02% bromophenol blue] for 5 minutes. Proteins were separated by 12% SDS-PAGE followed by the transfer onto a polyvinylidene fluoride membrane (Millipore, Bedford, USA). The blots were blocked and then incubated with biotin-labeled human IGF-II (1:500; BioIGF2-10; ibt-systems, Binzwangen, Germany). The binding proteins were detected by enhanced chemiluminescence using LuminataTM Forte (Millipore, Bedford, USA). Bands were visualized on a KODAK Image Station 4000MM (Molecular Imaging Systems, Carestream Health, New Haven USA) and quantified by using Gelanalyzer2010a software. Signal intensities were corrected for background and quantified using human recombinant standards as calibrators on each blot (R&D Systems, Wiesbaden-Nordenstadt, Germany). Curve fitting was performed by using a four parametric nonlinear regression (HILL equation) of each separate IGFBP. The calculation of the IGFBP concentrations in serum was performed using the software GraphPad Prism6 and corrected for dilution and volume/ lane of each sample.

All samples were measured in duplicate, and technicians were blinded to treatment assignment. The between- and within-plate coefficients of variation (CVs) for IGF-1 were < 6.8% and < 6.7%, respectively, and the CVs for low and high quality control IGFBP-3 samples were 19.2% and 14.9%, respectively. All but two of the 35 blinded duplicate quality control samples were measured in different IGF-1 plates or IGFBP-2 and 3 batches than the samples to which they were paired; for these duplicate pairs, the CVs for IGF-1 and IGFBP-3 were 14.7% and 22.7%, respectively. IGFBP-2 concentrations were below the limit of detection in 39 samples; the CVs for low and high quality control IGFBP-2 samples were 21.4% and 6.7%, respectively; and the CV for the duplicate pairs was 53%. Based on the large number of samples with values below the lower limit of detection and the large CV for the duplicate pairs, we excluded IGFBP-2 from subsequent data analyses.

The analytical range of IGFBP-3, defined by the highest and lowest concentration of recombinant standard used, was 159 – 10,000 ng/mL. The lower limit of quantification was 962.37 ng/mL for IGFBP-3, and the analytical sensitivity for IGF-1 was 0.09 ng/mL.

Statistical Analysis

Baseline characteristics of the treatment groups were compared using chi-square or Fisher's exact tests for categorical variables and analysis of variance (ANOVA) for continuous variables.

Of the initial 193 participants, blood samples for measurement of growth factors were available for 189 participants at baseline and 175 at follow-up. Primary analysis was based on the original treatment group assignment at randomization, regardless of adherence (intent-to-treat). IGF-1 and IGFBP-3 values were log-transformed before statistical testing to improve normality. Mean growth factor parameters were calculated for each treatment group

at baseline and 4 month follow-up. The molar ratio of IGF-1 to IGFBP-3 was calculated prior to log-transformation using the mass to molar conversion of 1 ng/mL IGF-1 = 0.130 nM for IGF-1 and 1 ng/mL IGFBP-3 = 0.036 nM IGFBP-3.

Treatment effects were estimated using a repeated-measures mixed linear model implemented using SAS Institute's Mixed Procedure (PROC MIXED) (SAS version 9.4 software; SAS Institute Inc., Cary, North Carolina). The model included visit, treatment groups, and a treatment group by visit interaction term. Because the growth factor values were natural log transformed, the visit- and treatment-specific geometric means, and the relative treatment effects, defined as $([\text{treatment group follow-up mean}] / [\text{treatment group baseline mean}]) / ([\text{placebo follow-up mean}] / [\text{placebo baseline mean}])$, were obtained directly from the Mixed model output. We also manually calculated the absolute treatment effect, defined as the "difference of differences," or $([\text{treatment group follow-up mean}] - [\text{treatment group baseline mean}]) - ([\text{placebo follow-up mean}] - [\text{placebo baseline mean}])$, using the geometric means for each treatment group.

We also conducted a cross-sectional analysis using multivariable-adjusted generalized linear models to investigate whether baseline concentrations of IGF-1 and IGFBP-3 were associated with sex, age, body mass index (BMI), regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) or aspirin, alcohol consumption, total energy intake, total fat intake, calcium intake, and red or processed meat consumption. Additionally, associations with milk product intakes were of particular interest, especially the non-calcium (and non-fat) component of milk, which contains IGF-1 as discussed above. To estimate the latter, we used residuals from linear regression models of total dairy and non-fat milk (as the dependent variables) with dietary calcium intake (as the independent variable). This method was modeled after the energy adjustment residual method.²⁵ Multivariate models were adjusted for sex, age, BMI, total energy intake, supplemental and dietary calcium as appropriate.

A two-sided *P* value of < 0.05 or a 95% confidence interval that does not include 1.00 was considered statistically significant. All statistical analyses were performed using SAS version 9.4 software (SAS Institute Inc., Cary, North Carolina).

RESULTS

At baseline, the mean age of the 193 study participants was 59 years, 63% were men, and 99% were white. Participants also consumed, on average, 800 mg of dietary calcium and 2.2 servings of dairy products daily. The treatment groups did not differ significantly on demographic, lifestyle, or dietary characteristics (Table 1). Adherence to follow-up visit attendance averaged 95.3% and did not differ among treatment groups. In each group, more than 98% of participants took 80% or more of their pills, and the mean percentage of pills taken was 97%.

The mean concentrations of IGF-1, IGFBP-3, and the IGF-1:IGFBP-3 molar ratio did not differ among treatment groups (Table 2). There were no appreciable effects of either 1g or 2g calcium supplementation on any of the growth factor concentrations. We also stratified by

age, sex, BMI, NSAID or aspirin use, smoking status, alcohol use, physical activity level, total energy intake, and dietary calcium intake, but the estimated treatment effects were similarly null across the strata (data not shown).

To gain potential insight into whether the null treatment effects were valid or possibly due to the effects of long-term storage on the blood samples, we analyzed baseline associations of participant characteristics with IGF-1, IGFBP-3, and the IGF-1:IGFBP-3 molar ratio (Table 3). IGF-1 and IGFBP-3 concentrations were 14.9% ($P = 0.02$) and 16.5% ($P = 0.01$) higher in men, respectively, and both were lower with increasing age ($P = 0.02$ and $P = 0.01$, respectively). IGF-1 concentrations were also 11.1% higher among participants in the higher total milk category ($P = 0.05$), but only an estimated 6.1% higher in the upper relative to the lower tertile of non-fat milk residuals ($P = 0.39$). IGFBP-3 concentrations were 17.7% higher among participants consuming 7 or more alcoholic drinks per week compared to those consuming none ($P = 0.04$); however, concentrations were not higher among those with moderate consumption. IGFBP-3 concentrations were also 13.0% lower in the upper tertile of total milk product residuals ($P = 0.04$), and IGF-1 was 7.3% lower in the upper relative to the lower tertile of total milk product residuals ($P = 0.28$).

DISCUSSION

In this analysis of a dose-response trial of calcium supplementation, we found no appreciable effects of either 1 or 2g elemental calcium daily on circulating concentrations of IGF-1, IGFBP-3, or the IGF-1:IGFBP-3 molar ratio in sporadic colorectal adenoma patients over a 4-month treatment period. However, consistent with previous findings, IGF-1 concentrations were higher with greater milk intakes, and IGF-1 and IGFBP-3 concentrations were lower in females and inversely associated with age.

To our knowledge, there are no previous clinical or epidemiological reports regarding a potential effect on or association of calcium supplementation with IGF-1 and IGFBP-3 concentrations among adenoma patients. However, four previously conducted placebo-controlled trials of calcium supplementation and bone health investigated changes in IGF-1 levels as secondary outcomes of interest. In two of the trials, no effect of calcium supplementation on IGF-1 concentrations among postmenopausal women was found after follow-up periods of 6 months and 2 years.^{26,27} In a third trial among 18 Japanese teenage girls over 8 months, only within-treatment group changes in IGF-1 levels were reported.²⁸ However, by our calculations, there was an estimated approximately 16% increase in IGF-1 levels in the calcium supplementation group relative to the placebo group. Based on the reported p-values, the estimated treatment effect was likely statistically significant since the IGF-1 levels statistically significantly increased in the calcium supplementation group and decreased in the placebo group. However, it is important to note that teenage girls are expected to have higher and fluctuating levels of IGF-1 during this growth and developmental phase. The fourth trial investigated IGF-1 levels in an intervention trial of bone health among Greek postmenopausal women by testing the effects of 600 mg supplemental calcium vs. 3 daily servings of low-fat dairy products fortified with calcium and vitamin D₃ (providing approximately 1,200 mg dietary calcium daily) vs. usual diet.²⁹ After 5 and 12 months, there were no differences in percent changes in IGF-1 among the 3

treatment groups. Although there is no epidemiological evidence of an association of supplemental calcium with circulating IGF-1 or IGFBP-3 concentrations, a cross-sectional study of men and women 20 years of age or older reported a positive association of serum calcium levels with circulating IGF-1 and IGFBP-3 levels.³⁰ However, serum calcium levels in the body are maintained under strict homeostatic control, and thus, may not necessarily reflect dietary intakes; therefore, the significance of this finding is unclear.

There is consistent, though limited, evidence that milk consumption may increase circulating IGF-1 levels. In a clinical trial conducted in adult men and women, there was a statistically significant increase in IGF-1 concentrations among those consuming 3 servings of non-fat or low-fat milk daily for 12 weeks compared to those not consuming milk.³¹ Positive associations of milk consumption and IGF-1 concentrations were also reported in observational studies,^{21,32–35} though associations of milk consumption with IGFBP-3 levels were primarily null. The findings across the few studies that reported an association of milk consumption with the IGF-1:IGFBP-3 molar ratio were inconsistent.^{32,35,36} In our study, we examined the milk product residuals as the non-calcium component of milk products and an indirect measure of IGF-1. Our findings suggest an inverse association of these residuals with IGFBP-3 concentrations. On the one hand, the latter finding would suggest higher concentrations of unbound (bioavailable) IGF-1 in the upper tertile of residuals; on the other hand, the residuals were similarly inversely associated with IGF-1 concentrations, although not statistically significant. However, given that this study was conducted from 1990 to 1993, milk may not have been produced using bovine somatotropin hormone, which was approved for use by the Food and Drug Administration in 1993³⁷ and is hypothesized to increase levels of IGF-1 in milk products. Thus, this is a potential reason why our estimate of IGF-1 from milk products, as estimated by both total milk product and non-fat milk residuals, were not positively associated with circulating IGF-1 levels.

Our findings of differences in IGF-1 and IGFBP-3 concentrations according to sex and age are consistent with those from some, but not all, observational studies. Our observed inverse association of IGF-1 and IGFBP-3 concentrations with age is consistent with previous studies,^{33,35,38–40} and lower concentrations of IGF-1 were consistently reported in women.^{35,36,38} However, the association of IGFBP-3 levels with sex is inconsistent among these studies. Our findings also suggest that IGFBP-3 levels may be positively associated with alcohol consumption. In an 8-week crossover trial among postmenopausal women (n = 53), IGFBP-3 concentrations increased with consumption of one but not two alcohol drinks daily.⁴¹ However, the association is unclear as limited observational studies reported inconsistent associations of IGFBP-3 concentrations with alcohol consumption.^{34,42}

To our knowledge, this is the first report on the effect of calcium supplementation on circulating levels of IGF-1 and IGFBP-3 among adenoma patients, and a strength of the study was the randomized, double-blind, controlled, dose-response design. However, there were also several limitations in this study. First, the blood samples collected at the baseline and follow-up visits were non-fasting samples. It is possible that circulating IGF-1 levels may be influenced by dietary sources of IGF-1, which are primarily animal products. Additionally, the time of day at which blood is collected may affect levels of IGF-1 and IGFBP-3.⁴³ Therefore, in future studies, it may be important to collect fasting blood samples

at a consistent time of day. Additionally, although numerous studies similarly examined the IGF-1:IGFBP-3 molar ratio as an indicator of IGF-1 bioavailability, to our knowledge, this ratio has never been experimentally validated as a reflection of IGF-1 bioavailability. A possible reason for this is that, to our knowledge, there is no current practical method to measure circulating free IGF-1, perhaps because it has a transient half-life. Because of this and since over 75% of the circulating (endocrine) form of IGF-1 is bound to IGFBP-3 in a 1:1 molar ratio,⁶ the IGF-1:IGFBP-3 molar ratio is currently the most widely used method of estimating IGF-1 bioavailability. Experimental validation of the IGF:IGFBP-3 molar ratio is needed.

In summary, although we found no appreciable effects of calcium supplementation on circulating levels of IGF-1, IGFBP-3, or the IGF-1:IGFBP-3 molar ratio among patients with previous sporadic adenoma, our results support the existing evidence that IGF-1 and IGFBP-3 concentrations are lower in females and inversely associated with age. Additionally, our results suggest a potential direct association of milk consumption with circulating IGF-1 levels. Further investigations are needed to investigate potential modifiable dietary and lifestyle factors that may impact the insulin-like growth factor system in the circulation and in normal colon tissue, which in turn may modulate risk of colorectal neoplasia.

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Abbreviations

ANOVA	analysis of variance
BMI	body mass index
bST	bovine somatotropin hormone
CV	coefficient of variation
HRP	streptavidin-horseradish peroxidase
HRT	hormone replacement therapy
IGF-1	insulin-like growth factor 1
IGFBP-3	insulin-like growth factor binding protein 3
NSAID	nonsteroidal anti-inflammatory drug
SE	standard error
95% CI	95% confidence interval

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Table 1

Selected baseline characteristics of study participants by treatment group.*

	Treatment Group			P value [†]
	Placebo (n = 64)	1 g Calcium (n = 62)	2 g Calcium (n = 63)	
Demographics				
Age, years	59.6 (9.1)	60.3 (9.0)	57.9 (10.4)	0.36
Male, %	65.6	61.3	61.9	0.86
White, %	98.4	100.0	100.0	1.00
Family history of colon cancer, % [‡]	26.6	24.2	30.2	0.75
Lifestyle Factors				
College education or higher, %	34.4	17.7	33.3	0.07
Current smokers, %	20.3	16.1	23.8	0.56
Alcohol, grams/day	11.1 (19.5)	13.7 (20.4)	7.7 (13.1)	0.19
Body mass index, kg/m ²	28.8 (4.4)	28.6 (5.7)	27.8 (4.6)	0.51
Physical activity, MET-hrs/week [§]	7.6 (12.9)	10.5 (15.4)	6.1 (11.6)	0.18
NSAID or aspirin use, %	26.6	33.9	25.4	0.52
HRT, % females	22.7	33.3	41.7	0.39
Dietary Intakes				
Total energy, kcal/day	2,102 (754)	1,979 (618)	2,102 (633)	0.50
Total fat, g/day	70.6 (29.2)	67.3 (26.5)	76.5 (26.7)	0.18
Dietary fiber, g/day	23.3 (9.4)	21.3 (6.5)	21.5 (8.9)	0.34
Total calcium, mg/day	885 (344)	786 (369)	855 (416)	0.33
Dietary calcium	851 (331)	737 (350)	811 (350)	0.17
Supplemental calcium	33.8 (71.5)	48.7 (120)	44.1 (185)	0.81
Total vitamin D, IU/day	334 (243)	298 (271)	314 (207)	0.70
Red meat, servings/day	0.6 (0.7)	0.7 (0.8)	0.7 (0.4)	0.89
Processed meat, servings/day	0.4 (0.5)	0.3 (0.4)	0.4 (0.6)	0.54
Total fruit & vegetable, servings/day	5.3 (2.4)	5.2 (2.2)	5.0 (2.9)	0.74
Total dairy, servings/day	2.3 (1.1)	2.2 (1.4)	2.2 (1.5)	0.75
High fat dairy	1.1 (1.0)	1.3 (1.2)	1.1 (1.1)	0.43
Low fat dairy	1.3 (1.0)	0.9 (0.9)	1.1 (1.0)	0.17
Cheese	0.6 (0.5)	0.6 (0.4)	0.7 (0.7)	0.71
Fermented dairy	0.7 (0.6)	0.8 (0.5)	0.8 (0.8)	0.61
Whole milk	1.1 (0.9)	0.8 (0.9)	0.9 (0.9)	0.23
Non-fat milk	0.03 (0.14)	0.09 (0.44)	0.03 (0.13)	0.39

Abbreviations: HRT, hormone replacement therapy; MET, metabolic equivalents of task; NSAID, non-steroidal anti-inflammatory drug.

* Values presented are mean (standard deviation) unless otherwise specified.

[†] P values from chi-square or Fisher's exact test for categorical variables and analysis of variance for continuous variables.[‡] Family history of a first-degree relative with colorectal cancer.[§] Physical activity calculated as MET-hrs. per week of moderate and vigorous activity.

Table 2

Changes in growth factor concentrations among colorectal adenoma patients in response to calcium supplementation.

Growth Factor	Baseline*			4-month follow-up*			Relative Treatment Effect [†]		Absolute Treatment Effect [‡]	
	n	Mean	SE	n	Mean	SE	Mean	95% CI		
IGF-1, ng/mL										
Placebo	64	108.1	1.1	60	104.1	1.1				
1 g calcium	62	109.9	1.0	58	109.9	1.0	1.02	0.93	1.12	3.97
2 g calcium	63	109.1	1.0	57	110.5	1.1	1.03	0.94	1.13	5.34
IGFBP-3, ng/mL										
Placebo	64	3,105	1.0	60	3,105	1.1				
1 g calcium	62	3,328	1.0	58	3,415	1.1	1.03	0.93	1.13	87.0
2 g calcium	63	3,133	1.1	57	3,184	1.1	1.00	0.91	1.10	50.8
IGF-1 : IGFBP-3										
Placebo	64	0.13	1.1	60	0.12	1.1				
1 g calcium	62	0.12	1.1	58	0.12	1.1	1.00	0.87	1.14	0.002
2 g calcium	63	0.13	1.0	57	0.13	1.1	1.03	0.90	1.18	0.01

Abbreviations: IGF-1, insulin-like growth factor 1; IGFBP-3, insulin-like growth factor binding protein 3 IGF-1:IGFBP-3, molar ratio of IGF-1 to IGFBP-3; SE, standard error; 95% CI, 95% confidence interval

* Geometric means

[†] Relative treatment effect from SAS Institute's Mixed Procedure (SAS version 9.4; SAS Institute, Car NC) defined as [(treatment group follow-up mean) / (treatment group baseline mean)] / [(placebo follow-up mean) / (placebo baseline mean)].

[‡] Absolute treatment effect calculated as [(treatment group follow-up mean) - (treatment group baseline mean)] - [(placebo follow-up mean) - (placebo baseline mean)].

Table 3

Mean baseline concentrations of growth factors by demographic, lifestyle, and dietary factors.*

Factor	Category [†]	IGF-1			IGFBP-3			IGF-1:IGFBP-3			
		N	Mean	SE	P [‡]	Mean	SE	P [‡]	Mean	SE	P [‡]
Sex	Male	117	115.9	1.0		3,371.6	1.0		0.12	1.04	
	Female	69	98.2	1.0	0.003	2,893.2	1.0	0.01	0.12	1.05	0.85
Age, years	<55	60	117.5	1.0		3,540.6	1.0		0.12	1.06	
	55 - 64	70	103.7	1.0		2,930.9	1.0		0.13	1.05	
	65	56	99.3	1.0	0.01	2,946.0	1.0	0.01	0.12	1.06	0.83
BMI, kg/m ²	<25	49	114.0	1.1		2,944.7	1.1		0.14	1.06	
	25 – 29.99	73	105.8	1.0		3,275.9	1.0		0.12	1.05	
	30	64	101.9	1.0	0.11	3,117.6	1.0	0.53	0.12	1.05	0.06
NSAID or aspirin use	Yes	54	101.3	1.1		2,930.7	1.1		0.12	1.06	
	No	132	109.0	1.0	0.21	3,207.0	1.0	0.13	0.12	1.04	0.81
Alcohol	None	54	103.6	1.0		3,048.5	1.0		0.12	1.06	
	1–6 drinks/wk.	87	105.5	1.0		2,986.4	1.0		0.13	1.05	
	7+ drinks/wk.	45	114.3	1.1	0.20	3,586.9	1.1	0.04	0.12	1.07	0.52
Total energy, kcal/day	Low	92	104.0	1.0		3,032.8	1.0		0.12	1.05	
	High	94	109.2	1.0	0.36	3,214.7	1.0	0.28	0.12	1.05	0.88
Total fat	Low	92	111.1	1.0		3,216.6	1.0		0.12	1.05	
	High	94	103.2	1.0	0.27	3,060.6	1.0	0.47	0.12	1.05	0.76
Total calcium	Low	92	105.9	1.0		3,256.0	1.0		0.12	1.05	
	High	94	107.7	1.0	0.78	3,024.3	1.0	0.21	0.13	1.05	0.19
Dietary calcium	Low	92	105.6	1.0		3,239.5	1.0		0.12	1.05	
	High	94	107.9	1.0	0.71	3,038.9	1.0	0.29	0.13	1.05	0.22
Red or processed meat	Low	91	111.1	1.0		3,124.3	1.0		0.13	1.05	
	High	95	103.0	1.0	0.17	3,140.7	1.0	0.93	0.12	1.05	0.21

Factor	Category [†]	IGF-1			IGFBP-3			IGF-1:IGFBP-3		
		N	Mean	SE	P [‡]	Mean	SE	P [‡]	Mean	SE
Total milk products	Low	92	107.3	1.0		3,179.5	1.0		0.12	1.05
	High	94	106.3	1.0	0.87	3,091.7	1.0	0.63	0.12	1.05
Total milk	Low	100	100.6	1.0		3,077.2	1.0		0.12	1.05
	High	86	111.8	1.0	0.05	3,178.3	1.0	0.56	0.13	1.04
Milk product residuals, tertiles	1	62	109.1	1.0		3,302.5	1.0		0.12	1.06
	2	62	109.7	1.0		3,239.7	1.0		0.12	1.06
	3	62	101.7	1.0	0.28	2,874.5	1.0	0.04	0.13	1.06
Non-fat milk residuals, tertiles	1	62	98.8	1.1		3,092.1	1.1		0.12	1.06
	2	62	118.1	1.0		3,396.9	1.0		0.13	1.06
	3	62	104.8	1.1	0.39	2,923.5	1.1	0.55	0.13	1.06

Abbreviations: BMI, body mass index; IGF-1, insulin-like growth factor 1; IGFBP-3, insulin-like growth factor binding protein 3; IGF-1:IGFBP-3, molar ratio of IGF-1 to IGFBP-3; NSAID, non-steroidal anti-inflammatory drug; OBS, oxidative balance score; SE, standard error.

* Models for all variables except sex adjusted for sex (M/F). Models for all variables except age adjusted for age (continuous). Models for all variables except BMI adjusted for BMI (continuous). Models for dietary variables also adjusted for total energy intake (continuous). Models for total milk products and total milk also adjusted for supplemental calcium (continuous). Models for milk product residuals and non-fat milk residuals also adjusted for dietary calcium (continuous).

[†] Low/high categories determined using sex-specific medians. BMI categories determined using World Health Organization classification of adult normal weight, overweight, and obesity.

[‡] P-values from analysis of variance.