HHS Public Access

Author manuscript

Cancer Epidemiol Biomarkers Prev. Author manuscript; available in PMC 2017 February 01.

Published in final edited form as:

Cancer Epidemiol Biomarkers Prev. 2016 February ; 25(2): 318–326. doi: 10.1158/1055-9965.EPI-15-0488.

Circulating biomarkers of gut barrier function: Correlates and non-response to calcium supplementation among colon adenoma patients

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Abstract

Background—Gut barrier dysfunction contributes to several gastrointestinal disorders, including colorectal cancer, but factors associated with intestinal hyperpermeability have been minimally studied in humans.

Methods—We tested the effects of two doses of calcium (1.0 or 2.0 g/d) on circulating biomarkers of gut permeability (anti-flagellin and anti-lipopolysaccharide [LPS] immunoglobulins [Igs], measured via ELISA) over a 4-month treatment period among colorectal adenoma patients in a randomized, double-blinded, placebo-controlled clinical trial (n = 193), and evaluated factors associated with baseline levels of these biomarkers.

Results—Baseline concentrations of anti-flagellin IgA and anti-LPS IgA were, respectively, statistically significantly proportionately higher by 11.8% and 14.1% among men, 31.3% and 39.8% among those with a body mass index (BMI) ≥ 35 kg/m^2, and 19.9% and 22.0% among those in the upper relative to the lowest sex-specific tertile of waist circumference. A combined permeability score (the summed optical densities of all four biomarkers) was 24.3% higher among women in the upper tertile of plasma C-reactive protein ($p_{trend} < 0.01$). We found no appreciable effects of supplemental calcium on anti-flagellin or anti-LPS Igs.

Conclusion—Our results suggest that 1) men and those with higher adiposity may have greater gut permeability, 2) gut permeability and systemic inflammation may be directly associated with

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Conflict of interests: None

This paper was presented orally, in part, at the 2015 AACR Annual Meeting (Minisymposium: Biomarkers and Cancer: New Insights).

one another, and 3) supplemental calcium may not modify circulating levels of gut permeability biomarkers within four months.

Impact—Our findings may improve understanding of the factors that influence gut permeability to inform development of treatable biomarkers of risk for colorectal cancer and other health outcomes.

Keywords

Calcium; biomarker; chemoprevention; clinical trial; gut permeability

Introduction

The gastrointestinal tract has the largest mucosal surface in the body interacting with the environment, and an intact gut barrier with selective permeability is key to balancing the absorption of nutrients and blocking harmful wastes, such as bacterial products (1). Abnormal gut barrier function contributes to several gastrointestinal disorders, such as inflammatory bowel disease (IBD), Celiac disease, food allergies (2), and colorectal cancer (3-5). There is also emerging evidence that individuals with prevalent colorectal adenomas are more likely to have higher plasma lipopolysaccharides (LPS) concentrations compared to healthy controls (6). Factors associated with gut hyperpermeability have not been well-characterized, although evidence suggests that diet, among other factors, may impact gut permeability, based on animal studies and very limited human clinical trials (2, 7).

Calcium is a plausible agent that may play a role in modulating gut barrier function since calcium can bind bile and fatty acids in the colon lumen by forming insoluble soaps, thus preventing them from oxidatively damaging the colonic mucosa and consequently producing inflammation (8-10), which, in turn, may help maintain the strength of the gut mucosal barrier. Our research group previously conducted a 6-month pilot randomized controlled trial among patients with previous colorectal adenoma, and found that among subjects treated with calcium (n = 23) compared to the placebo (n = 23), 8-hydroxydeoxyguanosine level (as a marker of oxidative DNA damage) in the normal-appearing colon tissue was reduced by 22% (11), and a comprehensive summary z-score of multiple plasma biomarkers of inflammation was reduced by 48% (12). Based on these data, we hypothesized that calcium may also favorably modulate gut permeability. The effect of calcium supplementation on gut permeability was previously tested in a very limited number of animal studies (13-15) and one pilot human clinical trial (n = 32) (16), and their results all support this novel hypothesis. However, to our knowledge, there are no reported full-scale clinical trials that directly tested the effect of calcium on gut permeability in humans.

To address these gaps in the literature, we measured circulating levels of flagellin- and LPS-specific immunoglobulins (Igs) IgA and IgG among patients with previous colorectal adenomas in a full-scale, randomized, double-blinded, placebo-controlled clinical trial (n = 193). Circulating levels of flagellin- and LPS-specific IgA and IgG may serve as markers of long-term systemic exposure to flagellin and LPS and may indicate altered adaptive immune responses related to colonic hyperpermeability (17-19). We evaluated factors associated with these circulating biomarkers of gut permeability at baseline (including major

demographic, diet and lifestyle factors, and systemic inflammation levels) and tested whether biomarker levels were affected by calcium supplementation over four months of treatment.

Materials and Methods

This study was an adjunct investigation using data and blood samples from a chemoprevention trial (1990 – 1994) in the Minneapolis, MN metropolitan area (20). The parent study was approved by the Committee on Use of Human Subjects in Research of the University of Minnesota. Written informed consent was obtained from each study participant.

Participant Population

Detailed information on study recruitment protocol, eligibility and exclusion criteria was published previously (20). Briefly, subjects aged 30 – 74 years who were in general good health and had a history of pathology-confirmed adenomatous polyps within the previous five years were recruited by project staff from the patient population of a major private-practice gastroenterology group in Minneapolis-St. Paul, MN. Exclusion criteria included contraindications to calcium supplementation or rectal biopsies; medical conditions, habits, or medication usage that would otherwise jeopardize safety, adherence, or interpretation of the study results, such as history of inflammatory bowel disease, familial polyposis syndromes, active liver or pancreatic disease, calcium supplement use, and supplemental daily intake of more than 400 IU of vitamin D; and failure to take > 80% of the prescribed tablets in a 1-month placebo run-in trial.

Clinical Trial Protocol

Potential participants were first invited for an eligibility visit to complete questionnaires and provide blood samples, after which those who appeared eligible entered a 4-week placebo run-in trial. Only participants without substantial perceived side effects and who had taken > 80% of their tablets in the 4-week placebo run-in trial were eligible for randomized assignment. Eligible participants (n = 193) then underwent a baseline visit and were randomly assigned (stratified by sex) to one of three parallel groups: a placebo control group (n = 66) and 1.0 g/d (n = 64) and 2.0 g/d (n = 63) elemental calcium supplementation groups. The supplement and placebo pills, prepared by SmithKline Beecham, Pittsburgh, PA, were identical in size, appearance, and taste. The calcium tablets were in the form of calcium carbonate and taken in two equally divided doses twice daily with food. The reasons for choosing calcium carbonate were described previously (20).

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

index [BMI] and waist-hip ratio) were assessed at baseline, several were reassessed at each follow-up visit, and all factors were reassessed at the final follow-up visit.

Peripheral venous blood samples were collected at the baseline and 4-month follow-up visits, after the subject sat upright with his or her legs uncrossed for 5 minutes. Blood was drawn into pre-chilled Vacutainer tubes for plasma and serum, and then immediately placed on ice and shielded from light. Tubes were immediately processed, plasma and serum were aliquotted into cryopreservation tubes, the air was displaced with nitrogen, and then the aliquots were immediately placed in a −80 °C freezer until analysis. Blood samples were available for 189 subjects at baseline and 174 subjects at follow-up.

Laboratory Protocol

Levels of flagellin- and LPS-specific IgA and IgG were measured via a previously described custom-made ELISA at Georgia State University (17-19). ELISA plates (Costar™) were coated overnight with laboratory-made flagellin (100 ng/well; prepared from *Salmonella typhimurium*, strain SL 3201 fljB$^{-/-}$ as previously described (21)) or purified *E. coli* LPS (2 μg/well; from *E. coli* 0128: B12, Sigma, Catalog No. 2887). Plasma samples diluted 1:200 were applied to wells coated with flagellin or LPS. After incubation and washing, the wells were incubated either with anti-IgG coupled to horseradish peroxidase (GE, Catalog No. 375112) or, in the case of IgA-specific antibodies, with horseradish peroxidase-conjugated anti-IgA (KPL, Catalog No. 14-10-01). Using the established platform, specificity of flagellin/LPS is observed when the signal is extremely low when using serum from germ free mice (very low flagellin- or LPS-specific immunoglobulins) and completely abolished using serum from RAG-1 knockout mice and germ free mice on an elemental diet (no flagellin- or LPS-specific immunoglobulins). The specificity of the anti-human IgA and anti-human IgG is in accordance to manufacturer's specifications, KPL and GE Healthcare Life Sciences, respectively. Quantitation of total immunoglobulins was performed using the colorimetric peroxidase substrate tetramethylbenzidine (TMB), and optical density (OD) was read at 450 nm and 540 nm (the difference was taken to compensate for optical interference from the plate), with an ELISA plate reader. Data are reported as OD corrected by subtracting background (determined by readings in blank samples) and are normalized to each plate’s control sample, which was prepared in bulk, aliquotted, frozen, and thawed daily as used. Standardization was performed using preparations of known concentrations of IgA, and IgG. The technician was blinded to treatment group and treated all samples identically. Baseline and follow-up samples from each participant were included in the same batch. The laboratory previously performed assays of these biomarkers in replicates with a very low coefficient of variation (CV < 5%); therefore, our samples were analyzed in singleton to minimize costs and time. The average within-batch CVs were 2.4%, 4.3%, 2.6%, and 4.6% for flagellin IgA, flagellin IgG, LPS IgA, and LPS IgG, respectively, based on three positive control samples included in each batch. The corresponding between-batch CVs were 4.1%, 8.2%, 15.8%, and 25.8% for flagellin IgA, flagellin IgG, LPS IgA, and LPS IgG, respectively. In addition, for quality control (QC), two duplicate plasma samples were measured in each batch. The average within-batch CVs were 4.5%, 6.6%, 4.2%, and 7.7% for flagellin IgA, flagellin IgG, LPS IgA, and LPS IgG, respectively. The corresponding

between-batch CVs using QC samples were 6.2%, 5.0%, 27.0%, and 32.5% for flagellin IgA, flagellin IgG, LPS IgA, and LPS IgG, respectively.

Plasma levels of the inflammation biomarkers were measured using electrochemiluminescence detection-based immunoassays (Meso Scale Discovery [MSD]) in the Emory Multiplexed Immunoassay Core (EMIC). All biomarkers were measured in duplicate, according to the manufacturer's protocol, and the technicians were blinded to the treatment group assignment. Eleven biomarkers were initially chosen to represent different aspects of the inflammatory response/immunomodulation in order to provide a more complete summary of systemic inflammation. Out of these biomarkers, we selected those with an average intra-assay coefficient of variation (CV) < 15% for further analysis, including C-reactive protein (CRP), interleukin (IL)-10, IL-12p40, IL-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF). Interferon gamma (IFN-γ), IL-17, and IL-4 were excluded (intra-assay CVs > 15%).

Statistical Analysis

Treatment groups were compared on baseline characteristics using analysis of covariance (ANCOVA) for continuous variables, and the chi-square or Fisher's exact test for categorical variables; sex was included as a covariate when appropriate. Pearson correlation coefficients were calculated for each pair-wise combination of the four gut permeability biomarkers. Associations of selected baseline demographic, diet and lifestyle factors, and circulating biomarkers of inflammation with gut barrier function biomarkers were assessed using ANCOVA, adjusted for sex and BMI as appropriate. To better present different aspects of inflammation, we created a baseline cytokine summary z-score, as the sum of the z values for each cytokine [$z = (x - \mu)/\delta$, where x is the natural log-transformed values for each individual marker, and μ and δ are the sex-specific mean and standard deviation of the natural log-transformed biomarker value, respectively, at baseline]. The z-score for IL-10 was included with a negative sign because of its anti-inflammatory properties (22).

The primary analysis of the effects of calcium on gut barrier function biomarkers was based on random assignment of treatment group regardless of adherence (intent-to-treat). Because the biomarker values were normally distributed, they were not log-transformed before statistical testing. Treatment effects on the biomarkers from baseline to 4-month follow up across the three treatment groups were compared using a mixed linear model for repeated measures data as implemented in SAS Institute's Mixed Procedure (SAS version 9.4; SAS Institute, Cary, NC). The model included as predictors the intercept, visit (baseline and 4-month follow-up), treatment groups (coded as dummy variables), and a treatment-by-visit interaction term. An absolute effect, obtained from the Mixed model, was defined as [(treatment group follow-up mean) − (treatment group baseline mean)] − [(placebo follow-up mean) − (placebo baseline mean)]. In order to provide a conservative estimate of the proportional change in the treatment group relative to that in the placebo group, we also calculated a relative effect, defined as (treatment group follow-up mean/treatment group baseline mean) / (placebo follow-up mean/placebo baseline mean). Its interpretation is somewhat analogous to that of an odds ratio (e.g., a relative effect of 1.10 would mean that

the proportional change in the treatment group was 10% higher than that in the placebo group).

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

Results

The mean age of the study participants was 59 years, 63% were men, 99% were White, and 28% had a family history of colorectal cancer in a first-degree relative. The baseline characteristics of the participants did not differ significantly across the three treatment groups (Table 1).

Among the 193 participants, measurements of the plasma biomarkers of gut permeability were available for 189 at baseline, and 174 at follow-up. The baseline gut permeability biomarkers were moderately to strongly correlated (Pearson correlation coefficients 0.20 – 0.67 for men and 0.37 – 0.80 for women), and the p-values for all pair-wise Pearson correlations were < 0.05 (Supplementary Table S1). As shown in Table 2, the baseline levels of anti-flagellin IgA and anti-LPS IgA were, respectively, statistically significantly proportionately higher by 11.8% and 14.1% among men (p value < 0.05) relative to women, 31.3% and 39.8% among those who were very obese (BMI ≥35 kg/m^2) relative to those who were underweight/normal weight (p_{trend} < 0.01), and 19.9% and 22.0% among those in the upper relative to the lowest sex-specific tertile of waist circumference (p_{trend} < 0.01). A combined permeability score (the summed optical density measurements from all biomarkers) was 24.3% higher among women who were in the upper relative to the lowest tertile of plasma C-reactive protein concentrations (p_{trend} < 0.01), but not among men (Table 3). No associations of any of the gut barrier function biomarkers were found with age, waist-hip ratio, cigarette smoking, alcohol drinking, NSAID use, or adenoma characteristics (Table 2), nor with physical activity, vitamin/mineral supplement use, intakes of fat, red/processed meat, and fruit/vegetable, or a comprehensive oxidative balance score (OBS, which reflects combined contributions of anti-oxidant and pro-oxidant diet and lifestyle exposures) (23, 24) (data not shown). Batch-adjustment did not change the results (data not shown).

Overall adherence to visit attendance was 95.3%, and did not differ among the treatment groups. The mean percentage of pills taken in each group was 97%, and > 98% of all participants in each group took > 80% of their pills. Changes in the gut barrier function biomarkers, alone or in combination, for each calcium treatment group relative to the placebo group, are shown in Table 4. We found no appreciable or statistically significant

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

Discussion

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

We found higher levels of anti-LPS and anti-flagellin Igs in men than in women. Overall, levels of anti-LPS and anti-flagellin Igs may reflect erosion of mucosal anatomic and immune barriers, gut bacteria composition and their ability to translocate across the gut, and immune responses against bacterial antigens. Because men generally have lower innate and adaptive immune responses than women (25), it is likely that men are systemically exposed to a higher level of bacterial products as a result of impaired gut barrier function and/or distinct microbiome profiles (26) potentially due to diet, lifestyle, or hormonal factors. Alternatively, there is evidence that given the same amount of *in vivo* LPS exposure, male mice produce higher levels of LPS-binding protein and higher inflammation mediators than female mice (27). While the exact biological mechanisms require further investigation, future observational epidemiologic studies for the association of gut permeability with various health outcomes may need to consider sex as an important confounder and/or effect modifier.

Our findings that BMI and waist circumference, a reliable predictor of visceral fat, are positively associated with colonic permeability is largely consistent with previous literature. Evidence from several human cross-sectional studies supports a positive association of obesity (especially abdominal obesity) with several intestinal permeability measurements, such as the sucralose-to-mannitol ratio, IgG against bacterial antigens, and LPS-binding protein (LBP) (28-30). One possible explanation is that obese individuals may have different gut microbiota and/or gut microbiome patterns (31); for example, obese individuals often consume a high-fat diet, which may favor the growth of gram-negative bacteria in the gut (32). Gram-negative bacteria may have a greater ability to translocate across the gut mucosa into the circulation compared to gram-positive microbes (33). Furthermore, LPS is a major component of the outer membrane of Gram-negative bacteria. Therefore, it is biologically plausible that obese individuals have higher levels of anti-LPS and anti-flagellin Igs. However, the temporal sequence of gut barrier dysfunction and obesity cannot be assessed in such cross-sectional studies. Results from a few animal and human trials suggested that gut barrier dysfunction and obesity could influence each other. For example, mice with induced metabolic endotoxemia (through infusion of LPS) experienced weight gain in 4 weeks, suggesting that the LPS system may trigger the onset of obesity (34). Conversely,

mice with induced-obesity had significantly higher IgG against bacterial extracts (29), and rats with transplanted visceral adipose tissue or that were injected with leptin had increased colonic epithelial permeability as measured by expression of trans-epithelial resistance and tight junction proteins, suggesting that obesity may induce gut barrier impairment (35). In humans, plasma LPS levels were higher in obese individuals (n = 49) than in controls (n = 17), but they were reduced after bariatric surgery; however, reduced LPS levels were not found with a preoperative weight-loss intervention, and the postoperative LPS reduction was not correlated with a BMI reduction, suggesting mechanisms beyond weight loss (36).

Our study provides some evidence that levels of systemic inflammation may be positively correlated with gut permeability. We previously hypothesized that oxidative damage and subsequent inflammatory responses in the gut result in damage to the gut barrier and increase gut permeability. Current evidence suggests that enhanced mucosal immune activities may also be a consequence of gut barrier dysfunction (1). For example, Hollander *et al.* found that compared to healthy controls, patients with Crohn's disease and their clinically unaffected relatives had similarly increased gut permeability, suggesting that gut barrier dysfunction is not secondary to intestinal inflammation (37). In experimental studies, translocation of flagellin across epithelia mediated Salmonella-induced mucosal inflammatory activities *in vitro* (21), *via* activating basolaterally expressed Toll-like receptor 5 (TLR5) (38), and systematic injection of flagellin in mice induced the expression of a panel of pro-inflammatory mediators such as cytokines (39). Gut permeability and inflammation are likely closely related in a complex manner, and may act together in the pathogenesis of metabolic disorders such as diabetes and obesity (34, 40, 41), both of which are associated with the incidence of several types of cancer, including colorectal cancer.

The effect of calcium on gut permeability has rarely been studied before. Bovee-Oudenhoven and colleagues conducted several controlled trials in rats, and reported that a high-calcium diet reduced the translocation of *Salmonella*, inhibited the increase in intestinal permeability as measured by urinary chromium EDTA (CrEDTA), and improved resistance to intestinal infection (13-15); they also found a similar effect of high-calcium milk relative to low-calcium milk against enterotoxigenic *Escherichia coli* (ETEC) infection in rats and a small group of men (n = 32) (16), but the potential interaction between calcium and other components in milk could not be excluded. We found no effects of calcium supplementation on immunoglobulins against selected bacterial products, possibly due to several reasons. First, calcium may simply have no important effect on gut permeability in humans. Second, the circulating biomarkers investigated in this study may not be the most direct measurement of gut permeability; however, emerging evidence suggests a positive correlation of antibodies against LPS and flagellin with serum fluorescein isothiocyanate–dextran (a direct measurement of intestinal barrier function) (42), or with LPS (43), and these antibodies are also elevated in patients with short bowel syndrome and Crohn's disease, conditions known to involve gut barrier dysfunction (17-19, 44). Third, although the treatment period of the original trial was 6 months, blood was only collected at baseline and month 4, since blood biomarkers were not the pre-specified primary outcomes of the trial. This treatment duration may be insufficient to observe an effect of calcium on these permeability markers, as antibodies against bacterial products can persist for several months (45, 46), and the effect

of calcium on gut barrier function may not be immediately accompanied by a decrease of antibody levels. Fourth, the original trial was conducted in the 1990s, so it is possible that the samples deteriorated over the years; however, we did not find strong evidence to support this. The samples were immediately processed and stored with no additional freeze-thaw cycles since the original storage, the levels of the inflammation markers were comparable to those in another trial with more recently collected blood samples (12), and anti-LPS and anti-flagellin Igs are stable over time (personal communication with A. Gewirtz) and, as described above, were associated with BMI as in other reported studies. Finally, chance remains a possible explanation.

Major strengths of our study include that it is a full-scale randomized, controlled trial with a dose-response component. Other strengths include the inclusion of novel gut permeability biomarkers and the excellent overall adherence to treatment. We also collected detailed questionnaire information and were able to evaluate associations of baseline demographic, diet, and lifestyle factors with gut permeability levels, which may provide insights for future epidemiological studies. Limitations of the study include the above-mentioned relatively short treatment period and long storage period of the blood samples. In addition, the gut permeability biomarkers were measured in singleton; however, based on previous assays on these same biomarkers we expect that our biomarker measurement reliability was high. The use of antibiotics may impact gut bacteria and subsequent immune responses against bacterial products. We excluded patients who were on antibiotics at baseline but lacked data on the use of antibiotics during the trial or during the year prior to the trial (which may have a long-term effect on the gut microbiota); however, antibiotic use is expected to be balanced among the three groups due to randomization. Also, this study is based on a population of patients with a history of colorectal adenoma who were participating in a chemoprevention trial, and thus our findings may have limited external generalizability. In addition, although there is evidence that colorectal adenoma patients have higher levels of plasma LPS than do healthy controls (6), whether they also have higher Igs against flagellin and LPS is unclear, and needs to be examined in a future case-control or cohort study. Furthermore, evidence from animal studies suggests that intestinal barrier dysfunction exists at the site of colorectal adenomas (47), but whether adenoma removal would improve or resolve such dysfunction is unclear and warrants further investigation.

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support: R03 CA184578 (V. Fedirko); R21 CA182752 (V. Fedirko); The Franklin Foundation (R.M. Bostick)

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Table 1

Selected baseline characteristics of the study participants (n = 193)[a]

Characteristics	Treatment group			P-value[b]
	Placebo (n = 66)	Calcium 1 g (n = 64)	Calcium 2 g (n = 63)	
Age, yrs.	60 (9)	60 (9)	58 (10)	0.37
Men (%)	64	63	62	0.98
White (%)	98	100	100	> 0.99
College graduate (%)	35	19	33	0.08
Employed (%)	52	45	56	0.48
Family history (%)	26	25	30	0.78
Take aspirin[c] (%)	21	27	16	0.34
Take non-aspirin NSAID[c] (%)	9	11	10	0.92
Currently smoke (%)	20	16	24	0.53
Alcohol intake, g/d	11 (19)	13 (20)	8 (13)	0.20
Body mass index, kg/m^2				
Men	28.0 (3.8)	29.0 (3.1)	28.8 (4.5)	0.47
Women	30.1 (5.2)	28.1 (8.4)	26.3 (4.4)	0.12
Vigorous/moderate physical activity, MET-hours/d	33 (21)	30 (22)	28 (21)	0.47
Dietary intakes				
Total energy, kcal/d	2,097 (753)	2,000 (627)	2,102 (633)	0.63
Total fat, g/d	64 (27)	62 (24)	70 (24)	0.19
Dietary fiber, g/d	24 (10)	22 (7)	22 (9)	0.33
Total vitamin D, IU/d	345 (251)	294 (268)	314 (207)	0.48
Total calcium, mg/d	884 (339)	787 (364)	855 (416)	0.33
Phosphorous, mg/d	1,359 (435)	1,248 (441)	1,327 (418)	0.34
Omega-3 fatty acids, g/d	0.2 (0.2)	0.2 (0.2)	0.2 (0.2)	0.41
Take any vitamin supplement(s) (%)	38	38	33	0.82
Biomarker measurements[d]				
Flagellin IgA	1.6 (0.5)	1.7 (0.5)	1.6 (0.5)	0.42
Flagellin IgG	1.7 (0.4)	1.8 (0.4)	1.6 (0.4)	0.07
LPS IgA	1.4 (0.6)	1.4 (0.5)	1.4 (0.5)	0.69
LPS IgG	0.8 (0.4)	0.8 (0.4)	0.9 (0.4)	0.77

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

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

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

[c] Regularly take once or more a week.

[d] The units for the permeability biomarkers is optical density (OD). The OD ranges at baseline among all participants were: 0 – 2.8 (Flagellin IgA); 0 – 2.8 (Flagellin IgG); 0 – 3.1 (LPS IgA); 0.1– 2.6 (LPS IgG).

Cancer Epidemiol Biomarkers Prev. Author manuscript; available in PMC 2017 February 01.

Table 2

Mean baseline plasma levels of gut permeability biomarkers by demographic and lifestyle factors[a]

		Flagellin IgA[b]			Flagellin IgG[b]			LPS IgA[b]			LPS IgG[b]			Permeability score[b,c]		
	N	**Mean**	**SE**	***P* value**	**Mean**	**SE**	***P* value**	**Mean**	**SE**	***P* value**	**Mean**	**SE**	***P* value**	**Mean**	**SE**	***P* value**
Age, yrs.																
< 55	61	1.55	0.06		1.64	0.06		1.32	0.07		0.87	0.05		5.37	0.18	
55 - 64	72	1.65	0.06		1.74	0.05		1.39	0.06		0.86	0.05		5.64	0.16	
≥ 65	56	1.66	0.07	0.21	1.67	0.06	0.68	1.40	0.07	0.37	0.81	0.05	0.42	5.54	0.18	0.49
Sex																
Male	119	1.71	0.04		1.73	0.04		1.46	0.05		0.83	0.03		5.73	0.12	
Female	70	1.53	0.06	0.01	1.65	0.05	0.22	1.28	0.06	0.02	0.86	0.04	0.62	5.32	0.16	0.05
BMI, kg/m^2																
< 25	49	1.47	0.07		1.63	0.06		1.18	0.07		0.76	0.05		5.04	0.19	
25 - 27.49	34	1.58	0.09		1.72	0.07		1.36	0.09		0.84	0.07		5.49	0.24	
27.50 - 29.99	41	1.61	0.08		1.70	0.07		1.40	0.08		0.94	0.06		5.64	0.22	
30 - 34.99	50	1.72	0.07		1.72	0.06		1.47	0.07		0.83	0.05		5.74	0.19	
≥ 35	15	1.93	0.13	< 0.01	1.70	0.11	0.34	1.65	0.13	< 0.01	0.98	0.10	0.08	6.26	0.35	< 0.01
Waist-hip ratio[d]																
Tertile 1	63	1.59	0.06		1.69	0.05		1.32	0.07		0.81	0.05		5.41	0.17	
Tertile 2	64	1.54	0.06		1.65	0.05		1.34	0.07		0.88	0.05		5.41	0.17	
Tertile 3	62	1.73	0.06	0.13	1.72	0.05	0.72	1.45	0.07	0.17	0.85	0.05	0.55	5.74	0.18	0.18
Waist circumference, cm[d]																
Tertile 1	64	1.51	0.06		1.65	0.05		1.27	0.06		0.76	0.05		5.19	0.17	
Tertile 2	63	1.55	0.06		1.70	0.05		1.29	0.06		0.90	0.05		5.44	0.17	
Tertile 3	62	1.81	0.06	< 0.01	1.71	0.05	0.35	1.55	0.06	< 0.01	0.87	0.05	0.10	5.95	0.17	< 0.01
Current cigarette smoker																
Yes	38	1.66	0.08		1.67	0.07		1.43	0.08		0.79	0.06		5.55	0.22	
No	148	1.61	0.04	0.59	1.68	0.04	0.86	1.36	0.04	0.48	0.87	0.03	0.24	5.52	0.12	0.93
Regular NSAID user[e]																
Yes	54	1.69	0.07		1.74	0.06		1.48	0.07		0.80	0.05		5.71	0.18	

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

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

[a] All means, standard errors, and p values were calculated using analysis of covariance (ANCOVA). Models for all variables but sex were adjusted for sex (men/women). Models for all variables but body mass index, waist-hip ratio, and waist circumference were also adjusted for BMI (continuous). P value is for trend if the explanatory variable has > two categories.

[b] The unit for any permeability biomarker alone or in combination is optical density (OD).

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

[d] Tertiles are sex-specific.

[e] Regularly take once or more a week.

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

Table 3

Mean baseline plasma levels of gut permeability biomarkers by sex-specific tertiles of systemic inflammation biomarkers[a]

		Flagellin IgA[c]			Flagellin IgG[c]			LPS IgA[c]			LPS IgG[c]			Permeability score[c,d]		
Categories[b]	**N**	**Mean**	**SE**	***P*** **trend**	**Mean**	**SE**	***P*** **trend**	**Mean**	**SE**	***P*** **trend**	**Mean**	**SE**	***P*** **trend**	**Mean**	**SE**	***P*** **trend**
Men and women combined																
CRP																
tertile 1	62	1.62	0.06		1.72	0.05		1.36	0.07		0.81	0.05		5.51	0.17	
tertile 2	64	1.52	0.06		1.63	0.05		1.27	0.06		0.85	0.05		5.27	0.17	
tertile 3	63	1.73	0.06	0.18	1.71	0.05	0.92	1.48	0.06	0.18	0.88	0.05	0.36	5.80	0.17	0.22
Z-score[e]																
tertile 1	61	1.67	0.06		1.63	0.05		1.38	0.07		0.80	0.05		5.48	0.18	
tertile 2	64	1.57	0.06		1.68	0.05		1.30	0.06		0.84	0.05		5.38	0.17	
tertile 3	64	1.63	0.06	0.69	1.74	0.05	0.14	1.43	0.06	0.60	0.90	0.05	0.15	5.71	0.17	0.36
Men																
CRP																
tertile 1	39	1.76	0.07		1.86	0.07		1.49	0.08		0.88	0.06		5.99	0.21	
tertile 2	40	1.64	0.07		1.60	0.07		1.36	0.08		0.82	0.06		5.43	0.21	
tertile 3	40	1.75	0.07	0.95	1.73	0.07	0.18	1.53	0.08	0.70	0.80	0.06	0.34	5.80	0.21	0.55
Z-score[e]																
tertile 1	39	1.73	0.07		1.65	0.07		1.40	0.08		0.78	0.06		5.56	0.21	
tertile 2	40	1.63	0.07		1.76	0.07		1.34	0.08		0.82	0.06		5.55	0.21	
tertile 3	40	1.79	0.07	0.55	1.77	0.07	0.26	1.64	0.08	0.04	0.91	0.06	0.13	6.10	0.21	0.07
Women																
CRP																
tertile 1	23	1.43	0.11		1.50	0.08		1.20	0.11		0.68	0.07		4.81	0.29	
tertile 2	24	1.37	0.11		1.71	0.07		1.18	0.10		0.88	0.07		5.13	0.28	
tertile 3	23	1.78	0.11	0.03	1.72	0.08	0.06	1.47	0.11	0.08	1.01	0.07	< 0.01	5.98	0.29	< 0.01
Z-score[e]																
tertile 1	22	1.63	0.12		1.64	0.08		1.43	0.11		0.84	0.08		5.54	0.31	
tertile 2	24	1.52	0.11		1.58	0.07		1.29	0.10		0.86	0.07		5.25	0.29	

Categories[b]	N	Flagellin IgA[c] Mean	SE	P_{trend}	Flagellin IgG[c] Mean	SE	P_{trend}	LPS IgA[c] Mean	SE	P_{trend}	LPS IgG[c] Mean	SE	P_{trend}	Permeability score[c,d] Mean	SE	P_{trend}
tertile 3	24	1.43	0.11	0.23	1.72	0.08	0.44	1.13	0.11	0.07	0.87	0.08	0.81	5.15	0.30	0.39

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

[a] All means, standard errors, and p values were calculated using analysis of covariance (ANCOVA). Models for all variables but sex were adjusted for sex (men/women). Models for all variables but body mass index, waist-hip ratio, and waist circumference were also adjusted for BMI (continuous).

[b] Tertiles are sex-specific.

[c] The unit for any permeability biomarker alone or in combination is optical density (OD).

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

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

Table 4

Effects of calcium supplementation on plasma concentrations of gut barrier function biomarkers[a]

	Baseline			4-month follow-up			Absolute treatment effect[b]			Relative
	n	Mean	SE	n	Mean	SE	Mean	SE	p value	Effect[c]
Flagellin IgA										
Placebo	64	1.60	0.06	59	1.58	0.06				
Calcium 1 g	62	1.71	0.06	58	1.72	0.06	0.03	0.04	0.40	1.02
Calcium 2 g	63	1.64	0.06	57	1.67	0.06	0.05	0.04	0.19	1.03
Flagellin IgG										
Placebo	64	1.69	0.05	59	1.69	0.05				
Calcium 1 g	62	1.79	0.05	58	1.80	0.05	0.00	0.03	0.88	1.01
Calcium 2 g	63	1.62	0.05	57	1.64	0.05	0.02	0.03	0.61	1.01
LPS IgA										
Placebo	64	1.37	0.06	59	1.34	0.07				
Calcium 1 g	62	1.40	0.07	58	1.43	0.07	0.06	0.03	0.09	1.04
Calcium 2 g	63	1.44	0.07	57	1.44	0.07	0.04	0.03	0.26	1.02
LPS IgG										
Placebo	64	0.83	0.05	59	0.87	0.05				
Calcium 1 g	62	0.83	0.05	58	0.84	0.05	−0.02	0.04	0.54	0.97
Calcium 2 g	63	0.87	0.05	57	0.87	0.05	−0.03	0.04	0.43	0.95
(Flagellin + LPS) IgA										
Placebo	64	2.97	0.12	59	2.92	0.12				
Calcium 1 g	62	3.11	0.12	58	3.15	0.12	0.09	0.06	0.17	1.03
Calcium 2 g	63	3.08	0.12	57	3.11	0.12	0.08	0.06	0.18	1.03
(Flagellin + LPS) IgG										
Placebo	64	2.52	0.08	59	2.56	0.08				
Calcium 1 g	62	2.62	0.08	58	2.64	0.08	−0.02	0.06	0.72	0.99
Calcium 2 g	63	2.49	0.08	57	2.51	0.08	−0.01	0.06	0.81	0.99
Flagellin (IgA + IgG)										
Placebo	64	3.29	0.10	59	3.27	0.10				
Calcium 1 g	62	3.50	0.10	58	3.52	0.10	0.04	0.06	0.54	1.01

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

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

[a] The unit for any permeability biomarker alone or in combination is optical density (OD).

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

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

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