Bowel Inflammation as Measured by Fecal Calprotectin: A Link between Lifestyle Factors and Colorectal Cancer Risk

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Abstract
The mechanisms by which the lifestyle risk factors obesity, physical inactivity, and low fiber intake predispose to colorectal cancer (CRC) are unclear. Chronic bowel inflammation predisposes to malignancy in cases of inflammatory bowel disease. Many lifestyle risk factors for CRC are associated with evidence of systemic inflammation as indicated by circulating levels of C-reactive protein (CRP), but it is unknown how this relates to inflammation at tissue level. Little is known about the degree of bowel inflammation in general population and the factors that affect it. Therefore, we aimed to assess the relation of levels of bowel inflammation in the general population and lifestyle risk factors for CRC, and to additionally assess whether these associations, if present, were attenuated by controlling for evidence of systemic inflammation.

Average CRC risk subjects (320) of either sex aged 50–70 were recruited in South London. A stool sample was provided for calprotectin measurement (a marker of bowel inflammation), serum for CRP, and a detailed dietary and lifestyle questionnaire completed.

There was a significant positive relationship between fecal calprotectin and increasing age (P = 0.002), obesity (P = 0.04), physical inactivity (P = 0.01), and an inverse relationship with fiber intake (P = 0.02) and vegetable consumption (P = 0.04). The relationship with obesity was attenuated by controlling for serum CRP.

Fecal calprotectin levels are associated with lifestyle risk factors for colorectal cancer. Low-level asymptomatic bowel inflammation may be the link between lifestyle and the pathogenesis of CRC, and circulating proinflammatory cytokines may be part of the mechanism for this link.

Introduction
Sporadic colorectal cancer (CRC) is the third commonest fatal malignancy in Western countries (1) accounting for >10% of all cancer deaths (2). In the United Kingdom alone CRC claims 20,000 lives/year (3). There is a significant heritable component to CRC, but this accounts for perhaps only 20% of cases.

Laboratory, nutritional, and epidemiological evidence implicates dietary factors in the pathogenesis of CRC. Global variation in the incidence of CRC (4), increase in incidence of CRC in several countries (5), and change in incidence on migration (6) all support the role of the environment. Fiber has been implicated as playing a role in pathogenesis with early population-based studies describing a reduced risk in populations consuming a high fiber diet (7); however, there is discrepancy between cereal fiber data (which is largely neutral), and fruit and vegetable fiber (which are largely protective; Ref. 8). Obesity increases the risk of a number of cancers including CRC, and in the European Union 21,500 cases of CRC per year are attributable to obesity (9). Physical activity has a protective effect on CRC (10). CRC occurs more commonly with increasing age. Cigarette smoking and alcohol consumption have been implicated as playing a role in the pathogenesis of CRC, but they are not well-validated risk factors. The mechanisms behind these associations are unclear.

Inflammation is a risk factor for a number of gastrointestinal and other malignancies. In subjects with chronic bowel inflammation, risk of CRC is elevated (11). Pharmacological suppression of this inflammation lessens the risk (12). In subjects without inflammatory bowel disease (IBD) regular consumption of anti-inflammatories (aspirin) reduces CRC risk (13). Sporadic CRC shares a number of biological similarities to IBD-associated malignancies, and IBD-associated colon cancer is strongly related to inflammation; this has lead to speculation that in non-IBD subjects the bowel is in a constant state of low level inflammation, as a result of interaction between the colonic flora and mucosa, and that this may predispose to CRC (14).

Physical inactivity, obesity, and increasing age are all risk factors for atherosclerosis, which is characterized by an inflammatory response within the blood vessel wall. These risk factors are also associated with evidence of systemic inflammation as indicated by serum C-reactive protein (CRP; Ref. 15). Serum CRP closely correlates with circulating levels of proinflammatory cytokines, interleukin-6 in particular (16). It is possible that as well as modulating an inflammatory disease within the blood vessel wall, that these risk factors could influence inflammation within the bowel wall, either through direct local effects or through effects on circulating proinflammatory cytokines.

Calprotectin is a 36 kDa neutrophil-derived protein, which can be quantified in the feces, and has become established as a marker of whole gut inflammation. Of all of the fecal markers of bowel inflammation available calprotectin is emerging as one of the most promising (17). In subjects with IBD, levels...
correlate to histological degree of bowel inflammation (18) and to 3-day excretion of indium-labeled granulocytes (19). Fecal levels have also been investigated as a potential marker for colon adenomas and carcinomas. In subjects with colonic neoplasms after surgical excision fecal calprotectin falls (20); however, in those with polyps following polypectomy, levels remain elevated (21). This suggests that in subjects with polyps there is an abnormal field defect throughout the bowel and not just at the site of adenoma formation.

The aim of this study was to look for possible associations between environmental risk factors for CRC i.e., age, fiber intake, obesity, and lack of physical exercise, with calprotectin as a marker of gastrointestinal inflammation in middle-aged average risk CRC subjects. We also aimed to determine whether these associations if present were attenuated by adjustment for serum levels of CRP, which may suggest that the relationships reflect circulating levels of proinflammatory cytokines, rather than local proinflammatory actions. We also aimed to determine whether fecal calprotectin in healthy subjects is a measure of inflammation by comparing it to two other fecal markers, a permeability marker (α 1 antitrypsin) and another neutrophil-derived protein (lactoferrin).

Materials and Methods

Subjects. We recruited a random population sample 50–70 years of age from the registers of general practices in South London. A total of 500 subjects were invited, and 325 (65%) responded. Subjects with IBD were excluded (5 subjects). A previously validated demographic and lifestyle questionnaire and a 23-point food frequency questionnaire were completed (22), the latter supplemented by questions about the number of portions/servings or pieces of fruit and vegetables consumed per day (23). A stool sample was provided as soon after this as possible (within 2 days of completion of the questionnaire). Informed consent in writing was obtained from each subject. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the local research and ethics committee.

Stool Sampling and Measurement of Calprotectin. The fecal calprotectin assay was carried out according to the manufacturer’s instructions (Calprest, Eurospital ASA, Trieste, Italy).

Briefly, fecal samples (40–120 mg) were collected with a disposable, breakable inoculation loop (10 µl; sterile, firm loop) and placed into a 14-ml disposable screw cap tube. The feces weight was measured and the loop handle broken off, leaving the loop and part of the handle inside the tube. Extraction solution (Calprest, Eurospital ASA) was added in a weight:volume ratio of 50:1. After 30-s agitation on a mixer followed by homogenization for 30 min at 14,000 rpm on a mechanical shaker, 1 ml of the homogenate was transferred to an Eppendorf tube and centrifuged for 20 min at 10,000 × g. The supernatant was collected for analysis.

The supernatant was assayed using a calprotectin ELISA according to the manufacturer’s instruction. The supernatants were diluted 1:50 with dilution buffer and for high concentration samples were diluted again at 1:250. Diluted supernatants (100 µl) were added to the microtiter plate wells, incubated at room temperature for 45 min on a shaker (600 rpm), and washed three times with washing solution. Affinity purified rabbit anticalprotectin antibodies conjugated with alkaline phosphatase were added and incubated for 45 min at room temperature on a shaker (600 rpm). After washing as above, 100 µl of substrate solution was added to each well followed by 100 µl 0.1 M NaOH after ~30 min. Optical densities were read at 405 nm. Calprotectin concentrations were calculated from the standard curve obtained from the kit standards. Interassay variation was 14.8% and intra-assay variation 1.9%.

Serum CRP was measured on a high sensitivity auto- analyzer (Immulyte 2000 auto-analyzer; Euro DPC Gwynedd) and expressed as mg/l, according to the manufacturer’s instructions. coefficient of variation was 3.1–8.7%.

A subset of 100 subjects had fecal α 1-antitrypsin (α1AT; a marker of gastrointestinal permeability; Ref. 24) and fecal lactoferrin (a neutrophil derived protein) assessed. Fecal lactoferrin was quantified using IBD-SCAN (Techlab, Blacksburg, VA) according to the manufacturer’s instruction. Briefly, 0.05 g of feces was added to an extraction diluent and mixed by vortex mixer. These samples were then stored at 4°C until the ELISA was performed. Before ELISA the samples were mixed by vortex again; the coefficient of variation was 7.4%–23.5%.

Fecal α1AT was measured using extracts from the calprotectin extraction method. These were analyzed by rate nephelometry using Beckman Image system (Beckman Coulter, Buckley, United Kingdom). Reagents were supplied by Beckman Coulter and used according to the manufacturer’s instruction; the coefficient of variation was 4.6%–5.6%.

Statistics. For the purpose of statistical analysis calprotectin results were log 10 transformed to correct for the positive skew of its distribution. Log fecal calprotectin was analyzed as the dependent variable in relation to age (continuous variable), body mass index (BMI; continuous variable), sex (male/female), physical activity (binary variable), pack years of cigarette smoking (continuous variable), units of alcohol consumed per week (continuous variable), serum CRP (continuous variable), and dietary measures (%). BMI was measured in kilogram weight/height in m² (all subjects were weighed and had their height recorded at the recruitment interview). Exercise was classified as any form of regular (at least once per week) physical activity carried out by the subjects that they regarded as in addition to their usual daily activity. Fruit and vegetable consumption was classified as number of pieces/ portions/servings per day; potatoes were not included. The percentage basic food groups (fiber, fat, protein, and carbohydrate) were calculated from the DINE questionnaire on the basis of the number of pieces/ portions/servings of the basic food group of interest divided by the total number of portions/pieces/servings of all of the food groups in the previous week. Stool form was self-reported by the subjects and was characterized as hard, pellets, thin, normal, mushy, or watery. Due to small numbers reporting certain types of stool form the groups were merged into normal, hard (hard or pellets), or soft (thin, mushy, or watery), with hard and soft being analyzed as dummy variables. Any nonsteroidal anti-inflammatory drug (NSAID), including low-dose aspirin, use in the week before the study was analyzed as a binary variable NSAID exposure being either present or absent. This was corrected for in the analysis, because NSAIDs can elevate fecal calprotectin levels (25). Cigarette smoking was analyzed as pack year smoking history (1 pack year = smoking 20 cigarettes/day for 1 year). Alcohol consumption was measured in units per week (1 unit of alcohol = 8 g) in the week before recruitment into the study.

For multiple regression analysis the relation of log 10 fecal calprotectin to age, BMI, sex, exercise, pack years of cigarette smoking, units of alcohol drunk per week, portions of fruit per day, portions of vegetables per day, and percentage of carbohydrate, fat, protein, and fiber were analyzed using Statview (SAS Institute Inc., Cary, NC)

Additional analyses were performed to control the asso-
associations between lifestyle risk factors and fecal calprotectin, and for serum CRP, the latter being log 10 transformed. Comparison of fecal α1AT, lactoferrin, and calprotectin was by Spearman’s correlation coefficient.

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Results

Calprotectin Levels. The median fecal calprotectin in our population was 27 μg/g with a range of 2–440 μg/g. Interassay variation was 14.8%, and the intra-assay variation was 1.9%. Twenty-two subjects provided a second specimen 10–14 days after the first; there was strong correlation between fecal calprotectin levels between each sample (r = 0.98; P < 0.0001). Seventy-nine subjects (24.7%) had fecal calprotectin levels above the normal range (<65 μg/g).

Demographic Details. One hundred sixty-three males (median age, 62) and 157 females (median age, 59) enrolled in the study. There was no difference in levels between males and females (geometric mean fecal calprotectin 32.4 μg/g and 33.5 μg/g, respectively). The distribution of the exposure variables of interest are shown in Table 1. The exposure variables were equally distributed between the sexes apart from BMI, pack year of smoking history, units of alcohol per week, and portions of vegetables per day; male subjects were heavier, had a higher cigarette exposure, consumed more alcohol, and ate less vegetables. Fig. 1 shows the distribution of fecal calprotectin in our population.

Stool Form and Fecal Calprotectin Levels. Subjects with self-reported hard stools had elevated fecal calprotectin levels compared with those with normal or loose stools (Table 2).

Diet, Lifestyle and Fecal Calprotectin Levels. Table 3 shows the relationship of factors to fecal calprotectin levels unadjusted, and the effect of adjusting for age, sex, stool form, serum CRP, and NSAID use. A significant independent relation was found with age, obesity, fiber consumption, physical activity, and serum CRP. Nonsignificant trends were seen with alcohol consumption and NSAID use. After additional correction for age, sex, stool form, and NSAID use the significant independent relationships were all maintained, and vegetable consumption became significant. These relationships are demonstrated in Fig. 2. After correction for age, sex, stool form, NSAID use, and serum CRP the relationship of fecal calprotectin and BMI was lost, and the relationships found for fiber intake, physical activity, and age all remained.

Alcohol consumption and NSAID use had no effect on fecal calprotectin in our study population.

Diet, Lifestyle, and Serum CRP Levels, and the Effect of Controlling the Effect of Lifestyle Risk Factors for CRC for Serum CRP. The effect of controlling the associations of the different risk factors for serum CRP is shown in Table 3. The relationship of obesity was accounted for by controlling for serum CRP levels, whereas the relation with physical inactivity, fiber intake, and age remained.

Comparison of Fecal Calprotectin with Lactoferrin and α1AT. Measurements for fecal α1AT were between 0.014 and 0.069 mg/g, and fecal lactoferrin 0.005–0.329 μg/g. Fifty percent fecal lactoferrin measurements were undetectable (<0.008 μg/g) making the assay unsuitable for the study of normal levels of bowel inflammation. There was no statistically significant relationship between fecal calprotectin and fecal α1AT (Rho = 0.24; P = 0.08). There was a moderate correlation between fecal calprotectin and lactoferrin (Rho = 0.41; P = 0.0007). There was no relationship between lactoferrin and α1AT (Rho = 0.15; P = 0.71).

Discussion

This is the first study to examine dietary and lifestyle risk factors for CRC and their effects on bowel inflammation as measured by fecal calprotectin. Several proposed environmen-
nal risk factors were found to be associated with fecal levels in a direction consistent with increased risk. The relationship of obesity was greatly attenuated by controlling for serum CRP. The degree of statistical significance for the relationship of each individual risk factor was modest, but the fact that all of the well-recognized environmental risk factors were associated lends weight to our hypothesis. The chance of all of the four main risk factors being associated by chance falls to 0.05 to the 4th power = 0.0000063.

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inflammation due to enhanced vagal and reduced basal sympathetic tone. This has been suggested to underlie the association of physical exercise with reduced serum levels of CRP (41). The autonomic nervous system is also a potent modulator of inflammatory responses at the tissue level (41), and this could be the mechanism for the association of exercise with reduced bowel inflammation.

The effects of dietary constituents on fecal calprotectin may be via the effects that they have on colonic bacteria. The colon is a reservoir to a huge volume and number of bacteria; dietary fiber reaches the colon where it is fermented by bacteria causing changes to the species distribution of the bacterial flora. A diet low in fiber and vegetables may promote the growth of more unfavorable microbial species.

In our study the colons of the subjects were not examined. Colonoscopic studies in asymptomatic individuals in the same age range of our study population have described a 10% prevalence of significant colonic neoplasms (adenomas ≥10 mm in diameter, villous adenoma, adenoma with high-grade dysplasia, or invasive cancer; 42). Some, but not all, of these lesions would cause alterations to fecal calprotectin levels (43) but are unlikely to explain the continuous relationships that we observed throughout the distribution of each of the risk factors. The fecal calprotectin levels in our study population were lower than published levels in subjects with IBD, but they do overlap, with 24.7% of our population having fecal calprotectin levels above the reference range.

The discovery that an easily measurable fecal protein reflects a number of lifestyle risk factors for CRC gives insight into the possible mechanism behind which the environment host interaction leads to CRC and provides a means by which environmental risk factors for CRC may be dissected from confounding factors. Prospective studies are required to determine whether bowel inflammation is indeed a risk factor for the development of CRC. This opens the way for studies to address the issue of whether modification of these risk factors reduces fecal calprotectin levels and influences subsequent CRC risk.

References
Lifestyle and Colorectal Cancer


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