Author's response to reviews

Title: Association of plasma endotoxin, inflammatory cytokines and risk of colorectal adenomas.

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Author's response to reviews: see over
November 12, 2012

Editor,
BMC Cancer

RE: “Association of plasma endotoxin, inflammatory cytokines and risk of colorectal adenomas”

Dear editors,

We thank the editor as well as the reviewers for their helpful comments on our manuscript titled “Association of plasma endotoxin, inflammatory cytokines and risk of colorectal adenomas”. We have revised the manuscript in response to the reviewers’ comments as detailed in the point by point response below. Changes in the manuscript are highlighted in yellow. Changes have also been made to tables, and this is included in the submission.

The manuscript has not been previously published and is not under consideration elsewhere. All authors approved the final version of the manuscript.

Thank you for your helpful comments, and we look forward to hearing your decision.

Sincerely,

Sincerely,

Temitope Keku, Ph.D, AGAF
Professor of Medicine
Adjunct Professor of Nutrition
Reviewer’s report  Reviewer #1.

General Required Changes.
1) Please change “levels” to “concentrations”. If you use “endotoxin” without levels, please change to “concentrations of endotoxin”.

Done.

2) As a general rule, P ≤ 0.05 is considered significant while 0.05 < P < 0.10 is considered a tendency toward significance. Please use these cut-offs to designate differences between groups in the manuscript.

Done.

3) I’m concerned that you equate cytokine expression of rectal mucosal biopsies with local inflammation. If you would have taken biopsy samples adjacent to adenomatous tissue, I would have seen the relevance to adenoma risk. Results from mucosal rectal biopsies, however, have not been shown in the literature closely related to the risk of colorectal adenomas. If you would have taken biopsy samples from the colon, I would have seen the relevance to plasma endotoxin values but the rectum is not a major site of fermentation.

Added to discussion: This is a point brought up by both reviewer #1 and #2. We added a section to address this issue in the discussion (page 17, line 372).

In this study, mucosal biopsies were obtained from the rectum while the adenomas were found in the colon. We and others have routinely sampled the rectum in human studies to assess biomarkers of colorectal adenoma and cancer risk. This is based on the idea of a “field effect” where events occurring in the rectum are reflective of events going on elsewhere in the colon (Martin et al, Gastro 2002; Keku et al, Cancer Epidemiol Biomarkers Prev 2005; Carroll et al, Cancer Prev Res 2011; Thompson et al, Gastro 2010; Barnes et al, Cancer Epidemiol Biomarkers Prev, 1999; Anti et al, Gut 1993). Ponz de Leon et al examined mucosal proliferation in different parts of the large bowel in subjects with colorectal polyps or cancer and normal controls (Cancer Res 1988). They found that there were no significant differences in cell proliferation between mucosal samples taken at various distances from the colorectal cancer margin suggesting that hyperproliferation of the entire colonic mucosa was common in patients with colorectal cancer. Further, we also have previously established that decreased apoptosis is a risk factor for adenomas, and lower rates of apoptosis can be detected from normal rectal mucosa distant from adenomatous tissue (Cancer Epidemiol Biomarkers Prev 2005). This field effect, where similar events occur at distant sites, in the intestine is not limited to cellular growth and apoptosis but has also been observed with bacterial composition. With regards to bacteria communities, Zoetendal et al demonstrated that predominant community of the bacteria in biopsy samples from all locations of the colon have very similar profiles even though the biopsies were not from the same area of the colon as where adenomas were found (Appl Environ Microbiol
Momozawa et al examined 7 sites in the gut and noted that there were no significant quantitative or qualitative differences in bacteria from ileum to rectum, suggesting that the majority of bacteria would be similar throughout the large intestine (PLoS One 2011). Thus, these studies suggest that rectal mucosal biopsies could be a good surrogate for biopsies adjacent to adenomas.

A) Please make a more convincing argument why rectal mucosa biopsies are a good indicator for local inflammation.

Several animal and human studies suggest a link between inflammation and colorectal adenomas and cancer. Mucosal inflammation of the rectum is common in ulcerative colitis, and ulcerative colitis has an increased risk of colorectal cancer. Thus, it is not improbable to evaluate mucosal inflammation in rectal biopsies in relation to adenomas. Taken together with our previous observations that rectal mucosal apoptosis predict adenomas elsewhere in the colon, we feel it is logical to examine markers of inflammation in rectal biopsies and assess their association with adenomas.

B) For Page 10 Line 16: Please change “At the local level” to “Rectal mucosa”.

Done.

C) Please discuss in the Discussion why rectal mucosa cytokine gene expression may be related with plasma endotoxin concentrations.

Added to discussion (page 14, line 293): We proposed that an increased proportion of gram negative bacteria in the adenomatous gut would lead to increased endotoxin release and thereby trigger inflammatory cytokine production. This, in turn, would promote leakiness of the intestinal mucosal barrier and cause translocation of endotoxin into the bloodstream. A recent study showed that intraluminal administration of LPS in the colon resulted in an altered local cytokine production suggesting that elevated LPS in the colon is able to cause intestinal inflammation (Im et al 2012)

We also added that one of the limitations of the study could be that we did not measure the endotoxin concentrations in the mucosa to compare (page 18, line 369)

D) Please include the site of mucosal biopsies as one of the limitations of your study.

While we understand the reviewer’s point, we feel that the rectal biopsies are a surrogate for colon biopsies and is reflective of events elsewhere in the colon, such as the bacterial composition, cellular proliferation and apoptosis as explained in the response to the question #3 by reviewer #1 (general required changes).

E) When appropriate specify that you examined “rectal mucosal inflammation” and not “mucosal inflammation” per se (page 3 Line 4).

Done.
4) Please do not consider results with a $P = 0.11$ as significant and different.

Done.

5) Endotoxin transfer in the blood stream can be caused by many factors besides presence of adenoma. As suggested, type of adenoma should affect whether endotoxin is transferred. Furthermore, tissue damages other than adenomas can increase endotoxin concentrations in plasma. Please include this into your discussion.

We agree that endotoxin transfer to the bloodstream could be caused by other reasons besides adenomas. We have addressed this in the discussion (page 17, line 354):

While endotoxin transfer to the bloodstream could be caused by other factors such as tissue damage, infection and other medical conditions that may cause systemic inflammation, all the subjects in our study were ambulatory and healthy enough to undergo outpatient screening colonoscopies. Also, even though we did not have detailed past infection histories, one of the inclusion criteria was that they had not taken antibiotics within twelve weeks prior, thus, ruling out recent bacterial infections contributing to inflammation.

We included a new Table 3 (page 23) comparing endotoxin concentrations by different adenoma characteristics.

6) The challenge with the LAL test in your study is not the sensitivity because you have sufficient concentrations of endotoxins in your samples, according to Table 2. A greater problem with LAL is the low repeatability (that is why I asked for the intra- and inter-assay CV) and the semi-quantitative nature of the test (it is a precipitation test with many serum components potentially interfering with the reaction). Please revise the section.

Added in methods (page 7, line 130): The intraassay and interassay coefficients were 4.3% and 17.2%, respectively.

(page 7, line 132) Quality control for plasma endotoxin assay. Blood samples were collected in EDTA containing tubes to prevent coagulation. Because LAL is composed of the coagulation system of the horseshoe crab, EDTA can inhibit LAL gel formation by chelating divalent cations in the LAL formulation, thus, depleting the lysate of the cations it needs to function properly. Therefore, samples were diluted in 10mM MgCl₂ to overcome the inhibitory nature of EDTA. This method was proven to pass the positive product control test by Lonza. Furthermore, plasma contains protein components that could inhibit the assay as well. Thus, to verify the lack of product inhibition, samples were tested for inhibition according to the manufacturer's instructions. Briefly, samples were either spiked with 0.4 EU/mL of endotoxin or unspiked. Endotoxin concentrations from both samples were determined. If the difference between spiked and unspiked samples equaled 0.4 EU/mL + 25%, the sample was considered uninhibited. We performed this on groups of samples and determined that plasma which contained hemolyzed red blood cells did not pass the test. Thus, only plasma samples with clear
yellow color passed the test and were included in our assay. With these measures, the components potentially interfering with the reaction were minimized.

Added in discussion (page 17, line 365): Even though we controlled for protein inhibition of the LAL endotoxin assay, it is possible that there were still components that could interfere with the reaction and could potentially obscure the distinction between cases and controls.

Minor required changes:
1) Page 2 Line 2: Please change “endotoxin was association with inflammation” to “concentrations of endotoxin were associated with indicators of systemic and local inflammation”
   Done.

2) Page 2 Line 8: Please change “endotoxin was measured” to “concentrations of endotoxin were measured”.
   Done.

2) Page 2 Line 8: Please change “We evaluated” to “We quantified concentrations of”.
   Done.

3) Page 2 Line 10: Please change “real time RT-PCR” to “quantitative RT-PCR”.
   Done.

4) Page 2 Line 10: Please change “IL-17” to “Interleukin 17”.
   Done.

5) Page 2 Line 13: Please change “than controls” to “than in controls”.
   Done.

6) Page 2 Line 15: Please change “pattern” to “patterns”.
   Done.

7) Page 2 Lines 16-17: Please change “in opposite directions” to “inverse”.
   Done.

8) Page 2 Line 18: Please change “r=0.35 P=0.0002” to “r=0.35, P=0.0002”.
   Done.
9) Page 4 Line 10: Please change “microbial products” to “microbial products,”.

Done.

10) Page 4 Line 16: Please change “adenomas” to “adenoma”.

Done.

11) Page 4 Lines 20/21: Please change the sentence.

Done.

11a) Page 5 Line 2: Please change “Gram” to “gram”.

Done.

12) Page 5 Line 4: Please change “and the release of LPS can cause” to “which can have”.

Done.

13) Page 5 Line 6: Please change “activation toll-like” to “activation of toll-like”.

Done.

14) Page 5 Line 8: Please change “LPS” to “Lipopolysaccharide”.

Done.

15) Page 5 Line 10: Please change “activate NF” to “activate the NF”.

Done.

16) Page 5 Line 13: Please change “that has” to “have”.

Done.

17) Page 5 Line 16: Please change “due to a shift toward” to “through a shift toward an”.

Done.

18) Page 5 Line 17: Please change “to increased” to “to an increased”.

Done.
19) Page 5 Line 20: Please insert a comma after “apoptosis”.

Done.

20) Page 5 Line 22: Please insert a comma after “inflammation”.

Done.

21) Page 5 Line 23: Please remove a comma after “cytokines”.

Done.

22) Page 6 Study Population: Please describe shortly the selection and matching criteria.

Added in Methods (page 6, line 101): Eligibility requirements were as follows: age > 30 years; proficiency in English to provide informed written consent and participate in a phone interview; a satisfactory preparation for colonoscopy and complete examination to the cecum; outpatient; and no history of familial polyposis, colitis, previous colonic resection, previous colon cancer or polyps, and had not taken antibiotics within 12 weeks prior to the colonoscopy.

This is a cross-sectional study, and the cases and controls were not matched. Case subjects were identified after the colonoscopy if they had an adenoma. This has been added to the Abstract and Methods sections (page 2, line 26, page 6, line 99, respectively).

23) Page 6 Line 4: Please change "Study population and .." to “The study population and.

Done.

24) Page 6 Lines 5 and 6: Please change the sentence to “Briefly, the subjects in our study, drawn from the Diet and Healthy Study V (DHSV), underwent screening colonoscopies at UNC Hospitals.”

Done.

25) Page 6 Line 7: Please change “have” to “had”.

Done.

26) Page 6 Line 8: Please remove “the”.

Done.
27) Page 6 Line 13: Please add a space before “cm” and add “the” after “from”.
Done.

28) Page 6 Line 14: Please change “in” to “at”.
Done.

29) Page 6 Line 22: Please add a comma before “then”.
Done.

30) Page 7 Line 2: Please change “plates” to “plate”. Please remove “the” and add a “the” after “to”.
Done.

31) Page 7 Line 9: Please add a sentence: “The intra-assay and inter-assay CV for plasma endotoxin concentrations were XX% and XX%, respectively.
Please see the response to question #6, reviewer #1 (general required changes).

32) Page 7 Line 10: Please change “real-time quantitative PCR (qPCR)” to quantitative real-time PCR (qRT-PCR)”.
Done.

33) Page 7 Line 11: Please change “Methods of RNA extraction and real time qPCR” to “Extraction of RNA and qRT-PCR”.
Done.

34) Page 7 Line 14: Please change “manufacture’s” to “manufacturer’s”.
Done.

35) Page 7 Line 15: Please change “was” to “were”.
Done.

36) Page 7 Line 17: Please change “1µg” to “One µg”.
Done.
37) Page 7 Line 20: Please change “Hydroxymethylbilane Sythase” to “hydroxymethylbilane synthase”.

Done.

38) Page 7 Line 20: Please change “7” to “seven”.

Done.

39) Page 7 Line 22: Please add a comma after “primer”.

Done.

40) Page 8 Line 7: Please add a comma before “and”.

Done.

41) Page 8 Line 8: Please change “Laboratories Inc)” to “Laboratories, Inc.”).

Done.

42) Page 8 Line 8: Please add a “the “ after “to”.

Done.

43) Page 8 Lines 10, 12, 13: Please change “levels” to “concentrations”.

Done.

44) Page 8 Line 11: Please change “by Mann-Whitney test” to “by the Mann-Whitney U test”.

Done.

45) Page 8 Lines 12-14: Please specify whether you used conditional or unconditional logistic regression. Please specify which subject characteristics were tested as potential confounders, what was the cut-off for inclusion of potential confounders to be included in the final model, and which subject characteristics plus their groupings were included into the final logistic regression model. Please specify which characteristics were tested for effect modification and how you tested for effect modification.

Done. Added to methods (page 10, line 207): Plasma cytokines, tissue cytokines, BMI, physical activity, smoking status and daily fat intake were tested as potential confounders. Each was added to a logistic regression model with case status as the response and upper or lower halves of endotoxin concentrations as the predictor. If the odds ratios (OR) for the concentrations of endotoxin changed by at least 10% after the
inclusion of the new variable in the model, then that variable was considered to be a potential confounder. After all such potential confounders were identified, they were all entered into the model along with the concentrations of endotoxin, and a backwards stepwise procedure was performed with endotoxin being forced into the model. Ultimately, we identified all tissue cytokines and dietary fat as confounders and ran the final logistic regression model with adjustment for these variables.

Previous Table 3, now numbered Table 2, was changed to reflect the new results (page 23).

46) Page 8 Line 15: Please change “cytokines” to “cytokine concentrations”.

Done.

47) Page 8 Line 16: Please change “Association” to “Associations”.

Done.

48) Page 8 Line 17: Please add a sentence and the following analysis: “In addition, we compared concentrations of plasma endotoxins and plasma cytokines or mucosal cytokines using a panel of cytokines using multivariate regression”. Please present your results in the Results section.

Added to Results (page 13, line 262): In addition, we examined the relationship between plasma endotoxin concentrations and a panel of plasma or rectal mucosal cytokines using multivariate regression. We found that those in the upper half of endotoxin concentrations were significantly more likely to have higher plasma IL-12 as compared to those in the lower half of endotoxin concentrations (OR=1.5, CI 95% 1.0-2.2) (Supplementary Table 1). Similarly, those in the upper half of endotoxin concentrations were significantly more likely to have higher mucosal IL-12 (OR=1.9, CI 95% 1.0-3.7) and mucosal IL-17 (OR=2.2, CI 95% 1.0-4.6) than those in the lower half of the endotoxin concentrations (Supplementary Table 1). No statistically significant results were found for the remaining cytokines.

49) Page 8 Line 17: Please add the following sentence and do the following analysis: “In addition, we compared concentrations of plasma endotoxins, plasma cytokines, and mucosal cytokines between cases with one versus multiple adenomas, cases with at least one adenoma of at least 1 cm in size versus all adenomas below 1 cm in size, and cases with at least one adenoma of over 25% of villous component or high grade dysplasia versus no adenoma having these characteristics, using the Mann-Whitney U test, respectively. Please present your results in the Results section.

We added a new Table 3 (page 23) analyzing endotoxin concentrations by adenoma characteristics as suggested by the reviewer.
We also assessed whether concentrations of plasma endotoxin were associated with adenoma characteristics. The average number of adenomas was 1.8 per person (range 1-9). The adenoma size distribution was as follows: small adenoma, 1-5mm, 66%; medium adenoma, 6-10mm, 25%; large adenoma, >10mm, 9% (Table 3). Endotoxin concentration was significantly higher in those with villous adenomas than tubular adenomas (p<0.05) (Table 3). However, there were no associations between adenoma size or number and endotoxin concentrations.

We compared plasma and rectal mucosal cytokines between cases with one versus multiple adenomas, cases with at least one adenoma of at least 1cm in size versus all adenomas less than 1cm in size, and cases with at least one adenoma with villous component versus all adenomas having only tubular components, using Mann-Whitney U test. In subjects with multiple adenomas as compared to those with only one adenoma, only median mucosal IL-12 level was significantly higher (1.09 vs 0.65, p=0.04). In subjects with villous adenomas, only median plasma IFN-γ was significantly lower than in those with only tubular adenomas (0 vs 1.64 pg/mL, p=0.02). None of the other comparisons for number of adenomas or adenoma type and plasma or rectal mucosal cytokines were statistically significant (results not shown).

P-values less than 0.05 were considered statistically significant while P-values greater than 0.05 but less than 0.10 were considered a trend toward significance.

P-values were adjusted for multiple comparisons via False Discovery Rate (Benjamini and Hochberg, J of the Royal Statistical Society 1995).
55) Page 9 Lines 8-10: Please change “than controls while at the tissue level, the mRNA gene expressions of” to “as compared to controls. Gene expression of …were greater in cases than in controls”.

Done.

56) Page 9 Line 10: Please add a comma before “and”.

Done.

57) Page 9 Line 12: Please change “was positive” to “was a positive”.

Done.

58) Page 9 Lines 15, 16: Please change “controls,” to “controls”

Done.

59) Page 9 Line 17: Please change “for the cases than controls” to “for cases than for controls”.

Done.

60) Page 10 Line 3: Please change “endotoxins concentration” to “endotoxin concentrations”

Done.

61) Page 10 Line 6: Please insert a comma after “medium adenoma”.

Done.

62) Page 10 Line 8: Please show the results for the association between plasma endotoxin levels and the number or size of adenomas using the suggested cut-off points for number and size of adenomas.

Please see the response to question #49, reviewer #1 (minor changes).

63) Page 10 Line 12: Please change “Specifically we tested the hypotheses” to “Specifically, we tested the hypothesis”.

Done.

64) Page 10 Line 16: Please add a comma after “IL-17”.

Done.
Removed IL-17 and showed only values that are significant and trending toward significance.

65) Page 10 Line 19: Please change “than controls” to “than in controls”.
Done.

66) Page 10 Line 20: Please change “were” to “are”/Done.

67) Page 10 Line 21: Please add a comma after “levels”, which need to be changed to “concentrations”.
Done.

67a) Page 11 Line 10: Please change “cytokines” to “cytokine”.
Done.

67b) Page 11 Line 10: Please change “exposures” to “exposure”.
Done.

68) Page 11 Line 12: Please change “el” to “et”.
Done.

69) Page 11 Line 13: Please add a comma after “IL-8”.
Done.

70) Page 11 Line 13: Please change “, and this” to “. This”.
Done.

71) Page 11 Line 16: Please change “Since” to “Because”.
Done.

72) Page 11 Line 17: Please change “have” to “had”.
Done.

73) Page 11 Line 18: Please change “be” to “have been”.

Done.

74) Page 12 Line 1, 2: Please change “endotoxins” to “endotoxin”.
Done.

75) Page 12 Line 1: Please change “But our results showed no” to “Our results did not show a”.
Done.

76) Page 12 Line 2: Please change “numbers” to “number”.
Done.

77) Page 12 Line 4: Please remove “of”.
Done.

78) Page 12 Line 5: Please change “as low” to “as its low”.
Done.

79) Page 12 Line 10, 12: Please insert a comma before “and”.
Done.

80) Page 12 Line 10: Please insert “that” before “studies”.
Done.

81) Page 12 Line 17: Please change “found positive” to “found a positive”.
Done.

82) Page 12 Line 18: Please insert a comma before “the”.
Done.

83) Page 12 Line 19: Please change “among cases than controls” to “in cases than in controls”.
Done.

84) Page 12 Line 21: Please insert “of rectal” before “mucosal”.
Done.

85) Page 12 Line 22: Please insert a comma before “especially”.

Done.

86) Page 13 Line 1: Please change “endotoxin” to “endotoxins”.

Done.

87) Page 13 Line 3: Please change “and dampening the systemic inflammation, as well as effect of role of the microbiota” to “and the dampening of systemic inflammation, as well as the effect of microbiota”.

Done.

88) References: Please insert the serial issue for references 7, 13, 21, 23, 26, 29, and 32.

Done.

89) Reference 7: Please remove the extra space before “97”.

Done.

90) Page 20 Line 2: Please change “Characteristics” to “characteristics” and end the title with a period.

Done.

91) Table 1: Please insert a column at the end for the P-values and insert the P-values.

Table 1 was revised to reflect this (page 21).

92) Table 2: Please add the units of measurement in the first column.

For clarity, Table 2 has been separated into 2 tables, now Table 4 and Table 5. The text has also been revised to reflect this change. Please see pages 24, 25.

93) Table 3 Title: Please change to “Table 3. Association between plasma endotoxin concentrations and presence of adenomas.

As stated in response to question #92, the previous Table 3 is now table 4. Title has been revised.
94) Table 3 first row: Please move “Endotoxin” one row down and remove the second row of the table.

Done.

95) Table 3 first column: Please provide the cut-off values in the first column.

Done.

96) Page 24, Lines 2, 5: Please change “correlation” to “correlations”.

Done.

97) Page 24 Line 3: Please end the sentence with a period.

Done.

98) Page 24 Line 4: Please change “0.01” to “0.05” and correct figure 1 accordingly.

Done.

99) Page 24 Line 7: Please change “0.04” to “0.05” and correct figure 2 accordingly.

Done.

Reviewer's report. Reviewer #2.

Major compulsory
1. The background focused on the role of the Gram negative bacteria in the dysbiosis associated to CRC while it has been shown in numerous studies that S. bovis and later S. gallolyticus are most strongly associated with CRC and both of them are Gram positive. They need to mention this as it is related to their research.

Added to introduction (page 4, line 65): An imbalance of the bacterial population in favor of pro-oncogenic bacteria could lead to abnormal proliferation of the colonic epithelium and adenoma formation (Sears and Pardoll, J Infect Dis 2011, Tjalsma et al, Nat Rev Microbiol 2012). Previous studies have reported an association between gram positive bacteria, Streptococcus gallolyticus (formerly S. bovis) and colorectal neoplasia (Abdulamir et al, Cancer Res 2011). We have previously demonstrated that increased abundance of Proteobacteria, decreased abundance of Bacteroides, and a disproportionate colonization of the gut with predominant Escherichia coli were associated with adenomatous states (Sanapareddy et al, ISME J 2012, Shen et al, Gut Microbes 2010). More recently, Kostic et al observed that Fusobacterium were
enriched in CRC, also supporting a role of gram negative bacteria in colorectal carcinogenesis (Genome Research 2012). As one of the mechanisms by which S. gallolyticus promotes development of colorectal tumors is via production of inflammatory cytokines by the release of their cell wall antigen (Abdulamir et al, Cancer Res 2011), bacterial dysbiosis that favors higher abundance of gram negative bacteria could contribute to formation of adenomas via increased endotoxin release and inflammation.

Methods
2. For ELISA, although they referred to a previous study, there is a need to mention the minimal detectable values for each cytokines detected by the kit used in this study depending on their own standard curve. Personally I prefer they explain the whole procedure again in this study in order to be more readily available for the readers.

Added to methods (page 8, line 149): Any samples with gross hemolysis or lipemia were not assayed. Briefly, plasma samples were thawed and centrifuged to remove any precipitates prior to running the assay. The plasma cytokine assay was performed using the Milliplex high sensitivity human cytokine kit for IL-4, IL-6, IL-8, IL-10, TNF-α, and IFN-γ (HSCYTO-60SK, Millipore, Billerica, MA) following the manufacturer’s recommendation. Briefly, plasma samples were mixed with fluorescently labeled, color coded microspheres in 96-well plates and incubated overnight at 4°C. The next day, plates were washed and a biotinylated detection antibody was added, followed by incubation with agitation for 1 hour at room temperature. Next, Streptavidin-Phycoerythrin was added to each well, followed by incubation for 30 minutes and another wash step. Finally, the reaction was read immediately on the Bioplex 200 System (Biorad, Hercules CA). Fluorescent intensities for the samples were derived by fitting on a standard curve. Each assay was run in duplicate with positive controls included in each batch. The average of two measurements was used for data analysis. Minimal detection levels were 0.13 pg/mL for IL-4, 0.10 pg/mL for IL-6, 0.11 pg/mL for IL-8, 0.15 pg/mL for IL-10, 0.05 pg/mL for TNF-α, and 0.29 pg/mL for IFN-γ. The intra-assay and inter-assay coefficients of variation were 4.16% and 9.12% for IL-4, 3.51% and 4.48% for IL-6, 3.26% and 6.48% for IL-8, 3.31% and 11.84% for IL-10, 3.49% and 3.78% for TNF-α, and 4.88% and 7.79% for IFN-γ respectively.

3. Again for the RT qPCR, authors are encouraged to mention the whole procedure. They did not mention whether they took into consideration the real duplication factor of their PCR reaction, or have they assumed it was x^2, ie. They need to measure the efficiency of PCR reaction. Any RT qPCR without calculating the PCR efficiency is considered invalid.

Added to Methods (page 9, line 186): Cycling conditions were: 1 cycle at 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 30 seconds. All samples were run in duplicate for both the target and housekeeping genes. The housekeeping gene was used for normalization. Pooled RNA from control subjects was included with each batch of RT-PCR reaction and served as a reference.
This pooled RNA also served as a calibration point across different batches of PCR runs.

4. Here I want to ask that RNA quality measurement by Agilent bioanalyzer was directly done after extraction or there was an intervening storage period. Authors need to mention that how long they stored RNA, if any, and was stored at what temp till being converted to cDNA. This is important as RNA is not as stable as cDNA, therefore, long periods of storage might affect the RNA integrity and quality. The quality of RNA must be mentioned at least briefly.

Added to methods (page 9, line 169): Rectal biopsies were placed in RNA Later (Qiagen, Valencia, CA) immediately after collection. RNA extraction was performed within 1 week of obtaining the tissue biopsies, and isolated RNA was stored at -80°C in small aliquots to prevent repeated freezing and thawing. Extraction of RNA and qRT-PCR were previously described. Briefly, RNA was extracted from tissue biopsies using the Qiagen RNeasy Protect Mini Kit following the manufacturer’s protocol (Qiagen, Valencia, CA). RNA purity and concentration were evaluated by Agilent Bioanalyzer (Agilent, Santa Clara, CA) as well as absorbance readings using the NanoDrop ND-1000 spectrophotometer immediately after the extraction (Thermo Scientific, Wilmington, DE). Only samples with a RNA integrity number (RIN) above 7 were used for RT-PCR assays (Agilent, Santa Clara, CA).

Results
5. Authors did not mention the limiting and exclusion criteria of their cases. The medical and infection history of the cases were not shown at all. This is a serious drawback in this study. How can we be sure that the claimed increase in inflammatory cytokines is not due other reasons rather than a possible colonic neoplasm and in the current shortage of patients history and exclusion criteria, how we can trust the correlations done with plasma endotoxin.

Please see the response to question #22, reviewer #1 (minor changes). We have included this as a limitation in the discussion.

Please see the response to question #5, reviewer #1 (general required changes).

6. The general characteristics of the study population mentioned in table (1) are not justified within the rationale of the study. Moreover, authors did not get use of these data for linking with the levels of endotoxin and inflammatory cytokines. As we know obesity, race, fat intake ..etc might have sort of relation to the studied targets of the study. Therefore, table 1 has lost its core importance. It is strongly advised that authors correlate or associate these criteria with the obtained values of the study.

Please see the response to reviewer 1, question #45. In doing the analyses, we tested for confounding by the physical characteristics and included confounders in our analysis.
7. Authors did not classify histologically the adenoma cases. It is well known for the experts in the field that benign adenomas are largely different in terms of prognosis, histopathology, aggressiveness, and transformation rate when compared to non-benign. And adenomas in this study must be classified into tubular, villous and tubule-villous and authors must try to find associations with inflammatory cytokines and endotoxin separately as well.

**Added to Results (page 11, line 231):** All adenomas were discovered in the colon, and only 5 subjects had adenomas with a villous component. We analyzed the data using only tubular adenomas, but results were largely unchanged from when we analyzed tubular and villous adenomas together. Thus, we present the data for all adenomas.

8. The other thing, authors did not mention the locations of adenomas and did not do statistics about their location, is it in the colon and which part or in the rectum?

In Table 3 (page 23), we noted the locations of adenomas and compared the endotoxin concentrations according to the adenoma locations. (See Reviewer 1, response to question #49).

9. Taking rectal samples of mucosa from the anal verge is not necessarily a good choice for adenoma. Rectal samples might not reflect the inflammatory state at or near adenomatous lesions especially we do not know the location of adenomas. Second, why authors did not take mucosal samples adjacent or near adenomas (<2cm) in order to detect the local mucosal inflammation at or near to adenomas which is much more precise than taking rectal mucosa from the anal verge or at least presenting 2 lines of investigation; distant mucosal and adjacent or close to adenoma mucosal investigation.

Please see the response to question #5 A and B, reviewer #1 (general required changes).

10. Moreover, since colonoscopy was done for detecting adenomas, I suppose adenomas were removed. Hence, why authors did not measure the cytokine levels at adenomatous tissue and relate it to the distant mucosal and plasma levels of cytokines and endotoxin, they need to justify why they didn’t do so

The adenomas found in patients are primarily used for diagnosis. In addition, most of the adenomas are small in size and not enough to use in our analyses.

**Minor**

1. In the abstract, the relationships among plasma endotoxin and plasma and mucosal cytokines expression is expressed in a not clear way, please clarify in more detail. Any abbreviation mentioned in the abstract must be defined.

Done.
2. The conclusion part of abstract seems not clear and not well written

Rewritten.

3. In the keywords must add Limulus Amebocyte Lysate

Done.

4. Many abbreviations are not defined at the first time of appearance such as IBD or UNC.

Done.

5. For the data analysis: the tests used are sound but authors need to justify why they used Spearman’s rank test and Mann Whitney tests instead of the parametric counterparts such as t-test. Were their data classified as non-parameteric? if so, what tests did they use to determine the normality of their data.

Done. Added in methods (page 10, line 199): Median concentrations of plasma endotoxin and inflammatory markers were compared between controls and cases by the Mann-Whitney U test as this data was highly skewed and not normally distributed. As a result, we used nonparametric approaches (Mann-Whitney U tests) which do not assume normality.

REFERENCES:


