



Serum Levels of Lipopolysaccharides and Risk of Advanced Colorectal Adenoma

Ellie Chen¹, Anisha Kalavar¹, Ngoc-Anh Bui-Thanh^{1,2}, Antone R. Opekun^{1,3,4},
Donna L. White^{1,3,4,5,6}, Daniel Rosen⁷, David Y. Graham^{1,3,4},
Rolando E. Rumbaut^{1,2}, Hashem B. El-Serag^{1,3,4,5,6} and Li Jiao^{1,3,4,5,6*}

¹Department of Medicine, Baylor College of Medicine, Houston, TX, USA; ²Center for Translational Research on Inflammatory Diseases (CTRID), Michael E. DeBakey VA Medical Center, Houston, TX, USA; ³Section of Gastroenterology and Hepatology, Michael E. DeBakey VA Medical Center, Houston, TX, USA; ⁴Texas Medical Center Digestive Disease Center, Houston, TX, USA; ⁵Center for Innovations in Quality, Effectiveness and Safety (IQuEST), Michael E. DeBakey VA Medical Center, Houston, TX, USA; ⁶Dan L Duncan Cancer Center, Baylor College of Medicine, Houston, TX, USA; ⁷Section of Pathology and Immunology, Michael E. DeBakey VA Medical Center, Houston, TX, USA

Abstract

Background and objectives: Production of lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria promotes the survival of cancer cells. Systemic level of LPS is considered a biomarker for microbial translocation. The association between LPS and the risk of colorectal tumors is not well known. The goal of this study was to examine the association between LPS serum levels and risk of advanced colorectal adenoma (ACA).

Methods: In this colonoscopy clinic-based case-control study, cases were male patients with a diagnosis of ACA, and controls were polyp-free male participants. Cases and controls were individually matched by age, ethnicity, and blood collection time. Information on demographics, lifestyle, and medical history was obtained using structured questionnaires. Serum levels of LPS were quantitated using the kinetic limulus amoebocyte lysate assay. Multivariable conditional logistic regression model was used to estimate the odds ratio and its 95% confidence interval of the ACA in association with serum LPS adjusting for cigarette smoking, body mass index, and medical history.

Results: We examined 43 cases and 43 paired controls, with a mean age of 62 years. There was no significant difference in serum LPS levels between the cases and controls (0.28 vs. 0.25 endotoxin units (EU)/mL, $P = 0.58$ for the non-parametric test). The adjusted odds ratio and its 95% confidence interval of ACA was 1.83 (0.40–8.24) in multivariable logistic regression model.

Conclusions: Serum levels of LPS were not statistically significantly associated with an increased risk of ACA in this preliminary study.

Keywords: Gut microbiome; Colorectal cancer; LPS; Endotoxin, Leaky gut; Microbial translocation; Biomarker.

Abbreviations: ACA, advanced colorectal adenoma; BMI, body mass index; CI, confidence interval; CRC, colorectal cancer; CV, coefficient of variation; EU, endotoxin units; LAL, limulus amoebocyte lysate; LPS, lipopolysaccharides; MEDVAMC, Michael E. DeBakey VA Medical Center; MET, metabolic equivalent of task; OR, odds ratio; TLR, toll-like receptor.

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*Correspondence to: Li Jiao, Center for Innovations in Quality, Effectiveness and Safety (IQuEST), Michael E DeBakey VA Medical Center, 2002 Holcombe Blvd, MS152, Houston 77030, Texas, USA. Tel: 713-440-4456, Fax: 713-748-7359, E-mail: jiao@bcm.edu

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Introduction

The gastrointestinal tract is the habitat of numerous bacteria of which some are known pathogens, including *B. fragilis*, *Clostridium*, and certain strains of *Escherichia coli*. The microbiome has multiple functions in metabolism, mucosal integrity, and immunity.¹ Prior studies have shown that an imbalance of the gut microbiome and the microbial metabolites can trigger dysregulation of various metabolic pathways that lead to colon cancer.^{2–5} For example, the Gram-negative anaerobic bacteria, *Fusobacterium*, are more abundant in colorectal cancer (CRC) tissue. *Fusobacterium nucleatum* creates an inflammatory environment that promotes the formation of colorectal tumors.⁶ Similarly, *Escherichia coli* cause genotoxic inflammation by attaching to the gut epithelium.^{7,8} Sev-

eral mediators for these oncogenic effects of the bacteria have been proposed. One of which is through lipopolysaccharides (LPS), which directly induce deleterious alterations of enterocyte membrane structure and function.

LPS, also referred to as endotoxins, are found in the outer membrane of Gram-negative bacteria. When Gram-negative bacteria undergo cell death or cell division, the bacterial membranes release LPS. Microbial translocation is defined as the passage of viable or non-viable microbes and microbial product such as LPS across an anatomically intact intestinal barrier to lamina propria and mesenteric lymph node, and possibly other tissue.⁹ The continuous presence of these products in the systemic circulation may lead to low-grade inflammation. As inflammation ensues, macromolecular LPS can be absorbed and frequently induce a toxic reaction by various receptor-mediated signals.¹⁰ Elevated LPS levels have been associated with induction of tumor necrosis factor alpha, leading to chronic inflammation and a weakened intestinal epithelial barrier,¹¹ and appear to be pathogenic factors in disorders such as irritable bowel disease and necrotizing enterocolitis.^{12,13} Animal studies have also shown that LPS may accelerate inflammation-related aging.¹⁴ Therefore, systemic levels of LPS may be a biomarker of microbial translocation and colorectal adenomas or CRC.

While several studies have examined the association between serum levels of LPS or markers of microbial translocation and risk of developing CRC,^{15,16} few have studied the association between LPS and its precursor lesion, namely advanced colorectal adenoma (ACA).¹⁷⁻¹⁹ Adenomas are the products of proliferation from dysplastic nonmalignant epithelial cells, which can progress into adenocarcinoma as deleterious mutations accumulate.

Therefore, we examined the association between serum levels of LPS and risk of developing ACA in a colonoscopy clinic-based case-control study using a kinetic assay. We hypothesized that serum levels of LPS are associated with an increased risk of ACA.

Methods

Study design and study population

The study cohort and comprehensive eligibility and exclusion criteria for this cross-sectional study have been previously reported.²⁰ The study was approved by the Institutional Review Boards of Baylor College of Medicine and Michael E. DeBakey VA Medical Center (MEDVAMC). All participants provided written informed consent for the study.

From 2013 to 2017, a total of 612 participants, aged 50 and 79 years, were recruited from the colonoscopy clinic at the MEDVAMC. A total of 55 patients had a diagnosis of ACA, which was defined as a polyp >1 cm found on colonoscopy or a colorectal adenoma with a villous component or high-grade dysplasia pathologically. Participants in the control group had no colorectal polyps found during the colonoscopy and had no history of colorectal adenoma in the past 3 years. Each control was individually matched to each case according to age (± 5 years), race/ethnicity, and sample collection time (± 3 months; and morning or afternoon collection).

Data collection

Patients were enrolled either on the day they were educated for bowel preparation or on the day of their colonoscopy. Information on demographics, lifestyle, and medical history was obtained through an interviewer-administered lifestyle questionnaire. The

detailed procedure has been previously reported.²⁰ The pathologist (Dr. Daniel Rosen) obtained the pathological features of the adenoma/polyp. The number of polyps/adenoma, location and size of the lesion, hyperplastic polyps, and high-grade dysplasia were documented for each patient.

Blood collection and LPS measurement

Fasting blood samples were collected on the day of the colonoscopy. Serum used for the LPS assay was placed into 7 mL anticoagulant-free BD vacutainer glass tubes previously baked to eliminate possible LPS contamination, and aliquoted into an endotoxin-free glass vial that was baked. These samples were stored at -80°C before testing for LPS using the quantitative Limulus Amebocyte Lysate (LAL) assay. The PYROGENT-5000 kinetic turbidimetric LAL assay (Lonza, Basel, Switzerland) is designed to test for endotoxin of Gram-negative bacteria in serum. The serum samples were diluted to a 1:10 ratio, heated, mixed with the LAL, and incubated for 10 m at 37°C , and a stop reagent was added to stop the reaction. A substrate solution was mixed with LAL solution and incubated at the same temperature for 6 m. A yellow color appeared if the endotoxin was present in the serum. The absorbance of the sample was measured through a spectrophotometer at wavelength of 340 nm. These absorbance values were then converted to concentrations in EU/mL and reported. The sensitivity of the assay ranges from 0.01 to 100 EU/mL. One EU is defined as the biological activity induced in the endotoxin test by about 120 pg LPS from *E. coli* O113H10:K.²¹

Quality control

The matched case-control pairs were assayed in the same 96-well plate. Each sample was assayed in duplicate. We included 5% of duplicate samples in each batch to monitor the batch variation. After examining the duplicate data, we found 11 case-control pairs (of 55 cases-control pairs) had a high ($>15\%$) coefficient of variation (CV) in either the case or control. These pairs were not included in the final analysis. We also eliminated one outlier of LPS value in controls. Therefore, we included 43 case-control pairs in the final analysis.

Statistical analysis

Data were analyzed using the STATA (StataCorp, College Station, TX, USA) software. The chi-squared test was used to test the difference in the distribution of categorical data between cases and controls. The paired *t*-test was used to test the difference in the means of continuous characteristics (*e.g.*, body mass index [BMI] and physical activity) between cases and controls. We used the Wilcoxon signed-rank test to compare the means of LPS levels between cases and controls, because the LPS levels did not follow the normal distribution as tested by the Kolmogorov-Smirnov test. The natural log-transformed LPS values were generated for the analyses where the normal distribution assumption should be met. We examined the associated between covariates and normalized LPS levels using the linear regression. Univariate and multivariable conditional logistic regression were used to estimate the odds ratio (OR) and 95% confidence interval (CI) for ACA in association with the transformed LPS value as the continuous variable. The covariates in the multivariable regression model included smoking, alcohol use, BMI, hypertension, and type 2 diabetes. We

Table 1. Characteristics of controls without polyps and cases with advanced colorectal adenomas

| Characteristics Mean (standard deviation) or % | Controls (n = 43) | Cases (n = 43) | P-value |
|--|-------------------|------------------|---------|
| Age (years) | 62.9 (6.7) | 61.9 (6.4) | 0.43 |
| Men, % | 100 | 100 | 1.00 |
| Non-Hispanic white, % | 62.0 | 60.4 | 0.35 |
| BMI, kg/m ² | 29.9 (5.5) | 31.6 (8.8) | 0.37 |
| BMI in categories, kg/m ² , % | | | 0.67 |
| <25 | 25.58 | 18.60 | |
| 25-<30 | 25.58 | 27.91 | |
| ≥30 | 48.84 | 53.49 | |
| Smoking status, % | | | 0.09 |
| Never | 44.2 | 23.2 | |
| Former | 37.2 | 44.2 | |
| Current | 18.6 | 32.6 | |
| Current alcohol drinking, % | 53.5 | 60.5 | 0.56 |
| History of polyp, % | 18.6 | 20.9 | 0.79 |
| Physical activity, MET-minutes per week | | | |
| Median (interquartile range) | 876 (198–3,066) | 738 (169-3,066) | 0.82 |
| Type 2 diabetes, % | 37.2 | 25.6 | 0.24 |
| Hypertension, % | 55.8 | 62.8 | 0.51 |
| Lipopolysaccharide (EU/mL) | | | |
| Median (interquartile range) | 0.24 (0.19–0.31) | 0.24 (0.18–0.35) | 0.58 |
| Mean (standard deviation) | 0.26 (0.01) | 0.28 (0.02) | 0.23 |
| Pathology of adenoma (median (range) or %) | | | |
| No. of adenoma | | 2 (1–6) | |
| Size of largest adenoma if multiple (cm) | | 1.2 (0.5–3.5) | |
| Having high-grade dysplasia, % | | 23.3 | |
| Having villous component, % | | 37.0 | |
| Serrated adenoma, % | | 5.0 | |

BMI, body mass index; EU, endotoxin unit; LPS, lipopolysaccharides; MET, metabolic equivalent of task.

also modeled the LPS levels as a dichotomized variable using the median in the controls as the cutoff point. Among those who had adenoma, we further tested whether transformed LPS levels differ by the characteristics of ACA according to the number of adenoma (more than 2 ACA or not), size of the adenoma (<2 cm vs. ≥2 cm), villous component (have vs. not have), and dysplasia (high grade dysplasia present or not). A *P*-value < 0.05 indicated statistical significance in a two-sided test.

Results

All participants were men with a mean age of 62 years. There was no significant difference in the distribution of BMI, history of polyps, physical activity, history of diabetes and hypertension, or alcohol use between 43 cases and 43 controls (Table 1). There was an insignificantly higher proportion of current smokers in the cases than controls (*P* = 0.09). Among those who were found to

have adenoma, 67% had one to two lesions, 19% had three to four lesions, and 14% had more than four lesions. A total of 29% had the size of adenoma greater than 2 cm, 37% adenoma had villous components, and 23% had high-grade dysplasia.

LPS was detected in all our samples. The absolute levels of LPS were slightly higher in cases (0.28 EU/mL) than controls (0.25 EU/mL) (*P* = 0.23) (Table 1). Figure 1 shows the pairwise comparison between the individually matched cases and controls using the untransformed value. Furthermore, the serum levels of LPS did not differ by smoking, alcohol use, and BMI in controls (*P* > 0.05) (data not shown). There was no statistically significant association between LPS and the risk of ACA (multivariable OR = 1.83, 95% CI: 0.40 to 8.24) (Table 2). We did not observe a significant association when LPS was modeled as a dichotomized variable (data not shown).

We further examined the LPS levels based on the characteristics of adenoma among patients with ACA (Fig. 2). Patients with a large sized adenoma (>2 cm), villous adenoma, or high-grade dysplasia

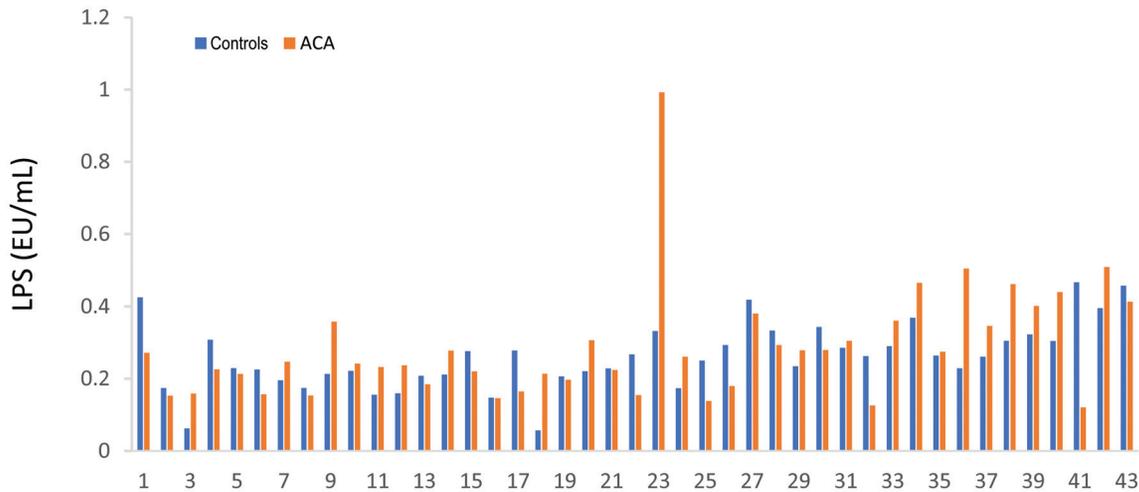


Fig. 1. Serum levels of LPS in 43 advanced colorectal adenoma cases and 43 polyp-free controls.

tended to have higher serum levels of LPS compared to patients with smaller, tubular adenoma, and adenoma without high-grade dysplasia, respectively ($P \leq 0.10$). However, the LPS levels did not differ by the number of adenoma ($P = 0.57$, data not shown).

Discussion

This small-scale case-control study showed that LPS levels were slightly higher in cases than controls, and slightly higher in ACA that was larger, with villous component, or with high-grade dysplasia than the small or tubular ACA. However, there was no statistically significant association between LPS and the risk of ACA. Larger studies are warranted to further investigate the association between the systemic levels of LPS and risk of colorectal tumors and understand the relevant clinical implications.

It is biologically plausible that LPS can contribute to colorectal carcinogenesis. LPS is classified as a pathogen-associated molecular pattern, which is recognized by pattern recognition receptors, such as toll-like receptors (TLRs), on enterocytes or other immune cells.²² LPS enter the intestinal capillaries after the death of Gram-negative bacteria in the gut.²³ An animal study showed that an increase in physiological concentrations of LPS led to increased permeability at the tight junction in cells.²⁴ The increased permeability further caused increased circulating levels of LPS, allowing for further inflammation²⁵ because the lipid A components of the LPS structure bound to the TLR-4/cluster of differentiation complex on innate immune cells in the circulation, and trigger the production of inflammatory cytokines.^{26,27} This signaling pathway

can lead to immune escape, uncontrolled tumor growth, and increased proliferation/metastasis of colorectal carcinoma.^{28–30}

In a European population, a positive association between elevated IgA and IgG anti-LPS immunoglobulins and an increased risk of developing CRC was shown.¹⁶ Another study of 138 cases and 324 controls found that higher concentrations of LPS as measured by the LAL assay were significantly associated with colorectal tubular adenomas.¹⁷ A case-control study in Korea, also used the LAL assay, found that 74 patients with colorectal polyps had higher levels of endotoxin than 71 controls (0.108 vs. 0.049 EU/mL, $P < 0.001$).¹⁸ However, we did not find a significant difference in the serum levels of LPS between ACA and polyp-free controls. Nevertheless, the LPS levels were higher in ACA cases, in patients harboring larger ACA or highly malignant ACA. Whether LPS can serve as a biomarker for colorectal tumor needs further investigation.

We showed that LPS can be detected in individuals without polyps after controlling for the possibility of endotoxin contamination to the best of our ability. To date, the physiological range of LPS has not been well established in healthy individuals. The clinical significance of systemic LPS is elusive. One study evaluated 38 studies that measured LPS levels in the general population.²¹ Among the 19 studies that reported an LPS level of EU/mL, the median level was 0.32 EU/mL, and the 25–75% interquartile range was 0.23–3.89 EU/mL. The median LPS value in poly-free participants (0.24 EU/mL) was in the range reported by these previous studies. Our study showed that the kinetic LAL assay can be used to test LPS in human serum in an epidemiologic study. The adequate dilution of the samples was critical in obtaining reproducible measurements.

We included obesity, smoking status, and alcohol as the confounding factors in the statistical model because they are known risk factors for CRC. Smoking was also a significant risk factor of ACA in our study. However, we did not observe an association between smoking status and LPS. While cigarettes do contain LPS and increased levels of lipopolysaccharide-binding protein are found in smokers, the association between serum levels of LPS and cigarette smoking is still unclear.^{31,32} Studies have also shown that excessive alcohol intake is significantly associated with elevated levels of serum LPS,³³ as well as a possible link between obesity and increased serum LPS.³⁴ We did not observe the association between serum levels of LPS and these host and environmental factors in our small-scale study.

Our study had several strengths. We used glass vials to store the

Table 2. Univariate and multivariate conditional logistic regression models for advanced colorectal adenomas in association with serum levels of LPS

| | OR | 95% CI | P-value |
|--------------|------|-----------|---------|
| Univariate | | | |
| LPS | 1.88 | 0.56–6.39 | 0.30 |
| Multivariate | | | |
| LPS | 1.83 | 0.40–8.24 | 0.43 |

LPS, lipopolysaccharide; OR, odds ratio; CI, confidence interval.

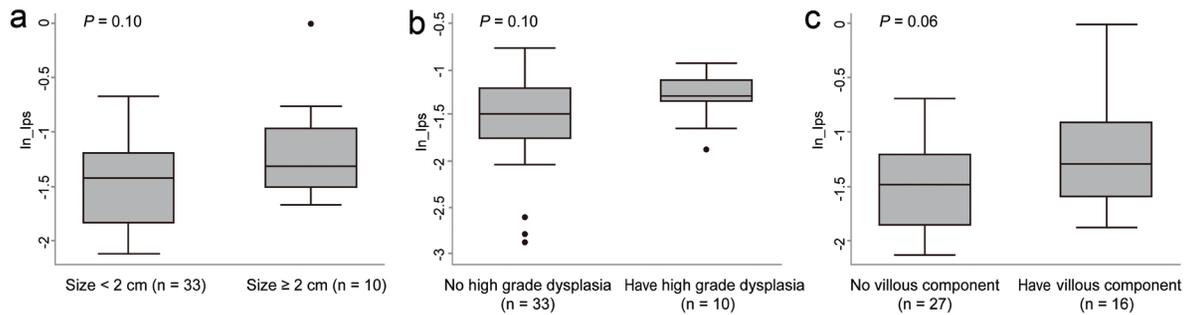


Fig. 2. Box-plot shows the difference in transformed LPS values based on the characteristics of the adenoma. *P* value was for the Student's *t*-test. Panel A, by adenoma size (<2 cm vs. ≥2 cm, *P* = 0.10); Panel B, by the degree of dysplasia (no vs. high-grade, *P* = 0.10); Panel C, by villous component (no vs. have, *P* = 0.06).

samples to prevent the possibility of the attachment of LPS to the plastic tubes. The glass vials were baked to prevent the contamination of endotoxin in the environment. We assayed the matched cases and controls in the same experiment batch to avoid the potential batch effect. We used the same lot of assay kit in the analysis to reduce the kit variation. In addition, those samples that had high CV were not included in the final analysis. Participants were excluded if they had a history of cancer, familial adenomatous polyposis, Lynch Syndrome, acute infectious disease, and antibiotic treatment in the past 3 months. Nevertheless, several limitations should be noted. The small sample size (43 paired samples) was the major limitation of the study. A larger sample size would have increased the statistical power of the data analysis. Because we enrolled the study participants from an elective colonoscopy clinic, the pool of the polyp-free controls was relatively small. Lastly, the study population consisted of only male veterans who may not represent women and the general population.

Future directions

A larger study is needed to determine whether LPS can be used as a biomarker of microbial translocation or leaky gut in population-based studies on colorectal polyp, benign adenoma, and adenoma with malignant potential. To date, the LPS assay has not been widely used in cancer epidemiologic research. Its clinical value should be explored further. Additional biomarkers can be incorporated to advance our understanding of the inflammatory nature of ACA. Finally, it has been shown that not all the components of LPS stimulate an immune response. Precise detection of the effective component of LPS may have greater clinical relevance.^{35,36}

Conclusions

Taken together, our study moves the field forward by showing that LPS can be detected in the serum of polyps-free individuals. We observed a nonsignificantly positive association between serum levels of LPS and ACA. The association between LPS and the risk of colorectal tumor should be further examined in larger studies using the same research approach established in the present study.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (LJ), acquisition of data (NAB, ARO, DR, DYG, RER, HBE, LJ), analysis and interpretation of data (EC, AK, ARO, DLW, DYG, HBE, LJ), drafting of the manuscript (EC, AK, LJ), critical revision of the manuscript for important intellectual content (ARO, DYG, DLW, HBE), administrative, technical, or material support, study supervision (RER, HBE, LJ).

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