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Bacterial translocation and risk of liver cancer in a Finnish cohort

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Abstract

Background: Elevated systemic exposure to gut-derived bacterial products has been associated with hepatic inflammation and chronic liver diseases, potentially increasing the risk of liver cancer. However, only one prior study prospectively examined exposure to bacterial products in the circulation and risk of liver cancer, with a relatively limited coverage of biomarkers.

Methods: We conducted a nested case-control study (224 liver cancer cases and 224 matched controls) in a large cohort of Finnish male smokers followed from baseline (1985-1988) to 2014. The associations between a panel of biomarkers for bacterial translocation and the risk of liver cancer were assessed using multivariable-adjusted conditional logistic regression. The biomarkers included immunoglobulin (Ig) A, IgG, and IgM against lipopolysaccharide (LPS) and flagellin, soluble CD14 (an LPS co-receptor), and the LPS-binding protein.

Results: Anti-flagellin IgA (OR=2.79 (95% CI=1.34-5.78, p_{trend}=0.01) and anti-LPS IgA 2.44 (95% CI=1.33-4.48, p_{trend}<0.01), were significantly associated with risk of liver cancer. When restricting the analysis to histologically-classified hepatocellular carcinoma, the ORs were 4.18 (95% CI=1.60-10.92, p_{trend}<0.01) and 2.48 (95% CI=1.16-5.29, p_{trend}<0.01), respectively. The

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results were not substantially changed after excluding cases diagnosed within the first five years of follow-up and those with hepatitis C virus infection.

Conclusions: Antibodies to flagellin and LPS were associated with increased risk of liver cancer.

Impact: Gut-derived bacterial translocation into the circulation may play a role in the development of primary liver cancer. Our findings could contribute to the understanding of primary liver cancer etiology and further prevention efforts.

Keywords

gut-liver axis; liver cancer; nested case-control study; bacterial translocation

INTRODUCTION

Liver cancer is the second leading cause of cancer mortality worldwide (1). The predominant histological type of liver cancer is hepatocellular carcinoma (HCC), accounting for over 85% of all liver cancers (1). The development of HCC is closely related to chronic inflammation (2). Major risk factors for HCC, including chronic hepatitis B and C virus (HBV and HCV) infections, consumption of aflatoxin contaminated foods, excessive alcohol intake, cigarette smoking, obesity, and diabetes, all cause chronic hepatic inflammation which can progress to liver disease and eventually to liver cancer (3).

It has been suggested that the gut microbiome and the translocation of gut-derived bacterial products into the circulation may contribute to a pro-inflammatory state in the liver that promotes liver disease (4). Although it is currently believed that the liver does not contain a microbiome of its own, it receives approximately 70% of its blood supply from the portal vein, which carries blood from the colon (5). Factors such as a high-fat diet, smoking, alcohol abuse, and intestinal disease can upset the balance between beneficial and potentially pathogenic bacterial species, creating a state of intestinal dysbiosis characterized by altered microbiota composition and decreased bacterial diversity. For example, increased abundance of Enterobacteriaceae, Veillonellaceae and Streptococcaceae, and decreased abundance of Lachnospiracea have been reported in association with cirrhosis (6, 7). When dysbiosis is coupled with subsequent gut barrier damage, the liver may be exposed to an elevated level of gut-derived bacterial products *via* the portal circulation (8). Murine studies have reported that exposure to the bacterial products lipopolysaccharide (LPS) and flagellin can cause inflammation and oxidative stress in the liver and may promote HCC (9–11). Similarly, evidence from human studies suggests that systemic exposure to these bacterial products may be positively associated with systemic inflammation (12, 13) and chronic liver disease (14–17). Thus far, the only study in humans to examine the association between antibodies (IgA and IgG, jointly) to LPS and flagellin and the risk of liver cancer reported a positive association (13). Whether individual immunoglobins (IgA vs IgG vs IgM) were associated with risk, however, has not been studied. In addition, an examination of the relationships of lipopolysaccharide binding protein (LBP) and soluble CD14 (sCD14) to liver cancer risk hasn't been previously reported. LBP, an acute-phase protein produced by hepatocytes in response to endotoxemia, binds LPS to form an LPS-LBP complex (18). The

LPS-LBP complex is then bound to sCD14, which triggers an inflammatory response that has been previously associated with liver injury, thus may be an indicator of increased risk of liver cancer (19–21).

In order to examination the association of these markers of bacterial translocation with liver cancer, we conducted a nested case-control study of 224 primary liver cancer cases (including 157 with confirmed HCC histology) and pair-matched controls within the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) cohort. The biomarkers examined included antibodies (IgA, IgG, IgM) against LPS and flagellin, and factors specifically produced in response to LPS, sCD14, and LBP (22).

METHODS

Study design

The ATBC study was a randomized controlled trial to test the effects of α-tocopherol and β-carotene on lung cancer incidence among male smokers in Finland (23). At trial baseline (1985-1988), 29,133 men aged 50-69 years, who smoked at least five cigarettes per day, were randomized to intervention or placebo. Potential study participants who reported during an interview that they had prevalent cancer (other than non-melanoma skin cancer), cirrhosis, chronic alcoholism, or other conditions that would limit their participation in the trial were excluded from participation (23). At enrollment, participants provided blood samples and completed a questionnaire that collected information on demographics, medical, dietary, and lifestyle factors. The trial ended in 1993, but participants continued to be followed for cancer incidence. This study was approved by the Institutional Review Boards of both the National Institutes of Health of the United States and the National Public Health Institute of Finland.

For this analysis, all cases of primary liver cancer (defined based on the International Classification of Diseases [ICD], version 9; topography codes 155.0 and 155.1, i.e., malignant neoplasms of the liver and intrahepatic bile ducts) diagnosed starting from the trial baseline through December 31, 2014 were identified through linkage to the Finnish Cancer Registry. Three cases were excluded because they did not have a stored serum sample. Among all cases of primary liver cancer, we additionally identified those with HCC histology using International Classification of Diseases for Oncology [ICD-O] morphology codes 8170-8175. Control men were selected from individuals with an available serum sample, who were alive and free of liver cancer at the time of the case's diagnosis. Controls were matched, pairwise, to cases on age at randomization assignment and date of blood collection. The final analytic cohort included 224 primary liver cancer cases (including 157 HCC cases) and 224 matched controls. The median length of time from study enrollment to liver cancer diagnosis was 15.6 years, with a range of 8.6 - 21.2 years.

Laboratory analysis

Serum samples were collected at baseline, aliquoted, and stored at −70°C. LBP and sCD14 were measured at the Frederick National Laboratory for Cancer Research. sCD14, assessed as pg/mL, was quantified using R&D Systems Quantikine kit (Cat# DC140). LBP, assessed

as μg/mL, was quantified using R&D Systems DuoSet enzyme-linked immunosorbent assay (ELISA) kit (Cat# DY870-05 and DY008). For both biomarkers, the serum samples were diluted 1:1000 in R&D Systems recommended diluent. Two recombinant controls from R&D Systems were included on each plate as quality control samples. All samples were tested in duplicate, and the average concentration value was used for additional analysis. Based on data from duplicate samples, the within-batch coefficient of variation (CV) for the markers was 7.7%, and the between-batch CV was 11.4%.

IgA, IgG and IgM against LPS and flagellin were measured at Georgia State University via a custom-made ELISA as previously described (24). Briefly, ELISA plates (Costar™ 3590) were coated overnight with laboratory-made flagellin or purified *Escherichia coli* LPS, and serum samples diluted 1:200 were applied to coated wells. After incubation and washing, the wells were incubated either with horseradish peroxidase-conjugated anti-IgM, anti-IgA, or anti-IgG. Quantitation of total immunoglobulins was performed using the colorimetric peroxidase substrate tetramethylbenzidine, and optical density was read at 450 nm and 540 nm. Data are reported as optical density corrected by subtracting background. The laboratory has extensive experience performing assays of these biomarkers and has consistently shown a very low CV in replicates; therefore, the samples were analyzed in singleton to minimize costs and time. For quality control, three duplicate samples were measured in each batch. The within-batch CV was <7.78 and the between-batch CV was 11.88. For all the bacterial translocation markers, samples were sent to the labs in matched pairs with case/control status blinded. The case/control pairs were run on the same plate.

In addition to the above biomarkers which were examined as the main exposures, we also measured HCV infection status (antibody to HCV), at the German Cancer Research Center as previously described (25). Briefly, antibodies to the core and NS3 proteins were analyzed using recombinant glutathione S-transferase (GST) fusion proteins in combination with fluorescent bead technology (multiplex serology). Reproducibility of this assay has been shown to be very high (kappa 0.98, 95% CI 0.94-1.00) (25). Samples that were positive for both antibody to HCV core and antibody to the NS3 proteins were considered to be anti-HCV(+), indicative of either a former or current infection. HBV infection status, as indicated by hepatitis B surface antigen positivity, was previously measured for 112 liver cancer cases and 269 individuals without liver cancer in the ATBC cohort (26); however, as <2% of liver cancer cases and <1% of liver cancer-free individuals were positive for HBV, HBV status was not determined for cases and controls in the current analysis.

Statistical analysis

We used conditional logistic regression to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the association between each biomarker and liver cancer risk. Biomarker levels were batch-adjusted using a normalization factor derived from quality control samples repeatedly measured across all plates (i.e., the difference between the mean value of quality control samples on all plates combined minus the mean value of quality control samples on an individual plate was added to individual values) and categorized into quartiles based on the distribution among controls.

In addition to matching on age and time of blood draw, we also controlled for the following confounders based on *a priori* knowledge: body mass index (BMI), education, smoking intensity (pack-years), alcohol intake, and history of diabetes or hypertension at baseline. Missing values for alcohol intake (15 cases and 9 controls) were imputed using the PROC MI procedure, using case status and adjustment factors (age, BMI, education, vocational trainings, smoking, diabetes, hypertension, and marital status; SAS Institute Inc., Cary, NC). We also evaluated original randomization arm, HCV status, and coffee intake as potential covariates, but these did not substantially alter the estimates and were not included in the final models. Furthermore, we evaluated effect modification by the following factors: age, BMI, smoking intensity, and alcohol intake, using likelihood ratio tests comparing models with and without the interaction term. Several sensitivity analyses were conducted, including 1) restricting the analytical cohort to histologically confirmed HCC cases and their matched controls; 2) excluding cases diagnosed within the first five years of follow-up; and 3) excluding cases with HCV infection.

As a secondary cross-sectional analysis, we calculated the mean and standard deviation (SD) of the level of each biomarker stratified by selected baseline characteristics of participants, including age, BMI, education, smoking intensity, alcohol intake, and coffee consumption. Because levels of the biomarkers were roughly normally distributed by visual inspection of the data, we did not perform natural logarithm transformation when calculating the means. All analyses were performed in SAS 9.3 (SAS Institute Inc., Cary, NC).

RESULTS

Selected characteristics of cases and controls are presented in Table 1. Compared to controls, cases were more likely to have more than an elementary school education, to be obese, to smoke and drink more heavily, to consume less coffee, and to have a history of diabetes, hypertension, and chronic HCV infection.

Table 2 shows a correlation matrix of the biomarkers included in the analysis. Across the immunoglobins of anti-LPs and anti-flagellins, there were moderate positive correlations between the IgA's (0.68) and the IgM's (0.47). Within the immunoglobulins of both anti-LPS and anti-flagellin, there were moderate positive correlations between IgA and IgG (anti-LPS=0.43, anti-flagellin=0.46).

Supplementary Table 1 shows the mean value of each biomarker measured according to selected characteristics among the controls, including age at randomization, BMI, education, intensity of cigarette smoking (pack-years), drinks of alcohol per day, and coffee consumption (g/day). Overall, the most significant p-values for trend were between sCD14 and alcohol consumption (p=0.02), anti-flagellin IgM and BMI (p=0.03) and LBP and smoking (p=0.04).

Table 3 shows the associations between each biomarker (categorized in quartiles) and the risk of primary liver cancer. In multivariable-adjusted models, the highest quartile of anti-flagellin IgA, compared to the lowest quartile, was associated with a nearly 3-fold increased risk of liver cancer (OR = 2.79, 95% CI = 1.34 to 5.78), and there was a statistically

significant linear trend (p_{trend} = 0.01). Similarly, there was association between anti-LPS IgA and liver cancer, with an OR comparing the highest to the lowest quartiles of 2.44 (95% CI = 1.33 to 4.48) and a statistically significant linear trend (p_{trend} < 0.01). The other individual biomarkers examined, including anti-LPS and anti-flagellin IgG and IgM, LBP, and sCD14, were not associated with the risk of liver cancer.

In sensitivity analyses, the strength of association for anti-flagellin immunoglobulins was stronger when restricting the analyses to HCC cases and controls (Supplementary Table 2). Comparing the highest to the lowest quartiles, the OR was 4.18 (95% CI = 1.60 to 10.92) for anti-flagellin IgA. Results for anti-LPS Immunoglobulins were not materially changed. Other sensitivity analyses, such as excluding 27 cases diagnosed within the first five years of follow-up or 10 cases with HCV infection, did not substantially change the main results. We also observed no evidence of interaction between each of the biomarkers and variables including age, BMI, smoking pack-years and drinks of alcohol per day ($p_{interaction}$ > 0.05).

DISCUSSION

In this large prospective cohort of Finnish male smokers, we observed significant associations between bacterial translocation biomarkers and risk of primary liver cancer. This study is among the first to address this novel research question, and the largest to date in terms of both sample size and biomarker coverage. Our results provide important insights into the role of bacterial products in the development of liver cancer.

In this study, we observed statistically significant associations between IgA against flagellin and LPS and risk of primary liver cancer. For IgG against flagellin and LPS, although results were not statistically significant, the OR for the highest quartiles were both around 1.5, suggesting possible elevated risk of liver cancer. As the IgA's and IgG's within anti-LPS and anti-flagellin were moderately correlated, however, the independence of the IgA and IgG results is not certain. Similarly, the moderate correlation between anti-LPS IgA and anti-flagellin IgA suggests that the IgA relationships to liver cancer may not be independent of one another. Previously, Fedirko *et al.* measured anti-flagellin and anti-LPS IgA and IgG in a prospective case-control study (139 matched pairs of cases controls) nested within the EPIC study, a large European cohort, and observed associations between each of the biomarker and risk of HCC (OR ranging from 4.13 to 8.67 in multivariable adjusted models, comparing extreme quartiles). Our results are generally consistent with the previous study, although the strength of association was more modest overall.

A notable difference between our study and the EPIC study is that our study population only consisted of male smokers, whereas the EPIC study included both sexes, regardless of smoking status. Indeed, it is biologically plausible to observe weaker associations between exposure to bacterial products and liver cancer among smokers, because smoking, besides being a risk factor itself for liver cancer (27), is associated with a broad range of alterations in systemic immune and inflammatory responses (28), and thus may obscure any additional immune/inflammatory responses caused by exposure to bacterial products. However, in the EPIC cohort, there was no suggestion of effect modification by smoking (13). Also, our study found no evidence of effect modification by pack-years of smoking. Nevertheless,

statistical power was limited for the assessment of effect modification by smoking in both studies, and future studies with sufficient sample sizes in each smoking category need to further evaluate whether the association differs by smoking status. Sex difference has also been reported in the immune defense against bacterial products (29) and immune responses in general (30). The EPIC cohort observed stronger association of antibodies against LPS and flagellin with risk of HCC in men than women; however, the heterogeneity was not statistically significant, perhaps due to the small number of female cases (13).

Biological mechanisms for the association between systemic exposure to bacterial products and liver cancer risk are not fully understood. However, elevated systemic exposure to bacterial products could be due to intestinal bacterial dysbiosis and gut barrier damage allowing an increased burden of bacterial products to translocate into the circulation. Increased bacterial translocation is common in persons with alcoholic liver disease (14), non-alcoholic fatty liver disease (NAFLD)(15, 16), and chronic viral hepatitis (17), indicating that bacterial products may serve as early indicators of liver damage. In murine models, the activation of Toll-like receptor 4 (TLR-4) by LPS, after complexing with LBP and sCD14, contributes to the promotion of HCC in chronically injured livers by increasing proliferative and anti-apoptotic signals (9, 10). In addition, high-dose flagellin administration in mice causes inflammation and oxidative stress in the liver, and induces injury through over-activation of TLR-5 (11). In addition, high concentrations of immunoglobulin A are present in the mucous membranes of the gastrointestinal tract and have been shown to be key in gut bacterial regulation in mice (31). These exacerbated proliferative, inflammatory, and oxidative responses may eventually result in the development of liver cancer.

One important question in the interpretation of our results and the previous findings in EPIC is whether elevated immune response against bacterial products merely reflected the presence of underlying liver diseases, as chronic liver diseases are associated with both systemic exposure to bacterial products and higher risk of liver cancer. In our study and the EPIC study, adjustment of chronic viral hepatitis infection did not materially change the results. Neither study had information on alcoholic or non-alcoholic fatty liver disease. Nevertheless, the EPIC study reported that adjusting for the Fischer ratio (a marker of liver dysfunction) did not alter the results. In our study, individuals with a history of cirrhosis or chronic alcoholism were excluded from the original trial. In addition, we adjusted for BMI, diabetes, and hypertension, all of which are linked to metabolic syndrome, thus the results may have been partially controlled for NAFLD. Therefore, we believe our observed associations are not solely due to confounding by underlying chronic liver diseases.

Due to the potential role of bacterial translocation as biomarkers of cancer risk in epidemiological studies, it is important to better understand variables (e.g., demographic, diet and lifestyle) associated with these biomarkers. Several cross-sectional analyses consistently reported higher levels of IgA and IgG against bacterial products or LBP among individuals with greater adiposity (12, 13, 32, 33). In addition, murine studies suggested that increased exposure to LPS may trigger weight gain (34), and induced-obesity could elevate IgG levels against bacterial extracts (32), indicating that bacterial products and obesity may mutually influence each other. In contrast, immunoglobulins against LPS and flagellin were

not correlated with BMI in our study. One possible explanation could be that among smokers, BMI is not a good proxy for adiposity; specifically, lower BMI among smokers may often indicate lower lean body mass but higher visceral adiposity and metabolic abnormalities (35). Therefore, the lack of association between BMI and systemic exposure to bacterial products in our study does not necessarily contradict previous findings, and in fact highlights the necessity of stratification by smoking status in future studies.

Our study is among the first to prospectively examine the associations between bacterial translocation and risk of liver cancer, with the largest sample size and most comprehensive coverage of biomarkers to date. Other strengths of our study include the long follow-up period (up to 29 years), availability of baseline fasting serum samples, detailed information on potential confounders including HCV infection, and use of ICD-O morphology codes to identify HCC cases. There are also several limitations. For example, there was only a single determination of biomarkers at baseline, and the temporal reliability of these biomarkers are not well established. A previous study demonstrated that the LBP had moderate test-retest reliability up to a nine-month period; however, it may be more desirable to include multiple measurements rather than a single measurement in future studies (36). In addition, there was no clinical determination of underlying fibrosis or cirrhosis at baseline. Our study population was confined to males and smokers, thus extrapolation to other populations should be done with caution.

In conclusion, our study indicates that biomarkers of bacterial translocation, specifically IgA against flagellin and LPS, may be associated with risk of primary liver cancer. Currently, there are limited data on biomarkers of liver cancer risk. Thus, our findings, if replicated in future studies, could better clarify the role of gut-liver axis, specifically gut-derived bacterial products, in liver cancer etiology, and may provide important insights to improve the prevention and risk prediction of liver cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ATBC	Alpha-Tocopherol, Beta-Carotene
CI	confidence interval
CV	coefficient of variation
HBV	hepatitis B virus
HCC	hepatocellular carcinoma

HCV	hepatitis C virus
Ig	immunoglobulin
LBP	lipopolysaccharide-binding protein
LPS	lipopolysaccharide
NAFLD	non-alcoholic fatty liver disease
OR	odds ratio
sCD14	soluble CD14
SD	standard deviation

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Table 1.

Participant characteristics in a nested case-control study of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) cohort

	Cases (n=224) N (%)	Controls (n=224) N (%)
Age at randomization in years, mean (sd)	57.4 (4.7)	56.8 (4.2)
Body mass index (kg/m^2)		
<25.0	63 (28.1)	90 (40.2)
25.0 - <30.0	111 (49.6)	104 (46.4)
≥30.0	50 (22.3)	30 (13.4)
Education[1]		
1	47 (21.0)	71 (31.7)
2	114 (50.9)	105 (46.9)
3	63 (28.1)	48 (21.4)
Cigarette smoking (pack-years)		
>0 – 24	53 (23.7)	71 (31.7)
25 – 34	43 (19.2)	54 (24.1)
35 – 44	50 (22.3)	52 (23.2)
≥45	78 (34.8)	47 (21.0)
Drinks of alcohol (per day)		
0	14 (6.7)	15 (7.0)
>0 - <1.0	70 (33.5)	108 (50.2)
1.0 - <2.0	59 (28.2)	54 (25.1)
≥2.0	66 (31.6)	38 (17.7)
Coffee consumption (g/day)		
0 - <200	29 (13.9)	13 (6.0)
200 - <500	80 (38.3)	78 (36.3)
500 - <1000	83 (39.7)	90 (41.9)
≥1000	17 (8.1)	34 (15.8)
Diabetes		
Yes	22 (9.8)	5 (2.2)
No	202 (90.2)	219 (97.8)
Hypertension		
Yes	56 (25.0)	44 (19.6)
No	168 (75.0)	180 (80.4)
Hepatitis C virus infection		
Yes	10 (4.5)	1 (0.4)
No	214 (95.5)	223 (99.6)
Randomization arm		
Placebo	55 (24.6)	60 (26.8)
α-Tocopherol	63 (28.1)	63 (28.1)
β-Carotene	54 (24.1)	60 (26.8)

	Cases (n=224) N (%)	Controls (n=224) N (%)
α-Tocopherol/β-Carotene	52 (23.2)	41 (18.3)

[1]Levels of education: 1) elementary school or less, no vocation training; 2) elementary school or less, vocational training; 3) more than elementary school

Table 2:

Correlation matrix of anti-LPS, anti-flagellin, sCD14 and LBP in a nested case-control study of the Alpha-Tocopherol, Beta-Carotene (ATBC) cohort

	anti-LPS			anti-flagellin			sCD14	LBP
	IgM	IgA	IgG	IgM	IgA	IgG		
anti-LPS IgM	1	−0.05708	0.05269	0.47430	−0.27636	−0.01915	0.10308	−0.06484
anti-LPS IgA		1	0.43014	0.03502	0.68036	0.34551	0.22324	0.18002
anti-LPS IgG			1	0.03697	0.13953	0.29785	0.17597	0.08992
anti-flagellin IgM				1	0.01780	0.34945	0.10371	0.07101
anti-flagellin IgA					1	0.46403	−0.00368	0.09803
anti-flagellin IgG						1	0.06070	0.09072
sCD14							1	0.31816
LBP								1

Table 3.

Associations between biomarkers of bacterial translocation and risk of primary liver cancer in a nested case-control study of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) cohort

	Cases N	Controls N	Minimally-adjusted[1] OR (95% CI)	Multivariable-adjusted[2] OR (95% CI)
anti-flagellin IgA				
Quartile 1	40	56	1.00 (ref)	1.00 (ref)
Quartile 2	43	55	1.14 (0.63, 2.06)	1.32 (0.68, 2.58)
Quartile 3	64	55	1.81 (1.02, 3.20)	1.75 (0.95, 3.21)
Quartile 4	76	56	2.93 (1.48, 5.79)	2.79 (1.34, 5.78)
p_{trend}			*<0.01*	*0.01*
anti-flagellin IgG				
Quartile 1	58	55	1.00 (ref)	1.00 (ref)
Quartile 2	50	56	0.85 (0.50, 1.46)	0.82 (0.44, 1.51)
Quartile 3	50	55	0.85 (0.49, 1.49)	0.76 (0.40, 1.44)
Quartile 4	65	56	1.23 (0.66, 2.29)	1.43 (0.71, 2.91)
p_{trend}			*0.68*	*0.33*
anti-flagellin IgM				
Quartile 1	61	56	1.00 (ref)	1.00 (ref)
Quartile 2	66	55	1.03 (0.62, 1.72)	0.96 (0.55, 1.69)
Quartile 3	45	56	0.72 (0.41, 1.27)	0.65 (0.34, 1.24)
Quartile 4	52	55	0.86 (0.50, 1.45)	0.94 (0.52, 1.69)
p_{trend}			*0.96*	*0.74*
anti-LPS IgA				
Quartile 1	41	55	1.00 (ref)	1.00 (ref)
Quartile 2	37	56	0.84 (0.48, 1.49)	0.79 (0.42, 1.49)
Quartile 3	40	55	1.10 (0.63, 1.92)	0.88 (0.47, 1.64)
Quartile 4	106	56	2.79 (1.61, 4.85)	2.44 (1.33, 4.48)
p_{trend}			*<0.01*	*<0.01*
anti-LPS IgG				
Quartile 1	54	55	1.00 (ref)	1.00 (ref)
Quartile 2	38	56	0.68 (0.39, 1.20)	0.71 (0.38, 1.32)
Quartile 3	48	56	0.97 (0.56, 1.68)	0.83 (0.45, 1.54)
Quartile 4	83	55	1.60 (0.94, 2.74)	1.62 (0.88, 2.97)
p_{trend}			*0.06*	*0.15*
anti-LPS IgM				
Quartile 1	52	56	1.00 (ref)	1.00 (ref)
Quartile 2	66	55	1.34 (0.75, 2.38)	1.06 (0.57, 1.99)
Quartile 3	47	56	0.88 (0.48, 1.62)	0.86 (0.44, 1.68)
Quartile 4	59	55	1.18 (0.63, 2.21)	1.15 (0.57, 2.31)
p_{trend}			*0.56*	*0.42*

	Cases N	Controls N	Minimally-adjusted[1] OR (95% CI)	Multivariable-adjusted[2] OR (95% CI)
LBP				
Quartile 1	52	56	1.00 (ref)	1.00 (ref)
Quartile 2	52	56	0.99 (0.58, 1.67)	1.14 (0.61, 2.10)
Quartile 3	52	56	1.06 (0.60, 1.85)	0.95 (0.50, 1.81)
Quartile 4	68	55	1.44 (0.81, 2.55)	1.16 (0.60, 2.24)
p_{trend}			*0.21*	*0.74*
sCD14				
Quartile 1	55	56	1.00 (ref)	1.00 (ref)
Quartile 2	51	56	0.94 (0.57, 1.56)	0.84 (0.47, 1.52)
Quartile 3	49	55	0.94 (0.55, 1.60)	0.76 (0.41, 1.41)
Quartile 4	69	56	1.31 (0.75, 2.29)	1.15 (0.60, 2.20)
p_{trend}			*0.60*	*0.81*

[1] Accounting for matching factors only (age at randomization assignment and date of blood collection

[2] Accounting for matching factors (age at randomization assignment and date of blood collection), and additionally adjusted for body mass index, education, smoking intensity, alcohol intake, and history of diabetes or hypertension at baseline

P values for linear trend were calculated using the Wald test, by including the continuous form of each biomarker in the model

Abbreviations: CI, confidence interval; Ig, immunoglobulin; LBP, lipopolysaccharide-binding protein; LPS, lipopolysaccharide; OR, odds ratio; sCD14, soluble CD14.