Lipopolysaccharide-binding protein, associated factors, and colorectal cancer

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A dissertation
submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

2016

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Program authorized to offer degree:

Public Health – Epidemiology

University of Washington

Abstract

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BACKGROUND - Recent research has pointed to a possible key role of gut microbial communities (GMC) in understanding the link between obesity, chronic inflammation, and the development of colorectal cancer (CRC) [1]. While some studies have demonstrated a strong association between obesity and circulating levels of lipopolysaccharide (LPS) [2–5], an endotoxin which is found on the outer cell wall of gram negative bacteria, the specific GMC associated with LPS concentrations under non-sepsis conditions remain unknown. LPS increases inflammatory response signaling, and may play a role in the pathogenesis of several adverse outcomes, including diabetes [2,5,6], inflammatory bowel disease [7–9], cardiovascular disease [10–12] and cancer [13,14]. However, no human studies have examined the relationship between lipopolysaccharide-binding protein (LBP; a marker of LPS exposure) levels and CRC risk. Thus,

the three studies within this dissertation aim to describe the association between LBP, the gut microbiome, and CRC risk as well as the reliability of LBP as a biomarker.

METHODS - We used several different studies to address the dissertation aims. We analyzed the temporal reliability of LBP measured in archived samples from participants in two studies (*Aim 1)*. In Study 1, 60 healthy participants (30 men and 30 women, aged 60-72 years) were recruited to have blood drawn at two time points: baseline and follow-up (either 3, 6, or 9 months) and evenly distributed as much as possible by time interval. In Study 2, we tested 24 individuals (8 men and 16 women, aged 20 to 40 years) with blood drawn 3-4 times over a 7-month period. LBP measured in archived plasma by ELISA was to evaluate within-person reproducibility over time. We evaluated the association of the GMC in stool, serum CRP concentrations, and adiposity with plasma LBP concentrations in 110 premenopausal (ages 40–45 years) women in the United States (*Aim 2)*. Multivariable linear regression analysis was used to assess the relation between LBP concentrations and adiposity. Structural equation modelling (SEM) was used to investigate the indirect effect of circulating LBP concentrations on the association between adiposity and CRP. We examined the association between colorectal cancer (CRC) and LBP in 1,638 participants (819 CRC cases and 819 controls, matched on age, sex, race, location, and time of blood draw, among other factors) from the Multiethnic Cohort study (MEC) (*Aim 3*). Conditional logistic regression models were used to estimate the multivariable-adjusted odds ratios (OR) and 95% confidence intervals (95% CI).

RESULTS - In *Aim 1* Plasma LBP concentrations showed low to moderate reliability in both Study 1 (ICC: 0.60, 95% CI: 0.43 to 0.75) and Study 2 (ICC: 0.46, 95% CI: 0.26 to 0.69). Restricting the follow-up period improved reliability. In Study 1, the test-retest reliability of LBP

over a three-month period was 0.68 (95% CI: 0.41 to 0.87). In Study 2, the ICC of samples taken ≤7 days apart was 0.61 (95% CI: 0.29 to 0.86). In *Aim 2* we found that while alpha diversity did not differ by LBP tertile, the beta-diversity was statistically significantly different between groups using unweighted Unifrac, but not weighted Unifrac. Several taxa, particularly those found in the Clostridia class may be more prevalent in women with low levels of LBP, while Bacteroides may be more prevalent with high levels of LBP. Additionally, findings from the current study suggested that LBP was associated with adiposity, but LBP did not mediate the association between adiposity and CRP. In *Aim 3* we did not find a statistically significant association between LBP and CRC. Compared to individuals whose LBP concentrations were in the lowest quartile, the ORs associated with second (range: 24.1-31.7 µg/mL), third (range: 31.8-42.6 µg/mL), and fourth (range: 42.7-107.5 µg/mL) quartiles were 1.23 (95% CI = 0.91-1.67), 1.36 (95% CI = 1.01-1.83), and 1.01 (95% CI = 0.73-1.39), respectively, (P_{trend} = 0.66).

CONCLUSIONS - Results of *Aim 1* suggest that plasma LBP may serve as a reliable marker in short-term studies; however, multiple samples may be needed in longitudinal studies to obtain more stable measures. Results from *Aim 2* suggest that there may be differences in distribution of rarer taxa between tertiles of LBP, but overall community structures do not differ between groups. Despite finding an association between inflammation, as measured by CRP, and LBP in *Aim 2*, results from *Aim 3* do not provide clear evidence of an association between plasma LBP concentrations and CRC risk. Additionally, there was no evidence of modification by BMI, dietary fiber intake and saturated fat intake, cancer site, or cancer stage.

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ACKNOWLEDGEMENTS

I would sincerely like to thank my committee members Johanna Lampe, Emily White, Polly Newcomb, Meredith Hullar, and Lianne Shepard for direction and support throughout my dissertation. Without the collective support of these committee members, this dissertation would not have been possible. I am grateful for their active involvement and insightful advice. I'd especially like to acknowledge the mentorship and guidance from Johanna Lampe, my committee chair, who helped me navigate the dissertation process and beyond. She was a constant source of encouragement through both the ups and downs of the dissertation and invested considerable energy and time in helping me to develop my dissertation and edit my manuscripts. Finally, I would like to recognize and thank my friends and family for their support during my graduate studies.

This work was supported by the National Institutes of Health grants K05 CA154337 (National Cancer Institute and Office of Dietary Supplements) and R01 CA142545, R25 CA094880, and P01 CA168530 (National Cancer Institute)

CHAPTER 1: Introduction

Introduction:

Recent evidence suggests a possible role of gut microbiota in the pathogenesis of obesity and its concomitant diseases. Obesity, especially visceral obesity, is associated with systemic microinflammation, or chronic low-grade inflammation [15]. Adipocytes and macrophages infiltrating visceral adipose tissue in obese participants are the source of circulating proinflammatory cytokines, such as TNF-α, IL-1, and IL-6 [15–17]. More recently, research has suggested that an alteration in gut microbial communities (GMC) escalates systemic microinflammation, at least in obese individuals [6].

Among different GMC found in the human gut, Cani et al identified lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, as an underlying factor of obesity-driven low-grade inflammation [18]. Lipopolysaccharide-binding protein (LBP), a protein that binds to LPS and transfers LPS monomers to CD14, is correlated with circulating levels of LPS [5]. LPS induces an increase of LBP production in the liver within 15 to 30 min after exposure [19], with a maximum serum level occurring after 24-48 hours [20]. Recent studies have shown that LBP, a marker of LPS exposure, is associated with high-fat diets and obesity [2,5,21–23]; however, relatively few studies have examined whether LBP levels vary by GMC distribution and adiposity.

Previous reports have shown a strong association between concentration of circulating LBP and obesity-associated metabolic disturbances [24,10]. Furthermore, LBP is associated with increased inflammatory signaling [18,25,26]. Several studies have shown that high-fat diets altered gut microbiota and intestinal permeability, subsequently promoting metabolic disturbances [21,27,28].

Additionally, LPS may play a role in the pathogenesis of several adverse outcomes, including diabetes [2,5,6], inflammatory bowel disease [7–9], cardiovascular disease [10–12] and cancer [13,14]. However, no human studies have examined the relationship between LBP (a marker of LPS exposure) levels and CRC risk. Furthermore, while LBP, may serve as a marker of chronic inflammation, no studies have examined the temporal reliability of the biomarker in a healthy population.

Evidence from *in vitro* studies has shown that LPS promotes cancer-cell survival and proliferation, angiogenesis, vascular permeability, and tumor cell adhesion [29–31]. While LPS is hypothesized to be associated with colorectal carcinogenesis, there are relatively few human studies which have examined this association and no studies which have examined the reliability of LBP as a biomarker in healthy individuals. Thus, this dissertation attempts to address these important gaps in knowledge by examining the association between the GMC and adiposity with plasma LBP concentrations and then examining whether increased plasma LBP concentrations are associated with CRC incidence. Additionally, we examine the reliability of LBP as a biomarker of risk.

Chapter 2, entitled "Reliability of plasma lipopolysaccharide-binding protein (LBP) from repeated measures in healthy adults" examines the temporal reliability of LBP measured in archived samples from participants in two studies.

Chapter 3, entitled "Association of plasma lipopolysaccharide-binding protein (LBP) with gut microbial communities and adiposity in premenopausal women" evaluates the association between GMC, adiposity, and circulating concentrations of LBP. In this cross-sectional study, we evaluated differences in GMC and adiposity by LBP tertiles.

Chapter 4, entitled “Plasma lipopolysaccharide-binding protein and colorectal cancer risk: a nested case-control study in the Multiethnic Cohort” examines the association between colorectal cancer (CRC) and LBP in 1,638 participants (819 CRC cases and 819 matched controls) from the Multiethnic Cohort study (MEC). This study aims to explore the role of LBP in CRC, as LPS is hypothesized to be associated with colorectal carcinogenesis, but relatively few human studies have examined this association.

In summary, the studies within this dissertation describe the association between LBP, the gut microbiome, and CRC risk as well as the reliability of LBP as a biomarker of risk. Understanding the complexities which drive LBP concentration and whether LBP is associated with CRC may help to identify individuals at high risk for CRC as well as potentially modifiable factors by which to decrease their risk.

CHAPTER 2: Reliability of plasma lipopolysaccharide-binding protein (LBP) from repeated measures in healthy adults

Title Page

Reliability of plasma lipopolysaccharide-binding protein (LBP) from repeated measures in healthy adults

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Acknowledgements:
This work was supported by grants from the National Institutes of Health (Grants K05 CA154337, R01 CA142545, R25 CA094880, and P01 CA168530)

Conflicts of interest:
The authors acknowledge they have no conflicts of interest.

Body word count: 1,490
Abstract word count: 253
Tables: 2
Figures: 1

Running title: Reliability of LBP in healthy adults

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Abstract

BACKGROUND – Plasma lipopolysaccharide-binding protein (LBP), a measure of internal exposure to bacterial lipopolysaccharide, has been associated with several chronic conditions and may be a marker of chronic inflammation; however, no studies have examined the reliability of this biomarker in a healthy population. We examined the temporal reliability of LBP measured in archived samples from participants in two studies.

METHODS – In Study 1, 60 healthy participants (30 men and 30 women, aged 60 – 72 years) had blood drawn at two time points: baseline and follow-up (either 3, 6, or 9 months). In Study 2, 24 individuals (8 men and 16 women, aged 20 – 40 years) had blood drawn 3-4 times over a 7-month period. We measured LBP in archived plasma by ELISA. Test-retest reliability (within-person reproducibility over time) was estimated by calculating the intraclass correlation coefficient (ICC).

RESULTS – Plasma LBP concentrations showed moderate reliability in Study 1 (ICC: 0.60, 95% CI: 0.43 – 0.75) and Study 2 (ICC: 0.46, 95% CI: 0.26 – 0.69). Restricting the follow-up period improved reliability. In Study 1, the reliability of LBP over a three-month period was 0.68 (95% CI: 0.41 – 0.87). In Study 2, the ICC of samples taken ≤7 days apart was 0.61 (95% CI: 0.29 – 0.86).

CONCLUSIONS – Plasma LBP concentrations demonstrated moderate test-retest reliability in healthy individuals with reliability improving over a shorter follow-up period. Results suggest that LBP may serve as a reliable marker in short-term studies; however, multiple samples may be needed in longitudinal studies to obtain more stable estimates.

Introduction:

Lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, has been implicated as an underlying factor of obesity-driven low-grade inflammation [18]. However, LPS, as measured by the Limulus Amebocyte Lysate (LAL) assay, is limited by its lack of sensitivity [32] . In addition, technical difficulties with the assay, the need to collect samples under LPS-free conditions, and fluctuations in LPS throughout the day make it is difficult to measure circulating LPS in large-scale studies [32,33]. Alternatively, lipopolysaccharide-binding protein (LBP), an endogenous protein that binds to LPS and transfers LPS monomers to "Cluster of Differentiation 14 "(CD14), has been used as a proxy for LPS concentrations [34].

Studies that have examined the temporal changes in circulating LBP in humans have focused on individuals with sepsis, as LBP concentrations increase dramatically following inflammatory reactions, such as bacteremia. Peak serum concentrations of LBP have been observed 6–12 hours after clinical suspicion of sepsis, and its half-life in serum is 12–24 hours [35]. Animal studies evaluating the temporal fluctuation in LBP levels have shown that serum levels peak shortly after bacteremia occurs, and remain elevated for up to 72 hours [34,36].

Circulating LBP levels have also been used as a proxy to assess chronic endotoxemia status and immune responses to it [5,2]. Recent studies have suggested that LBP concentrations are associated with high-fat diets and obesity [5,2,21]. Additionally, the results of several studies suggest that LBP may be associated with chronic diseases, including inflammatory bowel disease [8,37] coronary artery disease [10], and cancer [38]. Despite these promising results suggesting that LBP may be a marker for disease risk for a range of pathologic conditions, no studies have examined the temporal

reliability of the biomarker. It is important to establish the reliability of LBP, given that the biomarker is an acute-phase protein and may be influenced by short-term effects, such as diet or infection. Thus, the purpose of this study was to examine the reliability of LBP within healthy adults over time.

Methods:

The analyses were conducted using stored plasma samples collected as part of two completed studies: the Multiethnic Cohort (MEC) Reliability Study and the "Enzyme Activation Trial 2" (2EAT). Informed written consent was obtained from all study participants. The MEC includes men and women primarily from five different racial-ethnic groups (African Americans, Japanese Americans, Latinos, Native Hawaiians and whites in Hawaii and California) aged 45-75 at recruitment and aged 60 and older at blood draw. The MEC Reliability Study (designated Study 1) was an observational study designed to provide fasting repeat blood samples from a group of volunteers of the same ethnic and age groups as MEC participants in Hawaii [39]. Sixty healthy volunteers had blood drawn after an overnight fast at two different time points: baseline and follow-up (either at 3, 6, or 9 months) and evenly distributed as much as possible by sex and time interval.

The 2EAT study (designated Study 2) was a randomized, crossover, controlled feeding study designed to test the effects of vegetable diets on biotransformation enzyme activity and other biomarkers of cancer susceptibility in healthy adults [40]. Men and women, aged 20-40 years of white and Asian ethnicity, were recruited and randomized to four different controlled diet periods. Exclusions were made for health conditions known to influence inflammation, [e.g., chronic disease, medication use,

heavy alcohol consumption, smoking, and obesity (body mass index (BMI) > 30 kg/m^2]. There was a washout period of 21 days or longer between each controlled diet period. Blood was drawn in the morning after a 12 hour, overnight fast and plasma was obtained, aliquoted, and stored at -80˚C. For the current reliability study, we used blood samples drawn at baseline and at the end of each washout period (before the start of each controlled intervention period).

LBP concentrations were measured using a commercial ELISA kit (Cell Sciences Inc) designed to use either heparinized plasma or serum samples. Samples were diluted 1:1000 and the assay was conducted according to kit protocol with a standard curve of 5-50 ng/mL. Samples were run in duplicate. Based on blinded testing of quality control of samples, the median duplicate intra- and inter-assay coefficient of variation (CV) in our lab was 4.7 and 12.5%, respectively.

Statistical Analysis

LBP concentrations were log-transformed to normalize distributions. The temporal reliability was estimated by the intraclass correlation coefficient (ICC). We used the interpretation by Rosner, where the ICC is considered high at >0.75, moderate at 0.4 to 0.74, and poor at <0.40 [41,42]. The ICC was calculated using a multi-level measurement model where blood draws were nested within individuals. We also calculated the number of repeated measures that would be needed to yield a reliability measure (Cronbach α) of ≥0.75 using the *loneway* command. All analyses were conducted using Stata, v14.0 (StataCorp).

Results:

Table 1 gives the demographic characteristics of the participants in Studies 1 and 2. Table 2 gives LBP concentrations at each blood draw and the associated ICC

coefficients. Plasma LBP concentrations showed moderate temporal reliability in both Study 1 (ICC: 0.60, 95% CI: 0.43 – 0.75) and Study 2 (ICC: 0.46, 95% CI: 0.26 – 0.69) (Table 2). In Study 2, the mean time between baseline visit and follow-up visits 1, 2, and 3 was 18 days (range: 3 – 109 days), 71 days (range: 39 – 158), and 117 days (range: 76 – 196 days), respectively. Restricting the follow-up period to the shortest time period in each study (i.e., 3 months for Study 1 and ≤7 days for Study 2) improved reliability. In Study 1, the test-retest reliability of LBP over a 3-month period was 0.68 (95% CI: 0.41 – 0.87). In Study 2, the ICC of samples taken ≤7 days apart was 0.61 (95% CI: 0.29 – 0.86). Three measures of LBP would need to be averaged to achieve a reliability coefficient of 0.75 based on the results from Study 1 and four measures would be needed to obtain a reliability coefficient of 0.75 based on Study 2.

Discussion:

Plasma LBP concentrations demonstrated moderate test-retest reliability in healthy adults, with reliability appearing to be better in multiple blood samples collected over a shorter follow-up period. To our knowledge, this is the first study to measure the longer-term temporal reproducibility of LBP in a healthy population.

LBP is an acute phase protein that is synthesized in the liver upon exposure to LPS. LBP production is induced in the liver within 15 to 30 min after exposure to LPS [19], with a maximum serum concentration occurring after 24-48 hours [43]. While LBP may act as a proxy for LPS, it is important to note that LBP, in addition to binding to LPS, also binds to lipoteichoic acid, a major constituent of the cell wall of gram-positive bacteria, and is structurally similar to spirochetal glycolipids [44]. As such, LBP levels may be influenced by several different types of microbes in addition to gram-negative bacteria.

Circulating LBP concentrations vary in both acute (e.g., infection, injury, etc.) and chronic inflammatory conditions (e.g., autoimmune disease, obesity, cardiovascular disease, cancer). Therefore, it is important to determine whether circulating concentrations of the marker reflect only the short-term physiologic state or if they represent an individual's average concentration over time, relative to other individuals. LBP concentrations are thought to be affected by a wide range of covariates, including age, body mass, smoking, alcohol use, diet, particularly saturated fat and dietary fiber intake, physical activity level, and infection. For example, during an acute-phase reaction, such as septic shock, LBP concentrations can rise 10- to 50-fold [45] above baseline levels. While variations in LBP concentrations due to acute conditions are a concern when attempting to capture long-term levels of LBP, in our study, obtaining blood from healthy individuals after an overnight fast helped to minimize the influence of short-term factors which may affect LBP concentrations (such as diet and acute illness).

Additionally, while our results suggested only moderate temporal reliability, ICC estimates from the study were similar to other acute-phase protein markers [46]. For example, two studies measuring the test-retest reliability over 2 to 5 years of CRP yielded ICCs of 0.63 (no CI provided) and 0.62 (95% CI: 0.49 – 0.75) [47,48].

Advantages of this study include use of two studies, which included participants of different ethnicities and ages and followed individuals for varying time periods. While Study 1 followed individuals for a longer time period, Study 2 benefited from having 3 or more blood draws over varying time periods for each participant. Additionally, fasting samples were obtained for healthy individuals, at the same time of day, thus reducing the likelihood of obtaining a blood sample influenced by diet. The main limitation of our results is that the studies analyzed provided information on the

temporal reliability of LBP over weeks and months, while in certain types of studies, particularly long-term prospective studies, one needs to understand if the measures used reflect biomarker levels over years or decades. Moreover, not all participants completed all 4 diet periods in Study 2, resulting in fewer participants at the beginning of the fourth diet period. Stratified analyses examining reliability by race and sex were not possible given the small sample sizes in the studies. As such, additional studies in larger populations are warranted.

In conclusion, we found that circulating concentrations of LBP showed moderate temporal reliability up to a 9-month period. This suggests that a single measurement of these biomarkers may be used for risk assessment in short-term studies; longitudinal studies capturing exposure over a multiple-year period may be needed in order to obtain more stable estimates. Future studies are needed on the reliability of LBP measures over a longer duration, as are studies of the potential of LBP as a marker of chronic inflammation and cancer risk.

TABLES AND FIGURES

Table 1. Personal and study characteristics of participants from Study 1 and Study 2

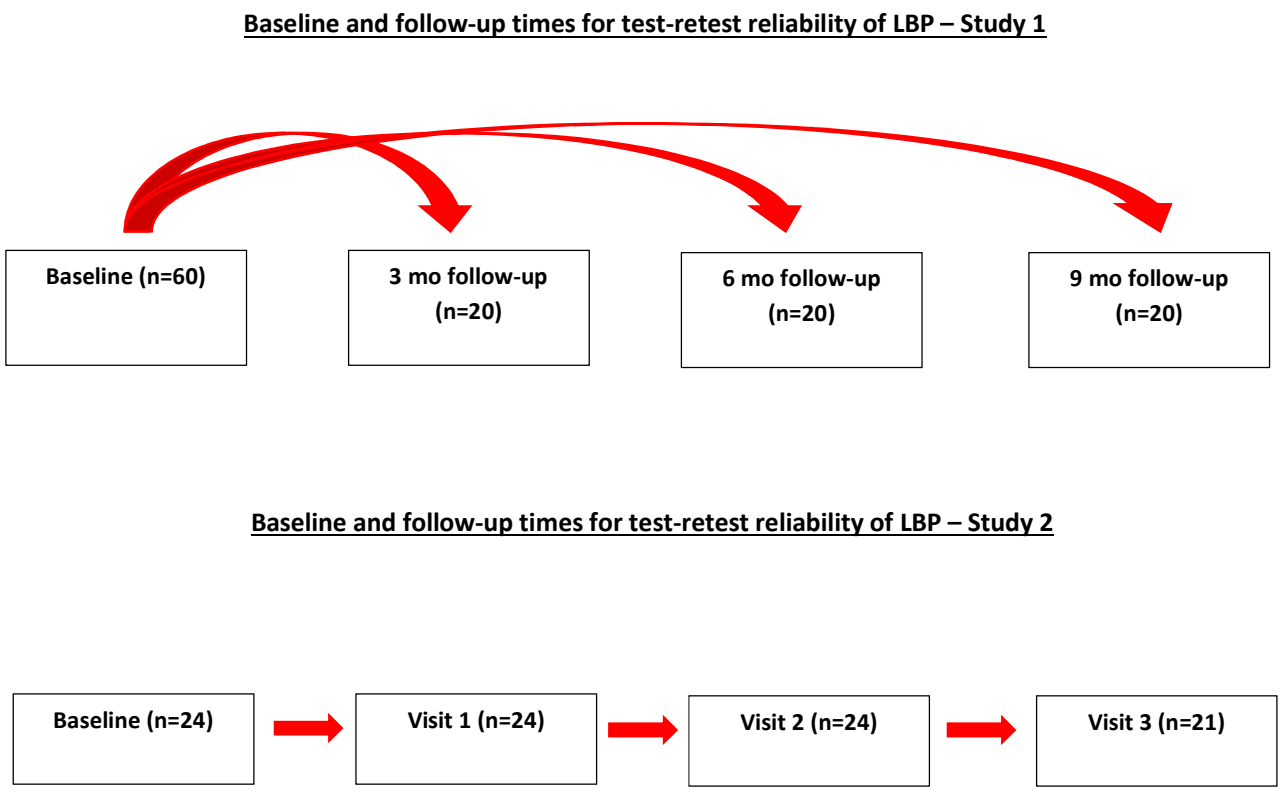
	N (%)
Study 1 (N=60)	
Sex	
Male	29 (48.3)
Female	31 (51.7)
Race/Ethnicity	
White	46 (76.7)
Japanese	12 (20.0)
Hawaiian	2 (3.3)
Age, years	
≤ 65	30 (50)
> 65	30 (50)
Study 2 (N=24)	
Sex	
Male	8 (33.3)
Female	16 (66.7)
Race/Ethnicity	
White	12 (50)
Asian	8 (33.3)
Other	4 (16.7)
Age, years	
≤ 30	11 (45.8)
> 30	13 (54.2)

Table 2. Mean concentrations and intraclass correlation (ICC) of circulating lipopolysaccharide-binding protein (LBP) at baseline and follow-up

	Mean concentrations (sd) µg/mL	**Range µg/mL**	**ICC (95% CI)**
Study 1			
Overall (n = 60)			0.60 (0.43 — 0.75)
Baseline	34.5 (13.4)	13.8 — 94.3	
Follow-up	34.4(13.9)	14.7 — 97.3	
3 months (n=18)			0.68 (0.41— 0.87)
Baseline	38.6 (18.2)	13.8 — 94.4	
Follow-up	37.6 (14.0)	17.0 —76.2	
6 months (n = 19)			0.51 (0.21 — 0.81)
Baseline	30.3 (8.3)	15.1 — 47.0	
Follow-up	30.9 (11.8)	14.7 —66.8	
9 months (n = 23)			0.52 (0.25 — 0.78)
Baseline	34.8 (12.0)	16.1 — 64.4	
Follow-up	34.8 (15.2)	21.0 — 97.3	
Study 2			
Overall (n = 24)			0.46 (0.26 — 0.69)
Baseline	27.3 (10.1)	15.8 — 50.0	

Follow-up visit 1 (n = 24)	24.6 (8.6)	13.5 — 37.6	
Follow-up visit 2 (n = 24)	28.2 (14.3)	14.4 — 79.3	
Follow-up visit 3 (n = 21)	27.6 (14.2)	13.3 — 77.8	
Visits ≤ 7 days apart (n = 14)			0.61 (0.29 — 0.86)
Baseline	26.7 (9.5)	15.8 — 49.0	
Follow-up visit 1	25.1 (8.7)	14.1 — 37.6	

Figure 1. Baseline and follow-up timelines



CHAPTER 3: Association of plasma lipopolysaccharide-binding protein (LBP) with gut microbial communities and adiposity in premenopausal women

Title Page:

Association of plasma lipopolysaccharide-binding protein (LBP) with gut microbial communities and adiposity in premenopausal women

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Acknowledgements:
This work was supported by the National Institutes of Health grants K05 CA154337 (National Cancer Institute and Office of Dietary Supplements) and R25CA094880 (National Cancer Institute)
Conflicts of interest:
The authors acknowledge they have no conflicts of interest.

Body word count: 3,606
Abstract word count: 370
Tables: 4
Figures: 3

Running title: GMC and Lipopolysaccharide-binding protein

Key words: Lipopolysaccharide-binding protein (LBP), gut microbial communities (GMC), CRP

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Abstract

BACKGROUND - While the mechanisms by which obesity increases cancer risk are unclear, some lines of evidence suggest that the gut microbial communities (GMC) may contribute to chronic inflammation in obese individuals through raised systemic levels of lipopolysaccharides (LPS). While some studies have demonstrated a strong association between obesity and circulating levels of lipopolysaccharide (LPS) [2-5], an endotoxin which is found on the outer cell wall of gram negative bacteria, the specific GMC associated with LPS concentrations under non-sepsis conditions remain unknown. Additionally, the pathway by which LPS drives systemic inflammation is unknown. Thus, in the current paper, we examine the relationship between plasma Lipopolysaccharide-binding protein (LBP; a measure of circulating LPS), GMC, and adiposity in a cross-sectional study.

METHODS - We evaluated the association of the GMC in stool and adiposity with plasma LBP concentrations in 110 premenopausal (ages 40–45 years) women in the United States. The GMC was evaluated using paired-end pyrosequencing of the 16S rRNA gene. Operational taxonomic units were identified at 97% sequence similarity. Taxonomic classification was performed and alpha and beta diversity in relation to LBP concentration was assessed. Multivariable linear regression analysis was used to assess the relation between LBP concentrations and adiposity. Structural equation modelling (SEM) was used to investigate the indirect effect of circulating LBP concentrations on the association between adiposity and CRP.

RESULTS - Alpha diversity did not differ by LBP tertile while the beta-diversity was statistically significantly different between groups using unweighted Unifrac, but not weighted Unifrac. Several taxa, particularly those found in the Clostridia class may be more prevalent in women with low levels of LBP, while Bacteroides may be more prevalent with high levels of LBP. Study results also showed that LPS concetrations were not correlated with plamsa LBP. LBP was associated with adiposity in the multivariable regression model, but the indirect effect of LBP on the association between adiposity and CRP was not statistically significant.

CONCLUSIONS –Overall community structures did not differ between tertiles of LBP, but there may have been differences in distribution of rarer taxa between groups. While LBP was associated with increased adiposity, it did not appear to mediate the association between adiposity and CRP concentrations.

Introduction:

Evidence suggests a possible role of gut microbiota in the pathogenesis of obesity and its concomitant diseases. Obesity, especially visceral obesity, is associated with systemic microinflammation (i.e. chronic low-grade inflammation) [15]. Adipocytes and macrophages infiltrating visceral adipose tissue in obese participants are a source of circulating pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6 [15–17]. More recently, research has suggested that an alteration in the gut microbial communities (GMC) escalates systemic microinflammation, at least in obese individuals [6].

Among different GMC found in the human gut, Cani et al identified lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, as an underlying factor of obesity-driven low-grade inflammation [18]. Lipopolysaccharide-binding protein (LBP), a protein that binds to LPS and transfers LPS monomers to CD14, is driven by circulating levels of LPS [34]. Exposure to LPS induces an increase of LBP production in the liver within 15 to 30 min [19], with a maximum serum level occurring after 24-48 hours [20]. Recent studies have shown that LBP, a marker of LPS exposure, is associated with high-fat diets and obesity [18,2,49,22,23]; however, relatively few studies have examined whether LBP levels vary by GMC distribution and adiposity levels. Previous reports have shown a strong association between concentration of circulating LBP, obesity-associated metabolic disturbances, and increased inflammatory signaling [18,25,26]. However, the interplay between obesity, the GMC, LBP, and inflammation has yet to be elucidated. Thus, the goal of the proposed study is to examine whether the GMC and adiposity levels differ by LBP

concentrations and whether LBP mediates the association between adiposity and inflammation, as measured by CRP concentrations.

Methods:

Study Participants

Participants were from the Equol, Breast and Bone (EBB) study, which has been previously described [50]. Briefly, participants in the EBB study were recruited from the Group Health Cooperative (GHC), a large mixed-model health care system in Washington State. Women were eligible if they were premenopausal, aged 40 to 45 years, and had received a screening mammogram at GHC prior to recruitment. Women were ineligible to participate if they were allergic to soy beans or soy protein; had been diagnosed with Crohn's disease or ulcerative colitis or had any part of their colon removed; had been diagnosed with breast cancer; were pregnant or planning to become pregnant; had a hysterectomy or any part of their ovaries removed; were perimenopausal (skipped ≥1 periods in the previous 12 months, or had irregular bleeding patterns); were currently using hormone therapy or oral contraceptive, had used them for ≥1 month in the past 12 months, or had used them in the 6 months before their screening mammogram; were currently taking antibiotics or had taken them for ≥1 month in the previous 12 months; and had ever taken tamoxifen or were currently taking raloxifene, bisphosphonates, or oral steroids.

After obtaining informed consent, EBB participants were mailed a health and demographics questionnaire and a physical activity questionnaire, to be completed prior to their clinic visit. At the clinic visit, weight and height were measured as well as body fat distribution (adiposity %) which was assessed using dual energy X-ray absorptiometry (DXA; Hologic Delphi, Hologic Inc.). Participants also provided a 50-ml sample of blood after fasting for at least 12 h at the clinic visit. All samples were

processed within 1 hour of collection, aliquoted into 1.8-ml tubes, and stored at –70°C. Date, time of collection, and time since last meal were recorded. Stool samples were collected in RNAlater and then stored at −80°C, as previously described [51,52]. Additionally, all participants were asked to complete a 3-day food record (3DFR) within two weeks of the clinic visit.

A total of 1,407 women were identified as potential participants from the Group Health Breast Cancer Screening Program. Of these women, 367 (26%) were found to be ineligible, 691 (49%) refused participation, and 146 (10%) were not able to be interviewed or scheduled for a clinic visit. Of the 203 EBB study participants, 110 completed a health questionnaire, provided stool and blood samples, and had body fat % measured from a DXA scan.

Biological Specimens

LBP concentrations were measured using a commercial ELISA kit (Cell Sciences Inc) and samples were diluted 1:1000 and the assay was conducted according to kit protocol with a standard curve of 5-50 ng/mL. CRP was measured from serum samples using a clinical chemistry analyzer (COBAS) in the FHCRC PHS Biomarker Lab. Serum CRP was measured using CRP Ultra Wide Range reagent (Genzyme Diagnostics) on a Roche Cobas Mira chemistry analyzer and read at 570 nm. Samples were run in duplicate, and the mean duplicate intraassay coefficients of variation (CV) were: 4.7% for LBP, and 4.1% for CRP. A blinded pooled serum sample was included in each batch to track plate-to-plate variation. The interbatch CV was 12.5% for LBP and the assay was conducted on once-thawed samples.

Microbiome bioinformatics analysis

DNA extraction and 16S rRNA sequencing methods used on EBB samples have been previously described [17]. Briefly, DNA was extracted from stool that had been

stored in RNAlater. The V1-V3 region of the 16S rRNA gene was amplified and sequenced using 454 pyrosequencing primers 27f and 519r. Sequences were compiled and processed using QIIME (v.1.8). Sequences were removed if they were < 200 bp or > 700 bp, had homopolymers >6 bp, more than one mismatch to the forward primer, more than one mismatch to the barcode, or more than six ambiguous bases. Sequences were truncated with a quality score sliding window of size 50 bp using a threshold of 25. Initial OTU generation was done using UCLUST within QIIME at 97% similarity. The OTU table was filtered using the QIIME script filter_otus_from_otu_table.py with --min_count_fraction set to 0.00005 as recommended in Navas-Molina et al. An additional filtering step set entries in the OTU table to zero if the number of observations was less than 10 per-sample, per-OUT. Additional OTU entries were filtered out if they were detected as chimeras using QIIME's identify_chimeric_seqs.py script with method blast_fragments. Sequences were aligned to the Silva 16S rRNA gene reference alignment using the NAST algorithm. Sequences that did not align to the appropriate 16S rRNA gene region were removed. The sequences were classified using MOTHUR's naive Bayesian Classifier trained against the SILVA database (release 111) clustered at the 97% similarity level. Classified sequences were assigned to phylum and genus-level phylotypes to characterize the community structure. OTUs were rarefied to 1,578 sequences per sample because uneven sampling depth biases alpha (within-person) and beta (between-person) diversity estimates.

Analysis of GMC functional profiles

Profiling phylogenetic marker genes, such as the 16S rRNA gene (as outlined above), is needed to understand the distribution of microbial communities, but does not provide direct evidence of a GMC's functional capabilities; as profiling 16s rRNA genes attempts to answer the question of "What bacteria are present?", profiling

functional genes, alternatively, attempts to answer the question of "What are the bacteria doing?". Thus, to gain insight into the functional differences in GMC among the study population, specifically functional genes associated with gram negative and gram positive bacteria (lipopolysaccharide biosynthesis and peptidoglycan biosynthesis, respectively), we used PICRUSt [53] to predict the metagenomic contribution of the communities observed (specifically LPS synthesis, and peptidoglycan biosynthesis). PICRUSt predicts metagenomic potential by imputing the available annotated genes within the KEGG catalogue, based on the presence/absence of OTUs of a 16S rRNA survey. PICRUSt has been utilized previously to describe differences in potential function within human samples and positively corresponds to actual metagenomic and metabolic comparisons.

Statistical Analysis

Diversity of the microbial community within an individual (alpha diversity), was calculated from OTUs (at 3% divergence) using the Shannon Index (Supplementary table). The Shannon Index takes into account both abundance and evenness of species present in the community. Box plots were created to graphically display the Shannon Index across tertiles of LBP.

Beta diversity estimates, which represent the similarity (or difference) in organismal composition between tertiles of LBP (i.e., between person/group variance) were based on weighted and unweighted UniFrac distance matrices that were generated in QIIME. The UniFrac approach [54] creates a combined phylogenetic tree and compares which branches individuals have in common. Unweighted UniFrac, a qualitative measure looking at the fraction of unique branch length, and weighted UniFrac, a quantitative measure based on relative abundance, may be viewed as complementary approaches that explore different aspects of how communities diverge,

with results from the weighted and unweighted analyses exploring the different factors affect the presence/absence and relative abundance of microbial lineages. As such, both weighted and unweighted Unifrac matrices were used in the analysis.

Multiple response permutation procedure (MRPP) [55], a non-parametric procedure for testing the null hypothesis of no difference between 2 or more groups, was conducted to test whether the composition of the GMC differed between tertiles of LBP. While there are several non-parametric methods for testing group differences (e.g., ANOSIM, MANOVA), MRPP was selected as it does not require distributional assumptions (multivariate normality, homogeneity of variances) which are usually not by with microbiome data. MRPP was performed with 1,000 permutations on UniFrac distance matrices.

Differences in the GMC (at both the phylum and genus level) by tertiles of LBP concentration were visualized by heatmap and by NMS ordination plots (Figures 1 & 2). Heatmaps were created from OTU relative percent fraction data that was arcsine square-root transformed.

Indicator species analysis (ISA) [56] complemented MRPP by assigning significant indicator values to bacteria taxa (at the genera level) that were indicative of community structure separation between LBP tertiles. ISA identifies species for each study group (based on LBP tertiles in the current study) that are indicative or representative of the community present in that group. The indicator species are usually either dominant species (highly frequent or abundant) or rare species in their particular habitat/group but are markedly less dominant or nonexistent in the other groups. To adjust for differences in per-subject sampling frequency, P-values were averaged over 1,000 bootstrap iterations with even per-subject sampling frequency. Bootstrapped P-values were adjusted for FDR using the Benjamini and Hochberg (B-H) method [57] based on the

total number of taxa after excluding those that represented < 1% average relative abundance.

The Kruskal Wallis (K-W) tests were used to assess whether phyla differed by tertile of LBP. K-W is a rank-based nonparametric test which is used to determine if there are statistically significant differences between two or more groups of an independent variable on a continuous (or ordinal) dependent variable. Additionally, we used K-W tests to examine whether the relative abundance of gram-negative bacteria (represented by the Firmicutes/ Bacteroidetes ratio and gram+/gram- ratio), and functional genes representing gram negative and gram positive bacteria (Lipopolysaccharide biosynthesis and peptidoglycan biosynthesis) differed between LBP tertiles. However, given that the Firmicutes/Bacteroidetes ratio may not accurately reflect relative abundance of gram-negative bacteria [58], we constructed a gram negative/gram positive ratio (+/- ratio) by categorizing genus-level taxa as either gram negative or gram positive. Spearman correlations (a nonparametric measure of rank correlation) were computed for LBP tertiles and GMC community ratios (Firmicutes/Bacteroidetes ratio and gram positive/gram negative bacteria ratio) to determine which ratio may better represent LBP concentrations. Additionally, a Spearman correlation was computed for LBP and LPS to determine if LBP concentrations accurately reflect circulating LPS concentrations.

Multivariable linear regression analysis was used to assess the relation between LBP concentrations and adiposity. As such, LBP concentrations (the dependent variable) were log-transformed to normalize the distribution. We fit a linear regression model for the association of log-LBP concentrations with percent body fat (tertile, using the first [lowest] tertile as reference, with tertiles based on overall sample distributions.), controlling for potential confounders which were determine a priori and

included: saturated fat intake (quartiles, using the first quartile as reference), dietary fiber intake (tertiles, using the first tertile as reference), alcohol consumption (g/day), race/ethnicity (Asian, non-Asian), physical activity (tertile, using the first [lowest] tertile as reference) and age (years). Covariates with >10% missing values were categorized as categorical variables. Individuals with missing covariates were dropped (n=8) in the regression model.

LBP Mediation of adiposity-inflammation association

Structural equation modelling (SEM) was used to investigate post-hoc the indirect effect of the LBP on the association between adiposity and CRP. LBP concentrations. SEM was chosen as it enables testing of non-straightforward patterns of relationships, and is therefore well suited to the management of cross-sectional data for inferential purposes. The total, direct, and indirect effects were estimated using the post-estimation command "estat teffects". The indirect effect of LBP on adiposity was recorded and represents the change in CRP for every unit change in adiposity that is driven by the LBP. We also calculated the proportion of the effect attributable to the mediator. This was calculated by dividing the coefficient of the studied effect (i.e., through the specific mediator) by the coefficient of the total effect of adiposity on CRP. If the indirect effect was significant (i.e. $P < 0.05$), then LBP was considered to be important in the interaction. Additionally, in a post-hoc analysis, we explored whether LBP concentrations mediated the association between adiposity and CRP.

All reported P-values are two-sided, and a P-value < 0.05 was considered statistically significant. All analyses were carried out using STATA 14 (StataCorp, College Station, TX), QIIME [22], R version 3.3.1 (http://www.r-project.org), vegan [59], ggplot2 [60] and labdsv [61].

Results:

Characteristics of the study population and macronutrient intakes according to tertiles of LBP concentrations are shown in Table 1. No substantial differences were observed in ethnicity, education, smoking history, age, dietary fiber intake, soluble and insoluble fiber intake. Compared to those in the lowest tertile, those in the highest tertile of LBP had a higher fat intake, saturated fat intake, adiposity, and CRP levels. In the current study, LBP was not correlated with LPS concentrations (Spearman correlation coefficient = 0.02, P=0.79)

Bacteria were distributed across phyla: Actinobacteria (0.2%), Bacteroidetes (30.4%), Cyanobactera (0.03%), Firmicutes (67.7%), Lentispharae (0.06%), Proteobacteria (1.2%), and Tenericutes (0.4%), and Verrucomicrobia (0.03%) (Table 2). Firmicutes decreased while Bacteroidetes increased with LBP concentration although these differences were not statistically significant. Additionally, the relative abundance of Tenericutes was statistically significantly different between LBP groups. Moreover, LPS biosynthesis and peptidoglycan synthesis were statistically significantly different by tertiles of LBP. However, neither the Firmicutes/Bacteroidetes ratio nor the gram positive/gram negative bacteria ratio were statistically significantly different across LBP tertile.

The bacterial alpha diversity was similar between groups (Tertile 1: 2.30; Tertile 2: 2.27; Tertile: 2.32) was not significantly different between tertiles of plasma LBP (Shannon Index, ANOVA, F=0.3356, n=110, P=0.72; Figure 1). Beta diversity was significantly different between tertiles of LBP using unweighted Unifrac (MRPP; A=0.0045666, P=0.027, 1,000 permutations), but no difference between tertiles of LBP was observed using weighted Unifrac (MRPP; A=0.0002919, p=0.20; 1,000 permutations). Heatmaps of GMC (phyla and genera-level) are presented in Figure 1.

Using Indicator Species Analysis (ISA), we found that 13 bacterial OTUs were associated with LBP. Of these, 12 phylotypes were "indicators" of low levels of LBP concentrations, and 1 was an indicator for the highest tertile of LBP (Table 3). Uncultured Christensenellaceae and unclassified Ruminococcaceae showed the highest indicator values for low LBP while Bacteroides was the strongest indicator for high LBP.

In the multivariable adjusted linear regression model, LBP was statistically significantly higher in the highest vs. lowest tertile of adiposity, measured as percent body fat (Beta coefficient = 0.128, 95% CI = 0.02-0.25, P=0.02); there was no association with the second tertile of adiposity (Beta coefficient = 0.14, 95% CI = -0.08-0.37, P=0.21).

The mediation analyses revealed no significant findings (Table 4). LBP was responsible for 16.5% of the association between adiposity and CRP, but this indirect effect was not statistically significant (P=0.15).

Discussion:

In this cross-sectional study, we evaluated differences in LBP by GMC and adiposity. We found that while the beta diversity was statistically significantly different between groups using unweighted Unifrac, gut microbial community structures did not differ when using weighted Unifrac. Differences in results may have been due to the fact that unweighted Unifrac is sensitive to rarer taxa while weighted Unifrac is not influenced by these taxa. Further analysis revealed that several taxa, particularly those found in the Clostridia class in the Firmicutes phylum may be more prevalent with low levels of LBP while Bacteroides may be more prevalent with high levels of LBP. In the lowest tertile, the highest indicator value belonged to the gram-positive Christensenellaceae genus, which has been found to be more abundant in lean

individuals [62]. Animal models have also shown that transplantation of Christensenellaceae into germ-free mice protected against weight gain [63] Alternatively, the highest indicator value in the highest tertile of LBP was Bacteroides, a gram-negative genus which has been associated with both obesity and IBD is previous studies [64,65]. Taken together, these findings point to an association between adiposity, increased levels of bacteria associated with obesity and inflammation, and increased levels of circulating LBP. However, independent replication is required as these associations were not established a priori and may have been due to chance, given that many taxa were examined, although p-values were corrected for multiple testing.

In the current study, there was no correlation found between LPS and LBP concentrations using a singular fasting blood sample. While some studies have shown a correlation between LBP and LPS [5,66], other studies have failed to find an association, noting that LBP is a results from exposure to LPS and, as such, LBP increase is delayed with respect to LPS [67]. LPS induces an increase in LBP production in the liver within 15-30 minutes of exposure [36]. Thus, measures taken from a single time point will not accurately capture both markers.

There were no differences observed in gram-negative/gram-positive ratios by LBP tertiles, despite using two measures of this ratio in the current study. This is the first study to have examined this association, however, the accuracy in using these ratios to describe the relationship of GMC to other factors has been questioned. While a number of studies have found that obese individuals have lower ratios of Bacteroidetes to Firmicutes [68–71], several large-scale analyses have found no relationship between obesity and the Firmicutes/Bacteroidetes ratio [72–74]. These observations suggest that no simple taxonomic signature of obesity, and, by extension, LBP, exists in the gut microbiome.

LPS biosynthesis and peptidoglycan synthesis were statistically significantly different by tertiles of LBP. Our finding that higher levels of both LPS biosynthesis and peptidoglycan biosynthesis corresponded to higher LBP tertile levels may help to explain why an association between the gram positive/gram negative ratios and LBP was not observed. However, it should be noted that peptidoglycan synthesis is not specific to gram positive bacteria and occurs with gram negative bacteria as well [75].

Consistent with previous studies [2,76], we found that percent body fat was statistically significantly associated with LBP concentration even after controlling for potential confounders. Additionally, similar to previous studies which found that high endotoxins concentrations can trigger local inflammation, or enter circulation and induce systemic inflammation through cytokine release [21,77], findings from the current study suggest that LBP is associated with CRP. However, in the current study, LBP did not mediate the association between adiposity and CRP, despite adiposity being statistically significantly associated with CRP. These results suggest that there might be alternative pathways, aside from LBP, by which adiposity leads to inflammation. Alternatively, current results may be due to use of CRP as the single marker of inflammation, as previous studies have used different markers, such as TNF-α and IL-6 [17,78]. Given that no other studies have examined whether LBP mediates the association between adiposity and inflammation, future studies are needed to confirm results.

Strengths of the study include use of a well-described population of pre-menopausal women and stringent exclusion criteria which removed factors that could potentially influence GMC (e.g., antibiotic use). Additionally, careful assessment of diet using a 3DFR, rather than a FFQ, limited the potential for measurement error due to poor recall. Furthermore, body fat was measured using DXA, providing a more accurate measure of

adiposity. However, DXA scan did not identify adipose tissue subtype, which may be differentially associated with circulating LBP concentrations [79]. Compared to subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT) is considered to have a higher levels of inflammation. Additionally excess VAT has also been linked to T2DM [80,81], insulin resistance [82], and other obesity-related diseases [83]. The two cross-sectional studies which have examined the association between LBP and adiposity subtype found that: 1) LBP was substantially increased in VAT and SAT and 2) LBP expression was significantly higher in obese vs. non-obese individuals [2].

Additional limitations of our study, beyond the size sample size, are the cross-sectional design and the methodology of microbiome analysis. Use of pyrosequencing rather than newer sequencing methods led to a fewer number of sequences per read and subsequently reads may not have provided as much coverage as reads sequenced using Illumina. Additionally, the annotations used in KEGG suggest the functional potential of the community, but the presence of these genes/functions does not mean that they are biologically active (i.e., they may not be transcribed). To collect this information, metatranscriptomics would have needed to be performed. As such, these issues limit the ability to detect associations and to extrapolate findings to other populations.

The current study found that GMC may differ in relation to LBP concentrations, and while LBP may be associated with CRP, it does not mediate the association between adiposity and CRP. Given that this appears to be the first study to examine LBP in relation to adiposity and GMC, validation of our findings in larger, more representative populations are needed.

Table 1. Selected Baseline Characteristics of EBB Participants by LBP Tertile

	Tertile 1 (0-14.9 µg/mL) n=36	Tertile 2 (15.0-22.1 µg/mL) n=37	Tertile 3 (22.4-94.6 µg/mL) n=37
Age, y	42.34 (1.3)	42.54 (1.4)	42.33 (1.4)
Ethnicity			
White	34 (97.1%)	30 (83.3%)	34 (94.4%)
Asian	0 (0.0%)	3 (8.3%)	1 (2.8%)
Other	1 (2.9%)	3 (8.3%)	1 (2.8%)
Education			
≤12 Years	0 (0.0%)	3 (9.1%)	2 (6.3%)
13-15 Years	9 (27.3%)	6 (18.2%)	10 (31.3%)
16 Years	10 (30.3%)	10 (30.3%)	10 (31.3%)
17+ Years	14 (42.4%)	14 (42.4%)	10 (31.3%)
Caloric Intake (kcal/day)	1,899 (343)	1,871 (446)	2,020 (437)
Fat Intake, (g/day)	71.9 (18.0)	71.7 (22.3)	81.2 (24.9)
% Fat of Diet	34.4 (8.3)	34.2 (6.1)	35.5 (6.6)
Saturated Fat Intake (g/day)	24.4 (7.9)	25.4 (3.8)	29.0 (10.8)
Dietary Fiber Intake (g/day)	21.1 (7.9)	19.3 (7.3)	21.1 (8.2)
Soluble Fiber Intake (g/day)	5.3 (2.0)	5.0 (2.1)	5.6 (2.4)
Insoluble Fiber Intake (g/day)	15.6 (6.1)	14.1 (5.5)	15.3 (6.4)
Alcohol (g/day)	8.3 (12.3)	7.2 (10.5)	8.8 (13.4)
History of smoking			
Yes	12 (34.3%)	11 (30.6%)	13 (36.1%)
No	23 (65.7%)	25 (69.4%)	23 (63.9%)
Physical Activity (METS/year)	79.52 (44.9)	105.06 (67.01)	95.92 (73.87)
Percent Body Fat (Tertiles)			
0 (0-29.4%)	15 (45.5%)	12 (36.4%)	7 (21.9%)
1 (29.5-37.3%)	11 (33.3%)	8 (24.2%)	12 (37.5%)
2 (>37.3%)	7 (21.2%)	13 (39.4%)	13 (40.6%)
CRP (mg/L)	0.95 (1.4)	1.4 (2.26)	2.9 (3.8)

Table 2. GMC distribution of EBB Participants by LBP Tertile

	Tertile 1 n=36	Tertile 2 n=37	Tertile 3 n=37	P for difference between groups*
Phylum (%)				
Actinobacteria	0.1	0.22	0.27	0.03
Bacteroidetes	34.6	27.0	29.6	0.14
Cyanobacteria	0.0	0.07	0.005	0.36
Firmicutes	63.8	70.9	68.3	0.16
Lentispharae	0.05	0.08	0.04	0.98
Proteobacteria	1.2	1.2	1.2	0.51
Tenericutes	0.2	0.6	0.5	0.25
Verrucomicrobia	0.02	0.03	0.03	0.77
Other	0.007	0.0	0.004	0.36
Functional Genes				
Peptidoglycan Biosynthesis	1263.1	1738.3	2080.9	0.05
Lipopolysaccharide Synthesis	254.8	287.6	339.0	<0.0001
Gram +/Gram - Ratios				
Firmicutes/Bacteroidetes Ratio	2.86	3.67	3.43	0.14
Gram + / Gram - Ratio	0.69	0.69	0.71	0.8

*Based on K-W Test

Table 3. *Posthoc* mediation analysis.

Exposure	Mediator	Outcome	Indirect Effect	Direct Effect	Total Effect	Relative %	P-value for Indirect Effect
Adiposity	LBP	CRP	0.50 (-0.18-1.19)	2.53 (0.79-4.98)*	3.03 (0.60-5.50)*	16.50	0.15

*P<0.05

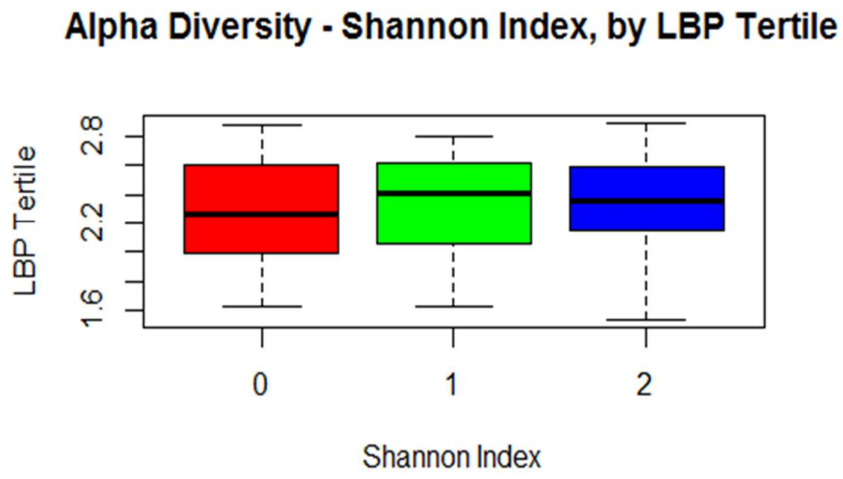
Table 4. Indicator Species Analysis – Genus Level

Indicator Species Analysis Results - Genus Level

	LBP Tertile	Indicator Value	Corrected P-value*
Firmicutes.__Clostridia.__Clostridiales.__Christensenellaceae.__uncultured	1	0.5924	0.01
Firmicutes.__Clostridia.__Clostridiales.__Ruminococcaceae.Other	1	0.5509	0.01
Firmicutes.__Clostridia.__Clostridiales.__Ruminococcaceae.__uncultured	1	0.5198	0.01
Firmicutes.__Clostridia.__Clostridiales.__Ruminococcaceae.__Incertae_Sedis	1	0.4627	0.05
Firmicutes.__Clostridia.__Clostridiales.__Ruminococcaceae.__Subdoligranulum	1	0.4559	0.02
Firmicutes.__Clostridia.__Clostridiales.__Veillonellaceae.__Phascolarctobacterium	1	0.4308	0.01
Tenericutes.__Mollicutes.__RF9.__uncultured_bacterium.Other	1	0.4058	0.01
Firmicutes.__Clostridia.__Clostridiales.__Ruminococcaceae.__Oscillibacter	1	0.3629	0.04
Firmicutes.__Clostridia.__Clostridiales.__uncultured.__uncultured_bacterium	1	0.3525	0.01
Firmicutes.__Clostridia.__Clostridiales.__Ruminococcaceae.__Anaerotruncus	1	0.3456	0.01
Tenericutes.__Mollicutes.__RF9.Other.Other	1	0.3054	0.01
Firmicutes.__Clostridia.__Clostridiales.__uncultured.Other	1	0.225	0.01
Bacteroidetes.__Bacteroidia.__Bacteroidales.__Bacteroidaceae.__Bacteroides	3	0.4713	0.02

*Benjamini-Hochberg corrected P-value

Figure 1. Alpha Diversity by LBP Tertile



	Overall	Tertile 1 n=36	Tertile 2 n=37	Tertile 3 n=37
Shannon Index	2.3	2.27	2.32	2.34

Figure 2. GMC by LBP Tertile

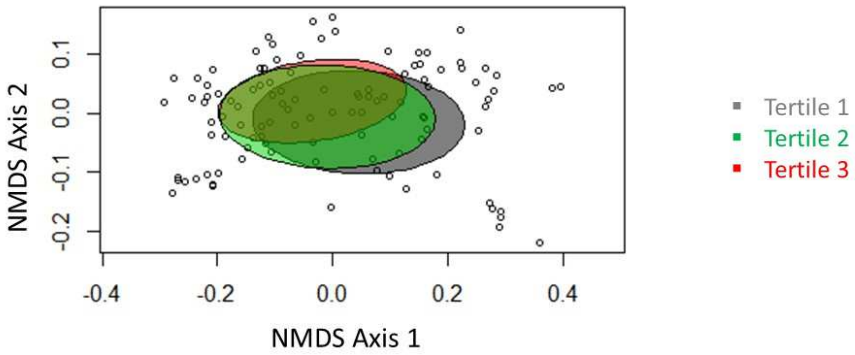
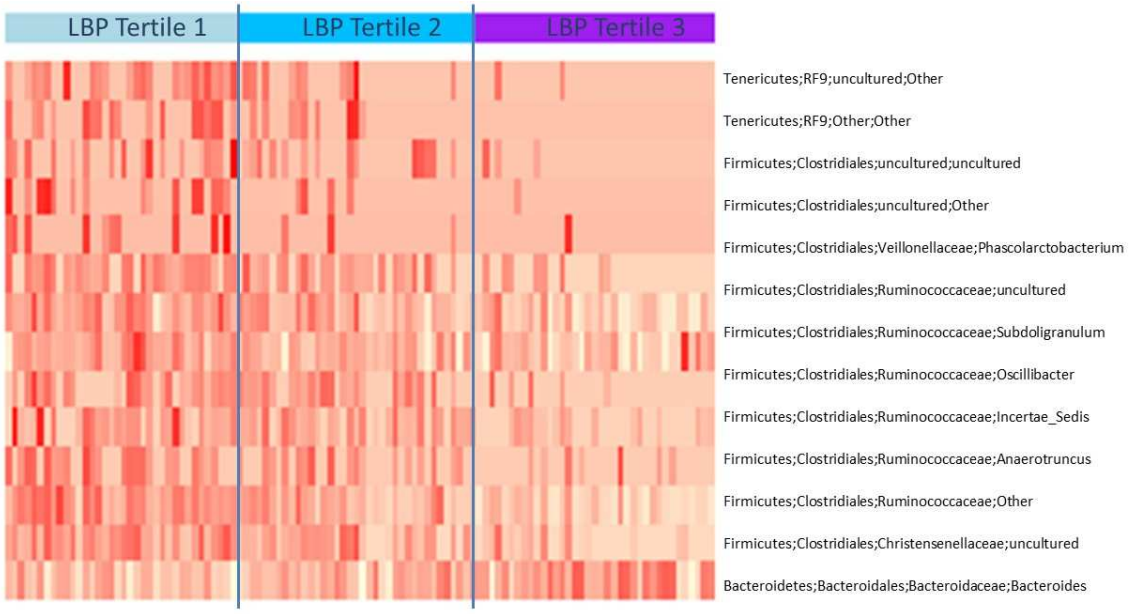


Figure 3. Heatmap of ISA Genera by LBP Tertile



CHAPTER 4: Plasma lipopolysaccharide-binding protein and colorectal cancer risk: a nested case-control study in the Multiethnic Cohort

Title Page:

Plasma lipopolysaccharide-binding protein and colorectal cancer risk: a nested case-control study in the Multiethnic Cohort

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Acknowledgements:
This work was supported by the National Institutes of Health grants, P01 CA168530 K05 CA154337 (National Cancer Institute and Office of Dietary Supplements) and R25CA094880 (National Cancer Institute)

Conflicts of interest:
The authors acknowledge they have no conflicts of interest.

Body word count: 3,295
Abstract word count: 209
Tables: 7
Figures: 0

Running title: Lipopolysaccharide-binding protein and colorectal cancer risk

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Abstract

BACKGROUND - Lipopolysaccharide (LPS), an endotoxin found on the outer cell wall of gram-negative bacteria, increases inflammatory response signaling and may play a role in the pathogenesis of several adverse outcomes, including inflammatory bowel diseases, cardiovascular disease, and cancer. While LPS is hypothesized to be associated with colorectal carcinogenesis, there are relatively few human studies which have examined this association.

METHODS - We examined the association between colorectal cancer (CRC) and lipopolysaccharide-binding protein (LBP), a marker of LPS, in 1,638 participants (819 CRC cases and 819 controls matched on multiple factors including age, sex, and race/ethnicity) from the Multiethnic Cohort study (MEC). Conditional logistic regression models were used to estimate the multivariable-adjusted odds ratios (OR) and 95% confidence intervals (95% CI).

RESULTS - Compared to individuals whose LBP concentrations were in the lowest quartile (2-24 μg/mL), the ORs associated with second (24.1-31.7 μg/mL), third (31.8-42.6 μg/mL), and fourth (42.7-107.5 μg/mL) quartiles were 1.23 (95% CI = 0.91-1.67), 1.36 (95% CI = 1.01-1.83), and 1.01 (95% CI = 0.73-1.39), respectively, (P_{trend} = 0.66).

CONCLUSIONS - This study did not find a statistically significant association between LBP (as a marker of LPS exposure) and CRC. Further studies are needed to understand the interplay between the gut microbiome, LBP, and CRC.

Introduction:

Colorectal cancer (CRC) is the fourth most common incident cancer in the United States, with an estimated 134,490 new cases expected to occur in 2016 [84]. Recent research has pointed to a possible key role of gut microbial communities (GMC) in understanding the link between obesity, chronic inflammation, and the development of CRC [1]. Among different GMC found in the human gut, Cani et al identified lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, as an underlying factor of obesity-driven low-grade inflammation [18].

LPS increases inflammatory response signaling, and may play a role in the pathogenesis of several adverse outcomes, including diabetes [22,6], inflammatory bowel disease [8], cardiovascular disease [10,26], and cancer [38,13]. LPS, and lipopolysaccharide-binding protein (LBP), a marker of LPS exposure, have been shown to reduce apoptosis [85,86] and increase proliferation in metastatic tumor cells [87,88] in both *in vitro* and *in vivo* experimental studies. A recent cross-sectional study of individuals undergoing colonoscopy found that those with higher plasma LPS concentrations were more likely to have adenomas compared to those with low LPS concentrations (OR 1.4, 95% CI 1.0-2.1), and this excess risk was independent of cytokine levels (TNF-α, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, and IFN-γ) [38]. Additionally, a recent prospective study showed that polymorphisms in the LBP gene (GA and GG genotypes of LBP rs2232596) correlated with increased CRC risk (odds ratio (OR) = 1.51, 95% confidence interval (CI): 1.15-1.99, P = 0.003; OR = 2.49, 95% CI: 1.16-5.38, P = 0.016, respectively) [89]. Several studies have shown that LBP polymorphisms (LBP SNPs rs2232613 and rs2232571) were associated with changes in plasma LBP concentrations [90–92], suggesting that the increased risk of CRC

associated with certain polymorphisms may be due to increased levels of circulating LBP.

In vitro studies have shown that LPS promotes cancer-cell survival and proliferation, angiogenesis, vascular permeability, and tumor cell adhesion [85,30,31,29], however, no epidemiologic studies have prospectively examined the association between LPS or LBP and CRC risk. Thus, the goal of this study was to examine whether pre-diagnostic plasma LBP concentration levels were associated with CRC incidence in a case control study nested within a prospective cohort study.

Methods:

Study Population and Overview

The Multiethnic Cohort (MEC) is a longitudinal study designed to investigate the association of dietary, lifestyle, and genetic factors with the incidence of cancer and other chronic diseases [39]. The MEC includes 215,251 men and women primarily from five different racial-ethnic groups (African Americans, Japanese Americans, Latinos, Native Hawaiians and whites in Hawaii and California [mostly in Los Angeles county]) aged 45-75 years at recruitment. The cohort was assembled in 1993-1996 by mailing a self-administered, 26-page questionnaire to individuals identified primarily through the driver's license files from Hawaii and California. The baseline questionnaire obtained information on demographics, medical and reproductive histories, cigarette smoking, medication use, family history of various cancers, physical activity, and quantitative food frequency questionnaire (FFQ). In Year 5 of the follow-up (1999-2001), a short questionnaire updated information on medical conditions and history, including screening tests for cancer. A ten-year update of the dietary and other exposure data was completed between 2003 and 2008 by re-administration of the full baseline questionnaire. These measures were obtained on 72% of the MEC participants

with biospecimens. The Institutional Review Boards at the University of Hawaii and the University of Southern California approved the study protocol.

LBP Exposure Assessment

Between 2001 and 2006, biospecimens, which included fasting blood samples, as well as a short questionnaire on current medication and dietary supplement use were collected from surviving members of the MEC in Hawaii and Los Angeles. This biorepository includes approximately 70,000 subjects who are broadly representative of all cohort members [93].

For this study, the concentration of LBP in plasma was measured in heparinized plasma using a commercial ELISA kit (Cell Sciences Inc) designed for either heparinized plasma or serum samples. Samples were diluted 1:1000 and the assay was conducted according to kit protocol with a standard curve of 5-50 ng/mL. Ten percent of samples were run in duplicate and laboratory personnel were blinded to the case-control status of the participants. Samples for matched cases and controls were positioned in random order next to each other in the same batch in order to reduce bias due to laboratory variation. Based on the approximately 10% QC duplicate samples, the intra- and inter- batch coefficient of variation percent (CV%) was 4.3 and 11.3, respectively.

Ascertainment of Case Status, Site and Stage

Identification of incident cancer cases was achieved by regular linkage with the Hawaii Tumor Registry (HTR), the Los Angeles County Cancer Surveillance Program (CSP), and the State of California Cancer Registry (CCR), all of which are NCI Surveillance, Epidemiology, and End Results (SEER) program [94]. Deaths in the cohort were identified by linkage to the state death-certificate files in CA and HI, and to the National Death Index for deaths occurring in other states. Colon cancer cases

included those with ICD-O-3 codes of C18.0-C18.9 and rectal cancer cases were those with ICD-O-3 codes of C19.9 and C20.9. Histologies >9000 were excluded. SEER summary stage was used, which defines localized cancer as cancer that is limited to the organ of origin, regional as beyond the original site to nearby lymph nodes or organs and tissues, and distant as cancer that has spread to distant organs or distant lymph nodes.

Individuals with a history of CRC prior to their phlebotomy date, based on the baseline questionnaire and/or linkage to the SEER registries, were excluded. A total of 846 colorectal cases with a diagnosis date prior to 10/2013 whose diagnoses occurred after blood collection were initially identified for LBP assessment. Two cases were ultimately discovered to have *in situ* tumors, as well as 25 cases with unspecified tumor stage were excluded (as we could not ascertain whether unspecified tumors were *in situ*) leaving 819 cases with invasive cancers of the colon and rectum.

Statistical Analysis

For this analysis, a nested case-control design was used, matching controls to CRC cases on a 1-1 ratio. For each case, a control pool was created consisting of cohort members who had donated specimens and who were alive and have never been diagnosed with CRC at the age of the case's diagnosis. Controls were matched to cases on birth year, location (Hawaii or Los Angeles), sex, ethnicity, age at phlebotomy (within ± 1 year), date of specimen collection (± 1 month), time of blood collection (± 2 hours) and time since last meal (± 2 hours). LBP was categorized into quartiles with the cutoffs for each quartile determined using control values. Conditional logistic regression with matched sets as strata was used to compute odds ratios (ORs) and 95% confidence intervals (CIs) for the relationship between LBP and CRC in the minimally-adjusted

model. Additional adjustment for covariates not matched on was performed in the multivariable model.

Covariates included in multivariable analyses were selected *a priori* (see Table 1), and included factors associated with LBP and/or CRC. The multivariable model included the following covariates evaluated at baseline: education (less than high school, high school graduation, some college, college graduate or higher), history of intestinal polyps (yes/no), diabetes (yes/no), history of CRC among first-degree relatives (yes/no), BMI (kg/m^2, categorized according to federal guidelines [95]: underweight (<18.5), normal weight (18.5–24.9), overweight (25–29.9), and obese (≥30), with normal weight serving as the reference group), aspirin frequency use (never, yes, yes but not current), alcohol consumption (g/day, categorized based on one standard drink equaling 14g alcohol and the Department of Agriculture and Department of Health & Human Services recommendation of no more than 1 drink/day for women and 2 drinks/day for men [96], females: non-drinker [0 g/day], 0.1 - <14g/day, and >14 g/day; males: non-drinker [0 g/day], 0.1 - <28g/day, and >28 g/day), relative density of dietary fiber intake (g/1,000 kcal/day), saturated fat intake (% of energy, calculated as 9*g saturated fat/kcal,), and physical activity (METS/day), dietary fiber intake, saturated fat intake, and physical activity were categorized into tertiles based on the distribution among controls.

Additionally, previous colonoscopy or sigmoidoscopy (yes/no) reported at the 5-year follow-up questionnaire, NSAID use at blood draw (yes/no), hormone replacement therapy use at blood draw (yes/no/refused/don't know), and smoking status at time of blood draw (never, current, former) were included in the multivariable model. We created a "missing" category for covariates with missing data in order to reduce the number of participants who would be dropped from the analysis.

Stratified analyses were performed to examine the association between LBP and CRC risk by BMI, dietary fiber intake, and saturated fat intake at baseline. BMI, as well as dietary fiber intake and saturated fat intake values, were dichotomized at the median value of controls. P-values for interaction were calculated by including a single cross-product term between LBP concentration, modeled as a linear categorical variable, and the binary variable (BMI, saturated fat, or dietary fiber) in a single model, adjusted for the covariates listed above.

We also evaluated heterogeneity of the LBP-CRC association by cancer site (colon vs. rectum), and cancer stage (local vs. regional/distal) at time of diagnosis. In the case of synchronous colorectal cancers (n=18), the tumor with the higher stage were used. Four individuals were excluded from the secondary analysis of LBP-CRC by tumor site as they had diagnosed tumors in both the colon and the rectum that were the same stage. Conditional logistic regression restricting cases to specific sites/stages was used to determine point estimates and corresponding 95% CI while unconditional logistic regression limited to cases (where regional/colon cancers were coded as cases and local/rectal cases were coded as controls) was used to determine statistical significance (P for difference) of subsite- and stage- specific differences. These regression models were adjusted for all covariates listed above as well as the matching variables sex, age, study site, and time since last meal. A sensitivity analysis, which excluded all cases diagnosed within one year of follow-up, was performed to address the possibility that preclinical CRC may influence circulating LBP concentrations. Further sensitivity analyses were performed by replacing baseline covariates with responses from follow-up questionnaire to examine any potential bias that may have occurred from the temporal discordance in blood and questionnaire collection - as the blood draw was drawn several years after the baseline questionnaire was administered.

Additionally, we also reanalyzed the association between LBP and CRC including of only those individuals with complete data to explore whether missing covariate data may have influenced results. A final sensitivity analysis dropping individuals with implausible dietary values (calories: <700 or <4200 kcal/day (n=126), alcohol: >100g/day (n=61) and weight measures (BMI: <15 or >50 (n=3), total dropped=144) was performed to examine bias introduced from individuals with extreme measurements.

All reported P-values are two-sided, and a P-value < 0.05 was considered statistically significant. All analyses were carried out using STATA 14 (StataCorp, College Station, TX).

Results:

Table 1 presents the distributions and means of the study covariates by matched case-control status (819 controls and 819 cases). Compared to controls, a history of diabetes was more prevalent among cases, while controls were more likely to be current smokers. Japanese represented the largest ethnicity among both cases (n=305) and controls (n=305), accounting for more than a third of each disease (i.e. case/control) group. Cases were more likely to be obese compared to controls, and a smaller percentage of cases had previously undergone sigmoidoscopy/colonoscopy compared to controls. The mean (± SD) LBP concentration level was 34.3 ± 14.3 μg/mL in cases and 34.6 ± 15.4 μg/mL in controls. Additionally, mean concentration levels differed slightly by ethnicity (Black: 37.6 ±17.9, Hawaiian: 31.9 ± 11.4 μg/mL, Japanese: 32.8 ± 13.5 μg/mL, Latino: 36.8 ± 15.8 μg/mL, White: 32.4 ± 12.6 μg/mL), by not by sex (Male: 34.4 ± 15.0 μg/mL, Female: 34.4 ± 14.7 μg/mL).

Associations of LBP concentrations and CRC risk are presented in Table 2. Overall LBP concentrations were not statistically significantly associated with CRC risk

in either the minimally adjusted or the multivariable adjusted models. In the multivariate adjusted model, the OR among those in the third quartile was statistically significantly higher than that in the lowest quartile (OR = 1.36; 95% CI = 1.01-1.83). However, there was no observed increased risk among those in the highest LBP quartile (OR = 1.01; 95% CI = 0.73-1.39) and the test for trend was not statistically significant (P_{trend} = 0.66).

Tables 3, 4, and 5 present analyses of the findings related to LBP concentrations, stratified by BMI, dietary fiber intake, and saturated fat intake, respectively. Among individuals in the high-BMI (> 26.12 kg/m²) group, the was a statistically significantly increased risk for those in the third quartile vs. first quartile (Q3: OR = 1.35; 95% CI = 1.01-1.81). Associations were slightly attenuated, albeit similar, in the low-BMI (≤ 26.68 kg/m²) group, and there was no evidence of heterogeneity between the groups ($P_{interaction}$ = 0.24). The association of higher LBP concentration and CRC risk was more apparent among individuals in the lower fiber category (vs. higher fiber), with a non-statistically significant trend observed across quartiles (P_{trend} = 0.10); however, there was no apparent heterogeneity between fiber groups ($P_{interaction}$ = 0.12). In both the high saturated fat and low saturated group, the positive association between LBP and CRP was most pronounced in the third tertile, but there were no apparent trends in CRC risk by LBP quartile in either group and no interaction between saturated fat intake and LBP on CRC risk ($P_{interaction}$ = 0.36). Tables 6 and 7 present analyses of LBP concentration and CRC by anatomic subsite, and cancer stage at diagnosis. While ORs across quartiles varied by cancer site (colon vs. rectum), these differences were not statistically significant (all $P_{difference}$ = 0.45). The association between LBP concentration and CRC risk was similar for local and regional/distant disease, with no evidence of heterogeneity (all $P_{difference}$ = 0.52).

In a sensitivity analyses, the associations between LBP concentration and CRC risk remained virtually unchanged after excluding cases diagnosed within one year of follow-up, after excluding individuals with extreme dietary and weight measurements, and after excluding those with missing covariate data (Supp Table 2).

Discussion:

Results from this study do not provide clear evidence of an association between plasma LBP concentrations and CRC risk. Additionally, there was no evidence of differential effects by BMI, dietary fiber intake, saturated fat intake, cancer site, or cancer stage. However, the stratified results in Tables 3-7 led to small numbers in some cells and limited power to detect main effects of LBP concentrations within subgroups or to detect significant interactions between groups.

Our results are not consistent with previous studies (prospective study using LBP gene polymorphisms and a cross-sectional study of individuals undergoing colonoscopy) on LPS, adenomas and CRC risk aforementioned in the introduction [38,89]. Differences in results may be due to use of LBP genes (not plasma LBP) in the first study and the cross-sectional nature of the second study, which measured LPS levels at the time of colonoscopy, and subsequent adenoma discovery [38]. Use of endogenously produced biomarkers such as LPS and LBP, which reflect the host's response to microbial products, can be directly influenced by various inflammatory factors; LPS transfer to the bloodstream may be caused by factors such as tissue damage, infection, or other medical conditions which may induce systemic inflammation, which would result in higher concentration levels in the adenoma cases as a byproduct, not a cause, of the adenoma in the second study. All of the participants in the current study were healthy at the time of blood draw, which, in turn, may have helped to reduce the likelihood that associations were confounded by illness or medical

conditions. Thus, differences in study results may be due to timing at which LBP concentrations were ascertained relative to the neoplasm diagnosis.

Additionally, the previous cross-sectional study measured LPS while our study measured LBP [38]. While LBP binds to LPS, the protein also recognizes the lipoteichoic acid of gram-positive bacteria as well as structurally similar spirochetal glycolipids [97,98]. However, it should be noted that the LBP assay is more reliable than the one used for LPS (given the large amount of interference in the Limulus Amebocyte Lysate (LAL) assay) [99]; moreover, in addition to difficulties with the assay, the need to collect samples under LPS-free conditions and high variability of LPS throughout the day may make LBP is a better marker [32,33]. As such, inconsistencies between current results and previous studies may have been due to inherent differences between the LPS and LBP molecules, as well as the assays used to measure them.

Current study findings may have been influenced by diet, as high fat, high caloric diet, and high carbohydrate diets have been shown to increase serum lipopolysaccharide levels. Human studies have shown that LPS concentrations are higher in subjects who consume a high-fat diet [100], with postprandial LPS concentrations rising significantly after a high-fat meal [27,28,101,102]. While the current study obtained blood from healthy individuals after an overnight fast to minimize the influence of short-term factors which may affect LBP concentrations, such as diet or illness, residual confounding may have persisted, as, at least in sepsis patients, LBP remains elevated for 12-24 hours following exposure – thus a high-calorie, high-fat meal the night before the blood draw may have led to a higher LBP concentration.

Additionally, it is important to note that LBP concentration range in the current population was well outside of normal healthy population limits. Based on previous literature, LBP concentrations in plasma range from 1 μg/mL to 24 μg/mL among

healthy individuals [49,103]. However, in the current population, LBP ranged from 1 µg/mL -107.5 µg/mL. LBP in the current population was normally distributed in both cases and controls, (as shown in supp figure 1). It is possible that the wide ranges may have been a consequence of processing times or long storage periods (samples were stored at -180˚C for roughly 10 years), although there are currently no studies examining how LBP measurements change in stored samples over time. The possibility that differences in processing times on case and control plasma samples led to higher LBP concentrations in some of the samples (i.e., processing times were different between cases and controls, where longer processing times led to erroneously higher values of LBP) may help to explain why a positive trend was seen between LBP concentration and CRC risk in the second and third quartile, but not the fourth (highest). Additional research is also needed to explain how LBP concentrations may change (i.e. degrade) in stored frozen samples over time.

Strengths of the study include its prospective design which allowed for LBP concentration levels prior to diagnosis of CRC to be measured, thus reducing bias resulting from reverse causality. Additionally, excluding cancer cases identified during the first year of follow up did not impact results, a finding that supports the likelihood that reverse causality did not influence point estimates. Lastly, our findings for LBP concentration and CRC by stage suggest that the associations were not stronger for those with more advanced stage, as would be expected if disease stage affected LBP.

Study limitations include the use of single measure of LBP. Given that LBP is an acute phase reactant and is influenced by a range of factors, one measure may not capture average long-term LBP concentrations within an individual. A previous study of the reliability of LBP showed that the intraclass correlation coefficient over a three month period ranged from 0.49-0.6,with shorter follow-up times corresponding to a

higher ICC [104]. Therefore, multiple measures would be optimal for precise LBP measurement, especially for longer periods of exposure. Future studies, using multiple LBP measures, are needed to understand the interplay between the gut microbiome, LBP, inflammation, and CRC.

Table 1. Baseline Characteristics of Colorectal Cancer Cases and Controls, MEC Participants

	Cancer Cases (n=819) N (SD or %)	Controls (n=819) N (SD or %)
Matched Factors		
Sex		
Male	453 (55.3%)	453 (55.3%)
Female	366 (44.7%)	366 (44.7%)
Age at blood draw, y	61.2 (8.3)	60.3 (8.2)
Ethnicity		
Black	160 (19.5%)	160 (19.5%)
Native Hawaiian	50 (6.1%)	50 (6.1%)
Japanese	305 (37.2%)	305 (37.2%)
Latino	172 (21.9%)	172 (21.9%)
White	132 (16.1%)	132 (16.1%)
Unmatched Factors		
Family History of CRC	85 (10.4%)	75 (9.1%)
LBP (µg/mL)	34.3 (14.3)	34.6 (15.4)
History of Colorectal Polyps Removed	54 (6.6%)	64 (7.8%)
Previous Colonoscopy or Sigmoidoscopy (at 5 year f-up questionnaire)	292 (35.6%)	326 (39.8%)
Alcohol Consumption (g/day)		
None	359 (45.1%)	365 (45.5%)
< 14 g/day	276 (34.7%)	287 (35.7%)
14-28 g/day	64 (8.0%)	67 (8.3%)
>28 g/day	97 (12.2%)	84 (10.5%)
Caloric Intake (kcal/day)	2,179 (1,043)	2,180 (976)
Saturated Fat Intake (relative %)	9.8 (2.9)	10.0 (2.9)
0 - 8.56	280 (35.2%)	265 (33.1%)
8.57 - 11.2	286 (34.9%)	271 (33.7%)
> 11.2	238 (29.9%)	267 (33.2%)
Dietary Fiber Intake (g/1,000kcal/day)		
0 - 9.39	284 (35.7%)	265 (33.1%)
9.40 - 12.72	220 (27.6%)	271 (33.7%)
> 12.72	292 (36.7%)	267 (33.2%)
Physical Activity (METS/day)		
0-1.50	270 (34.6%)	260 (33.6%)
1.51 - 1.75	297 (37.8%)	265 (34.1%)
>1.76	216 (27.5%)	249 (32.2.%)
History of Diabetes	974(11.5%)	61 (7.5%)
BMI (kg/m²)		
< 18.5	8 (1.1%)	9 (1.2%)
18.5 - 24.9	257 (31.2%)	322 (39.4%)
25.0 - 30.0	338 (41.1%)	332 (40.8%)
> 30.0	212 (26.2%)	150 (18.6%)
Smoking Status at blood draw		

Never	258 (41.6%)	216 (35.5%)
Former	332 (53.5%)	251 (41.3%)
Current	31 (5.0%)	141 (23.2%)
Years of School Completed		
< High School (11 years or less)	118 (14.7%)	117 (14.3%)
High School (12 years)	210 (25.3%)	191 (23.7%)
Some University (13-15 years)	258 (32.3%)	266 (32.5%)
University Graduate or higher (16+ years)	228 (27.6%)	239 (29.4%)
Aspirin Use		
Current Use	153 (19.5%)	124 (15.9%)
Former Use	158 (19.6%)	159 (20.5%)
No Use	499 (60.6%)	514 (63.5%)
Non-aspirin NSAID Use at blood draw	132 (16.2%)	138 (16.9%)
Estrogen Use at blood draw (Women only)	50 (6.0%)	76 (9.1%)

Table 2. Estimated Odds Ratios (OR) of Colorectal Cancer associated with LBP concentrations

			Cases	Controls	Minimally adjusted*	Multivariate adjusted**
LBP (quartiles)			N	N		
1	0-24.0	µg/mL	183	205	Ref.	Ref.
2	24 .1– 31.7	µg/mL	211	205	1.15 (0.87-1.50)	1.23 (0.91-1.67)
3	31.8 – 42.6	µg/mL	238	204	1.28 (0.99-1.67)	1.36 (1.01-1.83)
4	42.7 – 107.5	µg/mL	187	205	1.02 (0.76-1.36)	1.01(0.73-1.39)
P_{trend}					*0.63*	*0.66*

*Matched variables: age, location (Hawaii or California), sex, ethnicity, age at phlebotomy, date of specimen collection, time of blood collection, and time since last meal

**Additional covariates: education, BMI (kg/m2), physical activity, smoking status at time of blood draw, history of intestinal polyps, previous colonoscopy or sigmoidoscopy, diabetes, history of CRC among 1st degree relatives, NSAID use, aspirin use, alcohol consumption, dietary fiber intake, saturated fat intake and hormone replacement therapy use among women

Table 3. Odds Ratios (OR) of Colorectal Cancer Associated with LBP Concentrations, by BMI

	High BMI (> 26.12 kg/m²), N=883				Low BMI (≤ 26.12 kg/m²), N=755				
	Cases	Controls	OR[a]	(95% CI)	Cases	Controls	OR[a]	(95% CI)	P for interaction
LBP (quartiles)									
1	98	91	1.00	Ref	85	114	1.00	Ref	0.24
2	115	106	1.25	(0.92-1.70)	96	99	1.17	(0.86-1.58)	
3	143	103	1.35	(1.01-1.81)	95	102	1.26	(0.92-1.72)	
4	114	113	0.98	(0.71-1.34)	73	91	0.91	(0.63-1.31)	
P_{trend}				*0.33*				*0.87*	

[a] Matched on: age, location (Hawaii or California), sex, ethnicity, age at phlebotomy, date of specimen collection, time of blood collection, and time since last meal
Adjusted for education, BMI (kg/m2), physical activity, smoking status at time of blood draw, history of intestinal polyps, previous colonoscopy or sigmoidoscopy, diabetes, history of CRC among 1st degree relatives, NSAID use, aspirin use, alcohol consumption, dietary fiber intake, saturated fat intake and hormone replacement therapy use among women

Table 4. Odds Ratios (OR) of Colorectal Cancer Associated with LBP Concentrations, by Fiber Intake

	High Fiber (> 10.89 g/1,000kcal/day), N=836				Low Fiber (≤ 10.89 g/1,000kcal/day), N=802				
	Cases	Controls	OR[a]	(95% CI)	Cases	Controls	OR[a]	(95% CI)	P for interaction
LBP (quartiles)									
1	92	103	1.00	Ref	91	102	1.00	Ref	0.12
2	117	96	1.10	(0.80-1.53)	94	109	1.30	(0.95-1.78)	
3	110	97	1.36	(1.01-1.83)	128	108	1.60	(1.12-2.28)	
4	100	121	1.13	(0.80-1.59)	87	83	1.32	(0.84-2.09)	
P_{trend}				*0.41*				*0.10*	

[a] Matched on: age, location (Hawaii or California), sex, ethnicity, age at phlebotomy, date of specimen collection, time of blood collection, and time since last meal
Adjusted for education, BMI (kg/m2), physical activity, smoking status at time of blood draw, history of intestinal polyps, previous colonoscopy or sigmoidoscopy, diabetes, history of CRC among 1st degree relatives, NSAID use, aspirin use, alcohol consumption, saturated fat intake and hormone replacement therapy use among women

Table 5. Odds Ratios (OR) of Colorectal Cancer Associated with LBP Concentrations, by Saturated Fat Intake

	High Saturated Fat (Relative % > 8.94), N=820				Low Saturated Fat (Relative % ≤ 8.94), N=818				
	Cases	Controls	OR[a]	(95% CI)	Cases	Controls	OR[a]	(95% CI)	P for interaction
LBP (quartiles)									
1	78	85	1.00	Ref	105	120	1.00	Ref	0.36
2	102	103	1.15	(0.83-1.59)	109	102	1.26	(0.92-1.73)	
3	126	108	1.35	(1.01-1.82)	112	97	1.49	(1.06-2.09)	
4	97	121	1.06	(0.76-1.46)	90	83	1.16	(0.76-1.79)	
P_{trend}				*0.66*				*0.26*	

[a] Matched on: age, location (Hawaii or California), sex, ethnicity, age at phlebotomy, date of specimen collection, time of blood collection, and time since last meal
Adjusted for education, BMI (kg/m2), physical activity, smoking status at time of blood draw, history of intestinal polyps, previous colonoscopy or sigmoidoscopy, diabetes, history of CRC among 1st degree relatives, NSAID use, aspirin use, alcohol consumption, dietary fiber intake, and hormone replacement therapy use among women

Table 6. Odds Ratios (OR) of Colorectal Cancer Associated with LBP Concentrations, by Subsite

	Colon Cancer, N=651			Rectal Cancer N=164)			P for
	Cases	OR[a]	(95% CI)	Cases	OR[a]	(95% CI)	difference
LBP (quartiles)							
1	146	1.00	Ref	35	1.00	Ref	0.45
2	162	1.26	(0.89-1.79)	48	0.98	(0.46-2.10)	
3	186	1.42	(1.00-2.00)	52	1.16	(0.56-2.43)	
4	157	1.06	(0.74-1.52)	29	0.82	(0.33-2.01)	
P_{trend}			*0.553*			*0.88*	

[a] Matched on: age, location (Hawaii or California), sex, ethnicity, age at phlebotomy, date of specimen collection, time of blood collection, and time since last meal
Adjusted for education, BMI (kg/m2), physical activity, smoking status at time of blood draw, history of intestinal polyps, previous colonoscopy or sigmoidoscopy, diabetes, history of CRC among 1st degree relatives, NSAID use, aspirin use, alcohol consumption, dietary fiber intake, saturated fat intake and hormone replacement therapy use among women

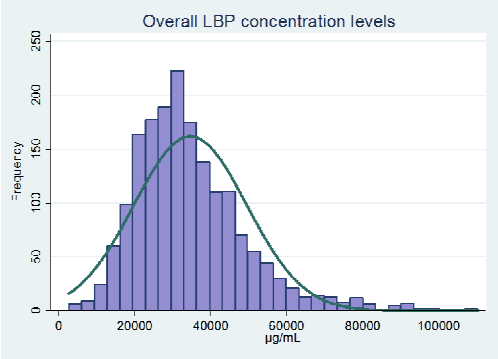
Table 7. Odds Ratios (OR) of Colorectal Cancer Associated with LBP Concentrations, by Stage

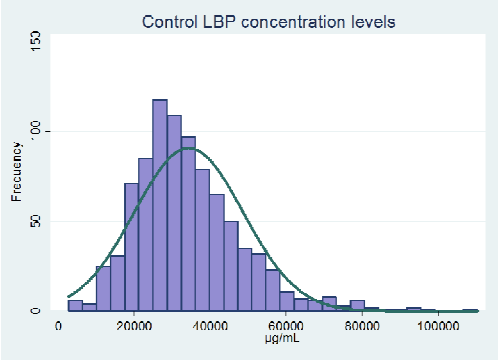
	Local, N=407			Regional/Distant, N=412			P for
	Cases	OR[a]	(95% CI)	Cases	OR[a]	(95% CI)	difference
LBP (quartiles)							
1	95	1.00	Ref	88	1.00	Ref	0.52
2	91	1.12	(0.70-1.80)	120	1.45	(0.94-2.22)	
3	123	1.37	(0.90-2.08)	115	1.41	(0.90-2.20)	
4	98	1.14	(0.72-1.80)	89	1.06	(0.64-1.71)	
P_{trend}			*0.36*			*0.78*	

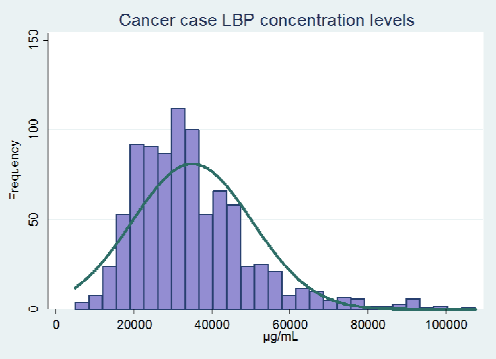
[a] Matched on: age, location (Hawaii or California), sex, ethnicity, age at phlebotomy, date of specimen collection, time of blood collection, and time since last meal
Adjusted for education, BMI (kg/m2), physical activity, smoking status at time of blood draw, history of intestinal polyps, previous colonoscopy or sigmoidoscopy, diabetes, history of CRC among 1st degree relatives, NSAID use, aspirin use, alcohol consumption, dietary fiber intake, saturated fat intake and hormone replacement therapy use among women

SUPPLEMENTARY TABLES AND FIGURES

Supplementary Figure 1. Distribution of LBP concentration values overall and by case-control status







Supplementary Table 1. Estimated Odds Ratios (OR) of Colorectal Cancer Associated with LBP Concentrations (removing cases diagnosed w/in 1 year of blood draw)

		Cases	Multivariate adjusted**
LBP (quartiles)		N	
1	0-24.7 µg/mL	158	Ref.
2	24 .8– 31.9 µg/mL	183	1.24 (0.89-1.74)
3	32.0 – 42.8 µg/mL	205	1.39 (1.01-1.92)
4	42.9 – 107.6 µg/mL	161	1.05 (0.74-1.49)
P_{trend}			*0.56*

*Matched variables: age, location (Hawaii or California), sex, ethnicity, age at phlebotomy, date of specimen collection, time of blood collection, and time since last meal

**Additional covariates: education, BMI (kg/m2), physical activity, smoking status at time of blood draw, history of intestinal polyps, previous colonoscopy or sigmoidoscopy, diabetes, history of CRC among 1st degree relatives, NSAID use, aspirin use, alcohol consumption, dietary fiber intake, saturated fat intake and hormone replacement therapy use

Supplementary Table 2. Estimated Odds Ratios (OR) of Colorectal Cancer Associated with LBP Concentrations (removing individuals with missing covariates)

		Cases	Multivariate adjusted**
LBP (quartiles)		N	
1	0-24.7 µg/mL	79	Ref.
2	24 .8– 31.9 µg/mL	82	1.18 (0.61-2.28)
3	32.0 – 42.8 µg/mL	116	1.32 (0.70-2.47)
4	42.9 – 107.6 µg/mL	98	1.06 (0.66-1.90)
P_{trend}			*0.72*

*Matched variables: age, location (Hawaii or California), sex, ethnicity, age at phlebotomy, date of specimen collection, time of blood collection, and time since last meal

**Additional covariates: education, BMI (kg/m2), physical activity, smoking status at time of blood draw, history of intestinal polyps, previous colonoscopy or sigmoidoscopy, diabetes, history of CRC among 1st degree relatives, NSAID use, aspirin use, alcohol consumption, dietary fiber intake, saturated fat intake and hormone replacement therapy use

Supplementary Table 3. Estimated Odds Ratios (OR) of Colorectal Cancer Associated with LBP Concentrations (removing outliers)

LBP (quartiles)		Cases N	Controls N	Multivariate adjusted**
1	0-23.9 µg/mL	166	188	Ref.
2	24 .0– 31.5 µg/mL	184	188	1.23 (0.88-1.72)
3	31.6 – 42.5 µg/mL	217	188	1.41 (1.01-1.95)
4	42.6 – 107.6 µg/mL	168	187	1.03 (0.72-1.46)
P_{trend}				*0.55*

*Matched variables: age, location (Hawaii or California), sex, ethnicity, age at phlebotomy, date of specimen collection, time of blood collection, and time since last meal

**Additional covariates: education, BMI (kg/m2), physical activity, smoking status at time of blood draw, history of intestinal polyps, previous colonoscopy or sigmoidoscopy, diabetes, history of CRC among 1st degree relatives, NSAID use, aspirin use, alcohol consumption, dietary fiber intake, saturated fat intake and hormone replacement therapy use

Supplementary Table 4. Odds Ratios (OR) of Colorectal Cancer Associated with LBP Concentrations, by Location

	California, N=822				Hawaii, N=816				
	Cases	Controls	OR[a]	(95% CI)	Cases	Controls	OR[a]	(95% CI)	P for interaction
1	81	73	1.00	Ref	102	132	1.00	Ref	0.01
2	95	83	1.31	(0.80-2.14)	116	122	1.13	(0.73-1.73)	
3	122	121	0.95	(0.60-1.50)	116	84	2.06	(1.29-3.26)	
4	113	134	0.66	(0.41-1.06)	74	70	1.28	(0.78-2.10)	
P_{trend}				*0.04*				*0.03*	

[a] Matched on: age, location (Hawaii or California), sex, ethnicity, age at phlebotomy, date of specimen collection, time of blood collection, and time since last meal
Adjusted for education, BMI (kg/m2), physical activity, smoking status at time of blood draw, history of intestinal polyps, previous colonoscopy or sigmoidoscopy, diabetes, history of CRC among 1st degree relatives, NSAID use, aspirin use, alcohol consumption, dietary fiber intake, saturated fat intake and hormone replacement therapy use

Supplementary Table 4. Odds Ratios (OR) of Colorectal Cancer Associated with LBP Concentrations, by Sex

	Men, N=906				Women, N=732				
	Cases	Controls	OR[a]	(95% CI)	Cases	Controls	OR[a]	(95% CI)	P for interaction
1	107	123	1.00	Ref	76	82	1.00	Ref	0.70
2	107	106	1.11	(0.74-1.68)	104	99	1.69	(1.00-2.85)	
3	128	107	1.37	(0.92-2.04)	110	98	1.53	(0.93-2.52)	
4	111	117	1.07	(0.71-1.62)	76	87	1.02	(0.60-1.86)	
P_{trend}				*0.49*				*0.87*	

[a] Matched on: age, location (Hawaii or California), sex, ethnicity, age at phlebotomy, date of specimen collection, time of blood collection, and time since last meal
Adjusted for education, BMI (kg/m2), physical activity, smoking status at time of blood draw, history of intestinal polyps, previous colonoscopy or sigmoidoscopy, diabetes, history of CRC among 1st degree relatives, NSAID use, aspirin use, alcohol consumption, dietary fiber intake, saturated fat intake and hormone replacement therapy use

CHAPTER 5: Conclusions

Conclusions and future research:

In this dissertation, we sought to test the association between the GMC and adiposity with plasma LBP concentrations and to determine whether increased LBP concentrations are associated with CRC incidence. Additionally, we also examined the reliability of LBP as a biomarker of risk.

In Chapter 2, we found that circulating concentrations of LBP showed moderate temporal reliability up to a 9-month period. This suggests that a single measurement of these biomarkers may be used for risk assessment in short-term studies; longitudinal studies capturing exposure over a multiple-year period may be needed in order to obtain more stable estimates. Future studies are needed on the reliability of LBP measures over a longer duration, as are studies of the potential of LBP as a marker of chronic inflammation and cancer risk.

In Chapter 3, we found that beta-diversity was statistically significantly different between LBP groups using unweighted Unifrac, but not weighted Unifrac. Additionally, results showed that several taxa, particularly those found in the Clostridia class may be more prevalent in those low levels of LBP while Bacteroides may be more prevalent with high levels of LBP. Adiposity was statistically significantly associated with LBP concentration even after controlling for covariates, and LBP did not mediate this association. Given that this appears to be the first study to examine LBP in relation to adiposity and GMC, validation of our findings in larger, more representative populations are needed. Furthermore, studies are needed to examine alternative pathways in which adiposity influences inflammation and future studies should examine whether specific taxa associated with high/low LBP concentrations are correlated with inflammatory markers.

Chapter 4 results did not provide clear evidence of a dose-response association between plasma LBP concentrations and CRC risk. Additionally, there was no evidence of differential effects by BMI, dietary fiber intake, saturated fat intake, cancer site, or cancer stage. Based on findings from Chapter 2, multiple measures may have been needed in order to obtain more stable estimates, given the longitudinal nature of the study. Additionally, LBP concentrations may have been influenced by the age of the MEC blood samples. As such, future studies are needed on the reliability of LBP measures over a longer duration, as are prospective studies on the GMC, LBP, and CRC.

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