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Precision Nutrition for the Management and Prevention of Chronic Disease: Exploring the Relationship Between Intestinal Permeability and High-Density Lipoproteins in Alzheimer's Disease

By

EDUARDO ROMO

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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Abstract

High-density lipoproteins (HDL), inflammation pathways, and associated proteins like lipopolysaccharide binding protein (LBP) play complex and interrelated roles in major age-related diseases including cardiovascular disease and Alzheimer's disease (AD). This dissertation integrates findings across multiple key studies to elucidate novel insights of the "lipoprotein-gastrointestinal-inflammation axis" underlying these conditions. By reviewing evidence on HDL glycoprotein alterations in disease, identifying elevated LBP in Alzheimer's patients and analyzing impacts of fiber supplementation on inflammation in preliminary trials, new insights emerge. These discoveries pave the way for improved risk prediction, prevention, and treatment strategies that leverage knowledge of HDL, LBP, and glycosylation aberrations. This body of work catalyzes future research to enable transformative advances against society's most pressing health burdens.

1. Chapter one reviews research on glycosylation of HDL-associated proteins, showing it is altered in diseases like cardiovascular disease (CVD) and has potential as a diagnostic biomarker. It discusses analytical methods for profiling HDL glycoproteins and evidence linking glycosylation to HDL function. The chapter emphasizes the need for large cohort studies on HDL glycan profiles and health outcomes.

2. Chapter two presents findings from an Alzheimer's study identifying significantly higher LBP concentrations in plasma fractions of apolipoprotein E (APOE)3E3 AD patients compared to APOE3E4 controls. LBP was associated with dementia severity and verbal

memory scores. This implicates LBP-driven inflammation in AD pathogenesis in genetically low-risk individuals.

3. Chapter three analyzes data from a prebiotic fiber supplementation trial, finding limited overall impacts of the fiber supplement on plasma concentrations of LBP and HDL function, but reductions in LBP selectively in those individuals with higher baseline LBP. Our findings suggest that some individuals who consume low-fiber diets have increased gut permeability, and for these individuals, even a low-dose daily fiber supplement can improve gut barrier function.

Together, these discoveries reveal new aspects of lipoproteins, LBP, and glycosylation in major age-related disease. This dissertation promotes future research leveraging this knowledge for enhanced prediction, prevention, and treatment of conditions like AD and CVD. Key priorities include large glycoprofiling studies, elucidating LBP's role in AD, and assessing early-life precision nutrition anti-inflammatory interventions in at-risk groups.

Chapter 1: Glycosylation of HDL-Associated Proteins and Its Implications in Cardiovascular Disease Diagnosis, Metabolism and Function.

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Abstract

High-density lipoprotein (HDL) particles, long known for their critical role in the prevention of cardiovascular disease (CVD), were recently identified to carry a wide array of glycosylated proteins, and the importance of this glycosylation in the structure, function and metabolism of HDL are starting to emerge. Early studies have demonstrated differential glycosylation of HDL-associated proteins in various pathological states, which may be key to understanding their etiological role in these diseases and may be important for diagnostic development. Given the vast array and specificity of glycosylation pathways, the study of HDL-associated glycosylation has the potential to uncover novel mechanisms and biomarkers of CVD and other diseases such as diabetes, kidney disease, liver failure, and Alzheimer's disease. To date, no large studies examining the relationships between HDL glycosylation profiles and cardiovascular outcomes have been performed. However, small pilot studies provide promising preliminary evidence that such a relationship may exist. In this review article we discuss the current state of the evidence on the glycosylation of HDL-associated proteins, the potential for HDL glycosylation profiling in CVD diagnostics, how glycosylation affects HDL function, and the potential for modifying the glycosylation of HDL-associated proteins to confer therapeutic value.

Keywords: High-Density Lipoproteins, Cardiovascular Disease, Glycosylation, Biomarker

Introduction

It has been established across multiple cohorts that high density lipoproteins (HDL) are atheroprotective (1–4). HDL are complex, heterogeneous nanoparticles, with various subclasses, comprised of numerous functional proteins and lipids (5), and have more recently been shown to be highly glycosylated (6) and structurally and compositionally variable in various physiological and pathological states (7,8). Owing to this high heterogeneity, HDL particles have diverse biological functions including immunomodulatory, anti-inflammatory, antioxidant, antithrombotic, and anti-proteolytic functions among others, which are dependent on their composition (9–13). Protein and lipid composition, as well as particle structure and size, are important known factors driving differences in HDL functional capacity. The role of glycosylation in the differential functionality of HDL particles has only recently started to emerge.

Protein glycosylation is generally an enzymatically driven post-translational modification of newly biosynthesized proteins that occurs in the endoplasmic reticulum and Golgi apparatus where sugars are attached to proteins by N- or O-linkages, forming glycans (14). N-glycans are attached to a nitrogen atom on the asparagine moiety of the protein whereas O-glycans are bound to the oxygen atom of either threonine or serine (15). Glycans contribute to various biological capacities including protein folding, receptor binding, enzyme activity, and physical properties by lending charge to the protein, and are vastly particular to the type, extent, and specific site of glycosylation (15–19). Protein glycosylation functions as a biological language and is important for biological particle self- and non- self-recognition, molecule transport, and endocytosis (20). In the last 8 years since it was first demonstrated that HDL are highly glycosylated, and

specifically sialylated particles (6,)(Figure1), there has been a steady increase in the evidence pointing to an important connection between the glycosylation of HDL-associated proteins, and the overall functionality of HDL particles. In this review paper we will discuss the current state of the evidence on the glycosylation of HDL-associated proteins, specifically, where we stand in terms of development of cardiovascular disease (CVD) diagnostics using HDL-glycosylation profiling, how glycosylation of HDL proteins affects HDL function, and the potential for modifying the glycosylation of HDL-associated proteins to confer therapeutic value.

HDL Glycosylation Profiling for Diagnostic Purposes

One of the problems with HDL particle analysis for diagnostic purposes has been the extreme complexity of these particles and the lack of resolution of older measurement tools. For example, although high HDL-cholesterol (HDL-C) concentrations have been found to be protective against CVD, several large recent studies demonstrated that the relationship between HDL-C concentration and adverse health outcomes tends to follow a U-shaped curve, with both low HDL-C and very high HDL-C being associated with increased cardiovascular (CV) events (21–23). Clearly, it is not simply the measurement of the total amount of cholesterol carried within HDL that is diagnostic, but rather some other aspect of HDL that is critical, whether it be compositional, structural, or functional.

For more sophisticated measurements of HDL structure, composition, and function, it is imperative to first isolate the HDL particles and purify them from other potentially contaminating components. Because HDL particles are so small (7-12 nm in diameter) as to overlap with many plasma proteins in terms of their size (e.g. ferritin), and because they are

close in density to other lipoprotein particles and even extracellular vesicles, they are difficult to isolate and purify. According to multiple proteomic studies HDL could carry as few as 12 key proteins or up to an excess of 200 proteins (24,25) depending on how they are isolated (24,26,27). Various methods, and combinations of these methods, have been used to isolate HDL including ultracentrifugation, size exclusion chromatography, immunoaffinity precipitation, and asymmetrical flow field flow fractionation. More recently, methods combining these different approaches have been used to improve the overall yield and purity of HDL particles while preserving their structural and functional integrity (28–30), including an optimized, validated method using sequential flotation density ultracentrifugation followed by size exclusion chromatography which yields highly purified HDL fractions (5).

Once HDL particles are isolated, the analysis of their glycosylation status can be performed. Pioneering work in lipoprotein glycobiology establishing analytical methods for profiling the glycome of HDL particles revealed for the first time that HDL has both N- and O-linked glycosylation and is distinctly highly sialylated (6). Glycosylation analysis is a complex chemical approach traditionally using mass spectrometry combined with various extraction methods such as enzymatic digestion, chemical cleavage, and liquid chromatography (31–35). HDL glycosylation can be profiled in several ways: 1) the glycans can be enzymatically or chemically cleaved, followed by mass spectrometry (6), 2) site-specific glycoprofiling can be performed by tandem mass spectrometry analysis of protease-digested samples (6,36,37), and 3) hydrophilic interaction liquid chromatography profiling can be performed, which uses a combination of the three main types of liquid chromatography for separation and profiling of glycan-containing peaks (38,39).

To date, no large studies examining the relationships between HDL glycosylation profiles and CVD outcomes have been performed. However, small pilot studies provide promising preliminary evidence that such a relationship may exist. For example, in a small pilot study performed by our group, differences in HDL glycan composition were able to differentiate between individuals at equal risk for CVD based on clinical parameters (i.e. total cholesterol, low-density lipoprotein-cholesterol (LDL-C), HDL-C, etc.) who were found to have arterial occlusion vs. not. (37). The role of HDL glycosylation in CV health is starting to be recognized as a promising new research field (40). Larger cohort studies investigating the relationships between HDL glycoprofiles and CV outcomes across factors including age, sex, and ethnicity are needed, and have the potential to add greatly to our ability to detect individuals at risk for CVD earlier when disease prevention measures are the most likely to be effective.

How Glycosylation of HDL Proteins Affects HDL Function

Most of the known HDL-associated proteins are glycosylated, and only a few are found to be non-glycosylated. In Table 1 we provide information on the N- and/or O-glycosylation status, sites of attachment, and number of unique glycans attached for several HDL proteins for which this information has been confirmed by extensive MS/MS analysis. We have also uncovered glycosylation sites in various other serum proteins including ApoB-100 but their presence may be due to contamination of trace amounts in the HDL fraction. Whereas there are many putative sites for O-glycosylation (i.e. any Ser or Thr residue) on any given protein, whether O-glycans are actually attached must be confirmed by advanced MS analysis. Thus, although several HDL-associated proteins, such as ApoC-I have Ser or Thr residues that could in theory be O-glycosylated, in Table 1 we report only those that have been demonstrated to be O-glycosylated

by MS measurement of isolated HDL fractions derived from a starting volume of 500uL of plasma. It is possible that some proteins (e.g. PLTP) are present at such low abundance in isolated HDL that their glycoforms fall below the limits of detection. Thus, to further investigate the glycosylation status of these low-abundance HDL proteins future experiments involving enrichment for these proteins will be required. Other proteins, such as ApoA-I, have been reported to be glycosylated in the literature, however, we do not include it in Table 1 because based on detailed MS analysis the O-glycosylation could not be confirmed. In the following section we review what is currently known about the impact of glycosylation of several key HDL-associated on overall HDL metabolism and function, for which there is currently information. As the field evolves this list will doubtless grow and a more comprehensive picture of the extent and diversity of glycans attached to HDL-associated proteins will emerge.

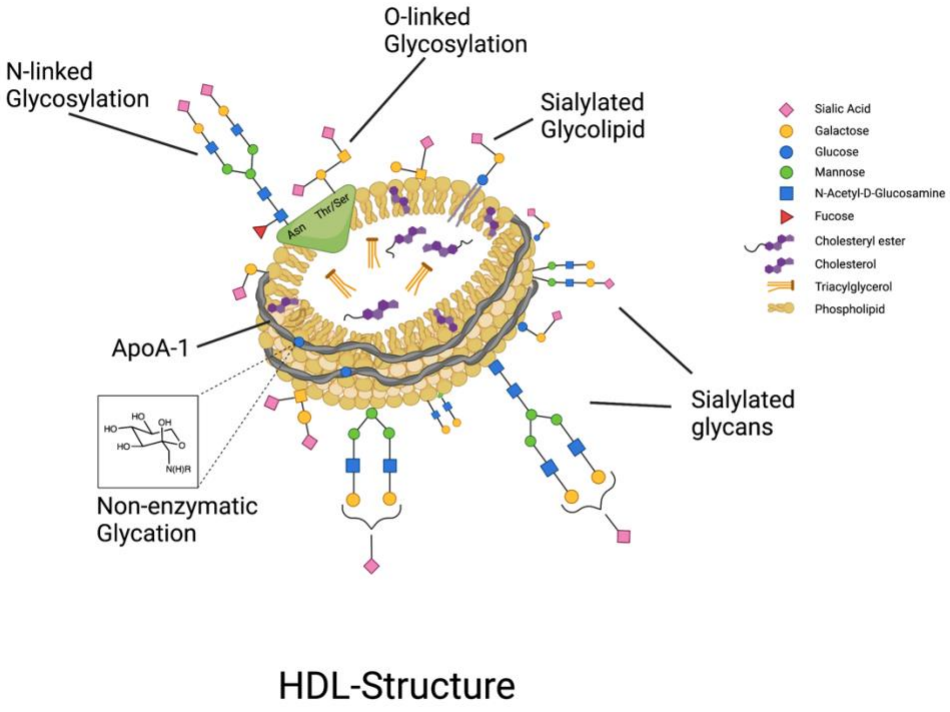

Figure 1: HDL particles are highly glycosylated, containing both glycoproteins that can be N- and O-glycosylated, and glycolipids, with glycan groups added enzymatically through highly regulated cellular processes. In contrast, non-enzymatic glycation of proteins such as ApoA-I can occur under conditions of hyperglycemia.

Apolipoprotein A-I (ApoA-I)

ApoA-I, the major structural, defining HDL apoprotein accounting for around 70% of total HDL protein mass, plays a key role in lipid and cholesterol metabolism and is highly associated with cardioprotection (41). Despite ApoA-I being reported to possibly be glycosylated (42–44) extensive mass spectrometry-based (MS)-based profiling demonstrated that there is no evidence of ApoA-I glycosylation (25). ApoA-I does not have the consensus sequence for N-glycosylation (AsnXxxSer/Thr/Cys, where Xxx can be any amino acid except proline), and whereas O-glycosylation is possible on any serine or threonine residue, detailed analysis of isolated HDL has not yielded any confirmed O-glycosylated peptides on ApoA-I. It is possible that ApoA-I O-

glycosylation can occur in certain conditions or disease states, however MS-based analysis has never confirmed the existence of this to date. ApoA-I can, however, be non-enzymatically or chemically glycated which has been found to likely be deleterious for HDL functionality as seen in diabetic and nephropathic patients (43).

Table 1: Glycosylation status of HDL-associated proteins with confirmed glycosylation sites.

Protein	N-glycans	O-glycans	Sites of Attachment
Alpha-1-antichymotrypsin (AACT)	8	0	Asn106, Asn127, Asn271
Alpha-1-antitrypsin (A1AT)	18	0	Asn70, Asn107, Asn271
Alpha-1B-glycoprotein (A1BG)	1	0	Asn179
Alpha-2-HS-glycoprotein (FETUA or A2HSG)	17	2	Asn156, Asn176, Thr346
Apolipoprotein A-II (APOA2)	0	4	Ser35, Ser88, Thr95
Apolipoprotein C-III (APOC3)	0	21	Thr94
Apolipoprotein D (APOD)	28	0	Asn65, Asn98
Apolipoprotein E (APOE)	0	40	Ser215, Thr307/Ser308*, Ser76/Thr83*, Ser129/Thr130*, Thr194, Ser197, Ser263, Thr289/Ser290* Ser296
Apolipoprotein F (APOF)	0	3	Ser269, Thr273/Thr27*
Apolipoprotein M (APOM)	9	0	Asn135
Clusterin (CLUS or APOJ)	10	0	Asn86, Asn291, Asn374
Complement C1s subcomponent (C1S)	2	0	Asn174
Complement C3 (C3)	4	0	Asn85
Hemopexin (HPX)	6	0	Asn187, Asn453, Asn240/Asn246*
Heparin cofactor 2 (HCF2)	2	0	Asn49
Kininogen-1 (KNG1)	4	0	Asn169, Asn205
Lecithin-cholesterol acyltransferase (LCAT)	1	0	Asn108
Serum amyloid A-4 (SAA4)	7	0	Asn94
Serum paraoxonase/arylesterase 1 (PON1)	8	0	Asn253, Asn324

Proteins included in this table include only those with glycosylation sites confirmed to actually

express glycans at those sites by mass spectrometry analysis of isolated HDL fractions from a starting volume of 500uL of plasma, as described in (25,87). HDL-associated proteins that have been reported to be glycosylated previously, and/or have putative sites but that either could not be confirmed by mass spectrometry or are present at low abundance such that they fall under the limit of detection, are not reported in this table.
*For these sites, the site of attachment could not be disambiguated thus both possible attachment sites are reported.

Apolipoprotein A-II (ApoA-II)

ApoA-II is the second most abundant HDL apoprotein, representing as much as 20% of total protein mass (41), and has been shown to have important implications for CV health though results were historically inconsistent and controversial. For instance, one early study showed that low serum ApoA-II was a marker of atheroprotection in patients with non-insulin-dependent-diabetes mellitus (45) but conversely another study showed that elevated levels of ApoA-II were proatherogenic (46). However, more recently a large prospective study (n=912) showed that ApoA-II was indeed inversely associated with future risk for coronary artery disease (CAD) and was exerting antiatherogenic properties (47). ApoA-II binds to phospholipid transfer protein (PLTP) on HDL (48), suggesting that it plays an important role in the remodeling of HDL particles. ApoA-II contributes to structural properties of HDL (49) and its presence on HDL enhances ATP-binding cassette transporter-1 (ABCA-1)-mediated efflux, suggesting that ApoA-II can contribute to structural changes in ApoA-I, and improve functionality of the HDL particle (50). Like ApoA-I, ApoA-II does not contain the consensus sequence for N-linked glycosylation, however it has been shown to be O-glycosylated (25,51). The glycosylation of ApoA-II contributes to its association affinities since sialylated ApoA-II preferentially associates with smaller HDL whereas non-sialylated ApoA-II associates with all sizes of HDL (52). In a recent study in patients who were equally at risk for CAD based on traditional biomarkers and who were then diagnosed as either having CAD or not using diagnostic coronary arteriography,

ApoA-II was significantly lower in CAD patients compared to patients without CAD (7). In children given a lipid rich dietary supplementation there was no difference in ApoA-II glycosylation between groups, but the analysis did confirm that ApoA-II indeed had multiple glycoforms (25). It is currently unknown what the role of glycosylation in ApoA-II function is, and whether the extent of sialylation drives the binding of ApoA-II to smaller HDL particles or whether higher sialylation is reflective of a particular pathway of metabolism that is linked with the production of small particles.

Apolipoprotein C-III (ApoC-III)

ApoC-III is a critical metabolic protein whose glycosylation status has long been known to be an important determinant of its function. ApoC-III is a small (8 kDa) O-glycosylated apoprotein whose glycans can be capped with 0, 1, or 2 sialic acids and thus is often denoted as, ApoC-III0, ApoC-III1, and ApoC-III2 accordingly. Because of the negative charge conferred by the sialic acids the ApoC-III glycoforms have differential migration on gel (18), which enabled the study of its glycosylation much earlier than more advanced MS-based tools became available. ApoC-III is synthesized in the liver and intestine and found on very-low-density-lipoproteins (VLDL), chylomicrons, LDL and HDL and is a multifunctional protein whose primary functions are to hinder apolipoprotein E (ApoE) mediated hepatic uptake of lipoproteins, and to inhibit lipoprotein lipase, a key enzyme that catalyzes the hydrolysis of triacylglycerols from lipoproteins to free fatty acids and monoacylglycerol fragments (53). ApoC-III has gained considerable attention due to its relationship with CV health and the strong correlation with ApoC-III overexpression and CVD due to its involvement in hypertriglyceridemia (54,55).

Though the association between elevated ApoC-III concentration and CVD has been established for some time, the focus has been primarily on the role of ApoC-III in VLDL metabolism, however, recently a relationship between ApoC-III and HDL has emerged. For example, CVD patients have increased HDL ApoC-III content (56,57). Changes in sialyation in the more common glycoforms of ApoC-III have been observed in multiple conditions including uremia, obesity, kidney disease, cancers and diabetes (8,58–61). The enzyme from the GalNAc-transferase family Golgi-localized polypeptide N-acetyl-D-galactosamine-transferase 2 isozyme (GALNT2) initiates the first step in the O-glycosylation of ApoC-III, as well as several other lipoprotein-associated targets including ApoE, PLTP, and angiopoietin-like 3 (ANGPTL3) (62). Loss of function of GALNT2 was found to be associated with extremely low HDL concentrations (63), highlighting the importance of O-glycosylation of critical apoproteins and related proteins involved in lipoprotein remodeling in HDL metabolism. Elevated circulating levels of triglycerides (TG) are a risk factor for CVD (64) which is positively correlated with circulating ApoC-III concentrations (65,66). High-throughput mass spectrometric immunoassay found that increased plasma TG levels were associated with higher ratio of ApoC-III1 over ApoC-III2 (67). Importantly, it is already well-known that the sialylation state of ApoC-III associated with LDL particles is responsible for its binding affinity to cell surface receptors, with ApoC-III2 being preferentially cleared by heparan sulfate proteoglycans and conversely ApoC-III1 being more effectively cleared by the LDL receptor and other receptors in the LDL receptor family (68). It is currently unknown whether and how the sialylation state of ApoC-III associated with HDL particles influences the binding of those HDL to cell surface receptors.

The glycosylation of ApoC-III is more complex than was previously thought. In addition to the known glycosite at position Alanine-74 (Ala)-74 (69) and the three possible non-sialylated and

sialylated glycans attached at this site (70), our group identified a total of 20 glycoforms most of which were fucosylated and nearly half were sialylated (15). Interestingly, 13 unique glycoforms of ApoC-III were significantly enriched in HDL particles compared to serum, with the HDL-associated glycoforms being more highly sialylated (15). These findings suggest that either ApoC-III glycosylation state modifies its affinity for a specific lipoprotein class, or that the metabolism of ApoC-III and its exchange between the circulating lipoproteins is reflected in its glycosylation. Research is needed to better understand the mechanisms driving these intriguing findings about the links between ApoC-III glycosylation and its association with HDL vs. the ApoB containing lipoproteins, and the unique role of ApoC-III in HDL particle metabolism.

In a recent study comparing the site-specific glycosylation of ApoC-III in patients across the spectrum from healthy, to those with metabolic syndrome to diabetic patients with chronic kidney disease on hemodialysis, ApoC-III was differentially glycosylated in patients with metabolic syndrome and diabetic hemodialysis compared to controls (37). Patients with chronic kidney disease who were on hemodialysis and patients with metabolic syndrome had HDL that were significantly more enriched in ApoC-III especially in di-sialylated ApoC-III (ApoC-III2) compared to the control group (37). Importantly, HDL ApoC-III glycosylation was able to distinguish between HDL that suppressed vs. increased IL-6 secretion by monocytes stimulated with lipopolysaccharide (LPS), when clinical biomarkers such as total cholesterol, LDL cholesterol, C-reactive protein (CRP), glucose and blood pressure were not discriminatory in this immunomodulatory ability (37). These intriguing preliminary findings suggest that ApoC-III glycosylation may play an important role in directing the immunomodulatory capacity of HDL particles.

Apolipoprotein E (ApoE)

ApoE may well be one of the most influential proteins in lipoprotein biology, and in metabolic health overall. Genome-wide association studies across multiple geographic regions have irrefutably identified APOE, which directs lipoprotein metabolism both peripherally and in the central nervous system, as the single strongest genetic marker of extreme longevity across multiple, multi-ethnic cohorts (69). APOE genotype is a major risk factor for a number of age-related pathologies including CVD and Alzheimer's disease (71,72). ApoE exists in three isoforms, ApoE2, ApoE3, and ApoE4, with ApoE4 conferring increased risk for both CVD and Alzheimer's (73–75). Importantly, it is well known that compared to ApoE3 the ApoE4 isoform has a reduced ability to induce cholesterol efflux (76,77), and has a higher binding affinity for VLDL than HDL particles, altering its metabolic fate (78). Unlike the intracellular fate of ApoB-100, which is largely degraded upon uptake via the LDL receptor, as much as 80% of ApoE internalized as part of VLDL particles is recycled and re-secreted as part of HDL particles (79). This recycling and re-secretion pathway is not exclusive to hepatocytes, and instead has been demonstrated to occur across a wide variety of cell types (79). Importantly, when internalized as part of TG-rich lipoproteins via receptors in the LDL receptor family, ApoE4 is more likely to be retained in the cell than recycled and re-secreted as part of HDL particles compared to ApoE3, resulting in diminished concentrations of ApoE4 in circulation and reduced cholesterol efflux (76). The endocytic vesicles involved in ApoE recycling were identified to contain sialyltransferase enzymes (80), suggesting that addition of sialic acid residues to ApoE glycan structures may be a critical step in directing ApoE from internalized TG-rich particles to re-secreted HDL particles. In support of this hypothesis, it has been found that HDL-associated ApoE is more highly sialylated than VLDL-associated ApoE (81).

ApoE was found to be glycosylated in 1979 (82), with 6 sialylated glycoforms identified (83). ApoE does not contain the consensus amino acid sequence for N-linked glycosylation, and instead is O-glycosylated with mucin-type glycans at the originally characterized site at Threonine194 (Thr194), which is not essential for ApoE secretion (84). More recently, additional glycosylation sites have been identified, including one at Thr212 (85), and 3 additional sites were identified at Serine290 (Ser290), Thr289 and Ser296 in ApoE secreted by macrophages isolated from peripheral blood mononuclear cells of a single donor with ApoE3/E3 genotype (86). It was recently shown that ApoE in fact has two more glycosites, for a total of 7 mucin-type O-glycosylation sites, with glycans ranging from simple GlcNAc to biantennary structures containing sialylation and fucosylation (87). Evidence regarding the importance of ApoE glycosylation in lipoprotein function is starting to emerge, building on the established evidence that ApoE structure impacts the metabolism of lipoproteins (76,88). An aberrantly glycosylated variant of ApoE causes defective binding to the LDL receptor (89). ApoE is highly sialyated when associated with HDL compared to serum, and its sialylation state is involved in mediating ApoE's binding affinity to HDL vs. VLDL (81,90). ApoE glycosylation was shown to be considerably different in cerebral spinal fluid (CSF) than in serum (91) and its extent of sialylation in CSF affects ApoE binding to amyloid beta, thus influencing the development of plaque formation and Alzheimer's disease (92) and suggesting that glycosylation of ApoE may be tissue-specific (91). Importantly, it was recently demonstrated that site-specific glycoprofiles of HDL-associated ApoE are correlated with HDL functional capacity (87), strongly suggesting that ApoE glycosylation is important for HDL function. ApoE isoform-specific glycoprofiling has not yet been performed and will likely be important in distinguishing ApoE genotype-specific effects on disease risk.

Alpha-1 antitrypsin (A1AT)

A1AT is an acute phase protein mainly synthesized by the liver, which acts as a protease inhibitor, and which has been shown to increase dramatically during inflammation and has also been found to persist post infection (93). Recent work showed that statins can also induce A1AT concentrations, and that association of A1AT with HDL protects the protein and enhances its anti-proteolytic activity in the context of the highly oxidative environment of the acute phase response (94). Post translational modifications of A1AT contribute to changes in conformation that may influence its function (95). Differential glycoforms of A1AT have been reported in patients with various types of lung cancers and are used in lung cancer diagnosis (96). Sialylation variations of A1AT have also been observed in patients with COVID-19 (97).

A1AT is N-glycosylated, and its site-specific glycosylation profiles differ when associated with HDL compared to serum (15). A1AT glycosylation is critical for its secretion by monocytes (98), is differential between serum and hepatocytes (99), and has increased fucosylated biantennary glycans in the serum of hepatocellular carcinoma patients (100). The site-specific glycosylation profiles of A1AT were highly differential between diabetic chronic kidney disease patients on hemodialysis compared to patients with metabolic syndrome and healthy controls: kidney disease patients had a higher proportion of monofucosylated to non-fucosylated glycans, and a lower proportion of di-sialylated glycans on A1AT (37). In the same study, HDL particles that attenuated the amount of Interlukin-6 (IL-6) secreted by LPS-stimulated monocytes had higher amounts of A1AT as well as lower amounts of several disialylated glycans across multiple sites, suggesting A1AT and its specific glycoprofile are involved in mediating HDL immunomodulatory function (37). A disialylated A1AT glycopeptide was also positively

correlated with cholesterol efflux capacity in healthy young adults (87), and in young children from Ghana (25). These findings suggest an important connection between HDL A1AT glycosylation, particularly disialylated A1AT glycans, and HDL functionality.

Alpha-2-HS-glycoprotein (A2HSG)

A2HSG is a hepatically derived protein found in plasma and associated with HDL particles (24). Several studies have shown that A2HSG is critically important for CV health (101–104), playing a particularly important role in preventing vascular calcification, and emerging as an independent risk factor of CVD and all-cause mortality (101). A2HSG is differentially glycosylated in patients with chronic pancreatitis and pancreatic cancer (105). Site-specific analysis of HDL-associated A2HSG revealed that it is highly sialylated and decorated with both N- and O-glycans at multiple sites (70). In patients with chronic kidney disease HDL were enriched with non-sialylated A2HSG, and non-sialylated A2HSG was enriched in HDL particles that enhanced IL-6 secretion by LPS-stimulated monocytes (37). Interestingly, A2HSG concentrations were lower in HDL compared to serum but specific glycoforms were significantly more enriched in HDL than in serum (15). Multiple A2HSG glycopeptides were positively correlated with HDL cholesterol efflux capacity and immunomodulatory capacity in healthy adults (87), and in young children in Ghana supplemented with a lipid-based nutrient supplement (25).

Lecithin-cholesterol acyltransferase (LCAT)

LCAT functions as a key enzyme in reverse cholesterol transport and HDL particle maturation by esterifying free cholesterol with a fatty acid from phosphatidylcholine (lecithin), which allows HDL particles to carry a larger cholesterol load as cholesteryl esters (CE) in the core of the particle (106). LCAT is strongly linked with CV health and disease (107). ApoA-I is a potent

activator of LCAT (108). Mutations in the LCAT gene lead to altered function of the enzyme resulting in elevated levels of TG and reduced HDL-C, which can lead to atherosclerotic pathology (109). The glycosylation of LCAT has been known since the 1990s, with both N-linked and O-linked glycoforms identified (110,111), and with important implications for LCAT function (112). The glycosylation of LCAT is critical for its structural stability and function (113). Loss of glycosylation at several sites resulted in loss of function but loss of glycosylation at site 408 increased the activity of the enzyme (114). Desialylation of LCAT by neuraminidase resulted in considerable alteration of LCAT activity, reducing cholesterol esterification and concomitantly reducing the size of HDL (115). Depending on LCAT glycotype LCAT binds preferentially to HDL or ApoB-containing lipoproteins (116). These findings provide strong evidence that LCAT glycosylation is imperative for overall lipoprotein metabolism as well as cholesterol efflux and transport globally, as well as metabolism and efflux capacity of HDL particles in particular.

Cholesterol Ester Transfer Protein (CETP)

CETP is a critical mediator of lipid transfer between HDL and ApoB-containing lipoproteins, which in the context of high TG concentrations, transfers CE from HDL in exchange for TG from ApoB-lipoproteins, thereby enriching HDL particles with TG and altering their metabolism (117,118). Loss of function genetic mutations in CETP and lower concentrations of CETP are associated with lower LDL-C and increased HDL-C, and lower risk of CVD, which has made CETP a major pharmacological target for CVD and atherosclerosis prevention (117,119). CETP is highly sialylated with four N-linked glycoforms (120). A major form of serum CETP lacking glycosylation at Asparagine341 (Asn341) was shown to have markedly increased functionality

compared to other forms (120,121). Defective sialylation of CETP in heavy alcohol drinkers showed a significant reduction in the function of CETP compared to controls (122). Patients with a congenital disorder of glycosylation of the glycosyltransferase enzyme beta-1,4-galactosyltransferase 1 have defectively glycosylated CETP with reduced functionality, and larger HDL than healthy controls (123). CETP is a minor component of HDL, whose function is to temporarily associate with HDL while bridging between the HDL and ApoB particle between which the exchange of lipids occurs, thus it is often missed as an HDL-associated protein depending on the HDL isolation method and sensitivity of the protein detection method (24). However, its importance in lipid metabolism and strong links with CVD make it an important protein whose content and glycosylation when associated with HDL particles is an area of focus for future studies.

Phospholipid transfer protein (PLTP)

The primary function of PLTP is to transfer phospholipids from ApoB containing TG-rich lipoproteins to HDL (124,125). As a key modulator of HDL size, composition, and concentration PLTP has gained considerable attention for its role in the development of CVD (126). PLTP overexpression has been reported to be an independent risk factor for CAD and is associated with type II diabetes and obesity (127). Two forms of PLTP have been described that have high and low phospholipid transfer activity, which may explain the conflicting findings of the association between PLTP and pro- vs. anti-atherogenic effects (128). Higher concentrations of the low-activity PLTP type may be the driver of the pro-atherogenic effects, and PLTP glycosylation may play a critical role in the function and activity of the protein. Human PLTP has 6 N-linked and 2 O-linked glycoforms (124). Multiple earlier studies showed that

tunicamycin treatment disrupts the ability of cells to secrete PTLP, suggesting glycosylation is necessary for synthesis and secretion (129,130). A later study confirmed that inhibition of PLTP N-glycosylation affected its structural stability and markedly reduced its ability to be excreted resulting in the non-glycosylated PLTP being intracellularly degraded (126). Much like CETP, PLTP is a protein that temporarily associates with HDL particles to mediate the exchange of material between HDL and ApoB-containing lipoproteins, thus the ability to detect its presence on HDL depends on the nature of the HDL isolation method. Although PLTP is a minor constituent of HDL particles and thus measuring its glycosylation may be limited without enrichment prior to analysis, its content and glycosylation profile are likely to be important factors in overall HDL metabolism.

Potential for modifying the glycosylation of HDL-associated proteins to confer therapeutic value

Given the growing evidence that HDL glycosylation may be critically involved in both metabolism and function, with implications for both CVD diagnosis and treatment, the potential for HDL-based therapeutics targeting HDL glycosylation is compelling. Strategies to reduce CVD risk and prevent or reverse CVD by increasing the concentration of HDL particles have been largely disappointing. Increasing the number of HDL particles through pharmacological means (e.g. CETP inhibitors, niacin), has met with some success, however the ability to further reduce residual CVD risk following LDL-lowering with statins has been difficult to achieve (131,132). Several additional HDL modifying therapies, including injection with reconstituted HDL particles, ApoA-I, as well as extracorporeal HDL lipid depletion, where HDL particles are removed from plasma, exogenously delipidated, and then reinfused, have similarly met with

modest success despite promising results in animal trials (133,134). Thus, novel therapeutic approaches to increase not just the concentration but also the function of HDL particles remain an important area of research. The potential for dietary and pharmacological strategies to improve HDL function via modulation of HDL glycoprofiles is tantalizing given the growing evidence of the importance of HDL glycosylation in its function. Several recent studies show promising results for the modification of HDL glycosylation through diet. Whereas the glycosylation of HDL-associated ApoE was not affected by a short-term intervention with Mediterranean vs. fast food diet, the glycosylation of HDL-associated ApoC-III was significantly altered in just 4 days (87). Specifically, disialylated ApoC-III (ApoC-III2) was increased after the Mediterranean diet whereas nonsialylated ApoC-III (ApoC-III0) was increased after 4 days of consuming a diet enriched in saturated fat and simple sugars and depleted in fiber (87). These alterations were associated with HDL cholesterol efflux capacity as well as immunomodulatory capacity (ability to suppress cytokine secretion in stimulated monocytes) (87). In young children in Ghana supplemented with a lipid nutrient supplement, HDL glycopeptides that were altered by the supplement were correlated with HDL cholesterol efflux capacity (25). There is also evidence that targeting GALNT2 activity may be a viable strategy to alter the glycosylation of HDL-associated proteins and thus increase HDL concentration and function (63,135). While this research area is very new, early tantalizing evidence provides support for the idea that the alteration of HDL glycoprofiles via dietary or pharmacological interventions may be a viable strategy for improving the functional capacity of HDL particles and thus improving CV outcomes.

Conclusions

While the study of HDL glycosylation is still in a nascent state, emerging evidence suggests that differential glycoprofiles of HDL-associated proteins may be diagnostic and may reveal new mechanisms in lipoprotein-mediated aspects of CVD. In order to uncover glycan-based disease biomarkers newly developed glycan analytical methods need to be applied to large, comprehensively characterized, and preferably genotyped cohorts with known CV outcomes. Basic cell and molecular biology studies are also needed to better understand how glycosylation affects HDL metabolism and function, so that the potential for modifying the glycosylation of HDL-associated proteins through intervention to confer therapeutic value can be realized. In the last 10 years there has been progress toward developing the fundamental methodologies for both the isolation of HDL from plasma and the analysis of HDL glycosylation especially using MS. This field is now ripe for major discoveries utilizing these tools in the areas of glycan-based HDL CVD biomarkers, novel CVD disease mechanisms, and ultimately, novel HDL-based therapeutics for cardioprotection.

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Chapter 2: Elevated Lipopolysaccharide Binding Protein in Alzheimer's Disease Patients with APOE3/E3 But Not APOE3/E4 Genotype

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Abstract

The role of lipopolysaccharide binding protein (LBP), an inflammation marker of bacterial translocation from the gastrointestinal tract, in Alzheimer's disease (AD) is not clearly understood. In this study the concentrations of LBP were measured in n=80 individuals: 20 apolipoprotein E (APOE)3E3 carriers with and 20 without AD dementia, and 20 APOE3E4 carriers with and 20 without AD dementia. LBP was found to be enriched in the 1.21–1.25 g/mL density fraction of plasma, which has previously been shown to be enriched in intestinally derived high-density lipoproteins (HDL). LBP concentrations were measured by ELISA. LBP was significantly increased within the 1.21–1.25 g/mL density fraction of plasma in APOE3E3 AD patients compared to controls, but not APOE3E4 patients. LBP was positively correlated with Clinical Dementia Rating (CDR) and exhibited an inverse relationship with Verbal Memory Score (VMS). These results underscore the potential contribution of gut permeability to bacterial toxins, measured as LBP, as an inflammatory mediator in the development of AD, particularly in individuals with the APOE3E3 genotype, who are genetically at 4-12-fold lower risk of AD than individuals who express APOE4.

Keywords: Alzheimer's disease, ApoE3E3 genotype, ApoE3E4 genotype, lipopolysaccharide binding protein, gut permeability

Introduction

Alzheimer's Disease (AD) is a devastating neurodegenerative disease affecting >5 million Americans, and growing in prevalence (1). There is currently no cure for AD, and late-stage treatments have proven ineffective at reversing the disease (2). In addition to the well-established mechanism of neurodegeneration linked with the accumulation of amyloid beta and phosphorylated tau in senile plaques in AD (3), impaired gut barrier function, which is linked with higher circulating concentrations of immunogenic endotoxins, has also been linked with AD (4–6). AD patients have been shown to have significantly higher concentrations of lipopolysaccharide (LPS) in plasma than age-matched controls, and inflammation caused by LPS has long been thought to play a major role in the onset and progression of AD (5,6). Apolipoprotein E (APOE) genotype is the single strongest genetic risk factor for AD, with the APOE4 allele increasing risk by 4-12-fold compared to APOE3 (7). While various functionalities related to the APOE4 protein have been implicated in AD pathophysiology (8–10) the etiology of AD in individuals who are not carriers of APOE4 is unclear. The hypothesis of endotoxin-mediated neurodegeneration suggests that endotoxin plays a major role in AD pathology by inducing systemic inflammation, degrading the blood brain barrier, and driving amyloid beta production and aggregation and TAU hyperphosphorylation, as well as activating brain microglia (6).

LPS is a potent immunogenic activator of the innate immune system, entering circulation from the intestine, mouth, or skin wounds, and is shuttled by LPS binding protein (LBP) to innate immune cell surface receptors CD14 and TLR4, eliciting inflammatory signaling and a strong immune response (11). Perturbation of intestinal mucosal homeostasis allows immunogenic

endotoxins like LPS to translocate across the gut barrier due to dysregulation of junctional complexes (12,13). Translocation of immunogenic endotoxins across a compromised intestinal barrier can trigger a chronic inflammatory response throughout the body that has been linked to an increased risk of various chronic and acute diseases including atherosclerotic cardiovascular disease, dementia, cancer, and sepsis (6,14–16). Interestingly, LBP has more recently been shown to be a powerful independent predictor of AD risk (17,18). LBP has also been shown to reverse the amyloid state of fibrin in people with type 2 diabetes and AD (19,20).

High-density lipoproteins (HDL) are heterogenous nanoparticles most commonly known for their role in cholesterol homeostasis but they also play a critical role in modulating TLR4-based inflammatory responses along with LBP by trafficking, neutralizing and clearing LPS (21,22). Recent findings indicate that HDL synthesized in the intestinal tract bind LBP to regulate LPS - mediated activation of liver inflammation (23). HDL-LBP-LPS complexes entering the fenestrated capillary and portal vein seclude LPS from hepatic TLR4+ macrophages, preventing excessive inflammation in a putative protective mechanism (23).

Intestinally derived HDL make up roughly 30% of the circulating HDL pool. (24) However, the function and composition of these intestinally derived particles has remained largely uncharacterized due to the difficulty in isolating them as a separate subclass from human plasma. However, recent evidence suggests that intestinally derived HDL are not only specialized but unique in their physicochemical characteristics. Using an in-situ perfusion model of mouse intestine, Yamaguchi et al demonstrated that intestinally derived HDL are smaller and denser than liver-derived HDL (23,25), and Andraski et al recently also demonstrated that in humans intestinally-derived HDL are smaller and denser using a stable isotope approach (26). We thus

hypothesized that LBP would be enriched in the 1.21–1.25 g/ml fraction containing dense HDL. We further hypothesized that LBP concentrations would be elevated in AD patients compared to non-demented controls, and that LBP concentrations would be associated with HDL function, APOE genotype and cognitive function.

Materials and Methods

Samples and Subjects

The detailed study design and the characteristics of the participants involved in this AD clinical trial have been described previously (7). Briefly, the study involved obtaining plasma samples from 194 participants who were part of the University of California, Davis Alzheimer's Disease Research Center biorepository. The participants were categorized into three groups including non-demented controls, patients with mild cognitive impairment (MCI), and patients with Alzheimer's disease dementia (AD). All participants had their APOE genotype determined. For this study a subset of n=80 samples out of the 194 were analyzed. The subset was selected randomly based on the following criteria: select n=20 participants with APOE3E3 genotype with AD dementia and n=20 without dementia, and n=20 participants with APOE3E4 genotype with AD dementia and n=20 without dementia such that the final groups each have equal numbers of male and female participants, and are as close as possible to equal average age. Cognitive assessments of the study participants were available and included the Spanish English Neuropsychological Assessment Scales (SENAS), the Clinical Dementia Rating (CDR) scale, and white matter hyperintensities identified through magnetic resonance imaging (7)

HDL Isolation

HDL particles were isolated from plasma through a two-step ultracentrifugation process, followed by size exclusion chromatography, as described previously (7,27). Briefly, a 500 µL aliquot of platelet-free plasma was subjected to ultracentrifugation using a Beckman Optima MAX-TL ultracentrifuge with a fixed angle rotor TLA-110 at 110,000 RPM for 0.5 hours using an Optiseal tube and a density cushion of 1.006 g/mL potassium bromide (KBr). This step isolated triglyceride-rich lipoproteins (TRLP, chylomicrons and very low-density lipoprotein (VLDL) particles) lighter than 1.006 g/mL. For the second ultracentrifugation at 110,000 RPM for 3.5 hours with a 1.210 g/mL KBr cushion, low density lipoprotein (LDL)/HDL particles denser than 1.006 g/mL but lighter than 1.210 g/mL were collected. The particles of density range 1.006 – 1.21 g/ml were then injected into a size exclusion chromatography (SEC) column to separate HDL, LDL, and albumin by size. Each fraction underwent buffer exchange using a 3 kDa molecular weight cut-off filter to remove the salt solution.

The remaining plasma protein fraction containing all plasma components > 1.21 g/mL in density was then further subjected to an additional ultracentrifugation step to obtain HDL particles within the density range of 1.21–1.25 g/mL. A solution was prepared by mixing 1200 µl of d=1.34 g/mL KBr and HPLC grade water with 2700 µl of the >1.21 g/mL plasma protein fraction, yielding a total volume of 3900 µl of d=1.25 g/mL solution. Using 4.7ml optiseal tubes, 800 µl of the prepared d=1.25 g/mL KBr solution was underlayed with 3900 µl of the d=1.25 g/mL density plasma protein solution and topped off with d=1.25 g/mL KBr solution. The samples were ultracentrifuged at 110,000 rpm for 6 hours using a Beckman Optima MAX-TL ultracentrifuge with a fixed angle rotor TLA-110. After ultracentrifugation, the top 2ml of the Optiseal tube, representing the d= 1.21–1.25 g/mL fraction was carefully collected. The fraction

was then concentrated and buffer exchanged to HPLC grade water by centrifugation at 4500 rpm for 7 minutes using an Amicon ultrafiltration unit. To ensure uniformity, HPLC grade water was added so that all samples had a final volume of 250 µl, which was then transferred to a storage tube and stored at -80C for subsequent analysis.

Analysis of LBP in Fractions

For the determination of LBP concentrations across all plasma fractions, all of the fractions obtained during the ultracentrifugation/SEC process were collected including the TRLP, LDL, intermediate density lipoprotein (IDL), large (HDL-L), medium (HDL-M), and small (HDL-S) HDL particles at 1.21 g/mL density, albumin (ALB), as well as the dense HDL (d=1.21–1.25 g/mL).

An initial assay was conducted on fractions TRLP, LDL, HDL-L, HDL-M, HDL-S, ALB, and 1.25 g/ml density fraction to determine where LBP was enriched. Given the variability in individual protein concentrations among the samples, the total protein concentration in each sample was optimized to reach concentrations that would fall into the quantifiable range for the Human LBP assay (Abcam LBP assay kit (ab213805). The optimal protein concentration range for the assay was determined to be that found in a 1:800 dilution of plasma. The microBCA (micro bicinchoninic acid) protein assay using the Thermo Scientific Micro BCA™ Protein Assay Kit (Catalog number: 23235) was used to determine protein concentrations in each fraction. Based on the protein concentration measurements each fraction/sample was diluted to obtain a protein concentration in range of the assay. The samples were then loaded into a 96-well plate, including the internal standards, and the assay was performed using the manufacturer's protocol. For the further analysis of the 1.21–1.25 g/mL fraction from all n=80 participants, an

additional quality control (QC) plasma sample was included on each plate to adjust for inter-plate variability.

Statistical Analysis

Statistical analysis were performed using the R programming language, version 4.2.2 (28). To compute the LBP index, LBP measurements were normalized, taking into account the QC values present on each respective plate. Normality of the data was examined through the Shapiro-Wilk test, while the Levene test was employed to ascertain the equality of variances among the groups. Because the LBP index did not meet the conditions for normality and variance homogeneity, the data were described in terms of median values, accompanied by the 25th and 75th percentiles for distribution.

Data visualization, including the creation of figures, was facilitated using the ggstatsplot package (29). In the context of the binary logistic regression model, several covariates were incorporated into the analysis, including age, sex, body mass index (BMI), documented history of diabetes, recorded history of hypercholesterolemia, and the APOE genotype. Covariates were included in the regression model if their P-value fell below the 0.1 threshold when considering participant characteristics within the combined group. Both unadjusted and adjusted P-values were subsequently reported.

To explore possible relationships between cognitive scores (verbal memory, executive function, spatial, semantic memory and clinical dementia rating scale) and the LBP index, partial

correlation analyses were conducted, adjusting for the influence of the APOE genotype. For the purposes of determining statistical significance, a threshold of $p < 0.05$ was applied.

Results

Demographic and Clinical Characteristics

Analysis of demographic and clinical characteristics revealed that controls were significantly younger compared to AD participants in the combined group (75.0 (69.0, 79.2) vs. 80.0 (76.5, 84.5) years, $P < 0.001$, **Table 1**). The same pattern was observed within the APOE3E3 subgroup. Notably, the controls had a significantly higher incidence of history of diabetes (37.5% vs 7.7%, $P = 0.002$) in the combined group compared to AD. This was also the case in the APOE3E3 subgroup (40.0% vs 0%, $p = 0.002$). The history of hypercholesterolemia was significantly higher in controls compared to AD (80.0% vs 56.4%, $p = 0.024$) in the combined group. There was no significant age difference within the APOE3E4 subgroup, and no significant differences were found in sex proportion, ethnicity, BMI, or history of hypertension in both the combined group and when stratified by diagnosis ($P > 0.05$).

Table 1. Participant characteristics

		Control	AD	p value
n, combined		40	39	n/a
	APOE3E3	20	20	n/a
	APOE3E4	20	19	n/a
Age, years, median (25^{th}, 75^{th})		75.0 (69.0, 79.2)	80.0 (76.5, 84.5)	**< 0.001**
	APOE3E3	75.0 (68.0, 78.0)	82.0 (78.8, 87.2)	**< 0.001**
	APOE3E4	75.5 (70.8, 82.0)	78.0 (75.0, 81.5)	0.216
Sex proportion, (male/female), combined		20:20	20:19	0.910
	APOE3E3	10:10	10:10	1.000
	APOE3E4	10:10	10:9	0.871
Ethnicity (African American/Asian/Hispanic/White), combined		10/0/10/20	6/1/9/23	0.356
	APOE3E3	2/0/7/11	3/1/5/11	0.809
	APOE3E4	8/0/3/9	3/0/4/12	0.153
BMI, kg/m^2, median (25^{th}, 75^{th})		28.6 (24.1, 31.2)	26.1 (23.4, 29.5)	0.093
	APOE3E3	28.2 (25.1, 30.1)	25.1 (22.9, 29.2)	0.181
	APOE3E4	29.6 (23.7, 32.0)	26.8 (23.4, 29.7)	0.339
History of Diabetes, %, combined		37.5%	7.7%	**0.002**
	APOE3E3	40.0%	0.0%	**0.002**
	APOE3E4	35.0%	15.8%	0.170
History of Hypertension, %, combined		77.5%	76.9%	0.951
	APOE3E3	75.0%	80.0%	0.705
	APOE3E4	80.0%	73.7%	0.640
History of Hypercholesterolemia, %, combined		80.0%	56.4%	**0.024**
	APOE3E3	70.0%	40.0%	0.057
	APOE3E4	90.0%	73.7%	0.184

Numeric variables were analyzed by using a two-tailed Wilcoxon Rank Sum Test and categorical variables were analyzed by chi-squared test.

LBP concentration across plasma fractions

ELISA was conducted on all isolated plasma fractions and the concentration of LBP in each plasma fraction was determined in pg/ug protein. LBP was enriched in the 1.21–1.25 g/mL fraction which was collected after a third ultracentrifugation at a density cutoff of 1.25 g/mL after removal of all d<1.21 g/mL lipoproteins (**Figure 1**). LBP was also detected in the d<1.21 g/mL large HDL fraction and LDL, however at 4–20-fold lower concentrations, respectively.

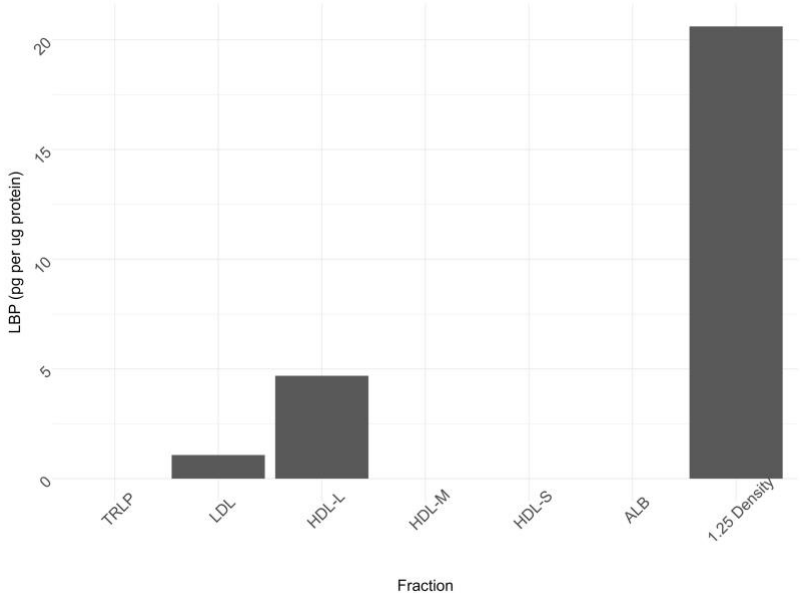

Figure 1: LBP as pg/ug protein. TRLP, triglyceride-rich lipoprotein; LDL, low density lipoprotein; HDL-L, large high-density lipoprotein; HDL-M, medium-high density lipoprotein; HDL-S, small high-density lipoprotein; Alb, albumin; 1.25 Density, fraction at d=1.21-1.25 g/mL.

LBP in AD patients vs. controls

In the unadjusted logistic regression model, a statistically significant positive association was observed between the LBP index and AD diagnosis. Specifically, for each incremental unit

increase in the LBP index, there was a significant increase in the likelihood of being categorized into the AD group (β = 0.57, p = 0.018) (**Figure 2, Table 2**). A significant difference in the median LBP index values was also noted between the control group (0.96, 25th-75th percentile: [0.45, 1.53]) and the AD group (1.57, 25th-75th percentile: [0.65, 2.28]), indicating an elevation of the LBP index in the AD group. However, after adjusting for age, BMI, APOE genotype, history of diabetes, history of hypercholesterolemia, and history of hypertension, the statistical significance of the LBP index diminished (β = 0.39, p = 0.147) (**Figure 2, Table 2**).

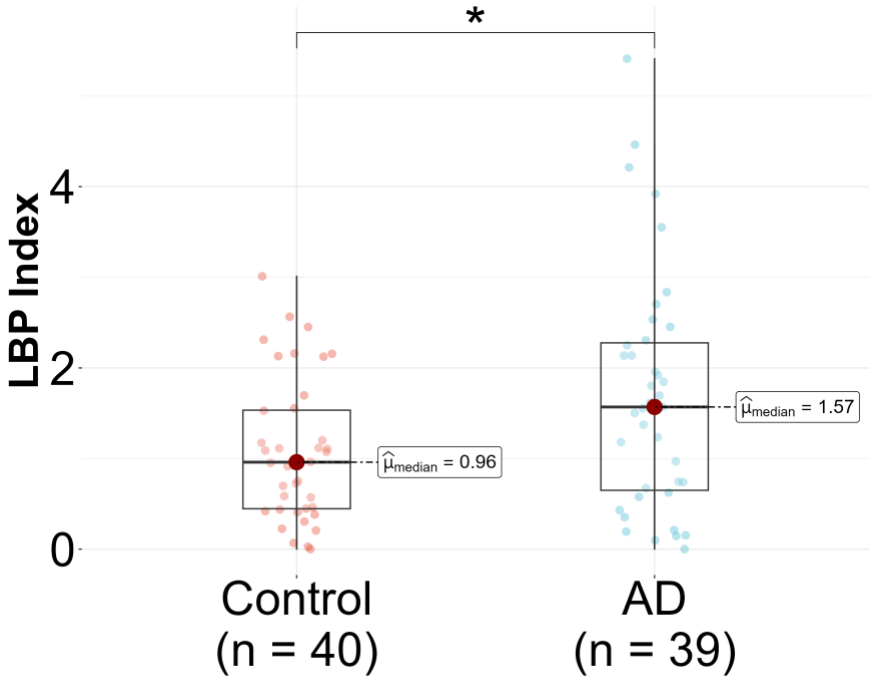

Figure 2: Lipopolysaccharide binding protein (LBP) in Alzheimer's disease (AD) patients compared to controls. LBP was measured in n=79 participants, 39 AD and 40 controls. Analysis was done in R utilizing logistic regression model.

Upon stratification by APOE genotype, a significant association between the LBP index and AD diagnosis was observed in the APOE3E3 subgroup. The median LBP index values for the control

and AD groups were 0.82 (25th-75th percentile: [0.43, 1.11]) and 1.83 (25th-75th percentile: [1.17, 2.58]), respectively (**Figure 3A, Table 2**). The unadjusted model exhibited a significant association (β = 1.3, 95% CI: [0.47, 2.41], p = 0.008), and this association remained significant after adjusting for covariates (p = 0.049) (**Table 2**). In the APOE3E4 subgroup no significant associations were found in either the unadjusted (p = 0.815) or adjusted models (p = 0.561) (**Figure 3B, Table 2**).

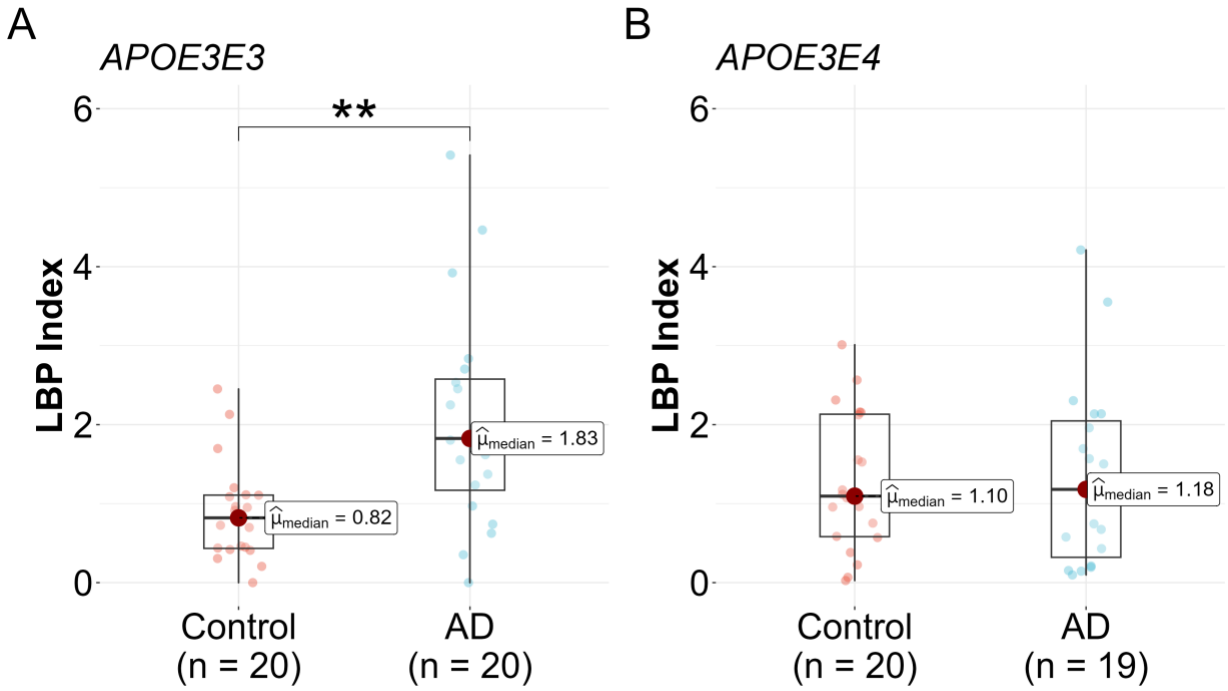

Figure 3: Lipopolysaccharide binding protein (LBP) in Alzheimer's disease (AD) patients compared to controls stratified by genotype. LBP were measured in n=79 participants, ApoE3E3 n=20 control and n=20 AD, and ApoE3E4 n=20 control and n=19 AD. Analysis was done in R utilizing logistic regression model.

Table 2. LBP Index in controls and Alzheimer's disease dementia patients

	Result by study group		Unadjusted Model		Adjusted model	
Outcome	**Control (*N* = 40)**	**AD (*N* = 39)**	***B*(95% CI)**	***P* value**	***B*(95% CI)**	***P* value**
LBP index [ab]	0.96 [0.45, 1.53]	1.57 [0.65, 2.28]	0.57(0.13, 1.08)	**0.018**	0.39(-0.11, 0.96)	0.147
APOE3E3+ APOE3E4						
APOE3E3[c]	0.82 [0.43, 1.11]	1.83 [1.17, 2.58]	1.3(0.47, 2.41)	**0.008**	2.69(0.56, 6.19)	**0.049**
APOE3E4[c]	1.10 [0.58, 2.13]	1.18 [0.32, 2.05]	0.07(-0.56, 0.72	0.815	-0.22(-1.00,0.50)	0.561

Abbreviations: AD = Alzheimer's disease dementia; *APOE* = apolipoprotein E.

[a]Values are represented as medians [25th, 75th].
[b]The adjusted model (logistic regression model) includes "group" (control and AD) as predictors and age, body mass index (BMI), history of diabetes, history of hypercholesterolemia, and *APOE* genotype as covariates.
[c]The adjusted model (logistic regression model) includes "group" (control and AD) as predictors and age, BMI, history of diabetes, and history of hypercholesterolemia as covariates.

LBP Index Correlations with Cognitive Measures

An examination of the associations between the LBP index and various cognitive, functional, and imaging scores, adjusted for APOE genotype, revealed significant inverse correlations between the LBP index and verbal memory score (VMS) (R = -0.42, 95% CI (-0.60, -0.20), p < 0.001, **Figure 4A**). Furthermore, the LBP index showed a positive correlation with the Clinical Dementia Rating (CDR) sum of boxes (R = 0.37, 95% CI (0.14, 0.57), p = 0.002, **Figure 4B**). These associations persisted after adjusting for the APOE genotype, with adjusted correlations remaining statistically significant for both VMS and the CDR sum of boxes. No significant association was observed between the LBP index and white matter hyperintensities volume.

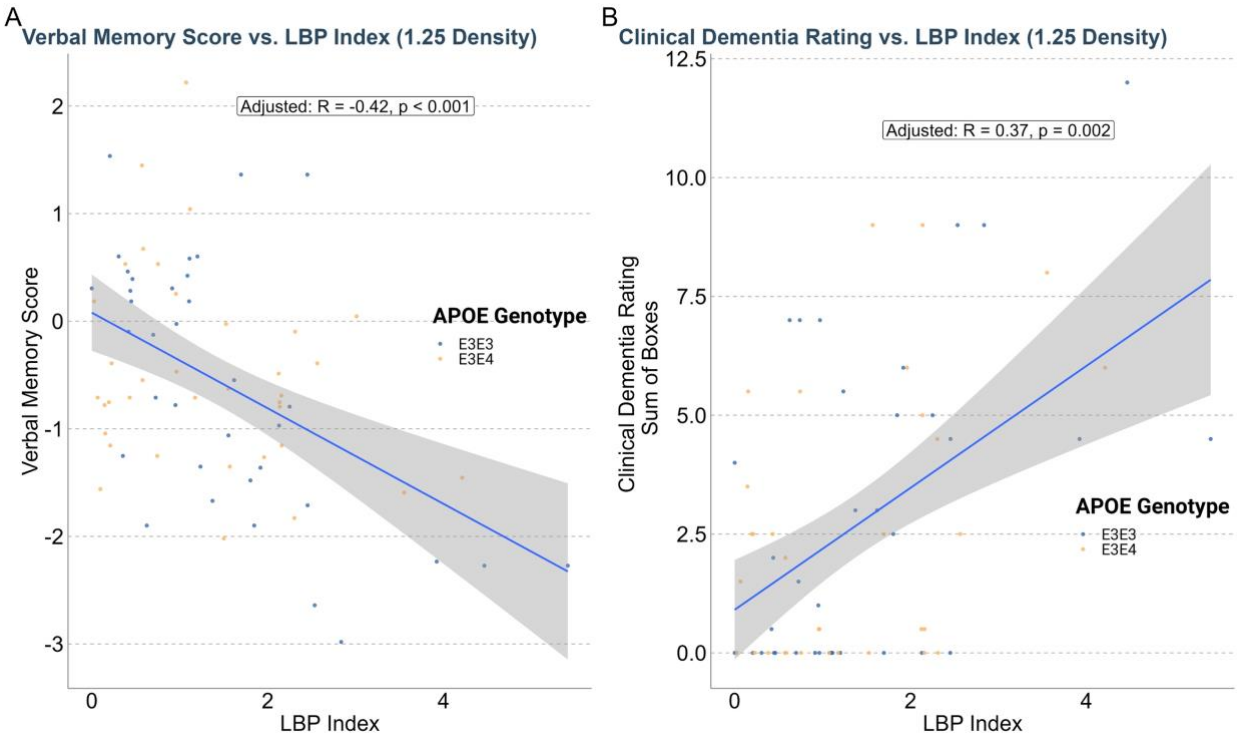

Figure 4: A) Verbal Memory Score vs LBP index. Correlation analysis were conducted and stratified by ApoE genotype. B) Clinical Dementia Rating vs LBP index. Correlation analyses were conducted and stratified by ApoE genotype

Discussion

In this study, we investigated the concentrations of LBP in a subset of an AD cohort including 20 APOE3E3 individuals with and 20 without AD dementia as well as 20 APOE3E4 individuals with and 20 without AD dementia. We specifically focused on the concentrations of LBP in the fraction of plasma at a density range of 1.21-1.25 g/mL, since LBP was found to be enriched in the dense HDL fractions which are also likely to be enriched in HDL of intestinal origin according to previously published papers (23,25). The key finding was that LBP was significantly elevated in APOE3E3 AD patients compared to APOE3E3 controls, even after adjusting for covariates. This elevation in LBP was not observed in APOE3E4 individuals. The

APOE4 allele is a well-established genetic risk factor for AD, whereas APOE3 is considered a neutral genotype (30). The fact that LBP is increased specifically in APOE3E3 AD patients points to intestinally derived inflammation as a potential contributor to disease development in those without the APOE4-mediated genetic predisposition.

LBP is an important marker for gut permeability as it correlates strongly with lactulose/mannitol ratio, the gold standard measure of gut permeability, making it a powerful tool for assessment (31). Although LBP is an indirect marker of LPS, which is cleared by the system rapidly, it remains detectable for up to 24 hours after exposure to LPS making it a robust marker of endotoxins and is used in research as an important, useful and specific marker of intestinal permeability and systemic inflammation (32)

Higher circulating LBP indicates increased endotoxin exposure, which can initiate neuroinflammatory processes implicated in AD (33). AD patients have been shown to have elevated LPS compared to controls and lateral sclerosis patients (5). It is unclear how endotoxins contribute to AD in humans but it is hypothesized that LPS degrades the blood brain barrier, allowing for translocation of endotoxins into the brain promoting the characteristic amyloid beta aggregation and tau tangles (5,6,20,34). In AD animal models injected peripheral LPS results in neuroinflammation, neuron and memory loss, amyloid beta aggregation and tau hyperphosphorylation.(35–39). Our study complements previous research showing elevated plasma LPS levels in AD patients (40,41). The study by Zhang et al (2009) shows significantly elevated LPS in plasma of patients with sporadic amyotrophic lateral sclerosis and AD patients compared to controls providing additional context to our observations regarding LBP's role in

inflammatory process and neurodegeneration.(5) Zhan et al (2016) demonstrated an increase in LPS within brain parenchyma and vessels of AD patients compared to controls and a colocalization of LPS with amyloid-beta plaques and around vessels which reinforces the potential link between gram-negative bacterial components and AD (40).

We also demonstrated correlations between LBP and clinical disease severity based on cognitive assessments. LBP concentrations exhibited a strong inverse relationship with VMS and a positive association with CDR. This aligns with prior studies correlating inflammatory biomarkers with rate of cognitive decline (42). The cognitive correlations lend further support to the relevance of LBP in AD pathogenesis. Taken together, these findings suggest endotoxin-induced inflammation could play a significant role in AD onset and progression.

Future studies are needed to measure LBP in larger cohorts of AD patients and controls, as well as individuals across the spectrum of AD progression and across age and APOE genotypes. Future studies are also needed to understand the factors contributing to increased LBP in the context of AD, for example the relationships with gut microbiome composition, particularly the presence of beneficial gut microbes known to be involved in supporting gut barrier function, diet, specifically the intake of fiber, and conversely factors that could be contributing to the loss of gut barrier function.

Conclusion

In summary, our key finding was a significant elevation in LBP concentration within the d=1.21-1.25 g/mL plasma fraction in APOE3E3 but not APOE3E4 AD patients compared to controls. This suggests circulating endotoxins and inflammation may play important roles in AD development among genetically low-risk individuals. The strong correlations between LBP and both VMS and CDR also indicate clinical relevance. These results shed light on a possible mechanism of inflammation that may be driving AD development in APOE3 carriers who are otherwise protected from AD compared to their APOE4 counterparts. Our study underscores the need to explore gene-environment interactions and consider novel systemic factors like LBP when investigating AD etiology, progression, and potential prevention strategies.

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Chapter 3: Effects of a Low Dose Prebiotic Fiber Supplement in Young, Healthy Adults Consuming Low-Fiber Diets on Lipopolysaccharide Binding Protein Concentrations

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Abstract

Although the beneficial effects of fiber supplementation on overall health and the gut microbiome are well-known, it is not clear whether a low-dose fiber supplement can also alter the concentrations of lipopolysaccharide (LPS) binding protein (LBP), a marker of intestinal permeability. We conducted a secondary analysis of a previously conducted study in which 20 healthy, young participants consuming a low-fiber diet at baseline were administered a daily dose of 12g of prebiotic fiber compared with a placebo over a period of four weeks in a randomized-order, placebo controlled, double-blinded, cross-over study. Changes in LBP concentrations and lecithin-cholesterol acyltransferase (LCAT) activity were measured, and correlations with clinical parameters, gut microbiome composition and function, and plasma metabolite profiles were explored. Fiber supplementation did not significantly alter LBP concentration or LCAT activity in the overall cohort. However, in a subgroup of individuals with elevated baseline LBP concentrations, fiber supplementation significantly reduced LBP from 9.27±3.52 to 7.02±2.32 ug/mL ($p=0.020$). Exploratory analysis found positive correlations between microbial genes involved in LPS synthesis and conversely negative correlations with genes involved in antibiotic synthesis and LBP. Positive correlations between LBP and multiple sulfated molecules including sulfated bile acids and perfluorooctanesulfate (PFOS), and ibuprofen metabolites were also found. Negative correlations between LCAT activity and several bacterial genes, for example, those involved in conferring antibiotic resistance were also found. These findings highlight multiple environmental and lifestyle factors such as exposure to industrial chemicals and medication intake, in addition to diet, which influence the association between the gut microbiome and gut barrier function.

Keywords: Lipopolysaccharide binding protein; prebiotic; PFOS; HDL; leaky gut

Introduction

Extensive evidence suggests that dietary fiber supplementation provides numerous health benefits. However, the majority of Americans consume less than half of the recommended amount of dietary fiber, with less than 5% meeting the recommended intake (1). Dietary fiber has been found to nourish and regulate microbial communities within the intestinal tract, significantly reducing the risk of all-cause mortality and disease (2–4). Beneficial gut microbes ferment fiber and play a vital role in promoting a healthy gut barrier by displacing pathogens and directly influencing mucosal homeostasis by synthesizing an array of metabolites that stimulate mucus production by goblet cells and regulate tight junction proteins (5–7). An impaired gut barrier is associated with the introduction of immunogenic endotoxins, including lipopolysaccharide (LPS), through transcellular and paracellular pathways, leading to disrupted mucosal homeostasis, inappropriate translocation of harmful particles, and dysregulation of cell adherence protein complexes (8,9). Such mechanisms have been observed in pathological conditions like obesity, type 2 diabetes, and Alzheimer's disease (10–12), but they are also linked to Western diets low in dietary fibers (13).

Evolutionary pressures have promoted highly conserved host mechanisms of the innate and adaptive immune systems to modulate endotoxins which include LPS binding protein (LBP), high density lipoproteins (HDL), macrophage and monocytes (14). HDL regulate cholesterol content in immune cells by influencing lipid raft composition, thus modulating signaling functions involving raft proteins such as toll-like receptors (15,16). HDL can also bind, deactivate, and clear LPS together with its associated protein LBP (14). When LPS is introduced into the system through the gut, gums, or a wound it is bound by LBP and trafficked to pattern

recognition receptors CD14 and toll-like receptor 4 (TLR4) on the surface of immune cells such as macrophage and monocytes, inducing an immune response resulting in the release of pro-inflammatory mediators (17). HDL can deactivate immune cells by transferring LPS from immune cells to HDL-associated LBP (18). HDL can also sequester, deactivate and clear LPS by binding LBP-LPS complex by enzymatic action of acyloxyacyl hydrolase (14,19). More recently, intestinally derived HDL have been shown to sequester LPS before it enters the portal vein, preventing recognition by TLR4 on macrophages and reducing overexpression of inflammatory molecules in the liver (14). Lecithin cholesterol acyltransferase (LCAT) plays an important role in the metabolism of HDL by esterifying and packing cholesterol into HDL particles. LCAT thus plays a critical role in the maturation and functional capacity of HDL particles (20,21).

LBP serves as a potent marker of endotoxin presence and has been shown to be an important alternative to the measurement of LPS since LPS is cleared relatively quickly whereas LBP plasma concentrations can persist for more than 24 hours after the onset of infection (22). LBP has thus become a specific marker of intestinally derived inflammation, and has also shown potential as a biomarker of disease risk in chronic diseases including cardiovascular disease and Alzheimer's disease (23,24). Differential concentrations have been reported in various pathological conditions including abnormally low LBP concentrations in Parkinson's disease related to gut permeability and intestinal microbial dysbiosis (25,26), and higher concentrations in diseases such as leptospirosis, tuberculosis, and Alzheimer's disease (27,28). LBP was recently shown to be a powerful independent predictor of Alzheimer's disease risk (24,29).

Our research aimed to uncover potential links between the function of HDL, as measured by LCAT activity, and gut permeability, as indicated by LBP concentrations, in the context of fiber supplementation in otherwise healthy individuals consuming a low-fiber diet. We hypothesized that 4 weeks of fiber supplementation in individuals with habitually low fiber diets would reduce LBP concentrations and improve HDL function. We also performed exploratory correlation analyses to determine whether LBP concentrations or LCAT activity were associated with gut microbiome composition and function, as well as plasma metabolomic profiles.

Methods and Materials

The complete study design and participant characteristics of the multi-omic analysis clinical trial were described previously (5). Briefly, the trial was a double-blinded, placebo-controlled, randomized order crossover trial with 20 healthy adult participants (n=10 men, n=10 women) aged between 18 and 45 years, all of whom had BMIs within the range of 23-32 kg/m2 and typically consumed less than 15 grams of fiber per day. Over a 4-week period, participants were provided with either a daily dose of 12g of a prebiotic fiber mix or a visually similar placebo, followed by a 4-week washout phase and then a switch to the opposite treatment for another 4 weeks. The prebiotic mixture was composed of fructooligosaccharides, resistant starch, sugarcane fiber, inulin, gum arabic, xanthan gum, and berry fruit powders. Stool samples were collected within 24h prior to the blood draw, and blood samples were collected after a 12h overnight fast at the onset and conclusion of each phase. Stool samples were analyzed by shallow shotgun metagenomic sequencing on an Illumina NovaSeq platform by Diversigen (Houston, TX, USA) to analyze the gut microbiome composition and the abundance of microbial genes.

Blood samples were analyzed through untargeted LC-MS metabolomics by Metabolon (Morrisville, NC, USA) to measure plasma metabolites, including microbially-derived metabolites. Additionally, anthropometric measurements, blood lipid and glucose concentrations, diet records, bowel movement patterns, and health questionnaire responses were collected. The clinical trial was registered at clinicaltrials.gov with the identifier NCT03785860. The study was approved by the Institutional Review Board of the University of California, Davis and written informed consent was obtained from all participants, following ethical guidelines (5).

Measurement of LBP

Concentrations of LBP were measured in plasma using the human LBP ELISA kit (Abcam, ab213805), following the manufacturer's protocol. Briefly, plasma samples were thawed, and aliquots were transferred to the provided microplate wells pre-coated with an LBP-specific antibody. After incubation and washing steps, a biotin-conjugated secondary antibody was added, followed by a streptavidin-HRP conjugate. Colorimetric detection was achieved with the addition of the substrate solution, and the reaction was stopped with the provided stop solution. Absorbance was measured at 450 nm using a microplate reader, and LBP concentrations were calculated using a standard curve generated from the provided LBP standards.

Measurement of LCAT Activity

Plasma samples were also used to assess HDL function by measuring LCAT activity. An LCAT Activity Assay Kit (MAK107) was employed according to the manufacturer's instructions. In

brief, plasma samples were thawed and mixed with the provided LCAT assay buffer and substrate. The reaction mixture was incubated at 37°C for a specific duration, and the reaction was stopped by adding the provided stop solution. The generated product was quantified by measuring the absorbance at 340 nm using a microplate reader. LCAT activity was calculated based on the change in absorbance and was expressed as units per mL of plasma.

Statistical Analysis

All statistical analyses were conducted using R software (version 4.2.2). For general analyses, a linear mixed model was employed with the lmerTest package (30) to account for repeated measures within the data. Post-hoc tests were performed on these models using the emmeans package (31). Exploratory analysis was conducted by dividing the participants into those with above-median vs. below-median LBP concentrations at baseline.

We performed a repeated measure correlation analysis using the rmcorr package (32) to explore relationships between metabolites, Operational Taxonomic Units (OTUs) from microbes and gene counts with LBP concentration and LCAT activity. Repeated measure correlation analysis enables us to evaluate the within-individual relationship across paired measures assessed on two or more occasions. Metabolites, OTU, and gene counts were log-transformed, followed by correlation analysis with LBP concentration and LCAT activity. The results were then adjusted for multiple comparisons using the Benjamini-Hochberg method, with statistical significance set at a p-value of less than 0.05. For the repeated measure correlation analysis of microbial gene abundance data, the edgeR package (33) was employed for gene preprocessing, including

normalization using the `calcNormFactors` function. As part of the preprocessing, genes with low abundance were excluded by filtering out those with a maximum count per million (CPM) value below a cutoff of 1. In other words, only genes with a maximum CPM value equal to or above 1 were retained in the dataset. Subsequently, limma-voom, a component of the limma package (34), was applied for data transformation. Limma-voom involves further filtering, where genes with low expression were removed based on their CPM values. The resulting dataset was then transformed using voom, a process that estimates the mean-variance relationship of log-counts and assigns precision weights to each observation. The transformed data, representative of normalized and filtered gene abundance, were then utilized in repeated measure correlation analyses with LBP ug/ml and LCAT activity.

Results

Table 1. Pre- and Post-Treatment LBP and LCAT Activity Levels in Placebo and Prebiotic Groups*

	Placebo		**Prebiotic**		**P-value**
Variable	Pre	Post	Pre	Post	
LBP (ug/mL)	5.99 ± 3.1	5.98 ± 3.21	6.62 ± 3.88	5.67 ± 2.65	0.446
LCAT Activity Index	1.053 ± 0.018	1.055 ± 0.024	1.047 ± 0.023	1.052 ± 0.02	0.718

*Data are shown as means ± SDs. Changes on pre- and post-treatment with placebo or prebiotic were compared with a linear mixed model (n = 20).

LBP concentrations did not differ significantly between the placebo and prebiotic arms ($p > 0.05$) and similarly, post-treatment LBP concentrations were not significantly different between the two groups ($p > 0.05$) (**Table 1**). Additionally, LCAT activity did not display significant differences between the placebo and fiber intervention arms before or after treatment ($p > 0.05$) (**Table 1**).

However, in participants with above-median pre-treatment LBP concentrations, a significant decrease in post-treatment LBP concentrations was observed following fiber supplementation ($p < 0.020$). This finding suggests that fiber supplementation may provide benefits to individuals with elevated LBP concentrations at baseline (**Table 2**).

Table 2. Participants stratified by above or below group LBP median at baseline*

	Above Median			**Below Median**		
Variable	Pre	Post	P-value	Pre	Post	P-value
LBP (ug/mL)	9.27±3.52	7.02±2.32	**0.020**	3.97±1.56	4.32±2.13	0.448
LCAT Activity Index	1.067 ± 0.01	1.057 ± 0.02	0.284	1.036 ± 0.01	1.036 ± 0.02	0.448

*Data are shown as means ± SDs. P-values were calculated from paired t-tests comparing pre- vs post-treatment lipopolysaccharide binding protein (LBP) or lecithin-cholesterol acyltransferase (LCAT) activity within each group ("above median" or "below median" baseline)

A significant positive correlation was found between LCAT activity and high-density lipoprotein cholesterol (HDL-C) ($r = 0.391$, p-value = 0.018), low-density lipoprotein cholesterol (LDL-C) ($r = 0.343$, p-value = 0.034), and total cholesterol concentrations ($r = 0.382$, p-value = 0.018), which remained significant after adjusting for multiple comparisons ($p < 0.05$) (**Table 3**).
A positive correlation between HDL-C and LBP concentration (measure $r = 0.271$, $p = 0.035$) was found, however it did not remain significant after correction for multiple testing, and no other statistically significant correlations were found between clinical characteristics and LBP concentration (**Table 3**).

Table 3. Association of clinical characteristics with LBP and LCAT activity

	LBP (ug/mL)				**LCAT Activity Index**			
Variable	Rmcorr	95% CI	P-value	Padjust	Rmcorr	95% CI	P-value	Padjust
Age, y	0.02	-0.23, -0.23	0.864	0.930	0.12	-0.14, -0.14	0.370	0.576
Weight, kg	-0.05	-0.3, -0.3	0.708	0.930	-0.22	-0.45, -0.45	0.087	0.244
Height, cm	-0.08	-0.32, -0.32	0.563	0.930	-0.08	-0.33, -0.33	0.527	0.726
BMI, kg/m2	0	-0.25, -0.25	1.000	1.000	-0.16	-0.4, -0.4	0.222	0.444
Systolic Blood Pressure, mmHg	0.1	-0.15, -0.15	0.424	0.930	0.07	-0.18, -0.18	0.571	0.726

Diastolic Blood Pressure, mmHg	0.13	-0.13, -0.13	0.320	0.930	0.17	-0.09, -0.09	0.194	0.444
Fasting glucose, mg/dL	0.02	-0.23, -0.23	0.857	0.930	-0.05	-0.3, -0.3	0.730	0.851
Fasting insulin, μIU/mL	0.07	-0.19, -0.19	0.607	0.930	-0.01	-0.26, -0.26	0.932	0.932
Total Cholesterol, mg/dL	0.13	-0.13, -0.13	0.334	0.930	0.38	0.14, 0.14	**0.003**	**0.018**
HDL cholesterol, mg/dL	0.27	0.02, 0.02	**0.035**	0.489	0.39	0.15, 0.15	**0.002**	**0.018**
LDL cholesterol, mg/dL	0.02	-0.23, -0.23	0.855	0.930	0.34	0.1, 0.1	**0.007**	**0.034**
Cholesterol:HDL	-0.04	-0.29, -0.29	0.753	0.930	-0.03	-0.28, -0.28	0.835	0.899
Triglyceride, mg/dL	0.04	-0.22, -0.22	0.782	0.930	-0.13	-0.37, -0.37	0.327	0.572
non-HDL	0.04	-0.21, -0.21	0.757	0.930	0.28	0.03, 0.03	0.032	0.112

*Repeated measures correlation (Rmcorr) was used to assess the within-individual association between clinical variables and outcomes (LBP or LCAT activity) over time. Rmcorr accounts for the non-independence of repeated pre- and post-treatment measurements from each participant, and estimates the common within-person correlation. 95% CI = 95% confidence interval. Padj = p-values adjusted for multiple comparisons using the Benjamini-Hochberg method. Bold padj < 0.05 indicates statistically significant correlation after adjustment. HDL, high-density lipoproteins; LDL, low-density lipoproteins.

In the assessment of relationships between 185 microbial genera and LBP, we found significant correlations between LBP concentrations and 3 microbes that were positively correlated and 2 that were negatively correlated ($P<0.05$), however these relationships were no longer significant after correction for multiple testing, and several were not present at detectable levels in the majority of participants (**Supplemental table S1**). Similarly, 11 microbes showed positive correlations with LCAT activity, while 3 were negatively correlated ($P<0.05$). However, these findings also lost significance upon multiple testing correction (**Supplemental table S2**).

Out of 890 plasma metabolites analyzed, 35 displayed positive correlations and 15 showed negative correlations with LBP concentrations ($P<0.05$), none of which remained statistically significant after multiple testing correction **(Figure 1, Supplemental table S3**). LBP concentrations were predominantly correlated with a variety of lipids and xenobiotics. Notably,

LBP was positively correlated with several sulfated bile acid species such as taurodeoxycholic acid 3-sulfate and taurocholic acid sulfate, the sulfated estrogen metabolite estrone 3-sulfate, as well as perfluorooctanesulfonate (PFOS), an industrial chemical pollutant that is also used in food packaging. Positively correlated metabolites also included those associated with xenobiotic metabolism pathways, including a positive correlation with multiple metabolites of ibuprofen. In contrast, negative correlations were observed between LBP and several palmitate-containing glycerophospholipids, exemplified by species such as 1-(1-enyl-palmitoyl)-GPC and 1,2-dipalmitoyl-GPC.

In relation to LCAT activity, 15 metabolites were positively correlated and 18 were negatively correlated ($P<0.05$), which did not remain statistically significant after multiple testing correction (**Figure 1, Supplemental table S4**). LCAT activity was similarly primarily associated with lipid metabolism, including positive correlations with lipids such as palmitate-, stearate-, oleate- and linoleate-containing glycerophospholipids and acylcholines, as well as with cholesterol and vitamin A (retinol).

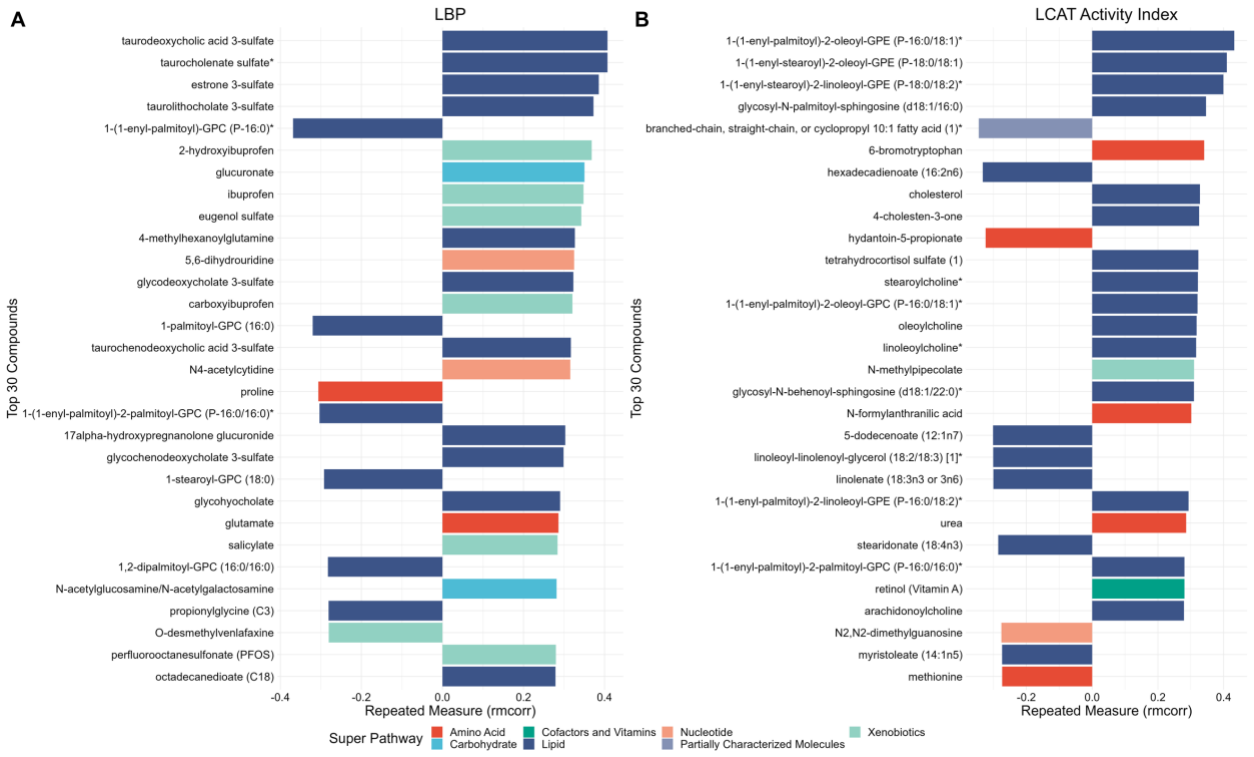

Figure 1: Correlation between LBP concentrations and LCAT activity and Plasma Metabolites. This figure displays the top 30 correlation of LBP concentrations and LCAT activity with 890 plasma metabolites. A) Highlighted are 35 metabolites with a statistically significant positive correlation and 15 with a negative correlation to LBP concentrations (P<0.05). B) 15 metabolites with a statistically significant positive correlation and 18 with a negative correlation to LCAT activity (P<0.05).

Out of 2,067 microbial genes, 14 displayed a positive correlation with LBP concentrations, while 8 exhibited an inverse relationship (P<0.05) which did not remain significant after multiple testing correction (**Figure 2, Supplemental Figure S5**). Among these, 3-deoxy-manno-octulosonate cytidylyltransferase (kdsB), which is involved in the synthesis of LPS by gram-negative bacteria, was positively correlated with LBP. Conversely, 2-deoxystreptamine N-acetyl-D-glucosaminyltransferase (btrM), responsible for the biosynthesis of butirosin, an aminoglycoside antibiotic complex that deactivates both gram-positive and gram-negative bacteria, was negatively correlated.

Two microbial genes were found to be positively correlated and 42 genes were negatively correlated with LCAT activity (P<0.05), none of which remained statistically significant after multiple testing correction (**Figure 2, Supplementary Table S6**). Notable genes inversely correlated with LCAT activity included UDP-N-acetyl-D-mannosaminuronic acid transferase (wecG), contributing to the synthesis of a lipid-linked intermediate in the formation of enterobacterial outer membranes, and sporulation kinase D (kinD), pivotal in activating a sporulation-regulatory protein. Furthermore, the vancomycin C-type resistance protein VanC (vanC), involved in conferring bacterial resistance to the antibiotic vancomycin, also displayed a significant negative correlation with LCAT activity.

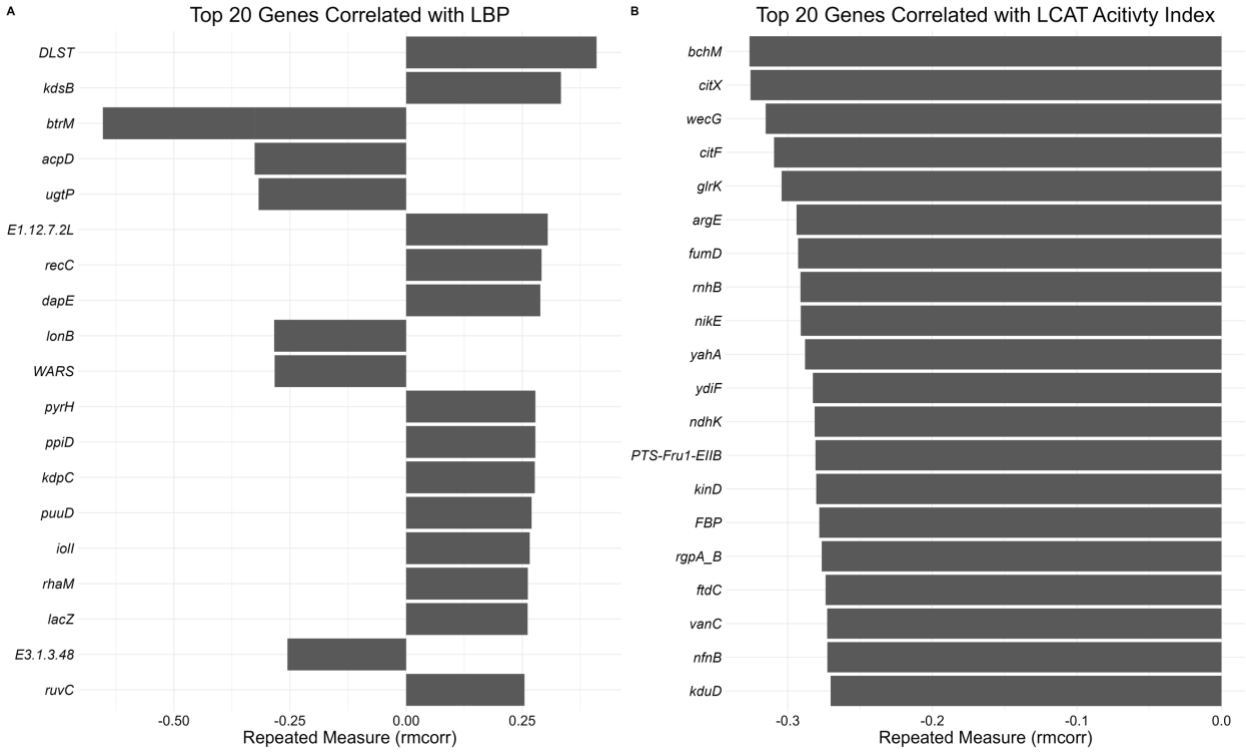

Figure 2: Correlations Between Microbial Gene Abundance and LBP concentrations and LCAT activity. The figure shows the top 20 genes. A) correlations with LBP (P<0.05) B) correlations with LCAT activity (P<0.05).

Discussion

In this study, the effects of prebiotic fiber supplementation on plasma LBP concentrations and LCAT activity in generally healthy, young individuals consuming a low-fiber diet were investigated. There were no statistically significant differences in LBP concentration or LCAT activity between the placebo and prebiotic arms. The lack of effect of the fiber supplement on LBP concentrations in the overall cohort may be due to several potential reasons including that a higher dose of fiber or longer intervention may be necessary to induce measurable effects, and that these generally young, healthy participants may not yet be experiencing the deleterious effects of a low-fiber diet that is seen in older adults or those who have already developed disease (35,36).

We performed an exploratory analysis dividing the group into those with above-median vs. below-median LBP concentrations at baseline, and found that in those participants with above-median baseline LBP, fiber supplementation significantly reduced LBP concentrations. Thus, even this low dose of fiber supplement, which was designed to achieve levels of fiber intake consistent with the recommended dietary allowance, benefitted young, healthy individuals who already had some degree of gut barrier impairment. Multiple cross-sectional studies have found plasma LBP concentrations to be negatively correlated with dietary fiber intake(36–38) as well as indices of healthy diet (e.g. the dietary inflammatory index) (39).

We performed exploratory correlation analysis to determine if there were any relationships between gut microbiome composition, function, or plasma metabolites and both LBP and LCAT activity, in order to elucidate some of the microbiome-associated factors that may influence gut

barrier function and HDL function. Notably, we found positive correlations between LBP and multiple sulfated molecules. Certain sulfated bile acids like taurolithocholic acid 3-sulfate are used to induce pancreatitis in mouse models, which may suggest a pathological role for this molecule (40). Interestingly, we also observed a positive correlation between LBP and PFOS, an industrial chemical that is used in a variety of industrial and consumer products (41), as well as in food packaging (42,43), and which has been shown to be associated with an array of negative health effects including high cholesterol, increased liver enzymes, thyroid disorders, immunotoxicity and cancer (44,45). Elevated concentrations of sulfated bile acids and sulfated estrone could indicate an increase in the activity of sulfotransferase enzymes in the liver, which are regulated by a wide variety of nuclear transcription factors (46) and which may be linked with the gut microbiome (47,48). The correlations between LBP and ibuprofen metabolites also suggest that non-steroidal anti-inflammatory medication use may also contribute to impairment of gut barrier function, as has been observed previously (49). Together these data point to an important potential link between xenobiotic exposure and detoxification, and gut permeability.

Furthermore, several associations between LBP concentrations and abundance of microbial genes were found. For instance, the positive correlation between kdsB, which is involved in LPS synthesis by gram-negative bacteria (50), and plasma LBP concentrations, suggests that in individuals whose microbiome in enriched in LPS synthesis genes there is a higher degree of LPS translocation across the gut barrier. On the other hand, the btrM gene, which is involved in the synthesis of an aminoglycoside that acts as an antibiotic (51,52) was negatively correlated with LBP, indicating that individuals whose microbiome is enriched in genes involved in the endogenous production of antibiotic molecules is more likely to have lower translocation of LPS

across the gut barrier. Together, these findings indicate that the background microbiome, both from a compositional and more importantly, from a functional perspective, is influential in determining the level of gut barrier permeability and thus the amount of translocation of pro-inflammatory LPS, which has a well-established, pro-inflammatory role across multiple chronic disease states (53–56).

When examining the relationships with LCAT activity, inverse correlations were found with bacterial genes like wecG and kinD, which are involved in the synthesis of enterobacterial cell wall components and sporulation proteins respectively (57,58). These observations suggest that the microbiomes of individuals enriched in enterobacteria and sporulating bacteria may be linked with decreased HDL functionality. The vanC gene, which enables bacterial resistance to the antibiotic vancomycin (59), was also negatively correlated with LCAT activity, suggesting that individuals whose microbiomes are enriched in antibiotic resistance genes, perhaps due to higher historical intakes of antibiotics, are also associated with decreased HDL functionality. These intriguing exploratory findings suggest that there are potential as yet underexplored relationships between antibiotic exposure, and increased abundances of certain classes of bacteria (e.g. enterobacteria and spore-forming bacteria) that may be linked with decreased HDL functionality, which merit further study.

Conclusion

Collectively, this secondary analysis study showed that a low dose fiber supplement taken for 4 weeks did not decrease gut permeability to LPS, as measured by plasma LBP, or improve HDL functionality, as measured by LCAT activity, in young, generally healthy individuals. However, those participants who had above-median concentrations of LBP at baseline did experience a reduction in LBP in response to the fiber supplement, indicating that even young, healthy adults can have some degree of gut permeability and that this can be ameliorated with a simple, low-dose, easy to use fiber supplement. Furthermore, exploratory correlation analyses uncovered potential links between gut microbiome function and both gut permeability and HDL functionality, highlighting the potential involvement of pathways in LPS synthesis, antibiotic synthesis, and antibiotic resistance. Exploratory analyses further revealed potential links between plasma metabolites related to xenobiotic detoxification and LBP concentrations, including industrial chemicals (PFOS), sulfated bile acids, and ibuprofen metabolites. These findings provide insights into connections between indicators of gut permeability, HDL function, and the gut microbiome—warranting future targeted studies to confirm findings and elucidate mechanisms. Our findings showcase the potential of affordable, easy to implement fiber supplementation as an approach to mitigate gut barrier dysfunction-associated inflammation in individuals who are low-fiber consumers, and point to potential environmental and lifestyle factors that could be contributing to impaired gut barrier function in addition to diet.

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Supplemental Table S1: LBP repeated measure correlation with top 50 microbes.

Genus	rmcor	p-value	padj
Other	-0.391	**0.002**	0.340
Sanguibacteroides	-0.348	**0.006**	0.549
Xylanimonas	0.284	**0.027**	0.997
Mobilibacterium	0.259	**0.044**	0.997
Faecalicatena	0.258	**0.045**	0.997
Solobacterium	-0.249	0.053	0.997
Mailhella	-0.248	0.054	0.997
Butyricicoccus	-0.247	0.055	0.997
Anaerofustis	-0.237	0.066	0.997
Merdimonas	-0.236	0.068	0.997
Bifidobacterium	-0.231	0.073	0.997
Haemophilus	0.227	0.079	0.997
Tannerella	0.227	0.079	0.997
Intestinibacter	-0.220	0.088	0.997
Fenollaria	-0.220	0.089	0.997
Peptoniphilus	-0.209	0.106	0.997
Neglecta	-0.197	0.129	0.997
Slackia	-0.188	0.147	0.997
Mogibacterium	0.187	0.150	0.997
Monoglobus	0.182	0.161	0.997
Mediterranea	-0.182	0.161	0.997
Rothia	0.181	0.163	0.997
Enterococcus	-0.181	0.163	0.997
Acidaminococcus	0.179	0.167	0.997
Lactobacillus	-0.176	0.175	0.997
Rikenella	-0.176	0.175	0.997
Agathobaculum	0.171	0.187	0.997
Streptococcus	0.170	0.191	0.997
Pseudomonas	-0.166	0.201	0.997
Aeromonas	0.164	0.206	0.997
Candidatus_Stoquefichus	0.164	0.208	0.997
Paraprevotella	-0.161	0.215	0.997
Olsenella	-0.159	0.220	0.997
Serratia	0.158	0.224	0.997
Massiliomicrobiota	-0.154	0.235	0.997
Subdoligranulum	-0.153	0.239	0.997
Blautia	0.151	0.245	0.997
Alistipes	-0.150	0.248	0.997
Catenibacterium	-0.144	0.269	0.997
Acetivibrio	-0.143	0.270	0.997
Provencibacterium	-0.143	0.273	0.997

Gemella	0.136	0.294	0.997
Dialister	-0.136	0.295	0.997
Eggerthella	0.135	0.298	0.997
Phoenicibacter	-0.134	0.301	0.997
Weissella	-0.131	0.315	0.997
Lachnospira	0.130	0.317	0.997
Fusicatenibacter	0.129	0.320	0.997
Lachnoclostridium	-0.129	0.322	0.997
Synergistes	-0.127	0.331	0.997

Supplemental Table S2: LCAT activity repeated measure correlation with top 50 microbes.

Genus	rmcor	pval	padj
Achromobacter	-0.316	**0.014**	0.613
Anaerotignum	0.298	**0.021**	0.613
Emergencia	0.295	**0.022**	0.613
Selenomonas	0.290	**0.025**	0.613
Turicibacter	0.284	**0.028**	0.613
Sellimonas	0.276	**0.032**	0.613
Dakarella	0.272	**0.036**	0.613
Eggerthella	0.272	**0.036**	0.613
Synergistes	-0.269	**0.038**	0.613
Shigella	-0.265	**0.040**	0.613
Massilioclostridium	0.265	**0.040**	0.613
Enterorhabdus	0.260	**0.045**	0.613
Libanicoccus	0.258	**0.047**	0.613
Dorea	0.258	**0.047**	0.613
Rikenella	-0.251	0.053	0.613
Collinsella	0.250	0.054	0.613
Mitsuokella	0.248	0.056	0.613
Butyricicoccus	-0.238	0.068	0.654
Adlercreutzia	0.234	0.072	0.654
Oscillibacter	0.229	0.078	0.654
Agathobaculum	0.227	0.081	0.654
Gordonibacter	0.222	0.089	0.654
Escherichia	-0.220	0.092	0.654
Gabonibacter	0.219	0.092	0.654
Listeria	0.219	0.093	0.654
Solobacterium	0.216	0.097	0.654
Erysipelatoclostridium	0.215	0.099	0.654
Murdochiella	0.215	0.100	0.654
Coprococcus	0.211	0.106	0.654
Dielma	-0.210	0.108	0.654

Lautropia	-0.209	0.110	0.654
Asaccharospora	0.204	0.117	0.678
Faecalicoccus	0.202	0.122	0.681
Monoglobus	0.197	0.131	0.713
Eisenbergiella	0.195	0.136	0.717
Gemmiger	0.192	0.142	0.728
Actinomyces	0.186	0.155	0.777
Merdibacter	0.184	0.160	0.779
Leuconostoc	0.182	0.165	0.783
Allisonella	0.179	0.170	0.783
Neglecta	0.178	0.173	0.783
Sutterella	-0.176	0.179	0.784
Anaerotruncus	0.175	0.182	0.784
Xylanimonas	0.170	0.194	0.817
Neisseria	-0.168	0.201	0.825
Fournierella	0.163	0.215	0.849
Sanguibacter	0.162	0.216	0.849
Megasphaera	0.160	0.221	0.853
Sanguibacteroides	-0.156	0.235	0.870
Clostridium	0.155	0.237	0.870

Supplemental Table S3: LBP repeated measure correlation with top 50 metabolites.

Metabolite Name	Super Pathway	Sub Pathway	Rmcorr	p_value	padj
taurodeoxycholic acid 3-sulfate	Lipid	Secondary Bile Acid Metabolism	0.407	**0.001**	0.502
taurocholenate sulfate*	Lipid	Secondary Bile Acid Metabolism	0.407	**0.001**	0.502
estrone 3-sulfate	Lipid	Estrogenic Steroids	0.386	**0.002**	0.519
taurolithocholate 3-sulfate	Lipid	Secondary Bile Acid Metabolism	0.373	**0.003**	0.519
1-(1-enyl-palmitoyl)-GPC (P-16:0)*	Lipid	Lysoplasmalogen	-0.369	**0.003**	0.519
2-hydroxyibuprofen	Xenobiotics	Drug - Analgesics, Anesthetics	0.368	**0.004**	0.519
glucuronate	Carbohydrate	Aminosugar Metabolism	0.350	**0.006**	0.667
ibuprofen	Xenobiotics	Drug - Analgesics, Anesthetics	0.348	**0.006**	0.667
eugenol sulfate	Xenobiotics	Food Component/Plant	0.343	**0.007**	0.680
4-methylhexanoylglutamine	Lipid	Fatty Acid Metabolism (Acyl Glutamine)	0.327	**0.010**	0.740
5,6-dihydrouridine	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.325	**0.011**	0.740
glycodeoxycholate 3-sulfate	Lipid	Secondary Bile Acid Metabolism	0.323	**0.011**	0.740
carboxyibuprofen	Xenobiotics	Drug - Analgesics, Anesthetics	0.321	**0.012**	0.740
1-palmitoyl-GPC (16:0)	Lipid	Lysophospholipid	-0.320	**0.012**	0.740

taurochenodeoxycholic acid 3-sulfate	Lipid	Secondary Bile Acid Metabolism	0.317	**0.013**	0.740
N4-acetylcytidine	Nucleotide	Pyrimidine Metabolism, Cytidine containing	0.315	**0.013**	0.740
proline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	-0.306	**0.016**	0.824
1-(1-enyl-palmitoyl)-2-palmitoyl-GPC (P-16:0/16:0)*	Lipid	Plasmalogen	-0.303	**0.017**	0.824
17alpha-hydroxypregnanolone glucuronide	Lipid	Pregnenolone Steroids	0.303	**0.018**	0.824
glycochenodeoxycholate 3-sulfate	Lipid	Primary Bile Acid Metabolism	0.299	**0.019**	0.842
1-stearoyl-GPC (18:0)	Lipid	Lysophospholipid	-0.292	**0.022**	0.842
glycohyocholate	Lipid	Secondary Bile Acid Metabolism	0.290	**0.023**	0.842
glutamate	Amino Acid	Glutamate Metabolism	0.286	**0.025**	0.842
salicylate	Xenobiotics	Drug - Topical Agents	0.284	**0.027**	0.842
1,2-dipalmitoyl-GPC (16:0/16:0)	Lipid	Phosphatidylcholine (PC)	-0.283	**0.027**	0.842
N-acetylglucosamine/N-acetylgalactosamine	Carbohydrate	Aminosugar Metabolism	0.282	**0.028**	0.842
propionylglycine (C3)	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	-0.281	**0.028**	0.842
O-desmethylvenlafaxine	Xenobiotics	Drug - Psychoactive	-0.281	**0.029**	0.842
perfluorooctanesulfonate (PFOS)	Xenobiotics	Chemical	0.280	**0.029**	0.842
octadecanedioate (C18)	Lipid	Fatty Acid, Dicarboxylate	0.279	**0.030**	0.842
ascorbic acid 2-sulfate	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	0.273	**0.033**	0.842
1-lignoceroyl-GPC (24:0)	Lipid	Lysophospholipid	-0.272	**0.034**	0.842
hexanoylglutamine	Lipid	Fatty Acid Metabolism (Acyl Glutamine)	0.271	**0.035**	0.842
dihydroorotate	Nucleotide	Pyrimidine Metabolism, Orotate containing	-0.269	**0.036**	0.842
palmitoyl sphingomyelin (d18:1/16:0)	Lipid	Sphingomyelins	-0.265	**0.039**	0.842
13-HODE + 9-HODE	Lipid	Fatty Acid, Monohydroxy	0.264	**0.040**	0.842
histidine	Amino Acid	Histidine Metabolism	-0.263	**0.041**	0.842
N,N,N-trimethyl-5-aminovalerate	Amino Acid	Lysine Metabolism	0.262	**0.042**	0.842
3-hydroxyadipate	Lipid	Fatty Acid, Dicarboxylate	0.261	**0.042**	0.842
glutamine conjugate of C7H12O2*	Partially Characterized Molecules	Partially Characterized Molecules	0.260	**0.043**	0.842
acetoacetate	Lipid	Ketone Bodies	0.259	**0.044**	0.842
3-carboxy-4-methyl-5-pentyl-2-furanpropionate (3-CMPFP)**	Lipid	Fatty Acid, Dicarboxylate	0.258	**0.044**	0.842
lignoceroylcarnitine (C24)*	Lipid	Fatty Acid Metabolism (Acyl Carnitine, Long Chain Saturated)	-0.257	**0.045**	0.842
decadienedioic acid (C10:2-DC)**	Lipid	Fatty Acid, Dicarboxylate	0.257	**0.046**	0.842

GlcNAc sulfate conjugate of C21H34O2 steroid**	Partially Characterized Molecules	Partially Characterized Molecules	0.254	**0.048**	0.842
glycerate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.254	**0.048**	0.842
1-linoleoyl-GPC (18:2)	Lipid	Lysophospholipid	-0.253	**0.049**	0.842
citrulline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	-0.253	**0.049**	0.842
1-methyladenosine	Nucleotide	Purine Metabolism, Adenine containing	0.253	**0.050**	0.842
oxalate (ethanedioate)	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	0.252	**0.050**	0.842

Supplemental Table S4: LCAT activity repeated measure correlation with top 50 metabolites.

Metabolite Name	Super Pathway	Sub Pathway	Rmcorr	p_value	padj
1-(1-enyl-palmitoyl)-2-oleoyl-GPE (P-16:0/18:1)*	Lipid	Plasmalogen	0.432	**0.001**	0.470
1-(1-enyl-stearoyl)-2-oleoyl-GPE (P-18:0/18:1)	Lipid	Plasmalogen	0.410	**0.001**	0.470
1-(1-enyl-stearoyl)-2-linoleoyl-GPE (P-18:0/18:2)*	Lipid	Plasmalogen	0.399	**0.002**	0.470
glycosyl-N-palmitoyl-sphingosine (d18:1/16:0)	Lipid	Hexosylceramides (HCER)	0.346	**0.007**	0.821
branched-chain, straight-chain, or cyclopropyl 10:1 fatty acid (1)*	Partially Characterized Molecules	Partially Characterized Molecules	-0.345	**0.007**	0.821
6-bromotryptophan	Amino Acid	Tryptophan Metabolism	0.341	**0.008**	0.821
hexadecadienoate (16:2n6)	Lipid	Long Chain Polyunsaturated Fatty Acid (n3 and n6)	-0.333	**0.009**	0.821
cholesterol	Lipid	Sterol	0.328	**0.011**	0.821
4-cholesten-3-one	Lipid	Sterol	0.325	**0.011**	0.821
hydantoin-5-propionate	Amino Acid	Histidine Metabolism	-0.324	**0.012**	0.821
tetrahydrocortisol sulfate (1)	Lipid	Corticosteroids	0.323	**0.012**	0.821
stearoylcholine*	Lipid	Fatty Acid Metabolism (Acyl Choline)	0.322	**0.012**	0.821
1-(1-enyl-palmitoyl)-2-oleoyl-GPC (P-16:0/18:1)*	Lipid	Plasmalogen	0.320	**0.013**	0.821
oleoylcholine	Lipid	Fatty Acid Metabolism (Acyl Choline)	0.318	**0.013**	0.821
linoleoylcholine*	Lipid	Fatty Acid Metabolism (Acyl Choline)	0.316	**0.014**	0.821
N-methylpipecolate	Xenobiotics	Bacterial/Fungal	0.310	**0.016**	0.829
glycosyl-N-behenoyl-sphingosine (d18:1/22:0)*	Lipid	Hexosylceramides (HCER)	0.310	**0.016**	0.829
N-formylanthranilic acid	Amino Acid	Tryptophan Metabolism	0.302	**0.019**	0.829
5-dodecenoate (12:1n7)	Lipid	Medium Chain Fatty Acid	-0.301	**0.019**	0.829
linoleoyl-linolenoyl-glycerol (18:2/18:3) [1]*	Lipid	Diacylglycerol	-0.301	**0.019**	0.829
linolenate (18:3n3 or 3n6)	Lipid	Long Chain Polyunsaturated Fatty Acid (n3 and n6)	-0.301	**0.020**	0.829

1-(1-enyl-palmitoyl)-2-linoleoyl-GPE (P-16:0/18:2)*	Lipid	Plasmalogen	0.293	**0.023**	0.829
urea	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.286	**0.027**	0.829
stearidonate (18:4n3)	Lipid	Long Chain Polyunsaturated Fatty Acid (n3 and n6)	-0.286	**0.027**	0.829
1-(1-enyl-palmitoyl)-2-palmitoyl-GPC (P-16:0/16:0)*	Lipid	Plasmalogen	0.281	**0.030**	0.829
retinol (Vitamin A)	Cofactors and Vitamins	Vitamin A Metabolism	0.281	**0.030**	0.829
arachidonoylcholine	Lipid	Fatty Acid Metabolism (Acyl Choline)	0.279	**0.031**	0.829
N2,N2-dimethylguanosine	Nucleotide	Purine Metabolism, Guanine containing	-0.276	**0.033**	0.829
myristoleate (14:1n5)	Lipid	Long Chain Monounsaturated Fatty Acid	-0.274	**0.034**	0.829
methionine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	-0.274	**0.034**	0.829
dihomo-linolenoyl-choline	Lipid	Fatty Acid Metabolism (Acyl Choline)	0.274	**0.034**	0.829
palmitoylcholine	Lipid	Fatty Acid Metabolism (Acyl Choline)	0.272	**0.036**	0.829
tiglyl carnitine (C5)	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.270	**0.037**	0.829
3-indoleglyoxylic acid	Xenobiotics	Food Component/Plant	0.268	**0.038**	0.829
linoleoyl-linoleoyl-glycerol (18:2/18:2) [2]*	Lipid	Diacylglycerol	-0.266	**0.040**	0.829
sphingomyelin (d18:1/21:0, d17:1/22:0, d16:1/23:0)*	Lipid	Sphingomyelins	0.264	**0.041**	0.829
fumarate	Energy	TCA Cycle	-0.263	**0.042**	0.829
gamma-tocopherol/beta-tocopherol	Cofactors and Vitamins	Tocopherol Metabolism	-0.263	**0.042**	0.829
glycerol	Lipid	Glycerolipid Metabolism	-0.262	**0.043**	0.829
linoleate (18:2n6)	Lipid	Long Chain Polyunsaturated Fatty Acid (n3 and n6)	-0.262	**0.043**	0.829
methylmalonate (MMA)	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	-0.262	**0.043**	0.829
N-acetyl-2-aminoadipate	Amino Acid	Lysine Metabolism	0.261	**0.044**	0.829
cis-4-decenoate (10:1n6)*	Lipid	Medium Chain Fatty Acid	-0.260	**0.045**	0.829
ceramide (d18:2/24:1, d18:1/24:2)*	Lipid	Ceramides	0.259	**0.046**	0.829
glycosyl ceramide (d18:1/20:0, d16:1/22:0)*	Lipid	Hexosylceramides (HCER)	0.259	**0.046**	0.829
21-hydroxypregnenolone disulfate	Lipid	Pregnenolone Steroids	-0.257	**0.048**	0.829
1-(1-enyl-palmitoyl)-2-linoleoyl-GPC (P-16:0/18:2)*	Lipid	Plasmalogen	0.257	**0.048**	0.829
N-behenoyl-sphingadienine (d18:2/22:0)*	Lipid	Ceramides	0.254	0.050	0.829
1-stearoyl-2-oleoyl-GPC (18:0/18:1)	Lipid	Phosphatidylcholine (PC)	0.253	0.051	0.829

trigonelline (N'-methylnicotinate)	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	0.252	0.052	0.829
1H-indole-7-acetic acid	Xenobiotics	Bacterial/Fungal	0.252	0.052	0.829

Supplemental Table S5: LBP repeated measure correlation with top 50 genes.

Gene Full Name	Gene Name	Rmcorr	p_value	padj
2-oxoglutarate dehydrogenase E2 component (dihydrolipoamide succinyltransferase)	*DLST*	0.410	**0.001**	1.000
3-deoxy-manno-octulosonate cytidylyltransferase (CMP-KDO synthetase)	*kdsB*	0.333	**0.009**	1.000
2-deoxystreptamine N-acetyl-D-glucosaminyltransferase / 2-deoxystreptamine glucosyltransferase	*btrM*	-0.326	**0.010**	1.000
2-deoxystreptamine N-acetyl-D-glucosaminyltransferase / 2-deoxystreptamine glucosyltransferase	*btrM*	-0.326	**0.010**	1.000
FMN-dependent NADH-azoreductase	*acpD*	-0.326	**0.010**	1.000
processive 1,2-diacylglycerol beta-glucosyltransferase	*ugtP*	-0.318	**0.013**	1.000
ferredoxin hydrogenase large subunit	*E1.12.7.2L*	0.305	**0.017**	1.000
exodeoxyribonuclease V gamma subunit	*recC*	0.291	**0.023**	1.000
succinyl-diaminopimelate desuccinylase	*dapE*	0.289	**0.024**	1.000
ATP-dependent Lon protease	*lonB*	-0.284	**0.027**	1.000
tryptophanyl-tRNA synthetase	*WARS*	-0.283	**0.027**	1.000
uridylate kinase	*pyrH*	0.278	**0.030**	1.000
peptidyl-prolyl cis-trans isomerase D	*ppiD*	0.278	**0.030**	1.000
K+-transporting ATPase ATPase C chain	*kdpC*	0.277	**0.031**	1.000
gamma-glutamyl-gamma-aminobutyrate hydrolase	*puuD*	0.270	**0.035**	1.000
2-keto-myo-inositol isomerase	*iolI*	0.266	**0.038**	1.000
L-rhamnose mutarotase	*rhaM*	0.262	**0.041**	1.000
beta-galactosidase	*lacZ*	0.261	**0.042**	1.000
protein-tyrosine phosphatase	*E3.1.3.48*	-0.255	**0.047**	1.000
crossover junction endodeoxyribonuclease RuvC	*ruvC*	0.255	**0.048**	1.000
chemotaxis protein CheD	*cheD*	-0.253	**0.049**	1.000
anaerobic dimethyl sulfoxide reductase subunit A	*dmsA*	0.253	**0.049**	1.000
[protein-PII] uridylyltransferase	*glnD*	0.252	0.050	1.000
UDP-GlcNAc:undecaprenyl-phosphate/decaprenyl-phosphate GlcNAc-1-phosphate transferase	*wecA*	0.250	0.052	1.000
UDP-GlcNAc:undecaprenyl-phosphate/decaprenyl-phosphate GlcNAc-1-phosphate transferase	*wecA*	0.250	0.052	1.000
NADH-quinone oxidoreductase subunit K	*nuoK*	-0.248	0.054	1.000
23S rRNA (adenine-N6)-dimethyltransferase	*ermC*	-0.247	0.055	1.000
N-acetylglucosamine kinase	*NAGK*	0.247	0.055	1.000
two-component system, cell cycle response regulator	*pleD*	-0.247	0.055	1.000
two-component system, OmpR family, sensor histidine kinase PrrB	*prrB*	-0.244	0.058	1.000
diaminopimelate epimerase	*dapF*	-0.244	0.058	1.000

acetolactate synthase I/III small subunit	*E2.2.1.6S*	-0.244	0.058	1.000
[glutamine synthetase] adenylyltransferase / [glutamine synthetase]-adenylyl-L-tyrosine phosphorylase	*glnE*	0.244	0.059	1.000
[glutamine synthetase] adenylyltransferase / [glutamine synthetase]-adenylyl-L-tyrosine phosphorylase	*glnE*	0.244	0.059	1.000
histidyl-tRNA synthetase	*HARS*	-0.243	0.059	1.000
fumarylpyruvate hydrolase	*nagK*	0.243	0.059	1.000
clostripain	*cloSI*	0.242	0.060	1.000
bis(5'-nucleosyl)-tetraphosphatase (symmetrical)	*apaH*	0.242	0.061	1.000
hydrogenase small subunit	*hyaA*	0.241	0.062	1.000
succinyl-CoA:acetate CoA-transferase	*aarC*	-0.240	0.062	1.000
arsenate-mycothiol transferase	*arsC*	-0.240	0.062	1.000
exoribonuclease II	*rnb*	0.240	0.063	1.000
bifunctional autolysin	*atl*	-0.240	0.063	1.000
bifunctional autolysin	*atl*	-0.240	0.063	1.000
acetyl-CoA carboxylase carboxyl transferase subunit alpha	*accA*	-0.239	0.064	1.000
acetyl-CoA carboxylase carboxyl transferase subunit alpha	*accA*	-0.239	0.064	1.000
cyclohexyl-isocyanide hydratase	*inhA*	-0.238	0.064	1.000
two-component system, sporulation sensor kinase E	*kinE*	0.238	0.064	1.000
sulfoquinovosidase	*yihQ*	-0.237	0.066	1.000
undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase	*arnC*	0.236	0.067	1.000

Supplemental Table S6: LCAT activity repeated measure correlation with top 50 genes.

Gene Full Name	Gene Name	Rmcorr	p_value	padj
magnesium-protoporphyrin O-methyltransferase	*bchM*	-0.327	**0.011**	0.545
holo-ACP synthase	*citX*	-0.326	**0.011**	0.545
UDP-N-acetyl-D-mannosaminouronate:lipid I N-acetyl-D-mannosaminouronosyltransferase	*wecG*	-0.315	**0.014**	0.545
citrate lyase subunit alpha / citrate CoA-transferase	*citF*	-0.310	**0.016**	0.545
two-component system, NtrC family, sensor histidine kinase GlrK	*glrK*	-0.304	**0.018**	0.545
acetylornithine deacetylase	*argE*	-0.294	**0.023**	0.545
fumonisin B1 esterase	*fumD*	-0.293	**0.023**	0.545
ribonuclease HII	*rnhB*	-0.291	**0.024**	0.545
nickel transport system ATP-binding protein	*nikE*	-0.291	**0.024**	0.545
c-di-GMP-specific phosphodiesterase	*yahA*	-0.288	**0.026**	0.545
acetate CoA-transferase	*ydiF*	-0.283	**0.029**	0.545
NAD(P)H-quinone oxidoreductase subunit K	*ndhK*	-0.281	**0.029**	0.545
PTS system, fructose-specific IIB component	*PTS-Fru1-EIIB*	-0.281	**0.030**	0.545
two-component system, sporulation sensor kinase D	*kinD*	-0.280	**0.030**	0.545
fructose-1,6-bisphosphatase I	*FBP*	-0.278	**0.031**	0.545
gingipain R	*rgpA_B*	-0.277	**0.032**	0.545

dTDP-3-amino-3,6-dideoxy-alpha-D-galactopyranose 3-N-acetyltransferase	*ftdC*	-0.274	**0.034**	0.545
D-alanine---D-serine ligase	*vanC*	-0.273	**0.035**	0.545
nitroreductase / dihydropteridine reductase	*nfnB*	-0.273	**0.035**	0.545
2-dehydro-3-deoxy-D-gluconate 5-dehydrogenase	*kduD*	-0.270	**0.037**	0.545
superoxide dismutase, Cu-Zn family	*SOD1*	-0.267	**0.039**	0.545
dTDP-L-rhamnose 4-epimerase	*wbiB*	-0.267	**0.039**	0.545
inorganic pyrophosphatase	*ppa*	-0.266	**0.040**	0.545
DNA polymerase III subunit epsilon	*dnaQ*	-0.264	**0.041**	0.545
5,6-dimethylbenzimidazole synthase	*bluB*	-0.263	**0.042**	0.545
oxalyl-CoA decarboxylase	*oxc*	-0.262	**0.043**	0.545
ATP-dependent RNA helicase RhlB	*rhlB*	-0.262	**0.043**	0.545
7-alpha-hydroxysteroid dehydrogenase	*hdhA*	-0.261	**0.044**	0.545
formiminoglutamase	*hutG*	-0.260	**0.044**	0.545
tRNA-splicing ligase RtcB (3'-phosphate/5'-hydroxy nucleic acid ligase)	*RTCB*	-0.260	**0.045**	0.545
hydroxyethylthiazole kinase	*thiM*	0.260	**0.045**	0.545
pimeloyl-[acyl-carrier protein] methyl ester esterase	*bioH*	-0.259	**0.045**	0.545
D-alanine---(R)-lactate ligase	*vanB*	-0.259	**0.046**	0.545
sulfofructose kinase	*yihV*	-0.259	**0.046**	0.545
S-formylglutathione hydrolase	*frmB*	-0.258	**0.047**	0.545
L-threonine kinase	*pduX*	-0.257	**0.047**	0.545
16S rRNA (adenine1518-N6/adenine1519-N6)-dimethyltransferase	*ksgA*	0.257	**0.047**	0.545
NADP-reducing hydrogenase subunit HndB	*hndB*	-0.257	**0.047**	0.545
tellurite methyltransferase	*tehB*	-0.257	**0.048**	0.545
heparan-sulfate lyase	*hepC*	-0.257	**0.048**	0.545
methylthioribulose-1-phosphate dehydratase	*mtnB*	-0.255	**0.049**	0.545
O-succinylbenzoate synthase	*menC*	-0.255	**0.049**	0.545
4-methylaminobutanoate oxidase (formaldehyde-forming)	*mabO*	-0.255	**0.049**	0.545
2,5-diketo-D-gluconate reductase B	*dkgB*	-0.255	**0.049**	0.545
phosphonate transport system ATP-binding protein	*phnC*	-0.253	0.051	0.545
4-hydroxybenzoyl-CoA reductase subunit beta	*hcrB*	-0.252	0.052	0.545
glycogen phosphorylase	*PYG*	-0.251	0.053	0.545
rhamnogalacturonan exolyase	*yesX*	-0.249	0.055	0.545
pullulanase	*pulA*	-0.248	0.056	0.545
D-proline reductase (dithiol) PrdB	*prdB*	-0.248	0.056	0.545

Conclusion

This dissertation aimed to elucidate the complex roles of high-density lipoproteins, lipopolysaccharide binding protein, and associated inflammatory pathways in cardiovascular disease and Alzheimer's pathogenesis. The three studies presented contribute new insights that will help guide future research and propel development of enhanced predictive measures, preventions, and treatments.