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The Intestinal Gut Microbiome as a Biomarker and Driver of Obesity and Non-Alcoholic Fatty Liver Disease

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

by

Tien Sy Dong

2020

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2020

ABSTRACT OF THE DISSERTATION

The Intestinal Gut Microbiome as a Biomarker and

Driver of Obesity and Non-Alcoholic Fatty Liver Disease

by

Tien Sy Dong

Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

University of California, Los Angeles, 2020

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Nonalcoholic fatty liver disease (NAFLD) affects almost 1 out of every 5 Americans. The development of NAFLD increases an individual's risk for cardiovascular disease, cirrhosis, and cancer. Given that there are few treatments available for NALFD, it is imperative to understand the features that can prognosticate and alter the progression of NAFLD. One area of research that has gained significant traction is the role of the gut microbiome in the development and progression of NAFLD. By utilizing a multi'omics approach, we discovered that the gut microbiome can affect obesity through alterations of the brain-gut axis. In a study of over 100 patients, we saw that the gut microbiome was highly associated to food addiction. Patients with food addiction had significantly lower abundances of *Bacteroides*, *Akkermansia*, and *Eubacterium*, and a higher abundance of *Megamonas*. This was associated with a reduction in a neuroprotective tryptophan-related metabolite, indolepropionate, and altered connectivity in the brain's reward regions. This

data suggests that the gut microbiome plays a role in eating behavior and is likely a modifiable risk factor for obesity. Furthermore, research has shown that the microbiome and metabolite profile of patients with NAFLD differs at each stage of the disease. Using a machine learning algorithm, we created and validated a classifier based on the gut microbiome that highly predicts advanced fibrosis in patients with NAFLD. To explore the causal effects of the gut microbiome on the development of NAFLD, we utilized the microbiome of bariatric surgery patients and transplanted them into antibiotic treated mice. Through this model, we see that an obese phenotype microbiome is able to induce significant weight gain and hepatic steatosis. Associated with these changes we see a significant alteration of the expression levels of natural killer T-cells, cytotoxic T-cells, Kupffer cells, and monocyte-derived macrophages. The data shows that not only can the gut microbiome prognosticate NAFLD, it can also alter the progression of NAFLD through changes of the innate immune system of the liver. This work shows the feasibility of the gut microbiome both as a biomarker but also as a source for potential novel therapeutics against obesity and NAFLD.

The dissertation of Tien Sy Dong is approved.

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2020

DEDICATION

I would like to dedicate this body of work to my wife, for who's unwavering support made this possible.

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ACKNOWLEDGEMENTS

Chapter 1 is a version of "Nonalcoholic Fatty Liver Disease and the Gut Microbiome: Are Bacteria Responsible for Fatty Liver?" The manuscript is published in *Experimental Biology and Medicine*, 2019 Apr; 244(6): 408–418. The work was funded by the National Institutes of Health grant T32 DK 07180 (TD) and Veterans Affairs grant CDAII IK2CX001717 (JJ).

Chapter 2 is a version of "A Distinct Brain-Gut-Microbiome Profile Exists for Females with Obesity and Food Addiction." The manuscript is in press in Obesity, May 2020. This research was supported by grants from the National Institutes of Health including K23 DK106528 (AG), T32 DK 7180-44 (TD), ULTR001881/DK041301 (UCLA CURE/CTSI Pilot and Feasibility Study; AG), R01 DK048351 (EAM); VA Career Development Award IK2CX001717 (JPJ); and pilot funds provided for brain scanning by the Ahmanson-Lovelace Brain Mapping Center. U.S. Provisional Patent No.: 62/885,010.

Chapter 3 is a version of "A Microbial Signature Identifies Advanced Fibrosis in Patients with Chronic Liver Disease Mainly Due to NAFLD." The manuscript is published in *Scientific* Reports, 10, 2771 (2020). https://doi.org/10.1038/s41598-020-59535-w. The work was funded by the National Institutes of Health grant T32 DK 07180 (TD), the UCLA GI Fellowship Seed Grant (TD), and Veterans Affairs grant CDAII IK2CX001717 (JJ).

Chapter 4 is an ongoing work and collaboration effort with the UCLA Center for Obesity and Metabolic Health. We would like to thank Arpana Gupta, Emeran Mayer, Cathy Liu, Jean Steins, Anna Baliokova, Yijun Chen, Erik Dutson, and Claudia Sanmiguel for their significant contribution. The work was funded by the National Institutes of Health grant T32 DK 07180 (TD), ULTR001881/DK041301 (UCLA CURE/CTSI Pilot and Feasibility Study; CS), R01 DK048351 (EAM), and the VA Career Development Award IK2CX001717 (JPJ).

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Chapter 1

The Modern Landscape of Nonalcoholic Fatty Liver Disease and the Intestinal Microbiome

INTRODUCTION

The human microbiome represents all microorganisms residing on or within the human body, including bacteria, archaea, fungi, protozoans, and viruses. The human microbiome has as the same number of cells and about 100 times more genes than the human body.[1–3] These genes encode a wide array of pathways that produce bioactive molecules that are derived from dietary or metabolic precursors.[4] While one of the main function of the gut microbiome is the fermentation and energy extraction of indigestible dietary fiber, many studies have connected the microbiome and its metabolites to the development of certain diseases such as obesity, metabolic syndrome, and nonalcoholic fatty liver disease (NAFLD).[5]

The incidence of NAFLD is rapidly growing in conjunction with the epidemic of obesity and metabolic disorders.[6] The risk factors associated with NAFLD include central obesity, insulin resistance, hyperlipidemia and metabolic syndrome. Data has suggested that NAFLD is more prevalent in men as compared to women and more prevalent in patients with Asian or Hispanic heritage.[7,8] NAFLD is now the one of the most common causes of chronic liver disease in the Western world and the top two reasons for cirrhosis and liver transplantation.[9,10] NAFLD is a term that encompasses two distinct diseases: nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). While patients with NAFL have only bland steatosis on liver biopsy, patients with NASH will also have lobular inflammation and/or hepatocyte ballooning, a sign of hepatocyte damage. Patients with NAFL will often remain stable for many years and will rarely ever progress further.[11,12] Patients with NASH, however, are more likely to progress to fibrosis, cirrhosis, and hepatocellular carcinoma.[11,13] Prior epidemiological studies have shown that patients with metabolic syndrome and insulin resistance were more likely to develop NASH than NAFL, and therefore are more likely to experience worse outcomes.[14] Because of the interplay between

the microbiome and energy metabolism, there have been many recent research studies that have looked into the relationship between the human microbiome and NAFLD development and progression.

In this chapter we will explore how diets associated with obesity and NAFLD affect the microbiome and how the microbiome in turn can influence the pathogenesis of these diseases. We will also review potential mechanisms and pathways that link the microbiome to the development and progression of NAFLD. Finally, we will discuss limitations of current research and explore potential future directions including therapeutic applications.

METHODS

A comprehensive literature review was performed from 1995 to the present using the following key terms in PubMed: Nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, cirrhosis, fibrosis, obesity, metabolic syndrome, diabetes, fat, adipose, bacteria, and microbiome. Emphasis was given for articles published within the last 5 years.

DIET AND THE MICROBIOME

Diet plays a critical role in the development of obesity, metabolic syndrome, and NAFLD. Epidemiological studies have consistently shown associations of diets high in fat and refined sugars with the incidence of obesity and NAFLD.[15] In experimental models, such diets have been shown to increase adiposity, hepatic steatosis, and inflammation.[16] Here we will discuss how the diets most commonly associated with NAFLD and obesity affect the gut microbiome.

Western Diet. A Western diet is often defined as a diet that is high in sugar, fat, processed meats, simple grains, while being low in fiber.[17] It has been linked to many negative health outcomes such as obesity, insulin resistance, metabolic syndrome, and NAFLD.[17,18] Studies have shown that a

Western diet can affect many different cells including endocrine cells, adipocytes, and hepatocytes. However, there is also a growing body of evidence that links the negative effects of a Western diet to changes in the microbiome.[17] Patients on this diet will often have a significantly lower microbial diversity and species richness than those on a more agrarian diet, features that are often associated with gut dysbiosis.[19] The Western diet microbiome is often described as having a higher abundance of Firmicutes with a relatively lower abundance of Bacteroidetes.[20] The high Firmicutes to Bacteroides ratio decreases in subjects who lose weight on either a carbohydrate-restricted or fat-restricted diet.[21] Ways by which the microbiome can cause metabolic changes in the host includes changes in short-chain fatty acid production, alterations in gut hormones like peptide YY and glucagon-like peptide, and activation of toll-like receptor signaling through the production of lipopolysaccharides or endotoxins. [22] At a genus level, a Western diet is associated with depletion of *Bifidobacterium* and *Lactobacillus* and enrichment of *Enterobacter*.[23] The role of the microbiome in mediating the link between a Western diet and obesity has been examined extensively in several mice models. Colonization of germ-free mice with the microbiota of obese mice (induced by leptin-deficiency or a Western diet) results in increased body fat accumulation compared to colonization with microbiota from lean controls.[24,25] Similarly, germ-free mice colonized with feces from obese humans had increased adiposity on a high fat diet compared to germ-free mice colonized with feces from lean humans in weight discordant twin pairs.[26] Moreover, mice deficient in Toll-like receptor 5 and inflammasome components develop susceptibility to Western diet-induced obesity that can be transmitted to other mice by fecal transplantation.[26]

<u>High Saturated Fat</u>. Similar to the Western diet, diets high in saturated fats can also have deleterious effects on health and the microbiome. Epidemiological studies have shown that diets

that are high in saturated and trans-fat are associated with obesity, cardiovascular disease, and NAFLD.[27,28] Mice that are fed a diet high in saturated fat develop similar hepatic steatosis and inflammation as seen in patients with NAFLD.[28] However, not all fats have similar results. Diets high in polyunsaturated fats, such as those seen in a Mediterranean diet, have been associated with reduced cardiovascular events and a lower prevalence of obesity.[26–28] To examine the role of different fats on the microbiome, one study of 88 patients with risk factors for metabolic syndrome were fed either saturated or monosaturated fats.[32] The authors found that a diet high in saturated fats led to a reduction of bacterial species richness and an overabundance of *Faecalibacterium prausnitzii*, changes that were not seen in patients taking monounsaturated fats.[32] However, this association with *F. prausnitzii* should not be construed as negative since the introduction of *F. prausnitzii* was protective against hepatic steatosis and adipose tissue inflammation in mice fed a high fat diet.[33] In one of the largest study examining the role of saturated and polyunsaturated fats, Menni *et al* demonstrated in 876 women that a diet higher in polyunsaturated fats were associated with a higher microbial diversity that was predominated by members of the Lachnospiraceae family.[34] These findings suggest that the specific composition of fat may be more critical than the total amount of fat. This idea is corroborated in a recent animal study. Mice that were fed on a lard fat diet had a greater abundance of *Bacteroides* and *Bilophila*, and a lower abundance of *Lactobacillus* and *Akkermansia*.[35] Mice on a lard fat diet also had higher toll-like receptor signaling, white adipocyte inflammation, and insulin resistance as compared to mice fed on a fish-oil based diet.[35] The fecal transplantation of the lard fat-associated microbiome into germ-free animals was able to replicate the donor's metabolic phenotype, suggesting that these pathways are in part mediated by the microbiome.[35]

ROLE OF THE MICROBIOME IN OBESITY AND INSULIN RESISTANCE

One of the early pivotal studies that linked the microbiome to the development of obesity came from Turnbaugh *et al*. in 2006.[24] They used 16s rRNA sequencing to demonstrate an increased ratio of Firmicutes to Bacteroidetes in obese humans and experimental mice on a high fat diet, and found that colonization of germ-free mice with this obesity-associated microbial profile could induce an obese phenotype in their germ-free animals. Since then, several other studies have shown that the microbiome was able to influence weight gain by affecting host gene expression, metabolic pathways, and even the gut-brain-axis.[36–39] These pathways include short-chain fatty acid signaling and lipopolysaccharide activation of toll-like receptors.[40] All of which can lead to altered gene expression, hormone secretion, and energy consumption in adipocytes and eventual changes in host metabolism.[40] Additionally, several papers have shown that the efficacy of surgical weight loss interventions may be in part mediated by shifts in the gut microbiome.[41] For example, a study in mice found that gastric bypass led to a persistent increase in *Escherichia* and *Akkermansia,* and that the microbial transplant from these mice into non-operated germ-free mice successfully transferred the donor phenotype.[42]

While obesity is a major risk factor for the development of NAFLD, another major risk factor is insulin resistance. As such a small double blind randomized trial was performed to examine the effects of probiotics and metformin on patients with NASH.[43] Sixty-four NASH patients were randomized to either a commercially available probiotic mixture with metformin or metformin alone for 6 months. The author showed that the addition of a probiotic led to a significantly greater reduction in serum liver enzymes and hepatic steatosis as determined by ultrasound. Similarly, another randomized control trial with 66 NASH patients showed that the oral supplementation with *Bifidobacterium longum* improved both liver enzymes and insulin resistance metrics.[44] This association between the microbiome and insulin resistance led Wu *et al*

to examine the effects of metformin on the microbiome of diabetic patients.[45] They found that not only does metformin greatly alters the microbiome, some of metformin's effects on the host was recapitulated by transferring this altered microbiome into germ-free animals. Wu's and prior studies provide support that the microbiome may play a role in insulin sensitivity and demonstrates how the microbiome can potentially alter the course of NAFLD through related pathways.

MICROBIOME ASSOCIATIONS ACROSS THE SPECTRUM OF NALFD

The growing evidence linking the microbiome to obesity spurred interest in the potential role of the microbiome in other metabolic diseases including NAFLD. Here we will review evidence from human studies for microbiome associations with NAFL, NASH, and NAFLD-related advanced fibrosis.

NAFL. Studies that have examined the microbiome profile of patients with NAFL as compared to either healthy controls or weight-matched controls have yielded variable results. Pediatric NAFL patients have been reported to have more *Prevotella* and less *Oscillospira* than matched controls.[46–48] In studies of adult NAFL patients, *Lactobacillus* and *Escherichia* have been enriched while *Coprococcus* and *Prevotella* have been depleted (**Table 1-1**).[49–52] These studies utilized 16s rRNA sequencing, which can only provide insight into composition (what bacteria are there) but not function (what products are made by bacteria that may affect disease). Three studies took a multi'omics approach combining microbiome sequencing with metabolomics analysis to evaluate potential microbial metabolic pathways promoting the development of NAFL.[51–53] Raman *et al found* 18 differentially abundant stool metabolites associated with NAFL in adults, including elevated levels of derivatives of butanoic, propanoic, and acetic acid.[53] Similarly, Da Silva *et al* found enrichment of propionate and isobutyric acid in the feces of NAFL patients.[51] These differences were associated with an increase in serum 2-hydroxybutyrate and L-lactic acid. The

most convincing data to date that links the microbiome to the development of NAFL comes from Hoyles *et al*.[52] This study assessed the hepatic transcriptome, gut metagenome, along with serum and urine metabolome of a cohort of non-diabetic obese women. NAFL was associated with increased serum levels of several branched-chain and aromatic compounds. Administration of one of these, phenylacetic acid, to mice colonized with human fecal microbiota triggered hepatic steatosis.

NASH. Studies characterizing the microbiome profile of NASH compared to NAFL or obese controls have found more consistent differences than has been seen for NAFL; however, there is still variability in the findings.[54] In children, patients with NASH generally had more *Ruminococcus*, *Dorea*, *Streptococcus* and *Escherichia* as compared to their obese counterparts.[46–48,55] In adults, patients with NASH had lower levels of *Faecalibacterium*, *Ruminococcus* and *Bifidobacterium*[51,56] and a higher level of *Lactobacillus*.[57] Few studies have examined fecal or serum metabolites distinguishing NASH from simple NAFL, most likely due to the fact that a diagnosis of NASH often requires a liver biopsy in order to distinguish it from NAFL. Del Chierico *et al* showed higher levels of 4-methyl-2-pentanone and 2-butanone in the serum of children with NASH.[47] Higher levels of 2-butanone was seen in the serum of adults with NAFL[52] but the functional significance of this metabolite is still unknown. In a cohort of 16 adults with biopsy proven NASH, patients with NASH had an increased ratio of primary to secondary bile acids, which the author correlated to an increased risk of hepatic injury.[58]

NAFLD-related Advanced Fibrosis. In contrast to NAFL and NASH, which have data from both children and adults, NAFLD-related fibrosis has only been studied in adults due to the slow progression of fibrosis. Advanced fibrosis, defined as a fibrosis stage > 2, is associated with a higher incidence of mortality and liver cancer.[59] Microbiome association studies of NAFLD-

related advanced fibrosis usually reports a decrease in microbial diversity, often due to an increase in gram-negative bacteria.[60–62] Multiple studies have found an association between advanced fibrosis and an overabundance of *Bacteroides* and *Escherichia,*[60–63] while associations with other genera such as *Prevotella* have been less consistent.[61,64] Utilizing metagenomic sequencing, which allows for species level resolution, Loomba *et al* showed that *Escherichia coli and Bacteroides vulgatus* were higher in patients with NAFLD-related advanced fibrosis.[62] They also examined serum metabolites and showed that 3-phenylproanoate was the metabolite with the highest fold increase in advanced fibrosis, though it did not reach significance. Recently, Caussy *et al* found an association between 3-(4-hydroxyphenyl)lactate, a microbial metabolite involved in amino acid metabolism, to patients with NAFLD-related advanced fibrosis.[63] This metabolite was also strongly correlated with several bacterial species that were also associated with hepatic fibrosis, including *Escherichia coli*, *Bacteroides caccae* and *Clostridium sp.*[62]

POTENTIAL MECHANISMS THAT LINKS THE MICROBIOME TO FATTY LIVER DISEASE

While recent human studies have provided meaningful insights into the composition and possible function of the microbiome in each stage in the development and progression of NAFLD, the findings are largely correlative and do not provide conclusive evidence of whether the microbiome is a critical driver of NALFD or simply responds to the altered diet and host environment associated with NAFLD. Mechanistic investigation supporting a causative role for the microbiome in NAFLD pathogenesis has largely depended upon animal models. The results of studies evaluating microbial composition and metabolites in animal models of NAFLD are summarized in **Table 1-2**.[24,65–72] Overall, studies involving inflammatory pathways such as toll-like receptor signaling, choline deficiency and bile acid metabolism have been linked to NASH

while pathways associated with short chain fatty acid and amino acid metabolism have been linked more to NAFL. Here we will review the potential mechanisms by which the microbiome influences NAFLD development.

Epithelial Barrier Function, Toll-Like Receptor Signaling and Endotoxemia. In adult patients with NAFLD as well as healthy patients on a Western diet, studies have shown that these patients were more likely to have a "leaky gut" characterized by higher intestinal permeability and altered tight junctions.[73,74] This disruption in the epithelial gut barrier leads to an increased translocation of bacterial products, like lipopolysaccharide (LPS), into the portal circulation, potentially inducing hepatic inflammation. One of the very first studies to causally link the microbiome to NAFLD demonstrated that mice lacking inflammasome components – which are important to intestinal barrier defense – developed dysbiosis and NASH. Transfer of this dysbiosis to wild-type recipients could induce NASH via an influx of toll-like receptor (TLR) agonist, specifically TLR4 and TLR9, into the portal circulation.[68] Rahman *et al* showed that fibrotic steatohepatitis induced by a high fat, high cholesterol and high fructose diet was exacerbated in mice lacking a gene involved in junctional adhesion molecules, an important component of the intestinal barrier. Administration of antibiotics improved liver histology in these knockout mice, suggesting that products of microbial metabolism crossing an impaired intestinal barrier mediated the phenotype.[75] There is also a significant role of the host immune system in modulating gut permeability. Beta7 integrin-deficient mice, which are deficient in intestinal immune populations requiring this integrin for chemotaxis, show decreased insulin resistance on a high fat diet.[77] Treatment of wild-type C57BL/6 mice on a high fat diet with a local gut anti-inflammatory medication, 5-aminosalicyclic acid, reversed diet-induced bowel inflammation and improved metabolic parameters.[77] The downstream effects of LPS translocation are mediated through induction of TLR signaling in the

liver. In several studies, LPS has been shown to induce TLR4, leading to increased NF-κB activation and cytokine production important to the progression from NAFL to NASH.[78,79] Unfortunately, a recent phase 2 trial did not show any significant benefit from TLR4 antagonism in NASH patients. Therefore, the clinical relevance of this pathway in remains unclear.[80]

Choline Deficiency. The relationship between choline deficiency and NAFLD development has been well established.[81] Deficiency in choline leads to abnormal phospholipid synthesis and alterations in VLDL secretion, eventually leading to hepatic steatohepatitis.[81] Recently, dietary choline bioavailability was shown to be reduced by the gut microbiome through the production of metabolites such as trimethylamine (TMA).[82] Several gut microbes are high utilizers of choline and only low abundance of these microbes is required to greatly reduce host choline levels.[83] Mice fed a high fat diet have been shown to have increased levels of gut microbes that metabolize choline and produce TMA.[84] TMA is converted to trimethylamine-N-oxide (TMAO) by liver flavin containing monooxygenase 3.[85] Elevated levels of TMAO are associated with cardiovascular disease, which potentially links the extrahepatic manifestations of NAFLD to microbial derived metabolites.[86] However, the role of circulating TMAO in NAFLD has not been well studied.

Short-Chain Fatty Acids. One of the major functions of the human microbiome is the fermentation of indigestible carbohydrates (e.g. fiber) to produce short-chain fatty acids (SCFAs). These SCFAs include acetate, propionate, and butyrate, and they act as a major energy source for intestinal epithelial cells. SCFAs also facilitate a wide array of biological activities including hormone production and gene regulation.[87] Obese individuals as well as individuals with NAFL have higher total levels of gut SCFAs as compared to lean controls.[51,53,88] The administration of inulin-type fructan prebiotics was associated with a reduction in SCFAs in obese women along with a

reduction of other metabolic markers.[89] Conversely, certain SCFAs may be beneficial against obesity and NAFLD. One mechanism by which SCFAs can affect the host is by binding to highly specific G-protein coupled receptors (GPR), which mediate distinct effects of each SCFA. For example, in a mouse model of diet-induced obesity, a mixture of SCFA predominantly made up of butyrate reduced hepatic expression of GPR41 and GPR43, two receptors that have been shown to promote hepatic lipid accumulation.[90,91] The positive effect of butyrate was further highlighted by Mattace Raso *et al* when they demonstrated that butyrate supplementation was able to improve hepatic steatosis induced in mice by a high fat diet.[92] Furthermore, fecal microbial transplantation from lean human donors to obese patients resulted in improved insulin sensitivity, which was associated with increased abundance of butyrate-producing bacteria.[93] The inconsistent findings on SCFAs is most likely due to the distinct biological effects of individual SCFAs on host metabolism.

Bile Acid Metabolism**.** The recent development and marketing of obeticholic acid, a farnesoid X receptor (FXR) agonist, underscores the importance of bile acids for host metabolism and health. Gut microbes play a critical role in the regulation of the bile acid pool through conversion of primary bile acids to secondary bile acids, which have distinct functional properties mediated by differential binding to bile acid receptors including FXR and G-protein coupled bile acid receptor 1 (GPBAR1).[94] In a murine model of NAFLD, animals with intestine-specific FXR disruption developed changes in their gut microbiome that were associated with reduced triglyceride accumulation in response to a high fat diet as compared to controls.[95] In mice treated with antibiotics, there was an increase in conjugated bile acid metabolites that inhibited intestinal FXR signaling.[95] GPBAR1 signaling was also found to be necessary for sustained weight loss and improved fatty liver in mice undergoing sleeve gastrectomy.[96] In humans, a phase 2 clinical trial

with obeticholic acid in patients with NASH showed improved NASH by histology after 72-weeks of treatment.[97] The administration of obeticholic acid also led to a reversible induction of gram-positive bacteria in the human small intestine and increased proportion of Firmicutes in mice.[98] While initial results are promising, ongoing studies and phase 3 trials are underway in order to better understand the complex relationship between the gut microbiome, bile acid synthesis, and FXR signaling.

Amino Acid Metabolism. The gut microbiome can also affect the synthesis and metabolism of aromatic and branched-chain amino acids (BCAAs). In patients with insulin resistance, *Prevotella copri* and *Bacteroides vulgatus* were identified as the main species associated with increased BCAAs and insulin resistance.[99] The authors also showed that mice gavaged with *P. copri* developed increased insulin resistance when fed a high fat diet as compared to controls.[99] In a recent study, Hoyles *et al.* demonstrated that phenylacetic acid, an aromatic amino acid derived from microbial metabolism, was strongly associated with hepatic steatosis in humans.[52] They also showed that the addition of phenylacetic acid in both primary human hepatocyte cultures and in mice models could trigger hepatic steatosis, implying a causal effect in NAFL.[52]

THERAPEUTIC IMPLICATIONS, LIMITATIONS, AND FUTURE DIRECTIONS

The growing evidence that links the human microbiome to NAFLD progression has motivated interest in the development of novel microbiome-related therapies for NAFLD. Microbiome-related interventions include gut-specific antibiotics, probiotics, prebiotics, and fecal microbial transplant (FMT).[54] However, large well-designed clinical studies examining microbiome-related interventions in NAFLD are lacking. Several randomized controlled trials involving probiotics in NAFLD have yielded conflicting results due to the lack of standardization across studies.[100] As of yet, no randomized controlled trial involving probiotics has shown any

significant changes in BMI.[100] But several small trials have shown a potential benefit of probiotics on such important markers as insulin resistance, ALT, AST, and even histology grade (**Table 1-3**).[43,44,101–103] For example, a small randomized trial with 66 patients showed that supplementation with *Bifidobacterium longum* and fructo-oligosaccharides improved insulin resistance, hepatic steatosis and NASH activity index after 24 weeks of treatment.[44] Whether these changes would hold up in larger trials is still unclear. There is also no data available yet about the role of FMT on NAFLD, but there are two actively recruiting clinical trials designed to address this question.[104,105] However, until there is a better understanding of the key mechanistic pathways by which the microbiome promotes NAFLD, the development of microbiome-related therapies will be limited. Nonetheless, a recent multi'omics study has provided initial support for the potential application of microbes and their metabolites as noninvasive biomarkers for diagnosis and prognostication of NAFLD.[62]

Despite major recent advances in microbiome research, the field is still in its infancy with many areas that can be improved upon. One of the main issues that have made it challenging to interpret the existing literature on the microbiome and NAFLD is variation in study design. Some studies utilize healthy controls, while others select BMI-matched controls. There is also a wide age range, including separate studies of pediatric populations and adults. In addition, there is wide variation in how diet is incorporated into the analysis (if at all) and how samples are collected and processed. This makes comparisons across all NAFLD studies difficult to perform. Moreover, early studies examining the gut microbiome and NAFLD were predominantly association studies. These studies are unable to differentiate whether the microbial profile described was a potential cause of NAFLD or rather a byproduct of the environment. Furthermore, relevant microbial metabolites that reach the liver may be produced primarily in the small intestine and/or proximal

colon, which may not be well represented by the microbiome and metabolome of feces. At this time, studies are shifting away from these types of analysis and are moving towards studies focusing on mechanistic pathways by utilizing humanized animals and multi'omics analysis.[106] By transplanting human microbiota into germ-free or antibiotic treated animals, researchers can establish causal relationship of dysbiosis with NAFLD development. By using a multi'omics approach that combines microbiome analysis with other fields like proteomics and metabolomics, for example, researchers can better dissect mechanistic pathways that may lead to NAFLD development and progression.

At this time, 16S rRNA sequencing is the most common method for microbiome analysis.[106] It is effective for defining microbial composition and taxonomy to the genus and to some extent species level, but does not provide functional data (i.e. presence of bacterial genes and their expression level). In order to achieve this level of specificity, shotgun metagenomic sequencing is required. Unfortunately, due to its high cost, the sequencing of bacterial metagenomes and transcriptomes is still out of reach for many. But with ongoing advances in sequencing technology, it is likely that the cost of this service will be low enough for more widespread use in the future, similar to the widespread adoption of 16s rRNA sequencing after the dramatic decrease in sequencing costs early this decade.[108]

CONCLUSIONS

In summary, both animal models and human studies have supported the relationship between the gut microbiome and development and progression of NAFLD. By affecting gut barrier function, TLR signaling, choline metabolism, bile acid synthesis, SCFA and amino acid production, the gut microbiome appears to play a critical and multifactorial role in NAFLD development. But despite the advances in technology and bioinformatics analysis, specific mechanistic pathways are not yet

clearly defined. Future large, longitudinal, prospective studies incorporating multi-omics analysis and germ-free animals are needed to better define the multifactorial host-microbiome relationship involved in fatty liver pathogenesis.

NAFLD Subtypes	Community Composition (Genera)	Fecal Metabolites	Serum Metabolites
NAFL	↑↓[p]*Bifidobacterium*[46,52] ↑[p]↓*Lactobacillus*[46,50,51,53] ↑↓[p]*Oscillobacter*[47,50,52,53] ↑[p]↓*Prevotella*[48,49] ↑*Roseburia*[53] ↑[p]↓*Ruminococcus*[47,49,51] ↑*Blautia*[47,49] ↑*Clostridium*[50] ↑[p]*Dorea*[47,53] ↑*Escherichia*[49,52] ↑*Streptococcus*[50] ↓*Alistipes*[50] ↓*Coprococcus*[5152] ↓*Faecalibacterium*[51] ↓*Odoribacter*[50] ↓[p]*Oscillospira*[47]	↑Acetic Acid[53] ↑Butanoic Acid[53] ↑Cholic Acid[58] ↑[p]Ethanol[48] ↑Isobutyric Acid[51] ↑Propanoic acid[53] ↑Propionate[51] ↓2-butanone[53]	↑[p]2-butanone[47] ↑2-hydroxy-butyrate[51] ↑Isoleucine[52] ↑Leucine[52] ↑L-lactic Acid[51] ↑Phenylacetic Acid[52] ↑Valine[52]
NASH	↑[p]↓*Ruminococcus*[46,51,57] ↑*Allisonella*[56] ↑*Blautia*[47,49] ↑*Clostridium*[58] ↑*Dorea*[46] ↑*Escherichia*[48] ↑*Lactobacillus*[46,57] ↑*Parabacteroides*[56] ↓*Bifidobacterium*[46,57] ↓*Coprococcus*[51] ↓*Faecalibacterium*[51,56,57] ↓*Oscillospira*[46]	↑Chenodeoxycholic acid[58] ↑Cholic Acid[58] ↑Lithocholic Acid[58]	↑[p]2-butanone[47] ↑[p]4-methyl-2-pentanone[47] ↑Ethanol[48]
NAFLD-Related Advanced Fibrosis	↑↓*Prevotella*[60,61] ↑↓*Ruminococcus*[60–62] ↑*Bacteroides*[60–63] ↑*Blautia*[61] ↑*Enterococcus*[61] ↑*Escherichia*[62,63] ↑*Klebsiella*[60] ↑*Lactobacillus*[61] ↑*Parabacteroides*[61] ↑*Roseburia*[61] ↑*Streptococcus*[61] ↓*Akkermansia*[61]		↑3-(4-hydroxyphenyl)-lactate[63] ↑3-phenyl-propanoate[62]

Table 1-1: Bacteria genera and fecal/serum metabolites associated with different stages of non-alcoholic fatty liver disease in human studies. [p] Denotes an association that has been reported only in pediatric cases of NAFLD.

NAFLD Animal Models	Community Composition (Genera)	Fecal Metabolites	Serum Metabolites
NAFL (high fat diet or leptin deficient mice)	↑*Bacteroides*[70] ↑*Barnesiella*[72] ↑***Bilophila***[7169] ↑***Dorea***[71] ↑*Helicobacter*[70] ↑*Oscillospira*[70] ↑*Roseburia*[72] ↑***Sutterella***[71] ↓↑*Allobaculum*[72] ↓↑*Lactobacillus*[67,72] ↓*Akkermansia*[69–71] ↓***Bifidobacterium***[71] ↓*Flavobacterium*[70] ↓*Marinitoga*[70] ↓*Parabacteroides*[70,71] ↓***Ruminococcus***[71]	↑Butyrate[24] ↓Deoxycholic acid (relative abundance)[71] ↓Hyodeoxycholic acid (relative abundance)[71]	Taurine conjugated bile acid[71]
NASH (NASH inducing diet, i.e. methionine-choline deficient diet)	↑(f) Bacteroidaceae[68] ↑(f) Erysipelotrichaceae[68] ↑(f) Porphyromonadaceae[68] ↑(f) *Clostridiaceae*[68] ↑***Alistipes***[65] ↑***Bacteroide***[65,66,68] ↑***Bilophila***[66] ↑***Blautia***[66] ↑***Parabacteroides***[68] ↑*Turicibacter*[68] ↓***Akkermansia***[66] ↓***Bifidobacteriu***[65,66] ↓***Desulfovibrio***[66] ↓***Enterorhabdus***[66] ↓***Lactobacillus***[68]	↑Hexadecane[65] ↑Tetracosane[65] ↓Arachidic acid[65] ↓Cholic acid[65] ↓Stearic acid[65]	

Table 1-2: Bacterial genera and fecal/serum metabolites associated with NAFL and NASH development in animal models. Wild-type mice on a control diet serve as the reference group.

Study	Number of Patients	Intervention	Major Findings
Aller *et al.*[101]	30	*Lactobacillus bulgaricus* + *Streptococcus thermophilus* vs placebo for 3 months	Decrease in ALT, AST, GGT
Alisi *et al.*[102]	44[p]	VSL#3 vs placebo for 4 months	Ultrasound improvement in fatty liver
Eslamparast *et al.*[103]	19	Synbiotic vs placebo for 28 weeks	Decrease in ALT, AST, GGT, CRP, TNFα, fibrosis score by transient elastography
Malaguarnera *et al.*[44]	66	Bifidobacterium longum + fructo-oligosaccharides + life-style modification vs life-style modification alone	Decrease in AST, LDL, CRP, TNFα, HOMA-IR, steatosis, and NASH activity index
Shavakhi *et al.*[43]	64	Protexin + Metformin vs Metformin alone	Decrease in ALT, AST, ultrasound grading of steatosis
Wong *et al.*[109]	20	Lepicol vs nothing	Decrease in intrahepatic triglyceride content as measured by proton-magnetic resonance spectroscopy

Table 1-3: Summary of randomized control trials involving NAFLD and probiotics. [p] Denotes a pediatric trial.

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Chapter 2

The Role of the Gut Microbiome on the Gut-Brain Axis in Relation to Obesity and Food Addiction

INTRODUCTION

Obesity has reached global epidemic proportions and has become the leading preventable cause of death, both in the United States and worldwide. Current estimated global prevalence rates are as high as 500 million adults who are considered obese, and these numbers continue to rise dramatically.[1] Obesity is associated with many comorbidities including numerous cancers and musculoskeletal disorders, diabetes, and premature mortality from cardiovascular disease (CVD).[2] In addition to health detriments, economic and social consequences of obesity are compounding. In 2011, medical costs associated with treatment of preventable diseases associated with obesity were estimated to increase by $48-66 billion/year in the U.S. alone, with an estimated 65 million more adults to become obese by 2030.[3]

Food addiction (FA) is a potential driver of obesity where the hedonic aspect of ingestive behaviors overrides the homeostatic mechanisms.[4] Overeating and sedentary lifestyles result in a positive energy imbalance, leading to adipose tissue accumulation. According to the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV), criteria for the diagnosis of substance addiction includes: 1) taking the substance in larger amounts than was intended, 2) inability to control its use, 3) taking the substance for a longer period of time than was intended, and 4) continued use despite adverse consequences.[5] For those that study FA, the *Yale Food Addiction Scale* (YFAS) has been developed and validated as a psychometrically sound measure to operationalize human cases of FA using the DSM-IV criteria for substance abuse.[4] While many believe that FA is a distinct entity from other behavioral eating disorders, the concept of food addiction is still a controversial issue even within the newest update of the DSM-V.[6]

Both obesity and FA can affect the connectivity of the brain. In several human cross-sectional studies, obesity has been shown to be significantly associated to alterations of the brain's

dopaminergic pathways in the brain's reward system (i.e. striatum, prefrontal cortical regions and amygdala).[7] These alterations are associated with dysregulation of reward sensitivity, motivation, and impulse control associated with unregulated ingestive behaviors.[7] FA has been shown to be correlated with neural activation in key reward brain regions which are activated in other addiction disorders and contributes to increased cravings, reduced inhibitory control related to ingestive behaviors, and higher levels of obesity.[8] Recent evidence has shown that obesity with hedonic or disinhibited eating patterns is associated with increased dopaminergic and GABA signaling both in animal and human models of food addiction and obesity.[6]

The gut microbiome is also another area that is gaining recognition as a significant player in the etiology of many diseases as well as obesity. Research has shown that deviation from a core, lean gut microbiome profile is reflective of obesity.[9] In addition to reduced bacterial diversity, there is alteration in bacterial gene representation and phylum-level modifications.[9] Obesity-associated gut microbiomes also show altered pathways of food metabolism. Correlations between obesity pathophysiology and the gut microbiome have been observed through metagenomic and biochemical analyses, demonstrating that obese gut microbiomes absorb energy at higher efficiencies than lean gut microbiomes.[10] This superfluous harvesting of energy results in accumulation of body fat. Existing studies have focused on the effect of short chain fatty acids (SCFAs) on ingestive behavior in animal models.[11] However, given our current understanding of the gut microbiome and obesity, there are very few studies that have examined the relationship between the gut microbiome and its metabolites with ingestive behaviors in obesity, especially in humans. Among these very few studies, tryptophan metabolites have been most closely implicated in modulating brain-gut-microbiome interactions within this context.[12]

Given these relationships, we aimed to test the hypothesis that the role of microbial profiles and tryptophan metabolites were significantly associated with food addiction as well as key reward regions of the brain in females with high BMI.

METHODS

Subject population

The prevalence of FA differs by sex, with females with obesity having a higher prevalence (15-30%) than males with obesity (~5%).[6] Due to the higher prevalence of FA in females,[6] we focused our analysis to female subjects. The sample was comprised of 105 right-handed female subjects, between the age of 18-50 years old without significant medical or psychiatric conditions. Medical and psychiatric conditions were screened using a standardized screening sheet and a physical exam by a trained registered nurse. Subjects were excluded for the following reasons: pregnant or lactating, substance use disorder, abdominal surgery, tobacco dependence (half a pack or more daily), extreme strenuous exercise (>8h of continuous exercise per week), current or past psychiatric illness and major medical (including inflammatory bowel disease, active malignancy, organ failure, and diabetes) or neurological conditions (including Alzheimer's disease, Parkinson's disease, history of stroke, traumatic brain injury, or seizure disorder). Subjects taking medications that interfere with the central nervous system or regular use of analgesic drugs were excluded. Since female sex hormones such as estrogen are known to effect brain structure and function, we only included females who were premenopausal. Subjects with hypertension, diabetes, metabolic syndrome or eating disorders were excluded to minimize confounding effects. No subjects exceeded 400lbs due to magnetic resonance imaging scanning weight limits. Subjects were also excluded if they had been on antibiotics or probiotics with 3 months of recruitment.

Multimodal magnetic resonance brain imaging (MRI), anthropometrics (height, body weight, and waist-hip ratio measurements, body mass index), measures of appetite and FA, and stool samples for 16S ribosomal RNA gene sequencing and metabolomics were collected.

All procedures complied with the principles of the Declaration of Helsinki and were approved by the Institutional Review Board at our institution (IRB # 16-000187).

Food Addiction Questionnaire

FA was assessed using the Yale Food Addiction Scale (YFAS) questionnaire, a 25-item scale developed to assess food addiction by assessing signs of substance-dependence symptoms in eating behavior.[13] This scale is based upon the substance dependence criteria as found in the DSM-4 (e.g., tolerance [marked increase in amount; marked decrease in effect], withdrawal [agitation, anxiety, physical symptoms], and loss of control [eating to the point of feeling physical ill]).[13] The YFAS questionnaire is a 25-question survey that measures several aspects of FA behavior: food dependence, withdrawal, tolerance, continued use despite problems, time spent eating, loss of control, inability to cut down, and clinically significant impairment. Food addiction was defined as having a YFAS symptom count $\geq$3 with clinically significant impairment or distress. Clinically significant impairment or distress was defined as having a at least one positive response to the following two questions in the YFAS questionnaire: "My behavior with respect to food and eating causes significant distress" and "I experience significant problems in my ability to function effectively (daily routine, job/school, social activities, family activities, health difficulties) because of food and eating," similar to previously published works.[6] The YFAS has displayed a good internal reliability (Kuder–Richardson α=0.86).[13]

Intestinal Microbial 16S rRNA Gene Sequencing

Stool was collected within 1 week of the patient's brain MRI scan. All samples were stored at -80^{o}C before 16S rRNA sequencing. DNA was extracted from frozen fecal samples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) with bead beating following the manufacturers protocol. The V4 hypervariable region of the 16S rRNA gene was amplified using the 515F and 806R primers. PCR products were purified by a commercial kit and sequenced on the Illumina HiSeq 2500 platform. The base pair reads were processed using QIIME v1.9.1 with default parameters. The taxonomic assignments of sequences were performed using closed reference operational taxonomic unit (OTU) picking in QIIME against the Greengenes database pre-clustered at 97% identity. OTUs were removed if they were present in less than 10% of samples.

Fecal metabolomics

Fecal samples were aliquoted under liquid nitrogen and shipped to Metabolon for processing and analysis as a single batch on their global metabolomics and bioinformatics platform. Data was curated by mass spectroscopy using established protocols and software as previously described. Because of our interest in the gut-brain axis in obesity, only tryptophan derived metabolites were examined.

Brain Magnetic Resonance Imaging

Whole brain structural and anatomical connectivity (diffusion tensor imaging; DTI) data was acquired using a 3.0T Siemens Prisma MRI scanner (Siemens, Erlangen, Germany). DTI measures the microscopic properties of white- matter and fiber track connectivity. Detailed information on the standardized acquisition protocols, quality control measures, and image preprocessing are provided in previously published studies.[14]

Acquisition:

Structural MRI. High resolution T1-weighted images were acquired: echo time/ repetition time (TE/TR) =3.26ms/2200ms, field of view (FOV)=220×220mm slice thickness=1mm, 176 slices, 256×256 voxel matrices, and voxel size=0.86×0.86×1mm.

Diffusion-weighted MRI. Diffusion-weighted magnetic resonance imaging was acquired according to two comparable acquisition protocols, in either 61 or 64 noncolinear directions with b=1000 s mm^{-2}, with 8 or 1 b=0 s mm^{-2} images respectively, TE/TR= =88ms/ TR=9500 ms, and FOV =256 mm with an acquisition matrix of 128x128, and a slice thickness of 2 mm, resulting in DTI data with 2mm isotropic resolution.

Quality Control and Preprocessing of images:

Structural images were included based on compliance with acquisition protocol, full brain coverage, minimal motion (Gibbs ringing), absence of flow/zipper, and minor atrophy/vascular degeneration. Preprocessing for quality control involved bias field correction, co-registration, motion correction, spatial normalization, tissue segmentation, Fourier transformation for frequency distribution, and specific quantitative checks for DTI images (apparent diffusion coefficient and fractional anisotropy [FA]) as previously described.[15] Preprocessing for diffusion-weighted imaging included visually checking for artifacts and motion on the raw diffusion weighted and b0 images, visual assessment of FA and mean diffusivity (MD) map quality, as well checking for physiologically feasible FA and MD values (FA of 0–0.1 and MD of 3–4 μm2/ms in ventricles, and FA of 0.6–0.9 and MD of 0.6–0.9 μm2/ms in splenium of corpus callosum). Maximum relative motion thresholds for translation and rotation for each direction (x, y, and z) were set at 2mm and 2°, respectively. No subjects presented with serious adverse imaging artifacts and no subjects exceeded motion thresholds.

Structural Image Parcellation and Anatomical Pairwise Network Construction:

T1-image segmentation and regional parcellation were conducted using FreeSurfer v.5.3.0[16,17] following the nomenclature described in the Destrieux and Harvard-Oxford subcortical atlas.[18] This parcellation results in the labeling of 165 regions, 74 bilateral cortical structures, 7 subcortical structures, the midbrain, and the cerebellum.[19]

Regional parcellation and tractography results were combined to produce a weighted, undirected connectivity matrix. The final estimate of white matter connectivity between each of the brain regions was determined based on the number of fiber tracts intersecting each region. Weights of the connections were then expressed as the absolute fiber count divided by the individual volumes of the two interconnected regions.[20]

Brain Regions of Interest

Based on previous research,[14] regions of interest were restricted to core regions of the reward network (basal ganglia: caudate nucleus, globus pallidum, putamen, thalamus, nucleus accumbens (NAcc), amygdala, and brainstem [including the substantia nigra/SN and ventral tegmental area/VTA]), as these regions have been implicated in brain-gut axis alterations associated with obesity.[21]

Statistical Analysis

Baseline demographic characteristics differences were compared using student's test for continuous variables and chi-squared test for categorical variables. Means are expressed with their respective standard deviation. Multilevel sparse partial least square linear discriminant analysis (sPLS-DA) was done to analyze microbiome data using the Mixomics package in R (http://www.R-project.org). sPLS-DA identifies OTUs that discriminated subjects with no FA from those with FA by simultaneously performing feature selection and modeling using lasso penalization. sPLS-DA operates using a supervised framework to find linear combinations of a

limited set of variables, here OTUs, that predicts *FA* status, similar to prior published works.[22] Microbial alpha diversity (i.e. diversity within a sample) were calculated in QIIME using OTU-level data rarefied to 34,222 sequences. The significance of differences in alpha diversity metrics - Faith's phylogenetic diversity (Faith's PD), Chao1, and Shannon index - was calculated by analysis of variance. Association of microbial genera with or without FA were evaluated using DESeq2 in R, which employs an empirical Bayesian approach to shrink dispersion and fit non-rarified count data to a negative binomial model. P-values for differential abundance were converted to q-values to correct for multiple hypothesis testing (< 0.05 for significance). Metabolomics data were normalized and then fitted to a gaussian model with the limma package in R. Brain imaging data was compared between individuals without FA as compared to those with FA using a generalized linear model in R and p-values adjusted using false discover rate for multiple comparisons. Because obesity is closely related to FA, microbial analysis, metabolomics, and brain imaging analysis was all adjusted for obesity by doing subgroup analysis of obese patients with or without FA, and normal weight individual versus patients with obesity without FA. A BMI of <25 was considered normal, a BMI $\geq$ 30 as obese. To determine microbial associations with brain imaging data and metabolomics, significant variables were dichotomized based on their median values before being applied to DESEq2 in R.

Random Forest Classifier

A random forest classifier was created in R to identify obese subjects with FA using the randomForest package (https://cran.r-project.org/web/packages/randomForest) with 1001 trees and mtry=2. The number of trees were varied from 100 to 10,000 at intervals of 500, and 1000 trees were selected as the parameter as it minimized the out-of-bag estimate of error. An odd number was used to prevent theoretical ties that may occur from forest generation. Similarly,

various mtry were selected and an mtry of 2 was used as it gave the highest area under the receiver operating curve (AUC). Features in the random forest classifier included OTUs that were significantly different by DESeq2 analysis and metabolites and brain imaging data that was statistically different in obese subjects with FA. The accuracy of the random forest classifier was estimated using a 5-fold cross-validation.

RESULTS

Patient Characteristics

105 female subjects were enrolled in the study. The average age was 32.4 years $\pm$ 10.2. Based on a YFAS symptom count of $\geq$3 with clinically significant impairment or distress, indicating the presence of FA, 19 subjects (18.1%) were identified with FA (**Table 2-1**). There was no statistical difference by age in subjects with FA as compared to those without FA. 89.5% (17/19) of the subjects with FA were obese as compared to 39.5% (34/86) of subjects without FA. The average BMI in subjects with FA was 35.6 $\pm$ 5.3 and the average BMI in subjects without FA was 29.1 $\pm$ 5.4 (p-value=0.0001). There were no statistical differences between subjects with FA or without FA in regards to race or ethnicity (p-value =0.55).

Gut microbial signature as it relates to food addiction

There were no statistical differences in beta-diversity using Bray-Curtis dissimilarity between obese subjects with or without FA. There were also no statistical differences in any alpha diversity metrics between the microbial samples of obese subjects with FA as compared to those without FA. However, there was a significant difference in alpha diversity metrics by race/ethnicity with Caucasians and African-Americans having higher diversity than Hispanics. After adjusting for race/ethnicity there were no differences in subjects with a BMI of $\geq$25 to normal

weight individuals by any alpha diversity metric. The taxonomic profiles of subjects with FA compared to subjects without FA on a phylum and genus level are summarized in **Figure 3-1a and 3-1b**, respectively. DESEq2 analysis of patients without FA identified 22 distinct OTUs that were associated with obesity as compared to normal weight individuals (**Figure 3-1c**). Six OTUs were negatively correlated and 16 OTUs were positively correlated to obesity. The four highest abundant OTUs belong to the genera *Bacteroides,* and *Prevotella.* The OTU with the greatest negative fold change was *Akkermansia muciniphila*. DESEq2 analysis of patients with obesity identified 15 OTUs that were associated with FA (**Figure 3-1d**). Ten OTUs were negatively associated with FA and 5 OTUs were positively associated with FA. The largest abundant OTU belonged to the genus *Bacteroides,* while the OTU with the largest positive fold change was *Megamonas* and the OTU with the largest negative fold change was *Eubacterium biforme*. Similarly to patients with obesity without FA as compared to normal weight patients, *Akkermansia muciniphila* was negatively associated with FA in patients with obesity.

Supervised learning methods were applied to identify a distinct microbial signature that differentiated between obese subjects and normal weight subjects without FA (**Figure 3-2a and 3-2b**) as well as obese subjects with or without FA (**Figure 3-2c and 3-2d**). Through the model, patients with normal BMIs were separated from patients with obesity by differences in OTUs belonging to such taxa as *Bacteroides*, *Blautia*, Lachnospiraceae, Ruminococcaceae, *Roseburia*, *Faecalibacterium* and *Clostridium*. In patients with obesity, over 30 different OTUs separated patients with FA from those without FA. Notable OTUs that distinguished patients without FA included *Eubacterium biforme* and *Bacteroides*. *Streptococcus* was the taxa with the largest contribution for obese subjects with FA.

Brain reward networks and food addiction

DTI pairwise MRI showed greater anatomical connectivity between the putamen (a key reward region) and the brain stem (Cohen's *d* =1.12, p.adj value= 0.0415) and intraparietal sulcus/transverse parietal sulcus (IntPS/TrPS) (Cohen's *d*=0.89, p.adj value=0.002) in obese subjects with FA compared to those subjects without FA (**Figure 3-3a, 3-3b, 3-3c**). Using DESEq2 analysis and dichotomizing the brain imaging data based on their respective median values, 17 OTUs were associated with an increase communication between the brain stem and the putamen (**Figure 3-3e**). Similarly, 10 OTUs were associated with an increase communication between the putamen and the IntPS/TrPS (**Figure 3-3f**). The OTU belonging to the genus *Megamonas* was positively associated with increase connectivity in both of these brain regions. Conversely, OTUs that belonged to *Bacteroides* and *Eubacterium* were negatively associated with the connections of both of these brain regions. *Akkermansia* was also negatively associated with the connection between the putamen and the IntPS/TrPS but was not associated with the connection between the putamen and the brain stem. There were no significant differences in DTI pairwise MRI brain imaging of patients with obesity without FA as compared to normal weight individuals. There were also no significant differences when comparing obese and overweight patients without FA to normal weight individuals.

Indolepropionate is associated with food addiction

By analyzing fecal metabolites that were related to tryptophan metabolism, we found that indolepropionate was negatively associated with FA in patients with obesity (Cohen's *d*, 0.74, p-value=0.045) (**Figure 3-3d**). By analyzing the level of indolepropionate with fecal microbiome data, we discovered 14 OTUs that were correlated with indolepropionate (**Figure 3-3g**). The highest abundant OTU that positively correlated to indolepropionate belonged to the genus *Bacteroides*. The highest abundant OTU that was negatively correlated to indolepropionate

belonged to the genus *Prevotella*. All the OTUs that were positively associated with indolepropionate belonged to *Akkermansia muciniphila* and *Bacteroides*. There were no significant differences in fecal tryptophan metabolites of patients with obesity without FA as compared to normal weight individuals. There were also no significant differences in fecal metabolites when comparing obese and overweight patients without FA to normal weight individuals.

Random forest classifier based on brain imaging, fecal metabolite, and 16S sequencing accurately identifies subjects with food addiction

Using the significant findings on brain imaging, fecal metabolite, and DESEq2 analysis of the fecal microbiome, a random forest classifier was created with a high accuracy for predicting obese subjects with FA behaviors. The AUC in 5-fold cross-validation was 0.81 (**Figure 3-4a)**. The contribution of each variable was expressed with a variable importance score, which measures the decrease in accuracy of the classifier if that feature was removed. The variables with the highest scores were those pertaining to brain imaging and indolepropionate. Seven OTUs also contributed significantly to the classifier and those OTUs belonged to Barnesiellaceae, Ruminococcaceae, *Desulfovibrio, Bacteroides, Eubacterium,* and *Megamonas* **(Figure 3-4b)**.

DISCUSSION

To our knowledge, this is the first study to utilize a systems biology approach to demonstrate associations between FA and changes in brain-gut-microbiome interactions by analyzing fecal microbes, metabolites, and anatomical connectivity (DTI) brain data. FA behaviors in females were associated with a distinct microbial profile, increased connectivity with the

putamen of the reward center of the brain, and a decrease in indolepropionate, a tryptophan derived microbial metabolite.

The study results indicated a strong negative association between *Bacteroides, Akkermansia, and Eubacterium* with FA. *Bacteroides* is the major genus belonging to the phylum Bacteroidetes. In both human and mouse studies, a rise in Bacteroidetes is often associated with a leaner phenotype.[10] In bariatric studies, subjects that had the most significant weight loss were those that had higher levels of *Bacteroides* and lower levels of *Prevotella*.[23] In a prospective study, *Bacteroides* species were higher in lean individuals and those subjects who were able to achieve weight loss as compared to subjects with obesity.[24] Whether the associations noted between *Bacteroides* and obesity are causative is still an area of active research. In our data, *Bacteroides* was positively associated with indolepropionate and negatively associated with brain regions related to FA.

Akkermansia was also another genus that was significantly associated with FA, brain imaging, and fecal metabolites. *Akkermansia* is a mucin-degrading bacterium that has been extensively studied for its protective role in metabolic syndrome and insulin sensitivity both in human and mouse studies.[25] In a study of 41 females with obesity undergoing calorie restriction, an increase in relative abundance of *Akkermansia* was associated with improved fasting glucose, waist-to-hip ratio, and subcutaneous adipocyte diameter.[26] This led to a recent phase 1 randomized double-blind, placebo- controlled clinical trial showing that *Akkermansia* supplementation in obese/overweight volunteers led to improved insulin sensitivity, reduced plasma cholesterol, and a trend towards decreased body weight and fat mass.[27]

However, unlike *Akkermansia* and *Bacteroides, Eubacterium biforme* has not been as well studied with regards to obesity or metabolic syndrome. In our study we show *Eubacterium* to be

negatively associated with FA as well as key areas of the brain reward network. Similar to *Bacteroides, Eubacterium* is known to be a significant producer of short-chain fatty acids.[28] Short-chain fatty acids is the by-product of bacterial fermentation of indigestible dietary fiber. The most abundant short-chain fatty acid is butyrate and several animal studies have shown that butyrate can be protective against obesity by increasing GLP-1, leptin release, and increasing fatty acid oxidation.[28] Butyrate is also able to communicate directly with the central nervous system by crossing the blood-brain barrier and activating the vagus nerve and hypothalamus. [28]

While *Akkermansia*, *Bacteroides*, and *Eubacterium* were negatively associated with FA, *Megamonas* was one of the few bacteria that was both positively associated with FA and an increased activity of the reward network of the brain. In human studies, *Megamonas* has been associated with an increase prevalence of prediabetes [29] and childhood obesity.[30] In context, these associations between the gut microbiome and obesity may be mediated through interactions involving the gut-brain axis.

In our study, we also saw that alpha diversity did not differ between subjects with or without FA but it was seen that alpha diversity did differ by race and ethnicity, which may be a reflection of dietary differences across these groups. Larger samples will allow for future analyses to account for cultural factors and for race and ethnicity differences.

Analysis of fecal metabolites revealed a negative association between indolepropionate and FA. Microbial analysis showed that *Bacteroides* and *Akkermansia* was positively correlated with indolepropionate while bacteria belonging to the phylum Firmicutes were negatively associated. This finding is in line with the numerous studies that have shown an increase in Firmicutes and a decrease in *Bacteroides* in patients with diabetes, metabolic syndrome and obesity.[31] Indolepropionate belongs to a larger class of tryptophan-derived metabolites termed “indoles.” In

contrast to other tryptophan derived metabolites (serotonin, kynurenine), which have also been implicated in brain-gut-microbiome interactions in obesity, indoles are the result of exclusively microbial metabolism, in which most undigested dietary tryptophan in the gut is converted to indoles.[32] The results we present here are consistent with our previously published work, where we describe associations between indoles on key regions of the extended reward network and both obesity and FA.[12] Indoles play an important role in modulating kynurenine synthesis, reducing central nervous system inflammation, improving the mucosal intestinal barrier, and altering GLP-1 secretion[33,34] all of which have been shown to be disrupted in states of obesity. Although indolepropionate has been less extensively studied, previous work has demonstrated a neuroprotective role of indolepropionate against Alzheimer's disease and neural oxidative stress.[35] Furthermore, a Finnish study of 200 subjects showed that a higher level of serum indolepropionate acid was associated with a reduce risk of type 2 diabetes.[36] This data suggests that indolepropionate may have both local protective effects on intestinal barrier function as well as remote effects on preserving β-cell function and central nervous system inflammation.

In this study we were also able to demonstrate that decreased fecal indolepropionate was associated with not only increased FA behaviors, but that this was related to increased connectivity between key reward regions involving the putamen. In line with the previous fecal microbiome data in FA and indolepropionate, we see a negative association of *Bacteroides* and *Akkermansia* to the connectivity between the putamen and the intraparietal sulcus. Normal eating behavior is under the control of the brain's homeostatic system and hedonic system, which includes regions involved in the processing of food-seeking behavior, inhibition, and integrating information to make decisions regarding food intake.[37,38] However, in both FA and obesity, activity within the extended reward network can override the homeostatic system.[8,37] This dominance of hedonic over

homeostatic influences on eating behavior has been related to the ubiquitous presence of cheap, highly palatable, high caloric foods, which are enhanced for taste and salience. This hedonic dominance not only leads to increases in cravings and ingestion of these foods, but environmental factors such as stress and adversity can serve as conditional cues for future food intake and long-term weight gain.[39] Some studies have indicated that overconsumption of highly palatable foods rich in calories, fat, and sugar reduce the reward thresholds of such foods when ingested, and therefore require a higher intake to generate the same satisfaction.[40]

Integrating the significant findings on brain imaging, fecal metabolites, and the fecal microbiome, we created a random forest classifier, which demonstrated a high accuracy for predicting obese individuals with FA behaviors. Next to indolepropionate and connectivity of key reward network region (the putamen), the bacterial genera with significant contribution to the classifier that were also significant in other analysis belonged to *Bacteroides*, *Eubacterium*, and *Megamonas*.

There are several limitations to our study. Because of the cross-sectional design the results only show associations between behavior, gut microbiome and brain structure. However, in the absence of a truly valid food addiction model in animals and the challenges of doing studies in humans that address the bidirectional BGM interactions, cross sectional studies are essential first steps to identify correlations within the BGM axis in humans. Another limitation is that this study enrolled only females, and due to the lower prevalence rates of FA in males compared to females, it would require larger sample sizes to observe the same effects in males. Lastly, this data should be validated in an external cohort to confirm the accuracy of the classifier.

In conclusion, food addiction refers to maladaptive ingestive behaviors resulting from a shift from primarily homeostatic to hedonic regulatory mechanisms of food intake which

primarily occurs in individuals with obesity. This shift reflects alterations at all levels of the BGM axis. The results of our study suggest that FA behavior may be mediated via effects of the gut microbiome and their metabolites on the reward centers of the brain (**Figure 3-5**). If confirmed in follow up studies, these findings suggest the possibility of targeting the brain-gut-microbiome axis to combat FA behavior and obesity.

Table 2-1: Baseline characteristics by food addiction

	No Food Addiction (No FA) (n=86)	**Food Addiction (FA) (n=19)**	**p-value**
Age (mean +/- SD) (yrs)	33.19 ± 10.31	28.57 ± 8.66	0.07
BMI (mean +/- SD)	29.1 ± 5.4	35.6± 5.3	**0.0001**
Normal Weight (n=16) %	18.60	0.00	
Overweight (n=38) %	41.86	10.52	
Obese (n=51) %	39.53	89.47	**0.0001**
YFAS Symptom Count (mean +/- SD)	1.15 ± 0.77	3.63 ± 1.16	**<0.0001**
Race/Ethnicity			
Hispanic (n=41) %	36.05	52.63	
Caucasian (n=28) %	23.26	42.11	
African American (n=13) %	12.79	10.53	
Asian (n=21) %	20.93	15.79	
Other (n=2) %	2.33	0	0.55

SD: Standard deviation

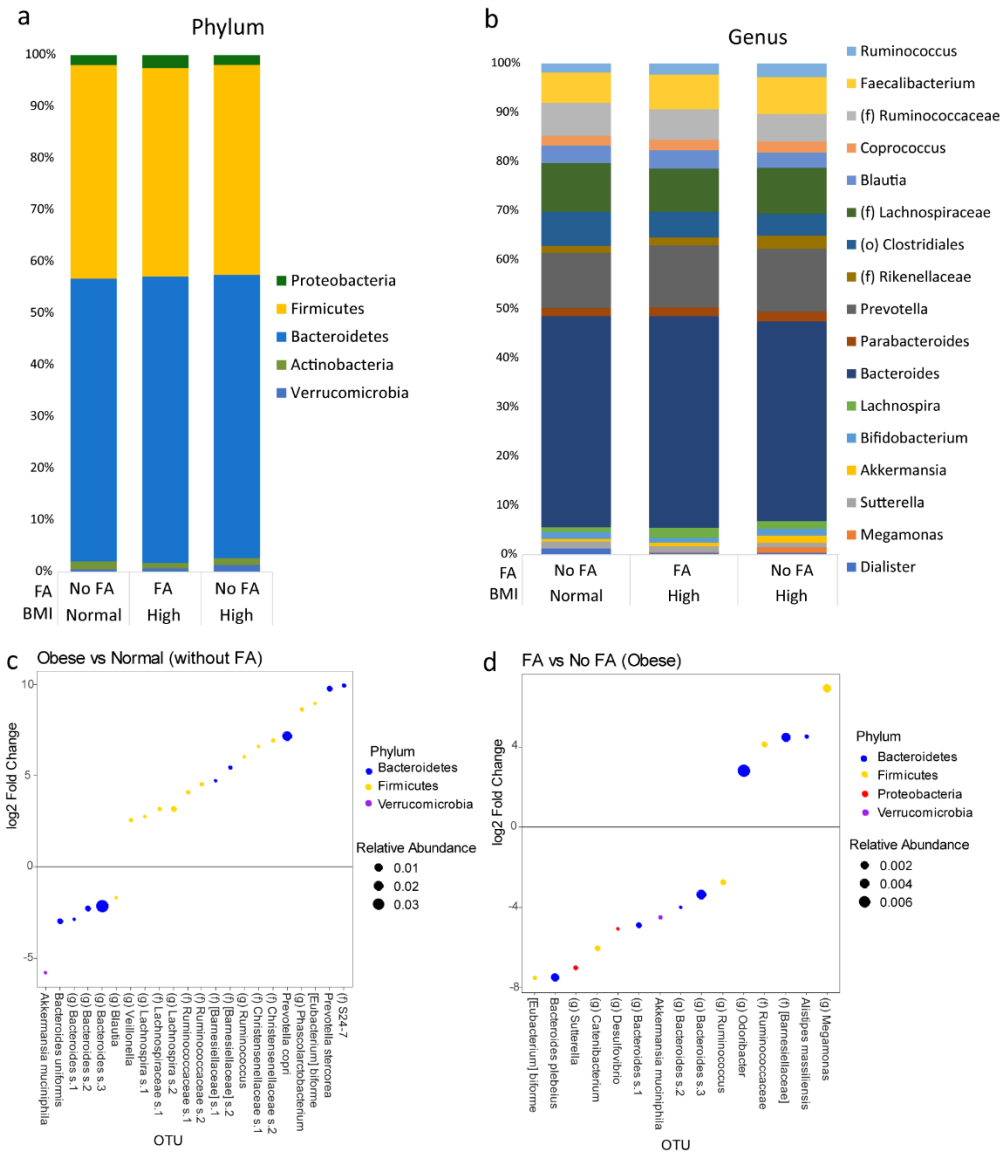

Figure 2-1: Several taxa are associated with food addiction. Taxonomic profiles between subjects with No Food Addiction (No FA) and Food Addiction (FA) on a (a) phylum and (b) genus level. Only taxa ≥ 1% relative abundance are shown. (c) DESEq2 analysis of patients without FA comparing those with obesity and those with normal BMI showing several OTUs associated with Obesity. (d) DESEq2 analysis of only patients with obesity showing several OTUs that are associated with FA.

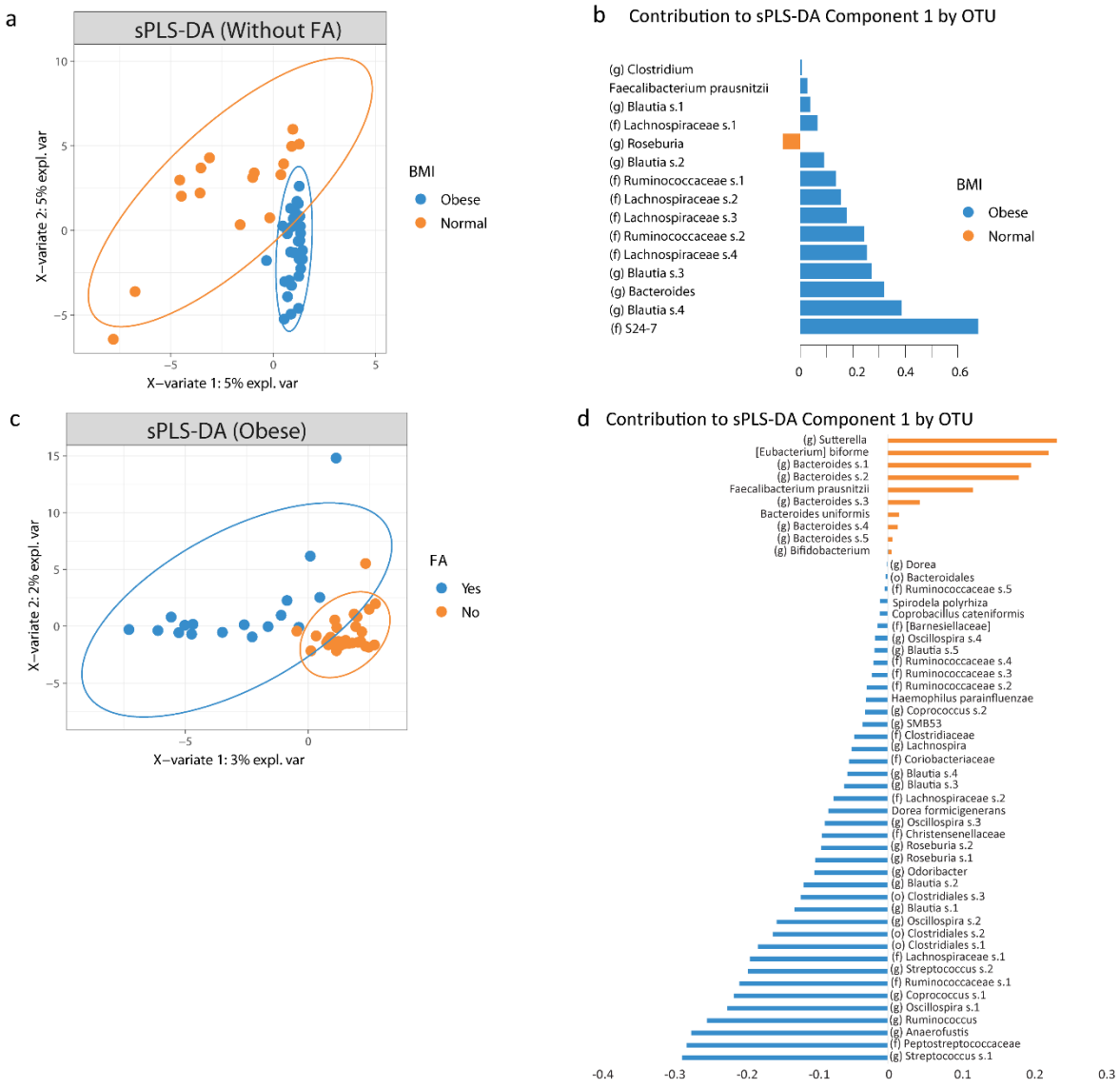

Figure 2-2: A distinct microbial profile differentiates subjects with obesity and food addiction from those without. A) Plot of the partial least square discriminant analysis of the gut microbiome composition between subjects with obesity without food addiction versus those with normal BMI and without FA along with their 95% confidence ellipses and B) contributing OTUs to component 1. C) Plot of the partial least square discriminant analysis of the gut microbiome composition between obese subjects with food addiction versus those without FA along with their 95% confidence ellipses and D) contributing OTUs to component 1.

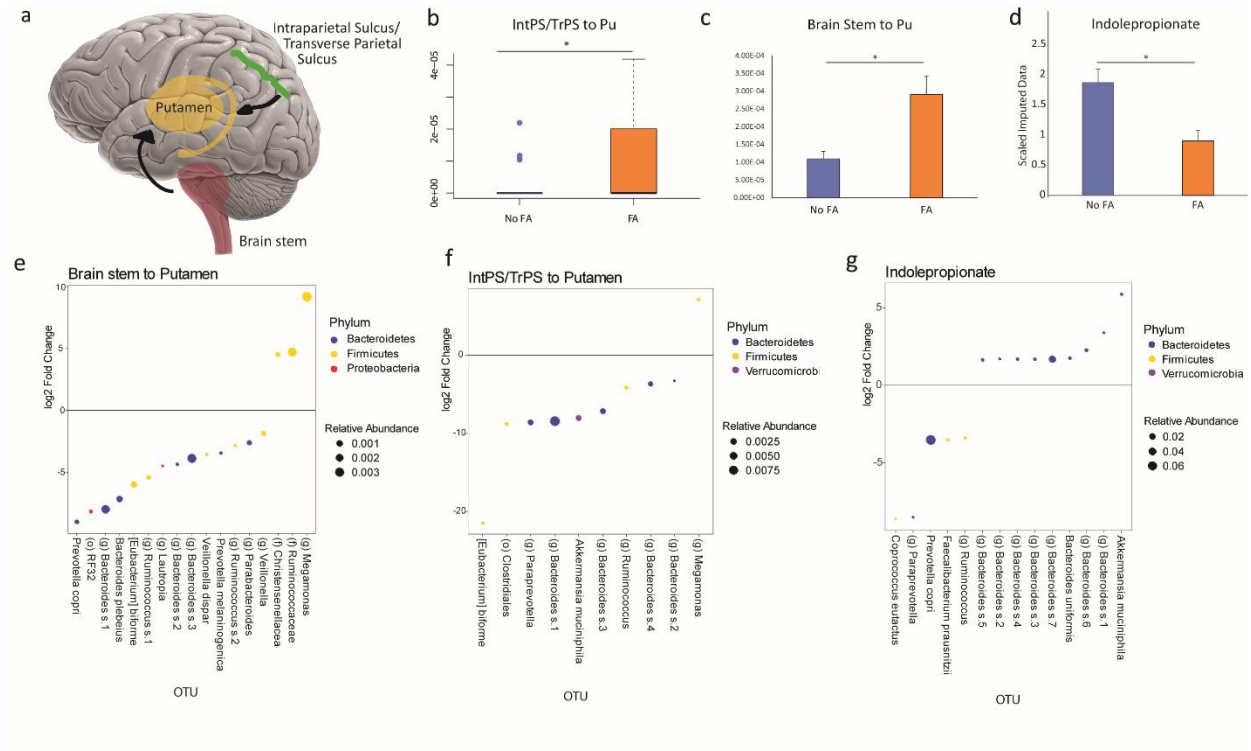

Figure 2-3: Brain imaging and indolepropionate is associated with food addiction. DTI pairwise showed that the communication between the intraparietal sulcus/transverse parietal (IntPS/TrPS) sulcus and brain stem to the putamen was positively associated with Food Addiction (FA) in patients with obesity. A) Schematic diagram of the significant brain region associated with food addiction and its quantification (B and C). D) Levels of fecal indolepropionate in obese subjects with or without food addiction (FA). E) DESEq2 analysis in obese subjects showing several OTUs associated with increase connectivity between the brain stem and the putamen. F) DESEq2 analysis in obese subjects showing several OTUs associated with increase connectivity between the IntPS/TrPS to the putamen. G) The OTUs that are correlated to fecal indolepropionate by DESEq2 in patients with obesity. IntPS/TrPS: intraparietal sulcus/transverse parietal sulcus. Pu: Putamen.

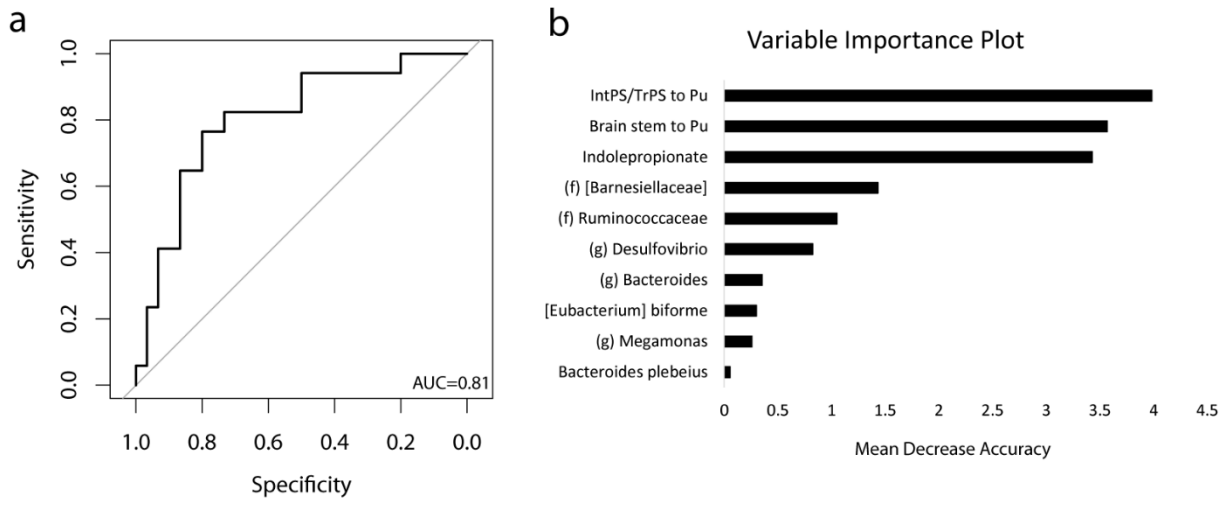

Figure 2-4: Combining fecal metabolite with 16S and brain imaging data, a highly accurate classifier is created that identifies subjects with food addiction (FA). A) ROC curve for the random forest classifier (AUCROC =0.81). B) Variable importance plot of each factor on the accuracy of the classifier. IntPS/TrPS: intraparietal sulcus/transverse parietal sulcus. Pu: Putamen.

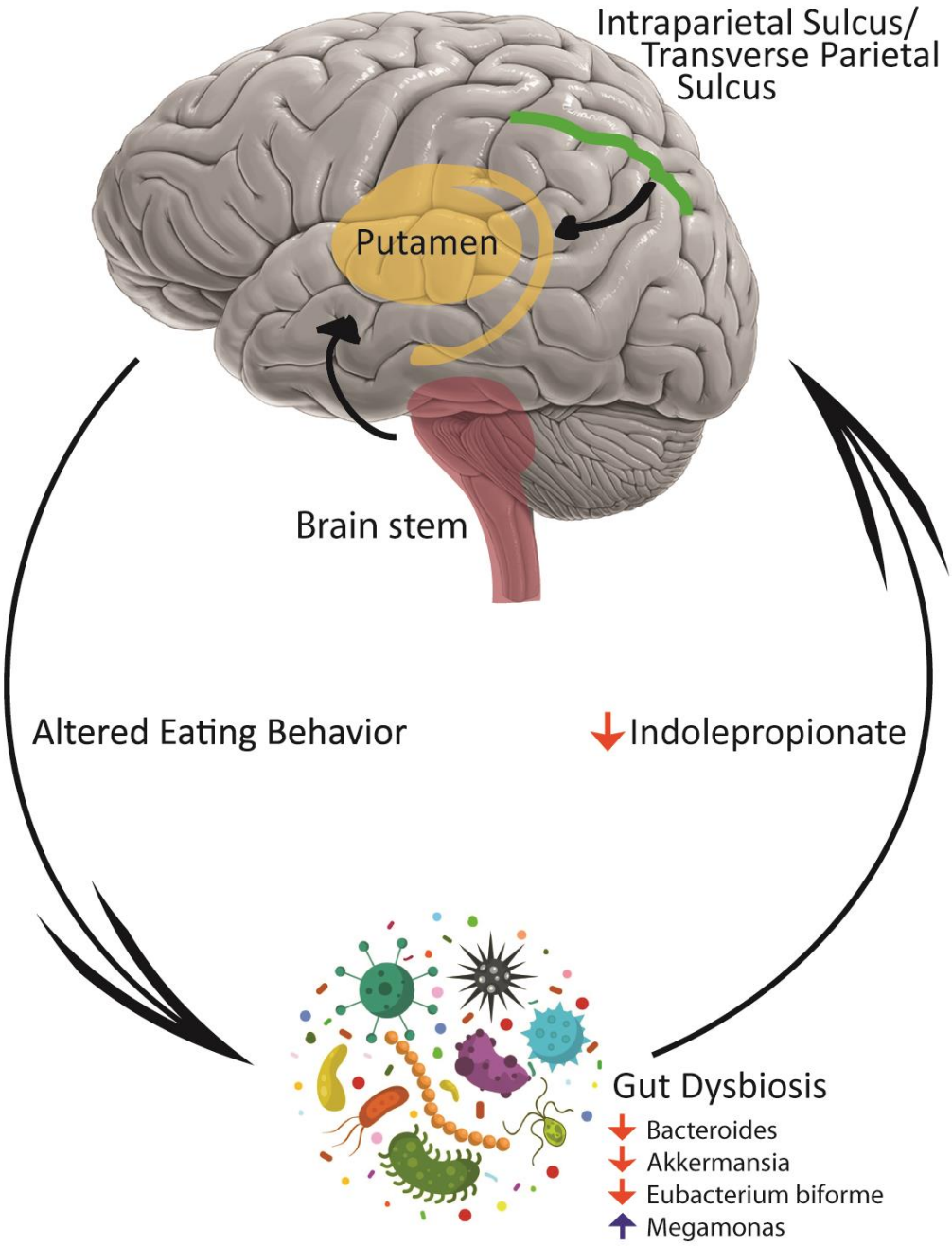

Figure 2-5: Proposed schematic diagram that connects the gut microbiome to food addiction (FA) via changes in metabolite and changes in connectivity of the brain's reward system.

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Chapter 3

The Intestinal Microbiome Identifies Advanced Fibrosis in Patients with Nonalcoholic Fatty Liver Disease

INTRODUCTION

Chronic liver disease is one of the most common medical conditions worldwide that affects as many as 840 million people with an estimated rate of mortality of 2 million deaths per year.[1] From 1999 to 2016, deaths from chronic liver disease in the US increased by 65 percent and deaths from liver cancer doubled.[2] This escalation has been attributed to factors such as increased alcohol use in younger Americans, increased intravenous drug use and rapidly rising rates of obesity in our society.[2]

One of the most challenging aspects of chronic liver disease is the identification of patients with liver fibrosis. The development of advanced fibrosis is a major predictor of liver-related morbidity and mortality.[3-5] Early identification of advanced fibrosis using non-invasive testing is a growing area of research in the field of hepatology.[4,6,7] The characterization of gut microbial biomarkers for advanced fibrosis has been a novel area of ongoing research. For example, Qin and colleagues in 2014 reported that an intestinal microbial signature was present in individuals with cirrhosis in a Chinese cohort as compared to healthy controls.[8] This study included different causes of cirrhosis including hepatitis C, hepatitis B, NAFLD, and alcoholic liver disease. Loomba *et al* in two separate studies was able to identify and validate a distinct microbial signature that was related to advanced fibrosis in patients with NAFLD.[9,10] However they did not explore other etiologies of chronic liver disease, so it is unclear at this time if this signature holds true for other causes of liver disease in western society. Given the association of the microbiome with chronic liver disease and cirrhosis, the aim of this study was to determine if specific fecal microbial profiles can be used as non-invasive biomarkers for advanced fibrosis in patients with varying etiologies of chronic liver disease.

METHODS

Patient Recruitment and Stool Collection

Patients with a diagnosis of chronic liver disease and undergoing ultrasound elastography were recruited prospectively from the VA Greater Los Angeles Healthcare System (VA) from 6/2017 to 6/2018. Chronic liver disease included patients with chronic hepatitis C virus (HCV) infection, chronic hepatitis B virus (HBV) infection, liver disease due to chronic alcohol use, primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), Wilson's disease, autoimmune hepatitis, hemochromatosis, and NAFLD. Patients were excluded if they were treated with antibiotics or probiotics within 3 months of enrollment, had only acute liver injury without any underlying chronic liver disease, treated HCV infection with sustained virologic response without any other forms of chronic liver disease, were on a specialized diet (e.g. gluten free, vegan, vegetarian, high protein), had a personal history of GI surgeries, irritable bowel syndrome or inflammatory bowel disease. Stool was collected within 7 days of their ultrasound elastography and placed into 95% ethanol and stored at -80^{o}C until processing. Patient information including age, gender, race/ethnicity, and comorbidities were also collected. For race and ethnicity, there were 5 categories with Hispanic as a separate category (i.e. non-Hispanic white, non-Hispanic black, Hispanic, Asian, and other). Co-morbidities were collected in order to calculate the Charlson comorbidity index, a validated score that assesses overall health and risk of 1-year all-cause mortality.[11] Stool samples from heathy control patients without any evidence of chronic liver disease were also collected. The study was approved by the Veteran's Affair Greater Los Angeles Healthcare System Institutional Review Board. All methods herein were performed in accordance with relevant guidelines and regulations. Verbal and written informed consent for study participation was obtained from all patients.

Liver Ultrasound Elastography

All patients with chronic liver disease underwent an ultrasound elastography using the FibroScan touch 502 machine (Echosens, MA, USA). All ultrasound elastographies were performed by trained technicians with over 100 scans of experiences each. Medium (M) and extra-large (XL) probes were utilized depending on the patient's body habitus according to manufacturer's protocol. Controlled attenuation parameter (CAP) score and liver stiffness were collected as non-invasive measurements of hepatic steatosis and fibrosis, respectively. All measurements were done at least 10 times at the same spot with interquartile range/median value less than 30% as per manufacturers guidelines. A CAP score of between 238 and 260 was given a steatosis grade of S1 representing 11-33% of fatty change in the liver, a score between 260 and 290 was given a grade of S2 representing 34-66% of fatty change, and a score higher than 290 was given a grade of S3 representing 67% or more of fatty change as per manufacturer's guideline. Standard cutoffs of liver stiffness as measured in kilopascals based on etiology of liver disease was used to determine extent of liver fibrosis (F0/F1 to F4).[12] Minimal fibrosis was defined as a score consistent with F0-F2 and advanced fibrosis was defined as a score consistent with F3-F4, similar to prior published studies.[10]

16S rRNA Sequencing

DNA was extracted from ethanol preserved stool using the Powersoil kit as per the manufacturer's instructions (MO BIO, Carlsbad, CA, USA). The V4 region of 16S ribosomal RNA was amplified and underwent paired end sequencing on an Illumina HiSeq 2500 (San Diego, CA, USA) as previously described.[13] The 253 base-pair reads were processed using QIIME 1.9.1 (San Diego, CA, USA) with default parameters.[14] The average sequence depth per sample was 45,560. Operational taxonomic units (OTUs) were picked against the May 2013 version of the Greengenes database, prefiltered at 97% identity. After removing OTUs that were present in fewer than 10%

of all samples, 1479 OTUs remained for analysis. Raw 16S rRNA sequence data were deposited under National Center for Biotechnology Information BioProject PRJNA542724.

Statistical Analysis

For demographic data, means are expressed along with their standard deviations and comparisons between means were performed using the Student's t-test. Categorical data were compared using the Pearson's chi-squared test.

For 16s rRNA sequencing data, alpha diversity metrics that included Chao1 (a metric for species richness), Faith's phylogenetic diversity, and Shannon Index (a metric that incorporates both species richness and species evenness) were computed using QIIME. The statistical significance of differences in alpha diversity metrics was calculated using a two-tailed t-test. Beta diversity, a metric of differences between samples, was calculated using the square root of the Jensen-Shannon divergence and visualized by principal coordinates analysis in R.[15] Univariate Adonis, a permutational analysis of variance, was performed using 10,000 permutations to test for differences in the square root of the Jensens-Shannon divergence across the following variables: age, gender, race/ethnicity, BMI, control/patient cohort, fibrosis as a binary categorical variable, steatosis grade, etiology of liver disease, and Charlson's comorbidity index. Only variables with a p-value <0.1 were used for the final multivariate analysis. This included steatosis grade, Charlson's comorbidity index, and fibrosis. Differential abundance testing was evaluated using DESeq2 in R, which employs an empirical Bayesian approach to shrink dispersion and fit non-rarified count data to a negative binomial model.[16] Variables listed in the multivariate analysis of DESeq2 were the same variables listed above for the multivariate Adonis analysis. P-values for differential abundance were converted to q-values to correct for multiple hypothesis testing (< 0.05 for

significance). All authors had access to the study data and had reviewed and approved the final manuscript.

Random Forests Classifier

A random forests classifier to predict advanced fibrosis was created in R using the randomForest package (https://cran.r-project.org/web/packages/randomForest) with 1001 trees and mtry = 2.[17] Features inputted into the random forest classifier were those associated significantly with advanced fibrosis as determined by multivariate DESeq2 models. The accuracy of the random forest classifier was estimated using a 10-fold cross-validation.

Predicted Metagenomics

Metagenomic data of each sample was inferred from 16S rRNA sequencing data by using PICRUSt 1.1.3 (http://picrust.github.io/picrust), a well validated tool designed to impute metagenomic data from 16S rRNA compositional data.[18] 16S rRNA sequencing data was inputted into PICRUSt and normalized by copy number using default parameters. The subsequent metagenes were then categorized by function using the KEGG database. Differences in predicted metagenes by advanced fibrosis were identified using DESeq2 with p-values adjusted for multiple hypothesis testing.

Validation Cohort

The findings of the random forest classifier were validated in a separate cohort of NAFLD patients recruited at the VA from January 1st, 2019 to October 1st, 2019. Inclusion and exclusion criteria were the same as above. All patients underwent stool collection and liver ultrasound elastography as described above. Demographic data, race, ethnicity, and comorbidities were

collected. In addition, all patients within this cohort filled out a validated diet questionnaire, the NIH Diet History Questionnaire III (DHQIII), at the time of their stool collection.[19]

RESULTS

Patient and Healthy Control Characteristics

Fifty patients with chronic liver disease and 25 healthy controls were recruited. Etiologies for liver disease included non-alcoholic liver disease (58.0%), hepatitis C (26.0%), hepatitis B (10.0%), and alcohol (6.0%) (Table 1). Nineteen patients had advanced fibrosis and 7/19 (36.8%) had F4 fibrosis. The healthy control cohort were younger on average than the patients with chronic liver disease and comprised of more females (p-value <0.001). The average Charlson's Comorbidity Index for the liver disease cohort was 4.33 $\pm$ 2.31. There was no difference in Charlson's Comorbidity Index between patients with advanced fibrosis as compared to those without advanced fibrosis. There was no difference in race/ethnicity between any groups and there was no statistical difference in etiologies of chronic liver disease by fibrosis stage.

Microbial Profiles Differs by Fibrosis Stage and Etiology of Liver Disease

In univariate analysis of beta diversity, only 3 variables had a p-value <0.1: steatosis grade, Charlson's comorbidity index, and the presence of advanced fibrosis. Therefore, these variables were used for the multivariate analysis. As demonstrated in the principal coordinates analysis plot (Figure 1A), the microbial profile of patients with advanced fibrosis differed significantly as compared to those with minimal or no fibrosis or healthy controls (p=0.003), while adjusting for the other covariates. In regards to alpha diversity metrics, patients with NAFLD and minimal or no fibrosis had a lower Chao1 index (species richness) and a lower Faith's Phylogenetic Diversity as compared to healthy controls and NAFLD patients with advanced fibrosis (Figure 1B). There

was no statistically significant difference in the Shannon Index (species richness/evenness) in any of the group comparisons.

The average taxonomic composition of chronic liver disease patients divided by etiology is summarized in Figure 2A on a phylum and genus level. The composite taxonomic summary of all patients with advanced fibrosis, minimal or no fibrosis, or healthy controls is shown in Figure 2B. Patients with alcoholic liver disease with F0-F2 fibrosis had a higher relative abundance of Bacteroidetes than any other group. Examining all patients with advanced fibrosis, there was a statistically higher abundance of *Prevotella* as compared to either healthy control or patients with F0-F2 disease as determined by differential abundance analysis adjusting for covariates.

Differential abundance analysis adjusting for covariates was also performed to compare patients with different etiology of liver disease to healthy controls (Figure 3). Because patients with alcoholic liver disease and patients with HBV infection only comprised of 8 patients, the analysis only focused on patients with chronic HCV infection and NAFLD adjusting for fibrosis and the other covariates listed above. Patients with HCV disease as compared to controls differed significantly across 25 different OTUs (a taxonomic unit roughly corresponding to species). An undefined species belonging to the family Rikenellaceae, two undefined species in the genus *Bacteroides*, and an undefined species in the genus *Dialister* made up the OTUs with the largest relative abundance (Figure 3A). NALFD patients had 34 separate OTUs that were differentially abundant from healthy controls (Figure 3B). The species with the highest relative abundance included *Prevotella copri*, an undefined species in the family Ruminococcaceae and an undefined species in the family Rikenellaceae. All 3 of these species were underrepresented in patients with NAFLD. Comparing NAFLD to HCV patients, there were 10 OTUs that were differentially abundant between the two groups. *Prevotella copri*, an undefined species belonging to the genus

Bacteroides, and an undefined species of the order Clostridiales made three most abundant OTUs. *Prevotella copri* was higher in patients with NAFLD adjusting for fibrosis stage, while the other two OTUs were higher in patients with HCV (Figure 3C).

Between patients with advanced fibrosis vs. minimal or no fibrosis, 26 OTUs were differentially abundant. The two most highly abundant differential OTUs were *Prevotella copri* and an undefined species belonging to the genus *Bacteroides*, both of which were elevated in patients with advanced fibrosis (Figure 4A). Examining differences between fibrosis stage within patients with HCV and with NAFLD, there were 12 OTUs and 23 OTUs that were differentially abundant, respectively. While *Prevotella copri* did have a higher relative abundance in HCV patients with advanced fibrosis, it did not reach statistical significance. Instead, two undefined species in the family Ruminococcaceae and *Akkermansia muciniphila* were the three differential OTUs with the highest abundance; all three were elevated in patients with HCV with advanced fibrosis (Figure 4B). In NAFLD patients, *Prevotella copri* was the predominant species and it was elevated in patients with advanced fibrosis (Figure 4C).

Predicted Metagenomic Profile Differs by Fibrosis

Metagenomic profiles were predicted for each sample from 16S rRNA compositional data using PICRUSt. The predicted metagenomic profile that differed between patients with advanced fibrosis as compared to those patients with minimal or no fibrosis is summarized in Figure 5. The average weighted Nearest Sequenced Taxon Index (NTSI) per sample was 0.08. Low scores indicate availability of closely related reference genomes and thus a higher quality of predictions.[18] While there was no overall large difference of the predicted metagenome between samples by fibrosis stage as represented by the principal coordinates analysis in Figure 5A ($p=0.34$), patients with advanced fibrosis did have a trend to have more bacterial genes present per sample (Figure

5B, p=0.09). From 16S rRNA compositional data, DESEq2 analysis of PICRUSt predicted metagenes showed 168 metagenes that were statistically differentially expressed in patients with advanced fibrosis as compared to those with minimal or no fibrosis. Categorizing these metagenes into functional categories showed 9 pathways that are different between the two groups. The pathways that were most different between the two groups were those involved in mineral absorption, arachidonic acid metabolism, carbohydrate digestion and absorption, and linoleic acid metabolism (Figure 5C).

A Microbial Signature Predicts Advanced Fibrosis

Using the 26 OTUs that were differentially abundant between patients with advanced fibrosis and patients with minimal or no fibrosis, a random forest classifier was created with high accuracy for predicting advanced fibrosis. The area under the receiver operating characteristic curve (AUROC) was 0.90 in 10-fold cross-validation (Figure 6A). The contribution of each OTU to the classifier was expressed as variable importance score, which measures the decreased accuracy of the classifier if that feature was removed (Figure 6B). The species with the greatest variable importance score was *Prevotella copri* followed by two undefined OTUs belonging to the genus *Lachnobacterium* and family Ruminococccaceae.

A Separate Cohort Validates the Finding that a Distinct Microbial Signature Predicts Advanced Fibrosis

In the validation cohort, there was no statistical difference between patients with advanced fibrosis as compared to minimal or no fibrosis in regards to age, gender, comorbidities, race, or dietary patterns (Table 2). Similar to the original cohort, a distinct microbial profile exists for patients with advanced fibrosis as compared to those with minimal or no fibrosis (Figure 7). In

univariate analysis of beta diversity, only age and advanced fibrosis had a p-value <0.1. Therefore, these two variables were used for multivariate analysis. Adjusting for age, the microbial profile of patients with advanced fibrosis differed significantly as compared to those with minimal or no fibrosis as demonstrated in the principal coordinate analysis plot (p=0.002). There was no statistical difference in Shannon index between patients with advanced fibrosis or those with minimal to no fibrosis in the validation cohort.

The average taxonomic composition by fibrosis category is summarized in Figure 7D and 7E, highlighting increased *Prevotella* in the advanced fibrosis group. Differential abundance testing demonstrated that 7 OTUs differed between patients with advanced fibrosis vs. minimal or no fibrosis. Of these, *Prevotella copri* was the most abundant and it was the only one that was enriched in those with advanced fibrosis. Applying the same random forest classifier trained on the initial cohort, microbiome composition had an AUROC of 0.82 for differentiating advanced vs. minimal or no fibrosis based on 10-fold cross-validation (Figure 7C).

DISCUSSION

This study yielded several important findings. In patients with chronic liver disease, we show that those with advanced stages of fibrosis have a distinct microbiome signature compared to those with lesser stages of fibrosis. This held true regardless of etiology of the liver disease and after adjusting for other covariates. These differences are characterized by an increase in the genus *Prevotella* and a decrease in *Bacteroides.* Furthermore, by using these microbial differences, a highly accurate model based on stool analysis can be created to identify those with advanced fibrosis.

We also show that microbial signatures differ across different etiologies of chronic liver disease. Similar to prior published works,[20,21] chronic HCV infection is associated with a decrease in the order Clostridiales and family Ruminococcaceae in patients with advanced fibrosis. This study also builds on prior data from NAFLD patients. Within our cohort the most abundant species that were significantly different between healthy controls and NAFLD patients while adjusting for the level of fibrosis were *Prevotella copri*, an undefined species in the family Ruminococcaceae, and an undefined species in the family Rikenellaceae. This is similar to other prior works showing a reduction of *Ruminococcus* and *Prevotella* in non-cirrhotic NAFLD patients.[9,22] *Prevotella's* reduction in non-cirrhotic NAFLD patients as compared to healthy controls is likely related to diet. Diets that are high in fats and animal protein as compared to diets that are rich in fiber has been shown to increase *Bacteroides* and decrease *Prevotella*.[23,24] This finding is therefore in line with previous works that has linked the gut microbiome to diet and non-cirrhotic fatty liver disease.

Though the idea of using stool as a novel biomarker for advanced fibrosis was recently explored and validated, it was only done in patients with NAFLD and did not include other etiologies.[9] In our cohort of racially diverse patients with varying etiologies of chronic liver disease, we show that the idea of using stool analysis to identify patients with advanced fibrosis is not only feasible but potentially highly accurate. While several other non-invasive methods are currently available for the diagnosis of advanced fibrosis including magnetic resonance elastography (MRE), transient elastography, and lab-based models, these modalities can have reduced accuracy in patients with diabetes or severe obesity.[25,26] Therefore, we propose that stool analysis can be a potentially accurate method when other modalities are limited. Combination of stool testing with other non-invasive tests including Fib-4 and NAFLD fibrosis scoring may also

prove to be an important clinical tool to identify those patients who are more likely to progress to advanced fibrosis or cirrhosis.

In our model, we show that *Prevotella copri* was the predominant species predictive of advanced fibrosis. This was also true in our validation cohort as well. While *Prevotella copri* is still present in normal healthy controls, it is significantly higher in patients with advanced fibrosis, a trend that is consistent across all etiologies of chronic liver disease. This is similar to Qin *et al* who showed that *Prevotella* was enriched in patients with cirrhosis as compared to healthy controls.[8] *Prevotella copri* is of great interest as it has been extensively studied in other inflammatory diseases.[27] It encodes a unique superoxide reductase which may provide resistance to or even the use of host-derived reactive oxygen species produced during inflammation.[28] Mice colonized with *P.copri* have increased inflammation in a colitis model induced by dextran sulfate sodium.[29] *In vitro* models have shown that *P. copri* can stimulate IL-6, IL-23, and IL-17, all cytokines associated with pro-inflammatory Th17 responses.[30] This has led many to believe that *P. copri* is a potential driver of inflammation and can even induce such inflammatory diseases as rheumatoid arthritis.[27] In a recent publication, *Prevotella copri* was also seen as the main bacteria associated with advanced fibrosis in NAFLD pediatric patients.[31]Our analysis also shows a distinct bacterial metagenomic profile for patients with advanced fibrosis. In our analysis, we show that the pathways that were most different between patients with advanced fibrosis compared to those without were related to mineral absorption, arachidonic acid metabolism, carbohydrate digestion and absorption, and linoleic acid metabolism. In mouse models of liver steatosis, linoleic acid was shown to be protective against inflammation by affecting PPAR-α and NF-κβ signaling.[32] The observed associations of *P. copri* and these functional pathways with advanced fibrosis provide preliminary evidence that the gut microbiome may contribute to the progression of liver fibrosis.

Therefore, it can be both a useful non-invasive biomarker as well as a potential target for future interventions.

We acknowledge that there were several limitations to this study. For example, we relied on FibroScan rather than liver histology to make the diagnosis of hepatic fibrosis. With the wide adoption of non-invasive testing for fibrosis, the use of liver biopsy is becoming less frequent. However, FibroScan is becoming a more widely accepted and accurate method for detecting the presence of hepatic fibrosis.[26] While other papers have mentioned that obesity might be a limitation of FibroScan, our facility and technicians had access to and were familiar with the XL probe, which has been proven to have improved diagnostics in obese patients.[26] Another limitation is that this is a single center VA study and so the generalizability of this study in other settings is still uncertain. While the multivariate analyses did not control for all factors that could affect the microbiome, including diet and medications, the corroboration of our findings in a separate validation cohort that accounted for diet strengthens the findings of our study. Furthermore, while we did attempt to represent a wide array of chronic liver disease, the majority of our patients had chronic HCV or NAFLD. A complete representation of all etiologies of chronic liver disease was unable to be accomplished due to the rarity of less common etiologies including autoimmune disease, Wilson's disease, hemochromatosis, PSC, PBC, and alpha-1 antitrypsin deficiency. Therefore, future studies will be needed in order to confirm that these findings apply to other chronic liver disease etiologies. Because this study is cross-sectional, it is unable to establish causality between the gut microbiome and hepatic fibrosis. Planned future studies will include the use of fecal metabolomics to examine the differential pattern of microbial derived metabolites in patients with advanced fibrosis and the use of animal models with microbial transplant or single bacteria gavage to understand the causal relationship between the gut microbiome and hepatic fibrosis.

In conclusion, there is a distinct microbial signature for patients with advanced fibrosis independent of liver disease etiology and other comorbidities. These results suggest that microbial profiles can be used as a non-invasive marker for advanced fibrosis and support the hypothesis that microbes and their metabolites contribute to hepatic fibrosis. Future studies should focus on the mechanism by which these microbial differences may contribute to the progression of fibrosis and if the models presented here are valid in other clinical subgroups.

	Control (n=25)	**F0-F2 (n=31)**	**F3/F4 (n=19)**	**p-value**
Age (yr) (SD)	35.7 (3.5)	58.7 (16.3)	66.2 (6.8)	**<0.001**
Male (%) (n=62)	52% (n=13)	88.9% (n=28)	100% (n=19)	**<0.001**
Charlson Comorbidity Index (SD)	N/A	3.9 (2.6)	5.1 (1.5)	0.13
Race/Ethnicity				
Caucasian (%) (n=26)	32.0% (n=8)	38.7% (n=12)	31.6% (n=6)	
African American (%) (n=26)	32.0% (n=8)	32.3% (n=10)	42.1% (n=8)	
Hispanic (%) (n=10)	8.0% (n=2)	16.1% (n=5)	15.8% (n=3)	
Asian (%) (n=7)	16.0% (n=4)	6.5% (n=2)	5.3% (n=1)	
Other/Unknown (%) (n=6)	12.0% (n=3)	6.5% (n=2)	5.3% (n=1)	0.89
Etiology of Liver Disease				
EtOH (n=3)		6.5% (n=2)	5.3% (n=1)	
HBV (n=5)		12.9% (n=4)	5.3% (n=1)	
HCV (n=13)		16.1% (n=5)	42.1% (n=8)	
NAFLD (n=29)	N/A	64.5% (n=20)	47.4% (n=9)	0.18

Table 3-1: Patient and healthy control characteristics. Fibrosis stage labeled from F0-F4. Minimal/no fibrosis: F0-F2; Advanced fibrosis: F3/F4; SD: standard deviation; EtOH: Alcohol, HBV: Hepatitis B virus, HCV: Hepatitis C virus, NAFLD: nonalcoholic fatty liver disease

Validation Cohort	**F0-F2 (n=27)**	**F3/F4 (n=10)**	**p-value**
Age (yr) (SD)	55.9 (11.0)	60.1 (6.7)	0.27
Male (%) (n=29)	77.8% (n=21)	80.0% (n=8)	0.63
Charlson Comorbidity Index (SD)	2.7 (1.3)	2.9 (0.9)	0.99
Race/Ethnicity			
Caucasian (%) (n=11)	29.6% (n=8)	30.0% (n=3)	
African American (%) (n=12)	40.7% (n=11)	0% (n=1)	
Hispanic (%) (n=12)	25.9% (n=7)	50.0% (n=5)	
Asian (%) (n=0)	0% (n=0)	0% (n=0)	
Other/Unknown (%) (n=2)	3.7% (n=1)	10.0% (n=1)	0.23
Dietary Data (intake per day)			
Alcohol (g)	1.81 (2.92)	1.59 (1.73)	0.82
Protein (g)	83.2 (42.4)	90.2 (44.5)	0.66
Total fat (g)	79.7 (50.9)	92.7 (63.1)	0.52
Total saturated fatty acids (g)	25.8 (16.0)	37.1 (33.5)	0.17
Total monounsaturated fatty acids (g)	29.4 (18.6)	31.4 (18.3)	0.78
Total polyunsaturated fatty acids (g)	17.4 (13.6)	16.0 (8.3)	0.78
Cholesterol (mg)	295.6 (197.0)	388.5 (221.0)	0.23
Carbohydrate (g)	244.9 (169.2)	247.9 (142.3)	0.96
Total sugars (g)	118.5 (97.0)	129.6 (88.9)	0.75
Dietary fiber (g)	21.6 (14.1)	20.8 (10.4)	0.87

Table 3-2: Validation cohort characteristics**.** SD: standard deviation

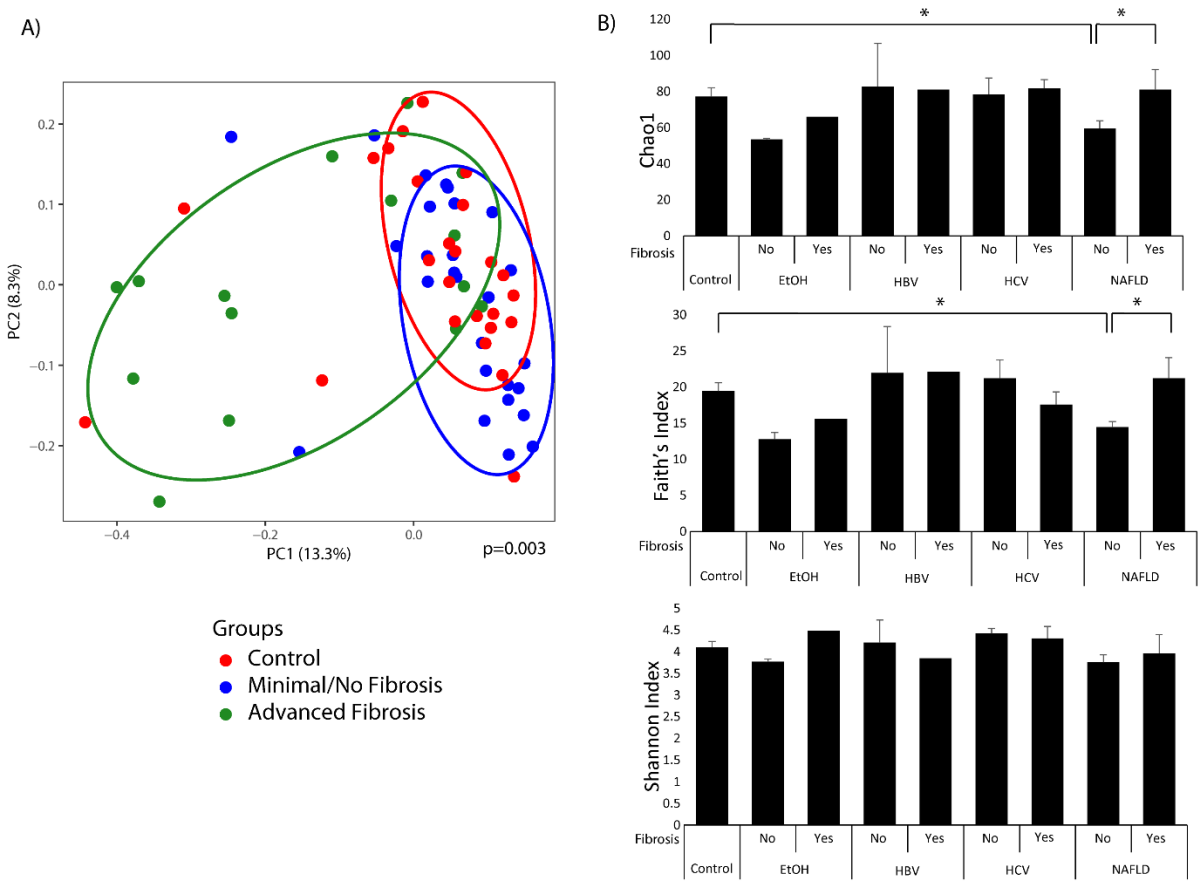

Figure 3-1: Patients with advanced fibrosis have distinct microbial composition and diversity compared to other liver disease patients or healthy controls. A) Beta diversity visualized by principal coordinates analysis plot of all patients colored by fibrosis stage or control group. B) Alpha diversity metrics by etiology of chronic liver disease and fibrosis stage. Chao1 is a metric of species richness, Faith's Index is a metric of phylogenetic diversity, and Shannon index is a metric of species richness/evenness. * represents comparison with p-value <0.05.

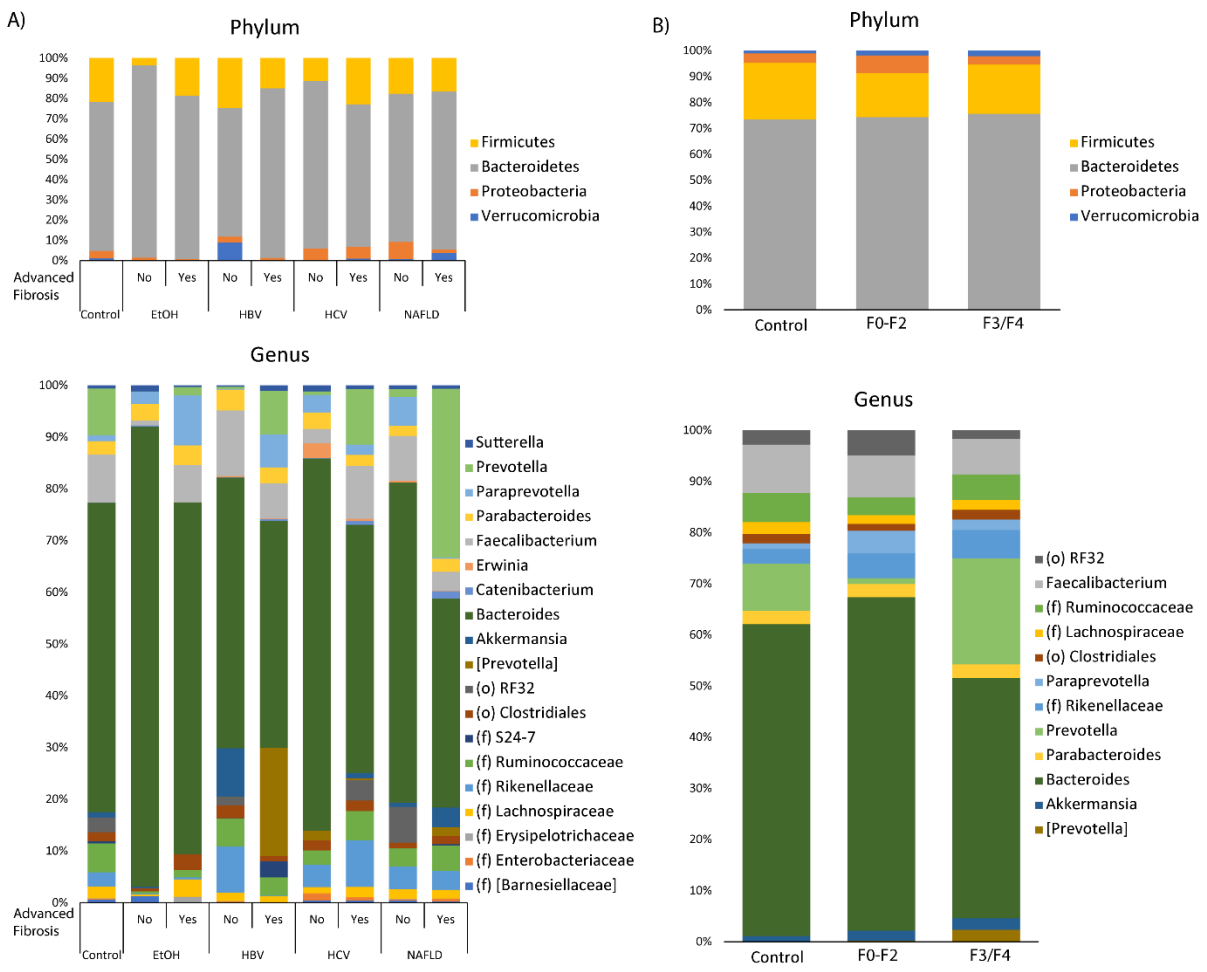

Figure 3-2: Taxonomic profiles categorized by etiology of chronic liver disease and fibrosis stage. A) Taxonomic profiles at the phylum and genus levels, divided by etiology of chronic liver disease and fibrosis stage. B) Taxonomic profiles by phylum and genus of patients with advanced fibrosis (F3/F4), liver patients with minimal/no fibrosis (F0-F2), and healthy controls. EtOH: Alcohol, HBV: Hepatitis B virus, HCV: Hepatitis C virus, NAFLD: nonalcoholic fatty liver disease

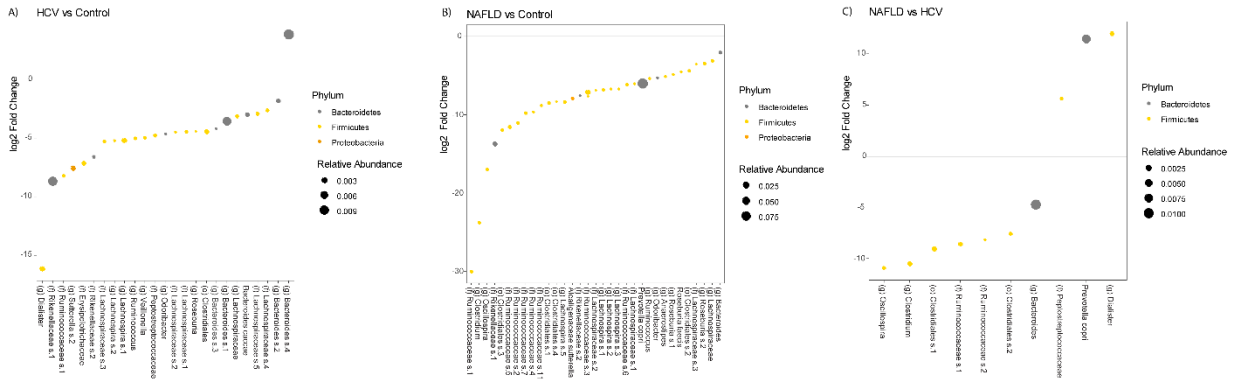

Figure 3-3: Microbial communities differ by etiology of chronic liver disease. DESEq2 differential abundance analysis comparing A) HCV patients to control, B) NAFLD patients to control, and C) NALFD patients to HCV patients controlling for fibrosis. HCV: Hepatitis C virus, NAFLD: nonalcoholic fatty liver disease

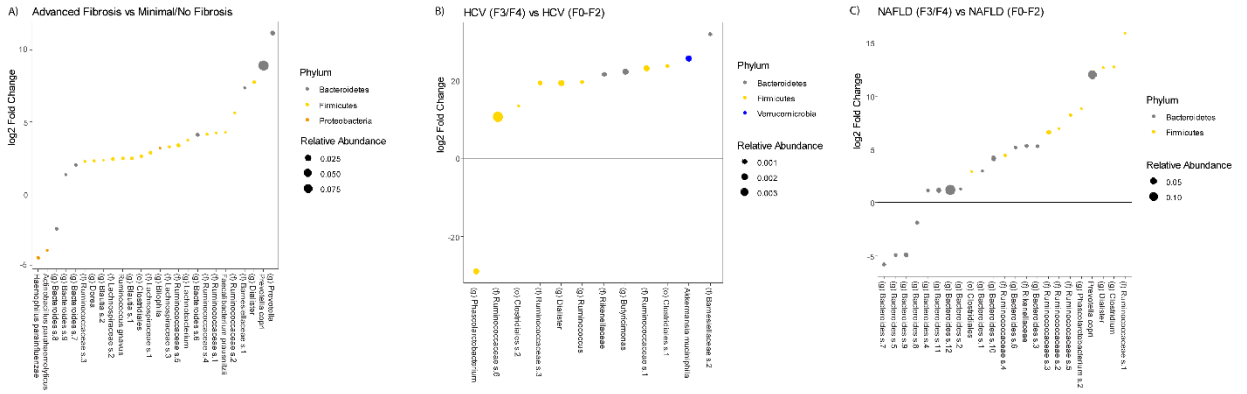

Figure 3-4: Microbial communities differ by fibrosis stage. DESEq2 differential abundance analysis comparing A) advanced fibrosis patients to minimal/no fibrosis patients, B) HCV patients with advanced fibrosis (F3/F4) to HCV patients without advanced fibrosis (F0-F2), and C) NAFLD patients with advanced fibrosis to NAFLD patients without advanced fibrosis. HCV: Hepatitis C virus, NAFLD: nonalcoholic fatty liver disease

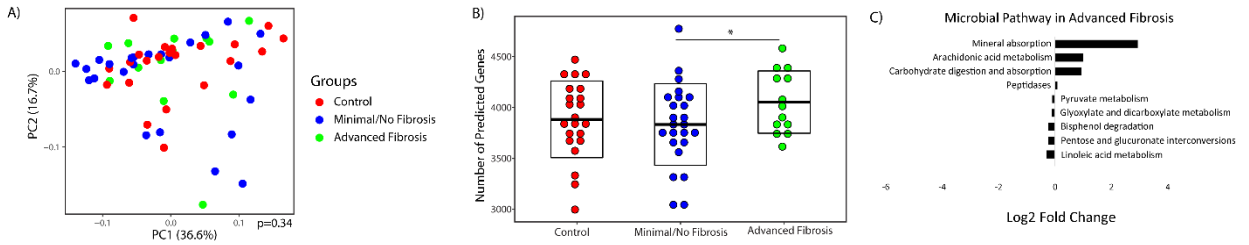

Figure 3-5: Predicted metagenomic differences by fibrosis stage. A) Principal coordinates analysis plot of predicted metagenomic profiles between samples by fibrosis stage. B) Number of predicted genes present per sample by fibrosis stage. Solid bar represents the mean and the box represents 1 standard deviation. *p=0.09. C) Differential abundance analysis (q<0.05) of predicted metagenes for advanced fibrosis categorized by KEGG pathways predicted by PICRUSt from 16S rRNA data.

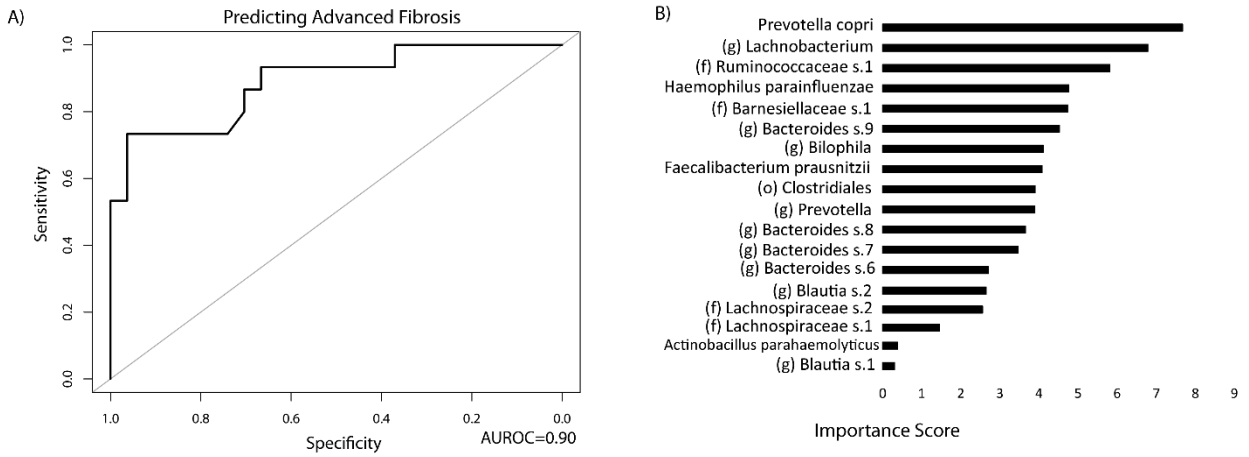

Figure 3-6: A distinct microbial signature can accurately identify patients with advanced fibrosis. A) Receiver operating characteristic curve of the random forests classifier for identifying patients with advanced fibrosis. B) Importance scores for features included in the random forests classifier for predicting advanced fibrosis.

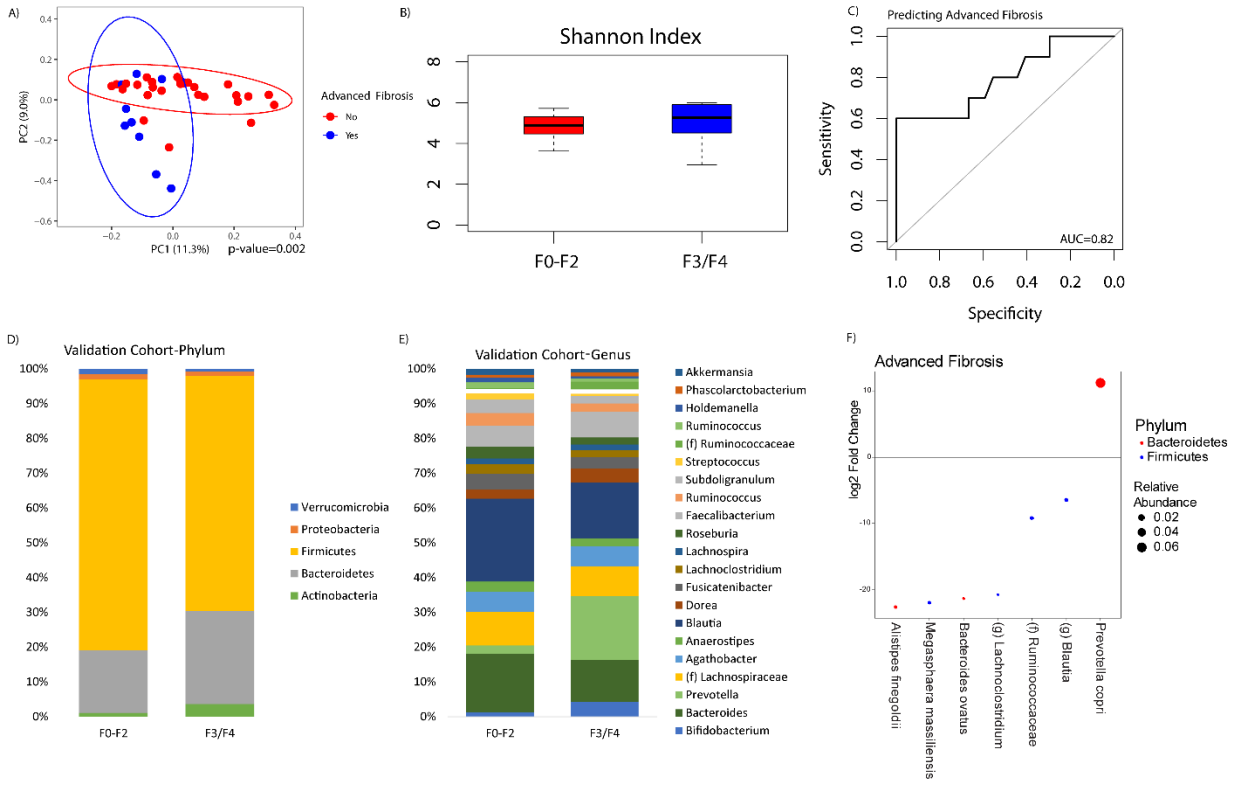

Figure 3-7: Validation of an advanced fibrosis microbial signature in a prospective study of NAFLD patients. A) Principal coordinate analysis plot of beta diversity between patients with minimal to no fibrosis (F0-F2) versus patients with advanced fibrosis (F3/F4) within the validation cohort. B) Shannon index of the validation cohort between patients with F0-F2 fibrosis and F3/F4 fibrosis. C) Validation of the random forests classifier as depicted by a receiver operating characteristic curve. D-E) Taxonomic profiles by phylum and genus of patients with minimal/no fibrosis (F0-F2) and patients with minimal/no fibrosis (F0-F2) within the validation cohort. F) DESEq2 differential abundance analysis comparing advanced fibrosis patients to minimal/no fibrosis patients in the validation cohort.

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Chapter 4

Bariatric Surgery Creates Long-term Changes in the Human Gut Microbiome and Alters the Progression of Nonalcoholic Fatty Liver Disease through Natural Killer T-Cell Expression

INTRODUCTION

Alongside obesity, the prevalence of nonalcoholic fatty liver disease continues to rise and it is estimated to now affect nearly 1 out of every 4 Americans.[1] To date, however, there are no well-established treatment for nonalcoholic fatty liver disease other than weight loss. While dietary interventions are important, many patients have difficulty maintaining a diet long enough in order to achieve sufficient weight loss. For that reason, bariatric surgery is one of the few proven treatments available for long-term weight loss in patients with obesity.[2] Bariatric surgery induces changes in the gastrointestinal tract anatomy, physiology and luminal environment, which in turn significantly affect the gut microbiome.[3,4] Roux-en-Y gastric bypass (RYGB) and laparoscopic sleeve gastrectomy (LSG) have similar effects on weight loss, despite the fact that they entail very different rearrangements of the GI tract anatomy and physiology.[5,6] This may reflect a shared mechanism via the gut microbiome, which plays an important role in regulating body weight and insulin resistance. Both gastric bypass and sleeve gastrectomy are associated with reduced hunger scores and changes in food preferences including a marked reduction in preference for high-calorie foods.[6] Bariatric surgery is also associated with a marked reduction in cytokines such as interleukin-8 and tumor necrosis factor-α (TNF-α).[7,8] Several studies performed in mouse models have shown that the gut microbiome affects feeding patterns and satiety signaling to the brain.[9-11]

To date, the handful of studies in animal models and humans have consistently shown changes in microbiota composition after RYGB and LSG, including increased abundance of Proteobacteria and decreased Firmicutes.[4,12] However, it is still not well understood how these shifts in gut microbiota composition contribute to weight loss and improvement in hepatic steatosis, inflammation, and fibrosis.

One potential pathway that can connect the gut microbiome to fatty liver disease is the host innate immune system. While the liver is a key player in lipid metabolism and detoxification, it is also an important regulator in host inflammation. The liver is known to house a myriad of immune cells including natural killer T-cells, macrophages, lymphoid and other non-lymphoid cells.[13] Being the first organ upstream to the GI tract, the liver is exposed to numerous antigens, pathogens, and bacterial signaling molecules. The dysregulation of this cross-talk between the gut, liver, and the host immune system has been implicated as a potential driver of insulin resistance and fatty liver disease progression.[13]

In this study, we will examine the effects bariatric surgery on the gut microbiome in humans and host inflammation. We will then explore the causal link between the gut microbiome after bariatric surgery on the pathogenesis of nonalcoholic fatty liver disease via the host immune system by employing the use of antibiotic-treated mice.

METHODS

Patient Recruitment

The basis of the human data is based on a pre-established cohort of female patients who were about to undergo laparoscopic sleeve gastrectomy (LSG). The cohort included only adult obese female patients who met clinical criteria for bariatric surgery. Prior to LSG, fecal and blood samples were collected before surgery and every 6 months post-surgery up to 1 year. Fecal and blood samples were collected while fasting as well as 30-minutes and 60-minutes post-feeding of a standardized 500 calorie meal. Clinical criteria for bariatric surgery were having a BMI $\geq$ 40, or more than 100 pounds overweight, or a BMI $\geq$ 35 with at least one obesity-related comorbidities, including type 2 diabetes, hypertension, hyperlipidemia, obstructive sleep apnea, nonalcoholic

fatty liver disease, gastric acid reflux, asthma, debilitating arthritis, and severe urinary incontinence due to obesity.[14] Patients were excluded from the study if they were not deemed a good surgical candidate or if they did not consent for blood or fecal sampling.

16S rRNA Sequencing

DNA extraction was performed using the ZymoBIOMICS DNA Microprep Kit (Zymo Research, Irvine, CA, USA) per the manufacturer's protocol. PCR amplification of the V4 region of the 16S ribosomal RNA gene was followed by a 250x2 paired-end read on an Illumina HiSeq (Illumina, San Diego, CA, USA), as previously described.[15] The sequences were processed using the DADA2 pipeline in R, and SILVA 132 database was used for taxonomy assignment. Next, data were incorporated into QIIME 2 version 2019.10. Amplicon sequence variants were filtered out if they were not present in at least 15% of all samples. Sequence depths ranged between 4,398 and 335,645 per sample with a median value of 183,882.

Metabolomics

Fecal and serum samples were aliquoted under liquid nitrogen and shipped to Metabolon for processing and analysis as a single batch on their global metabolomics and bioinformatics platform. Data was curated by mass spectroscopy using established protocols and software as per Metabolon.

Gastrointestinal Hormones and Serum Cytokines

Plasma levels of gastrointestinal metabolic hormones were measured using a human metabolic hormone magnetic assay kit (Millipore) according to the manufacturer's instructions. The hormones in the panel included amylin, c-peptide, gastric inhibitory peptide, glucagon-like peptide-1 (GLP-1), glucagon, insulin, leptin, peptide YY (PYY), Resistin, ghrelin, and pancreatic

polypeptide. Each sample was assayed in duplicate on a 96-well plate. All samples were processed in one batch and read via a Luminex 100 reader (Luminex, Austin, TX). Analysis of quality control standards provided in the kit matched expectations, and the assay had an inter-assay precision of <25% and an intra-assay precision of <7%. A similar assay was done for serum cytokines. The panel for serum cytokines included interleukin-6 (IL-6), tumor-necrosis factor alpha (TNF-α), and monocyte chemoattractant protein-1 (MCP-1), IL-8, IL-1 beta, hepatocyte growth factor, and nerve growth factor.

Animal Study

Antibiotic Treatment: Because germ-free animals are born and raised in a germ-free environment, their native immune system is innately different than the immune system of wild-type animals.[16] For this reason, antibiotic-treated C57Bl/6 mice and not germ-free C57Bl/6 mice were chosen as the model for fecal transplantation. Four human donors (pre- and post-bariatric surgery) from the above cohort were selected for fecal transplantation into antibiotic treated mice. The donors were selected as each one of them had documented fatty liver disease either by clinical notes or by radiology before bariatric surgery that improved after bariatric surgery. Two cocktails of antibiotics were used and placed into their drinking water at a concentration of 1 mg/ml per antibiotics. The first cocktail was a cocktail that was designed to have minimal systemic absorption (non-absorbable) that consisted of ertapenem, neomycin, and vancomycin. The second cocktail consisted of antibiotics with higher systemic absorption (systemic) which included ampicillin, cefoperazone, and clindamycin. Four-week-old weight-matched mice were treated with a 7-day period of non-absorbable antibiotics, followed by a 7-day period of systemic antibiotics, and then again by a 7-day period of non-absorbable antibiotics. After each 7-day period, the mice were given 2 days of rest with sterile water. This protocol was shown to give stable long-term

engraftment of human microbiome samples into mice comparable to germ-free animals.[17] After 2 days of rest after the last cycle of antibiotics, the mice were then gavaged with human fecal samples as previously described every other day for a total of three transfers.[18] The samples used were samples before surgery and samples 6-months post-surgery. A total of 6-10 mice were used per fecal sample. Immediately after gavage, the mice were given either a high fat (40% by kcal), high fructose (20% by weight), high cholesterol (2% by weight) (HF diet) (Research Diets, #D18061301) or a standard diet (10% fat by kcal, 0% fructose, and 0% cholesterol) (Research Diets, #D19082701) (SD) for 12 weeks. A negative control group was also present in each food group. These group of mice (n=4 each) were gavaged with only media without any fecal material. Food was double irradiated and packaged into individual 1-kg bags. A new bag was used at each cage change. All mice were housed on a single rack and only one person could change the food, water, and bedding of these mice over the 12 weeks to minimize cross-contamination.

Glucose Tolerance and Body Composition Testing: At the end of the 12 weeks, glucose tolerance testing was performed on each mouse by administering 2 grams/kg of glucose intraperitoneally and measuring serum glucose via a glucometer (Aimstrip plus, Fisher Scientific) at time 0, 30 minutes, 60 minutes, and 90 minutes. Two days after the glucose tolerance testing, the mice were then placed into an EchoMRI machine to measure total body fat and lean body mass.

Tissue and Blood Collection: Portal vein blood was collected by dissecting the portal vein and drawing up the blood via a capillary tube (Fischer, catalogue #22260950). Serum was then collected via heart puncture. The liver was then collected after perfusing the liver with 15 ml of sterile PBS via the inferior vena cava. The GI tract, spleen, gonadal fat, mesenteric fat, and dorsolumbar subcutaneous fat were also collected.

Cholesterol Testing: Cholesterol assay was performed using a total cholesterol-HDL and LDL/VLDL kit as per manufacturer's protocol (Abcam, catalogue #ab65390).

Staining and Quantification: Oil red O (Abcam) staining was done on frozen OCT embedded tissue as previously described.[19] Oil red O quantification was performed by using ImageJ and averaging the percent of area stained across 4 liver sections per mice. Hematoxylin and eosin staining were performed by the histology core at the University of California, Los Angeles. NAFLD activity score based on the hematoxylin and eosin staining was calculated by a blinded pathologist for each mice.

Flow cytometry: Immune cells were isolated from the liver through mechanical disruption as previously described.[20] Briefly, a section of the liver was pressed through a 70-micron filter into freshly prepared RPMI media with 10% fetal bovine serum. This solution was then wash and pelleted at 2000 rpm for 5 minutes at room temperature. The pellet was then placed into a 35% Percoll solution with RPMI and centrifuged at 2000 rpm for 20 minutes without any program deceleration. The pellet was then resuspended in 15 ml tubes with 3-5 ml of red cell lysis buffer for 5 minutes. RPMI with 10% fetal bovine serum was then added and the solution was then washed and pelleted again. The pellet was then resuspended with a 1:1 solution of PBS and FACS buffer and passed through a 30-micron filter for cell surface staining. Spleen tissue was processed similarly except without the Percoll gradient. Cells were first blocked with Fc block for 30 minutes at 4 degrees Celsius. The following antibodies were used for cell surface staining: AF488-Ly6C, AF647-F4/80, APC/Cy7-CD45.2, BV650-CD3, BV750-CD11b, PE-Nk1.1, PE/Dazzle-NKp46, PerCP/Cy5.5-CD4, BV421-CD45R, BV510-CD8a. All antibodies were purchased from BioLegend and were used at a dilution of 1:1000. Cells were stained for 30 minutes at 4 degrees

Celsius. Flow cytometry was performed on a BD Bioscience LSRII and data was analyzed using FlowJo.

Statistical Analysis

Human demographic data were expressed as means with standard deviation or as percentages. Differences in means were calculated using the Student's t-test. Differences in categorical data was performed using the fisher's exact test. Sustained weight loss at 1-year was defined as having at least a 20% reduction in body weight from baseline.

Plasma levels of hormones, cytokines, and cholesterol were compared using analysis of variance. All p-values were adjusted for false discovery rate.

Microbiome data was analyzed after sequence variant generation and filtering. Beta diversity was calculated using the DEICODE plugin in QIIME2. DEICODE uses a robust Aitchison distance metric that has been shown to have higher discriminatory power than other metrics such as Bray-Curtis or UniFrac.[21] Alpha diversity was also calculated via QIIME2 by using the Shannon index (a metric of evenness) with data rarefied to 4397 sequences. The differences in beta diversity was calculated using a permutation multivariate analysis of variance via the Adonis package in R. Statistical testing for alpha diversity was performed by using analysis of variance. Differential abundance testing was evaluated using DESeq2 in R, which employs an empirical Bayesian approach to shrink dispersion and fit non-rarified count data to a negative binomial model. P-values for differential abundance were converted to q-values to correct for multiple hypothesis testing (< 0.05 for significance).

For metabolomics, the data was normalized by using the scaled imputed data as provided by Metabolon. Differential abundance testing was performed using multiple analysis of variance corrected for false discovery rate.

Correlations between metabolomics, hormone profile, cytokine data, and microbiome analysis were performed using spearman's correlation and corrected for false discovery rate. Correlation networks were visualized using Cytoscape (Systems Biology, Seattle).

RESULTS

Outcomes After Bariatric Surgery: Eighteen female patients were recruited for the study. The mean age was 37.1 ± 9.36 years old (**Table 4-1**). The mean BMI before surgery was 44.7 ± 4.9 kg/m^2 and the mean weight before surgery was 118.5 ± 18.8 kg. Unsurprisingly, there was a significant decrease in both weight and BMI post-surgery as compared to their baseline with an average weight loss of 28.8 kg (**Figure 4-1**). The average patient lost 24.3 ± 5.4 % of their body weight at 6-months and 25.4 ± 5.8% at 1-year. In addition to weight loss, patients also had significant improvements in regard to their fasting glucose, c-reactive protein (CRP), and lipopolysaccharide binding protein (LBP) as compared to baseline (**Figure 4-1)**. Other inflammatory markers such as TNF-α and IL-6 were also significantly decreased after bariatric surgery as compared to their baseline values (**Figure 4-2**). There was no significant differences pre- or post-surgery regarding MCP-1, IL-8, IL-1 beta, hepatocyte growth factor, and nerve growth factor. The only gastrointestinal hormone that had a significant difference after bariatric surgery was leptin (**Figure 4-3**), with leptin showing significantly lower levels post-surgery.

Microbiome Analysis: The gut microbiome of patients pre- and post-surgery differed significantly (**Figure 4-4A**) across time (p-value <0.001) after adjusting for individual variations. There was no statistical difference across time in regard to alpha diversity. The taxonomic profiles of patients pre- and post-surgery staggered by sustained weight loss at 1-year is summarized in Figure 4-4C. Because most of the weight loss occurred at 6-months, we focused our analysis by comparing samples from 6-months post-surgery to baseline values. In patients that did not achieve sustained

weight loss (i.e. non-responders), a group belonging to *Ruminococcus* was overrepresented and a group belonging to Lachnospiraceae was underrepresented in patients 6-months post-surgery as compared to baseline. Conversely, in patients that did have sustained weight loss (i.e. responders), *Sutterella* and *Streptococcus* were overrepresented at 6-months. Similar to the non-responders, a group belonging to Lachnospiraceae family was underrepresented (**Figure 4-4D**).

Metabolomics Profiles Changed After Surgery: Forty-six serum metabolites were significantly different between the three different time points as determined by DESEq2: Pre-surgery, 6-months post-surgery, and 1-year post-surgery. These metabolites are summarized in Figure 4-5. Thirty-one of these metabolites belong to pathways involving lipid metabolism.

Baseline Microbiome Profile Predicts Sustained Weight Loss After Bariatric Surgery: While there was a significant difference in the gut microbiome of patients across time, there was also a significant difference when you examined the baseline gut microbiome of patients who had sustained weight loss and those that did not (**Figure 4-6**) (p-value=0.018). There was no difference in alpha diversity between these two groups. DESEq2 analysis shows that patients with sustained weight loss had 18 bacterial taxa that were overrepresented and 18 bacterial taxa that were underrepresented as compared to those without sustained weight loss. Of the 18 bacterial taxa that were overrepresented, *Blautia caecimuris*, *Parabacteroides distasonis*, and *Bacteroides vulgatus* were the three taxa with the highest relative abundance. Of the 18 bacterial taxa that were underrepresented, Alistipes obesi, Ruminococcaceae UCG-002, Phascolarctobacterium succinatutens, Alistipes ihumii, and Lachnospiraceae NK4A136 group were the taxa with the highest relative abundance. By using a random forest classifier, the microbiome had an area under the receiver operator curve (AUROC) of 0.98. The classifier that was based on microbiome data had the highest accuracy at predicting sustained weight loss at 1-year as compared to a classifier

based solely on baseline fasting hormone data (AUROC 0.68), baseline cytokine data (AUROC 0.61), and demographic information (AUROC 0.55). Lachnospiraceae NK4A136 group was the taxa with the greatest importance value in the classifier. A correlation network between microbial data and serum metabolite and hormones is summarized in Figure 4-7.

Microbial Transplant from Patients Pre- and Post-Bariatric Surgery Induces Significant Weight Changes in Antibiotic-Treated Mice: Not surprisingly, mice fed on a HFHF diet had significantly higher weight gain than mice fed on a SD. However, mice that were transplanted with the fecal microbiome of patients before bariatric surgery gained significantly more weight both on a SD and a HFHF diet as compared to mice that were transplanted with fecal material from the same patient post-surgery (**Figure 4-8**). This weight gain was not associated with any significant changes in cumulative food intake over the same period. Consistent with the weight gain, EchoMRI results showed that the mice on a HFHF diet had a significantly higher percentage of body fat and a lower percentage of lean body mass. Mice that were transplanted with the fecal microbiome of patients before bariatric surgery had significantly higher body fat and lower mean body mass a SD and a HFHF diet as compared to mice that were transplanted with fecal material from the same patient post-surgery. Mice fed on a HFHF diet also had higher liver mass as compared to those with SD. There was no significant difference in liver weights of any group while on a SD, but while on a HFHF diet, the mice with pre-bariatric microbiome had significantly heavier livers than the mice with post-bariatric microbiome.

Microbial Transplant from Patients Pre- and Post-Bariatric Surgery Alters Insulin Resistance and Cholesterol: Mice who were fed on a HFHF diet, had significantly worse glucose tolerance than the mice on a SD. In line with the human study, the mice with pre-bariatric microbiome had worse glucose tolerance than the mice with post-bariatric microbiome. This

difference, however, only occurred on the SD and not on the HFHF diet (**Figure 4-9**). This was similar to the results with cholesterol. Mice who were fed on a HFHF diet, had significantly higher cholesterol levels than the mice on a SD. The mice with pre-bariatric microbiome had higher levels of total cholesterol and higher levels of LDL/VLDL than the mice with post-bariatric microbiome. This difference, however, only occurred on the SD and not on the HFHF diet.

Microbial Transplant from Patients Pre- and Post-Bariatric Surgery Significantly Altered the Progression of Nonalcoholic Fatty Liver Disease: Mice on a HFHF diet had significantly worse steatosis (**Figure 4-10**) as determined by oil red o staining as well by NAFLD activity score (**Figure 4-11**). The mice with pre-bariatric microbiome had worse steatosis both on a SD and a HFHF diet as compared to their pos-bariatric counterparts. These mice also had worse NAFLD activity score both on a SD and a HFHF diet, but it only reached significance while on a HFHF diet.

The Microbial Differences Present in Pre- and Post-Bariatric Surgery Significantly Alters the Immunophenotype of the Liver: A HFHF diet significantly decreases the levels of NKT cells within the liver as compared to a SD alone (**Figure 4-12**). Furthermore, the microbiome of pre-bariatric surgery patients significantly lowers the expression levels of NKT cells in the liver as compared to their post-bariatric counterparts. This was seen in both on a SD as well as a HFHF diet. The microbiome of pre-bariatric surgery patients also significantly increases the expression levels of CD8+ CD4- T cells in the liver as compared to their post-bariatric counterparts both on a SD and a HFHF diet. A HFHF diet also significantly increases the expression level of Kupffer (CD11b (intermediate +) F4/80+) cells in the liver as well the levels of monocyte-derived macrophages (CD11b (High +) F4/80 +) (**Figure 4-13**). While a HFHF diet increased the levels

of these immune cells, the microbiome of post-bariatric patients was able to decrease them both, though this was only seen in the HFHF diet.

DISCUSSION

Bariatric surgery remains one of the best long-term treatment for obesity. Similar to prior published data, bariatric surgery provides long-term weight loss and reduces the pro-inflammatory state that is associated with obesity.[22] In our study we show that patients who undergo laparoscopic sleeve gastrectomy was able to achieve on average 25.4% of total body weight loss at 1-year. This was associated with a decrease in CRP, LBP, TNF-α, and IL-6. The likely decrease in these inflammatory markers is most likely due to a decrease in adipose tissue. Previous studies examining adipose tissue have shown that IL-6 and TNF-α expression is higher in adipose tissue of obese patients as compared to lean adipose tissue.[23,24] In other prospective studies examining bariatric surgery, researchers have found that with bariatric surgery and a decrease in adiposity IL-6 and TNF-α decreases both in the serum as well as in the adipose tissue.[22] Severe obesity is also marked by severe insulin resistance and leptin resistance.[25] Here we show that with a reduction in weight as caused by bariatric surgery, insulin resistance is improved and basal leptin levels are lowered.

While laparoscopic sleeve gastrectomy may not alter the GI tract as much as a Roux-en-Y gastric bypass, it is still able to cause significant long-term shifts in the gut microbiome. In our study we see that patients that did not meet the criteria for sustained weight loss, had less Lachnospiracea than at baseline and patients that had sustained weight loss had less Lachnospiraceae and more *Sutterella* and *Streptococcus* than at baseline. We also see a trend of increasing *Bacteroides* over time in the patients with sustained weight loss. In studies examining the gut microbiome and obesity, both Bacteroides and Sutterella have been shown to be negatively associated with obesity,

while bacteria like *Lachnospira* have been positively associated with obesity.[26,27] Therefore, the reduction of Lachnospiraceae over time with an increase in *Bacteroides* and *Sutterella* are within line with previously published microbial data. The exact mechanism by which the gut microbiome can cause obesity is still an active area of research. It is likely multifactorial that includes bile acid modulation, farnesoid X receptor signaling, alterations in the brain-gut axis, and increase energy extraction.[28-30]

However, a novel area by which the gut microbiome is gaining traction is in its role as a potential biomarker. Here we show that gut microbiome at baseline is an extremely accurate biomarker at predicting sustained weight loss of at least 20% body weight at 1-year post-bariatric surgery. We see that patients who are likely going to have sustained weight loss as compared to those that are not have very distinct gut microbiome at baseline. At baseline, those that had a better response to bariatric surgery had higher levels of *Bacteroides* and *Parabacteroides* and lower levels of *Alistipes*. Both Bacteroides and Parabacteroides have been shown to be anti-inflammatory by being able to promote regulatory T-cells and IL-10.[31,32] Conversely, *Alistipes* has been shown to be associated with type 2 diabetes.[33] The high accuracy of the gut microbiome to predict response to bariatric surgery suggests the idea that a person's innate microbiome can potentially enhance the effect seen by bariatric surgery. This suggests that the gut microbiome may be used as a potential biomarker for success with bariatric surgery. But it also suggests that the gut microbiome could be potentially altered before surgery as a method to increase the effectiveness of bariatric surgery. Future interventional trials will be needed to test this hypothesis.

We then showed the causal link between the gut microbiome with obesity and nonalcoholic fatty liver disease by transplanting the microbiome of 4 donors before and after their surgery into antibiotic treated mice. The results clearly show that the microbiome of patients before bariatric

surgery was able to increase the weight and body fat as compared to the microbiome after surgery. These changes were also associated with worse nonalcoholic fatty liver disease, worse insulin resistance, and worse cholesterol profile. In a landmark paper by Tremaroli *et al* they showed similar results of weight gain in germ-free mice transplanted with pre-bariatric microbiome and weight loss in germ-free mice transplanted with post-bariatric microbiome while on a standard diet.[34] In their study, there was no difference in food intake, respiratory quotient, or activity level in mice transplanted with control microbiome versus those transplanted with post-laparoscopic sleeve gastrectomy microbiome.[34] Our study corroborates that finding. Therefore, without changes in food intake or energy expenditure to explain the differences in weight gain, we propose that the differences are due to changes in energy extraction between the two microbiome populations. This idea is corroborated by the finding that the microbiome of pre-bariatric surgery patients and obese patients have a higher capacity to extract energy from the diet as evident from an increase concentration of fecal short chain fatty acids.[34,35] Furthermore differences in the microbiome can also lead to differences in bile acid concentration. Previous studies have shown that leaner phenotypes and patients who have undergone bariatric surgery have higher levels of bile acids.[34] These bile acids in turn can bind to such receptors like the Farnsenoid X receptor (FXR), which has been of great interest lately with the introduction of obeticholic acid (an FXR agonist) for the potential treatment for NAFLD.[36] We postulate that these differences in energy extraction and bile acid composition are contributing factors to the differences in weight seen in mice transplanted with pre-bariatric microbiome versus those transplanted with post-bariatric microbiome.

However, NAFLD is not merely a disease of increase fat. It is a disease that often requires multiple hits that usually involves increased inflammation.[37] Many studies to date have shown that an obese microbiome is often associated with increased gut permeability.[38,39] This “leaky gut” can lead to

increased bacterial translocation and antigen presentation in the gut as well as in the portal circulation leading to the liver.[39] This is one of the few studies that have looked into the connection between the gut microbiome and the immune system of the liver. Here we show that pre-bariatric surgery microbiome leads to an increase in adiposity in the liver and a significant decrease in NKT cells and an increase in cytotoxic T cells, Kupffer cells, and monocyte derived macrophages. This is in line with several other studies that have examined the immune system of the liver in regards to NAFLD.[40] In animal models of obesity, CD8+ T cells are often increased in visceral adipose tissue and may even proceed the infiltration of inflammatory macrophages.[41] In liver disease, cytotoxic T-cells are categorized as proinflammatory and their activation is often a hallmark of the progression from bland steatosis to steatohepatitis.[40] The pro-inflammatory state of the pre-bariatric microbiome is further exemplified by the increase level of Kupffer cells and monocyte-derived macrophages in the liver. One potential mechanism that can link the gut microbiome to the activation of Kupffer cells is toll-like receptor (TLR) signaling. TLR are part of a family of pattern recognition receptors that recognize bacterial, viral, and fungal ligands.[42] TLR can be activated by LPS and this activation can lead to an increase in nuclear factor κβ and Kupffer cell activation.[43] Furthermore, Kupffer cells can directly lead to the depletion of NKT cells via IL-12 expression.[44] The reduction of NKT cells in the liver of mice transplanted with pre-bariatric microbiome is in line with several obesity-related animal models.[40] NKT cells almost exclusively respond to lipid antigens and can be both proinflammatory or anti-inflammatory in nature.[44] In previous studies regarding both humans and mice, NKT cells are often depleted in favor of pro-inflammatory macrophages.[45] Many other studies have shown that NKT cells can have an attenuating effect on the development of NAFLD by both reducing steatosis as well as inflammation.[40,45,46] Future studies are needed to examine if these effects on the immune system

of the liver are mediated by increase adiposity or mediated by increase inflammatory signaling from the gut microbiome or both.

Our study shows that the gut microbiome is a critical player in the development and progression of obesity and NAFLD. The gut microbiome at baseline highly predicts the level of response to bariatric surgery and that the changes in the gut microbiome via bariatric surgery is sufficient to induce changes in steatosis and inflammation in the liver irrespective of diet. This implies that targeting either the gut microbiome or its downstream effects is a feasible and valid method for the treatment of both obesity and NAFLD.

Average (SD) (n=18)	Pre-Surgery	Post-Surgery (6 mo)	P-value
Age (yr)	37.1 (9.36)	37.1 (9.36)	NA
BMI	44.7 (4.9)	33.9 (4.8)	<0.001
Weight (kg)	118.5 (18.8)	89.7 (16.9)	<0.001
Race/Ethnicity			
Non-Hispanic White (%)	44.4		
African American (%)	5.6		
Asian (%)	11.1		
Hispanic (%)	38.9		NA

Table 4-1: Patient characteristics before and after bariatric surgery

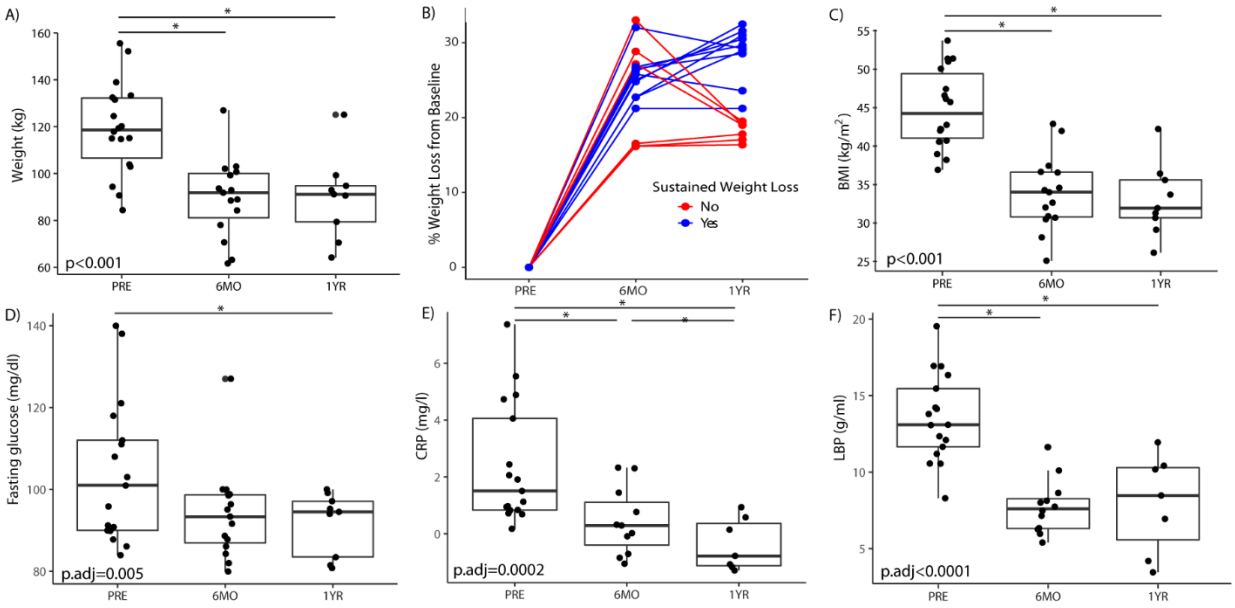

Figure 4-1: Bariatric surgery leads to significant weight loss, improved insulin resistance, and reduce inflammation. Weight change before and after bariatric surgery by (A) absolute weights, (B) percent weight loss from baseline and (C) BMI. Sustained weight loss was defined as at least 20% weight loss at 1-year post surgery. D) Fasting glucose before and after surgery. E) C-reactive protein (CRP) serum levels before and after surgery. F) Lipopolysaccharide binding protein (LBP) serum levels before and after surgery. *Signifies p-values <0.05.

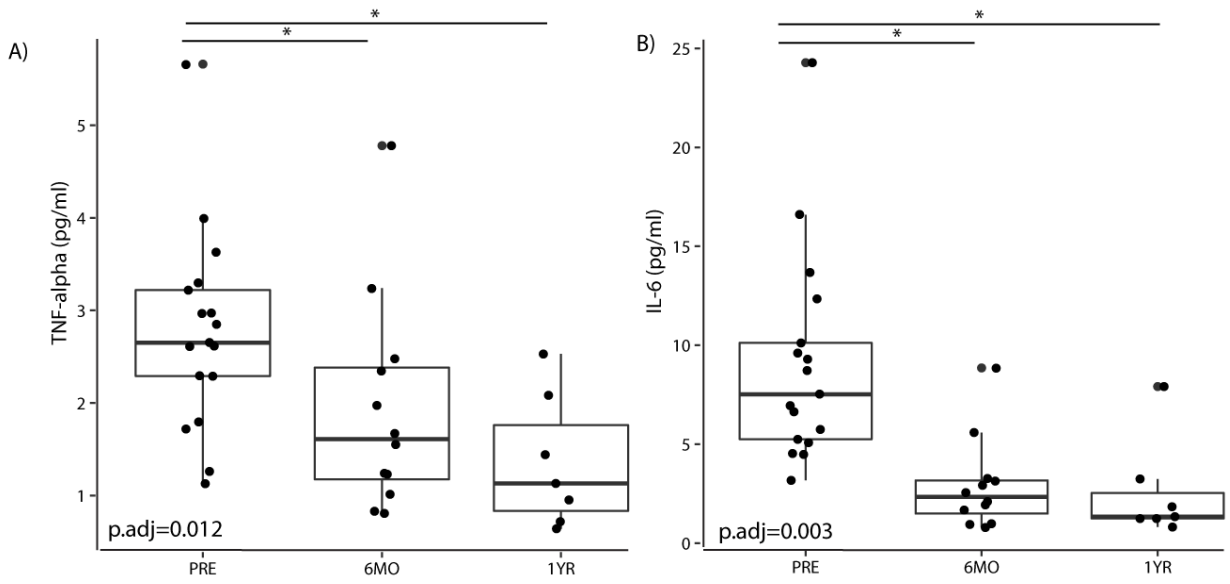

Figure 4-2: Inflammatory cytokine are reduced after surgery. A) Tumor necrosis factor (TNF) alpha and (B) IL-6 serum levels before and after surgery. *Signifies p-values <0.05.

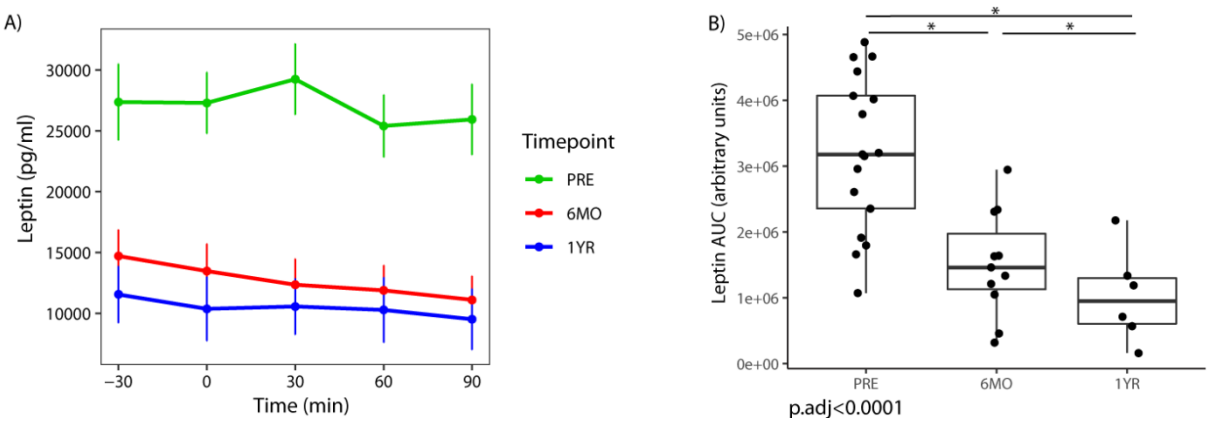

Figure 4-3: Leptin resistance decreases after bariatric surgery. A) Serum leptin levels before and after surgery as measured while fasting (-30) and post-prandially starting at 0 min time point. B) Area under the curve (AUC) for the graph. *Signifies p-values <0.05.

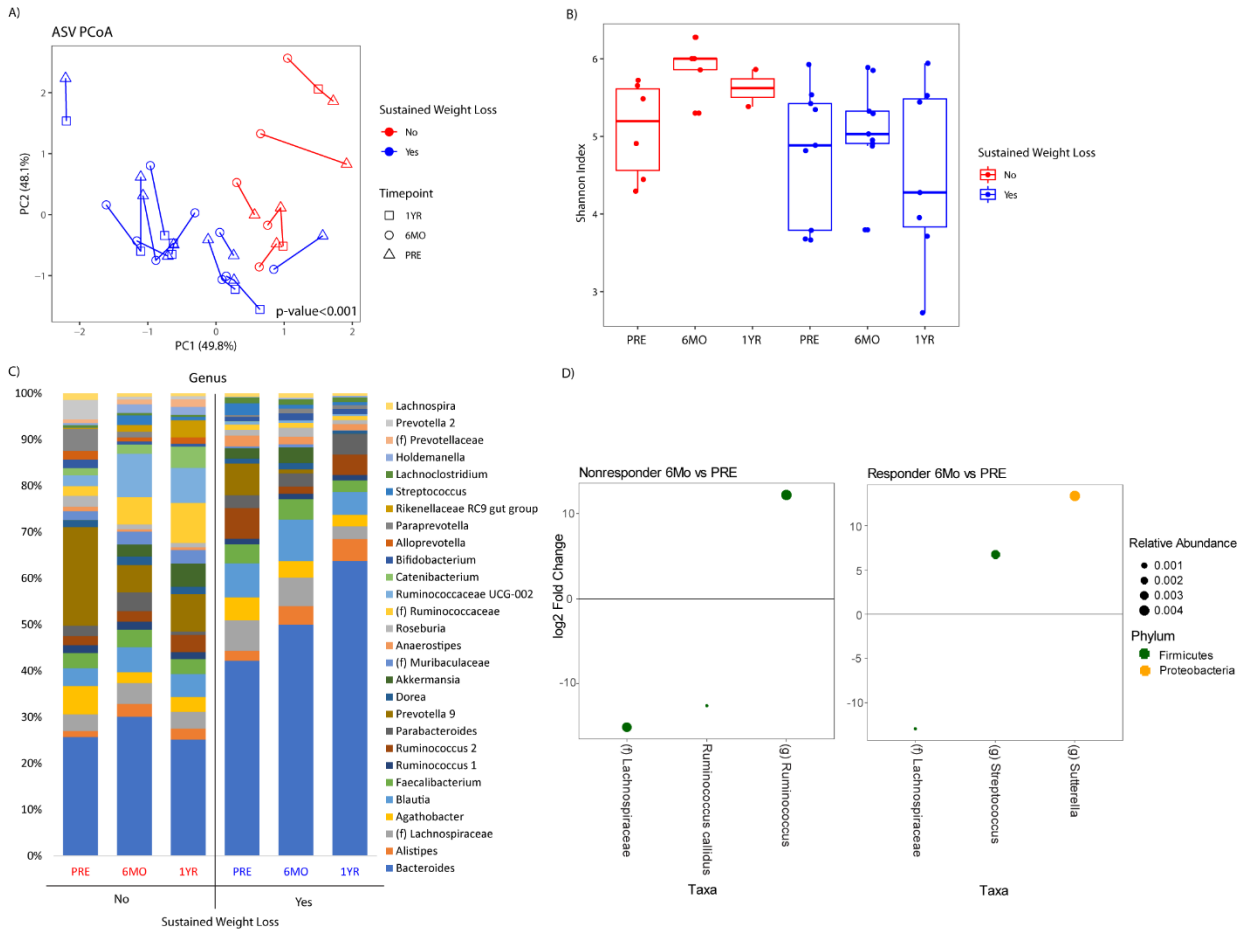

Figure 4-4: Microbiome is altered by bariatric surgery. A**)** Prinicipal coordinate plot colored by sustained weight loss with lines connecting patient samples across time. B) Alpha diversity as measured by Shannon Index which measures species evenness and abundance. C) Taxonomic plots by sustained weight loss over time. D) DESEq2 analysis showing taxa that are differentially abundant at 6 months as compared to baseline in patients with sustained weight loss (Responder) and those without sustained weight loss (Nonresponder).

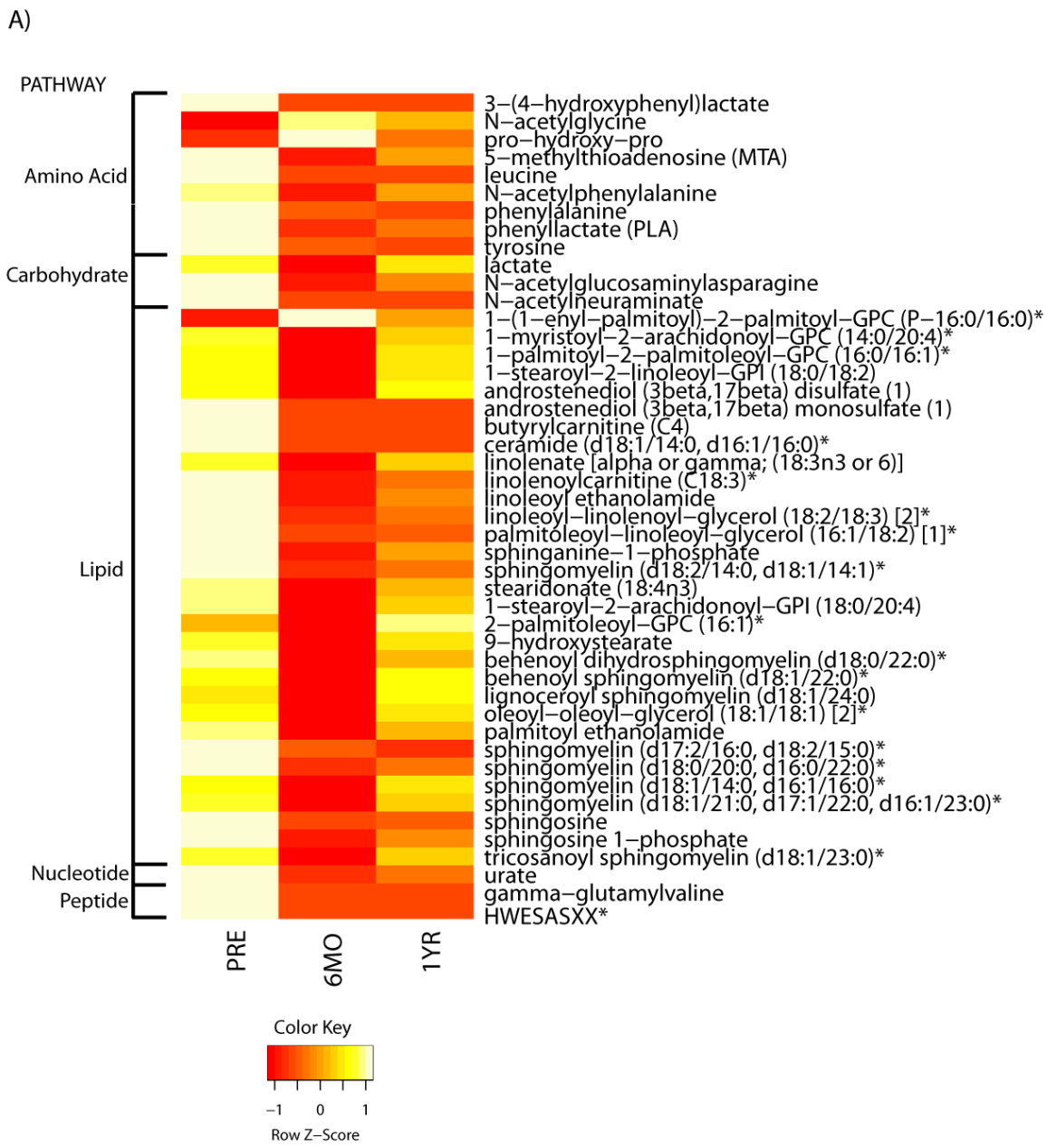

Figure 4-5: Heat map of differentially abundant serum metabolites as determined by DESEq2 across time.

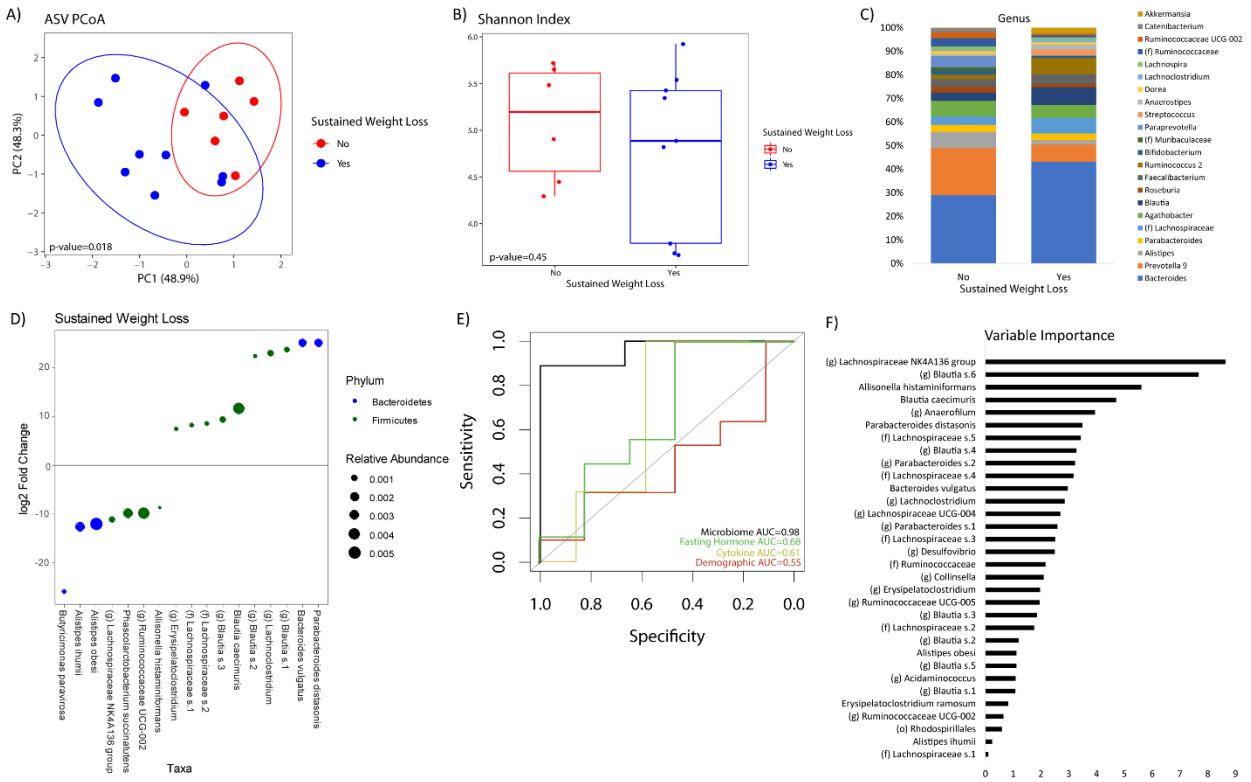

Figure 4-6: Baseline microbiome predicts sustained weight loss at 1-year post-bariatric surgery. A) Principal coordinate plot of the gut microbiome at baseline colored by patients achieving sustained weight loss. B) Alpha diversity as measured by Shannon Index which measures species evenness and abundance. C) Taxonomic plots by sustained weight loss D) DESEq2 analysis showing taxa that are differentially abundant between those with sustained weight loss and those without. Only those with a relative abundance of >0.0006 are shown. E) Receiver operator curves for random forrest classifier for classifier based on microbiome data, fasting hormone data, cytokine data, and demographic data. F) Variable importance plot of microbial random forest classifier.

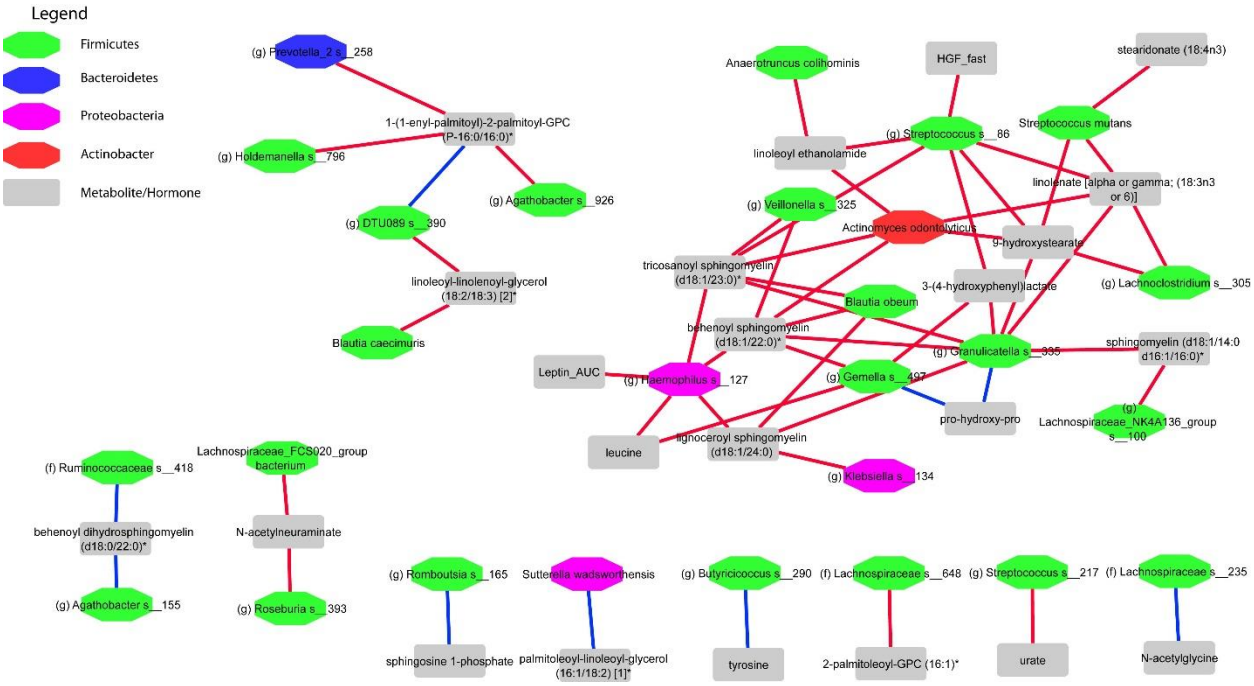

Figure 4-7: Correlation network of bacterial taxa to serum metabolite and serum hormone. Red color indicates a negative correlation. Blue indicates a positive correlation.

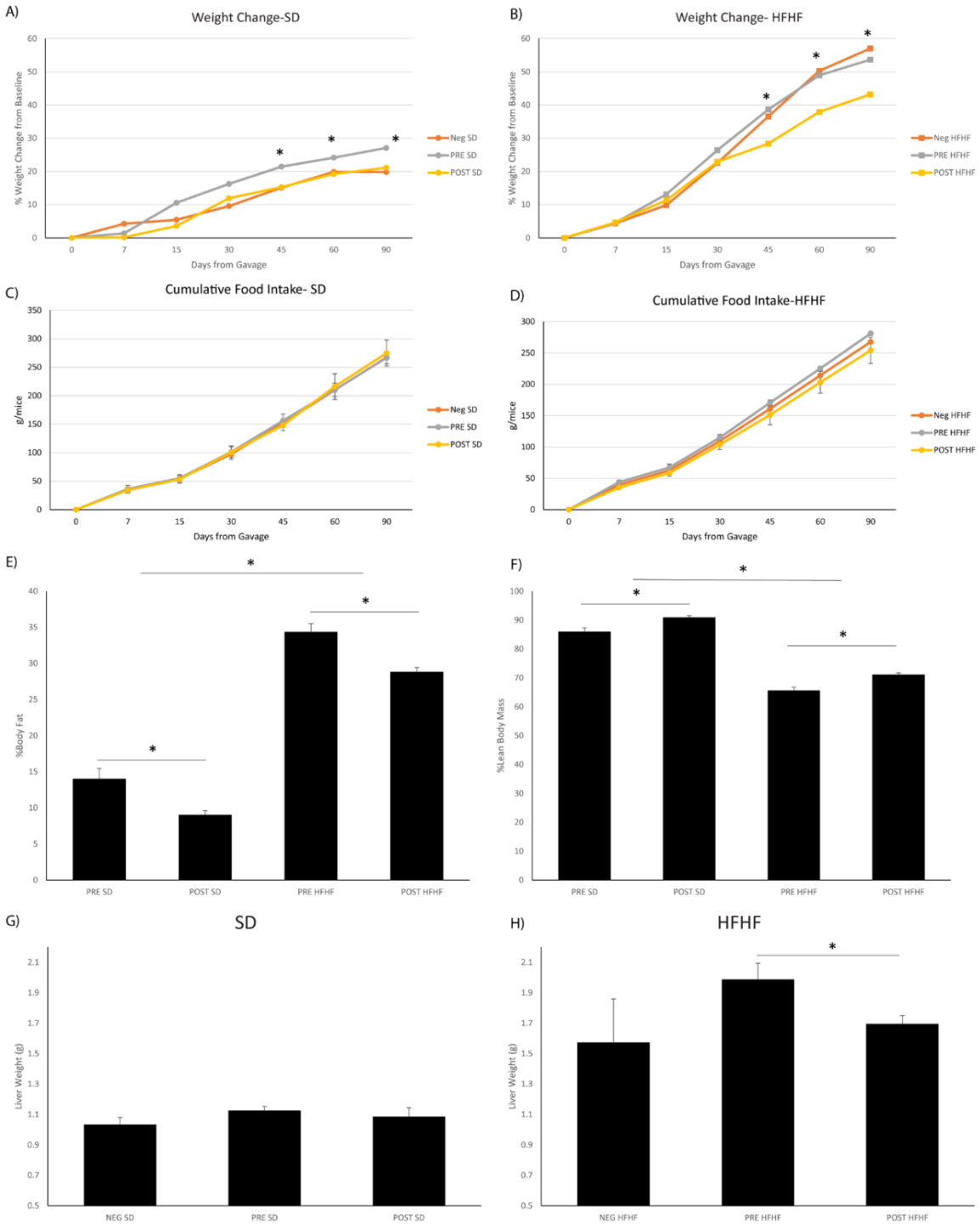

Figure 4-8: The gut microbiome of patients pre-bariatric surgery causes significant weight gain while on a standard diet (SD) or a high fat, high fructose, high cholesterol (HFHF) diet. Weight change over time of mice transplanted with pre- or post-bariatric surgery microbiome or mice

given negative control on a (A) SD or (B) HFHF diet. Cumulative food intake of the different mice on a (C) SD or (D) HFHF diet. EchoMRI results of mice as measured by (E) percent body fat or by (F) percent lean body mass. Liver weight of the different groups on a (G) SD or (H) HFHF diet. *Signifies p-value <0.05.

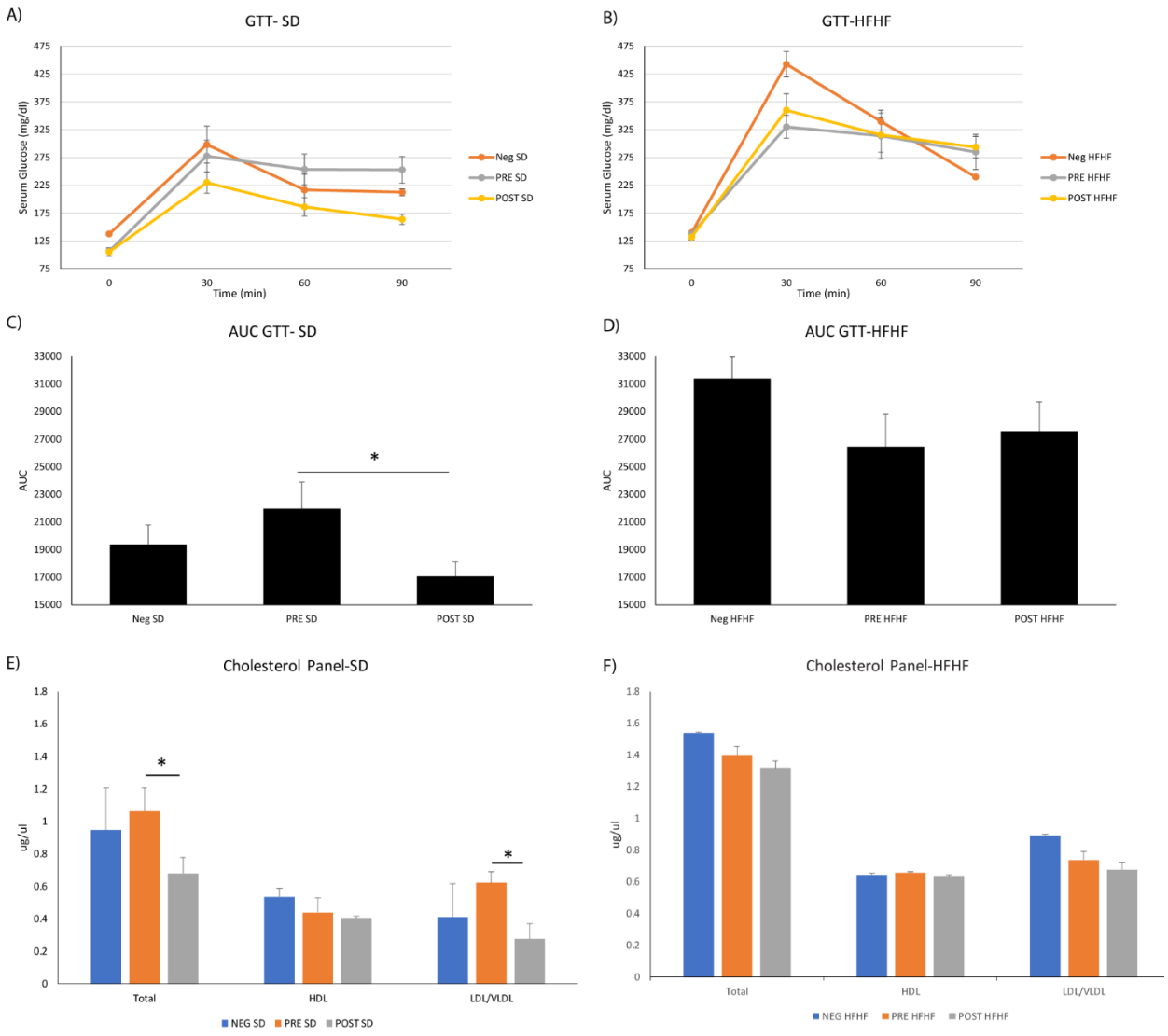

Figure 4-9: The gut microbiome of patients pre-bariatric surgery causes significant changes in insulin resistance and cholesterol while on a standard diet (SD) or a high fat, high fructose, high cholesterol (HFHF) diet. Glucose tolerance testing (GTT) of the different mouse groups on a (A) SD or (B) HFHF diet. (C-D) Area under the curve (AUC) of the respective graphs in A and B. Serum cholesterol level of the different mouse groups on a (E) SD or (F) HFHF diet. *Signifies p-value <0.05.

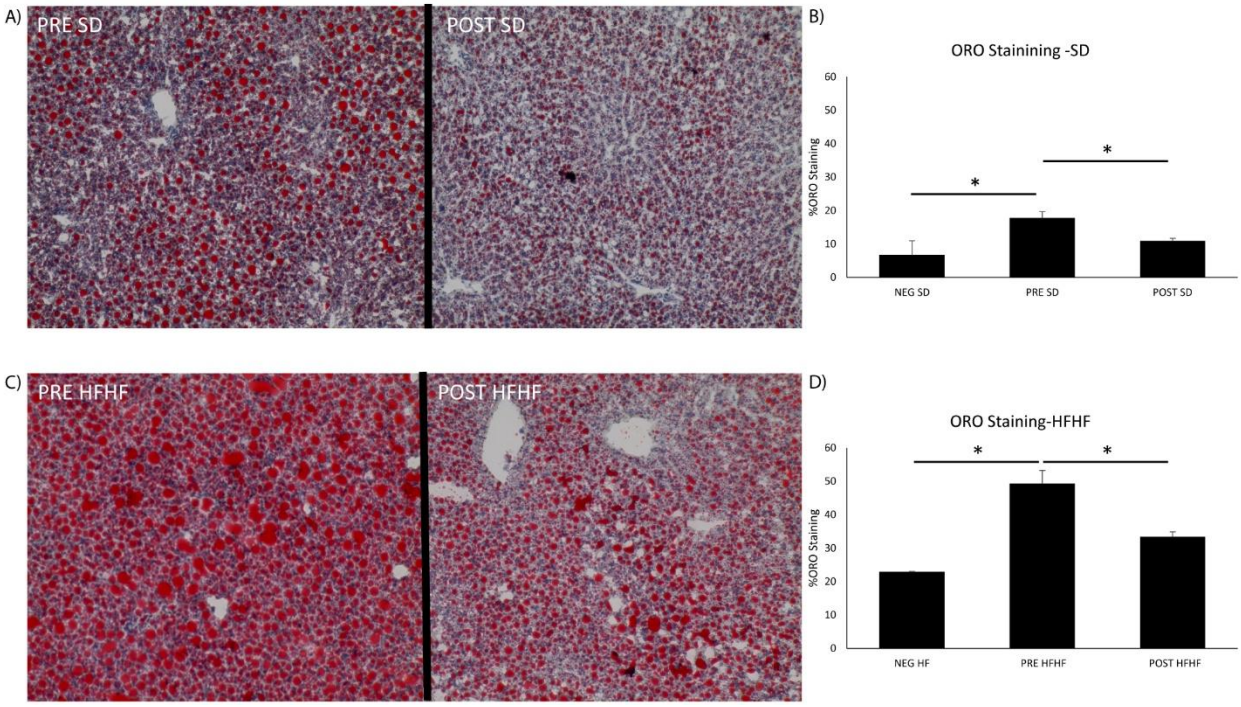

Figure 4-10: The gut microbiome of patients pre-bariatric surgery causes significant steatosis in the liver of mice on both a standard diet (SD) or a high-fat, high-fructose, high-cholesterol (HFHF) diet. Oil red o staining of liver tissue of mice transplanted with pre-bariatric surgery microbiome or post-surgery microbiome on a (A) SD or a (C) HFHF diet. B,D) is the percent of oil red o (ORO) staining. *Signifies p-values <0.05.

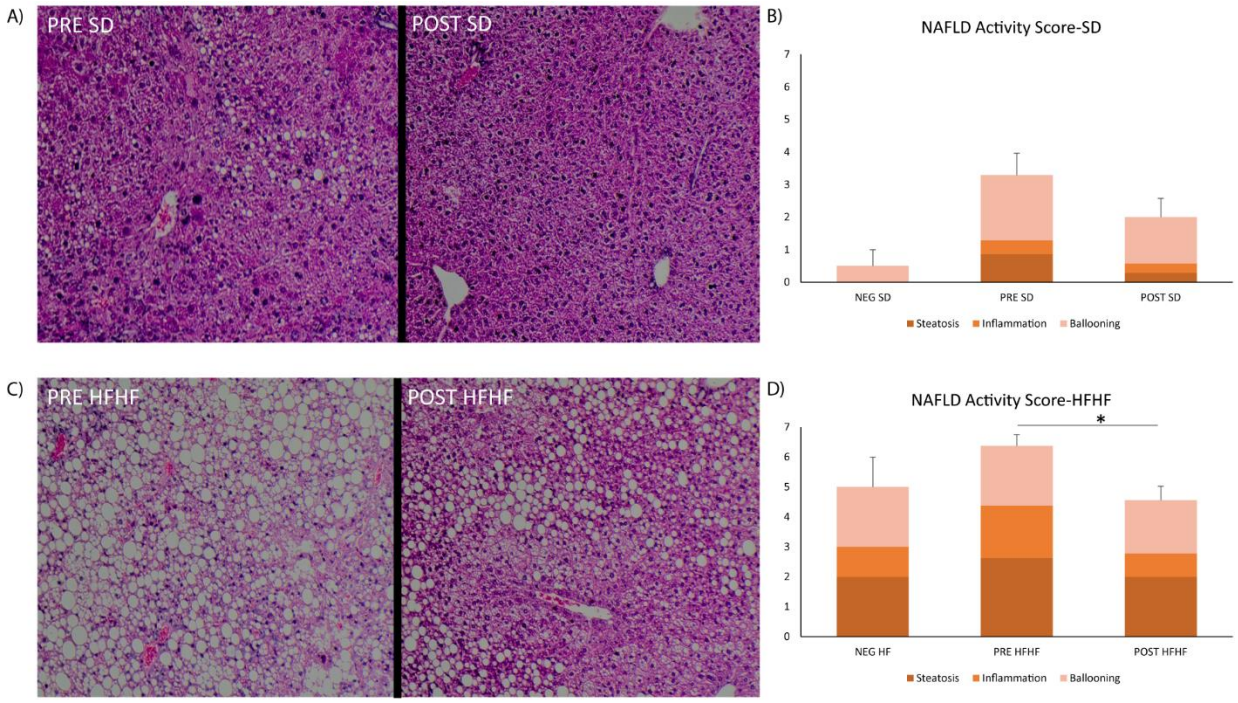

Figure 4-11: The gut microbiome of patients pre-bariatric surgery causes significant nonalcoholic steatohepatitis as measured by the NAFLD Activity Score on both a standard diet (SD) or a high-fat, high-fructose, high-cholesterol (HFHF) diet. Hematoxylin and Eosin staining of liver tissue of mice transplanted with pre-bariatric surgery microbiome or post-surgery microbiome on a (A) SD or a (C) HFHF diet. B,D) is their respective NAFLD activity score colored by the different sections that make up the score: steatosis, inflammation, ballooning. *Signifies p-values <0.05.

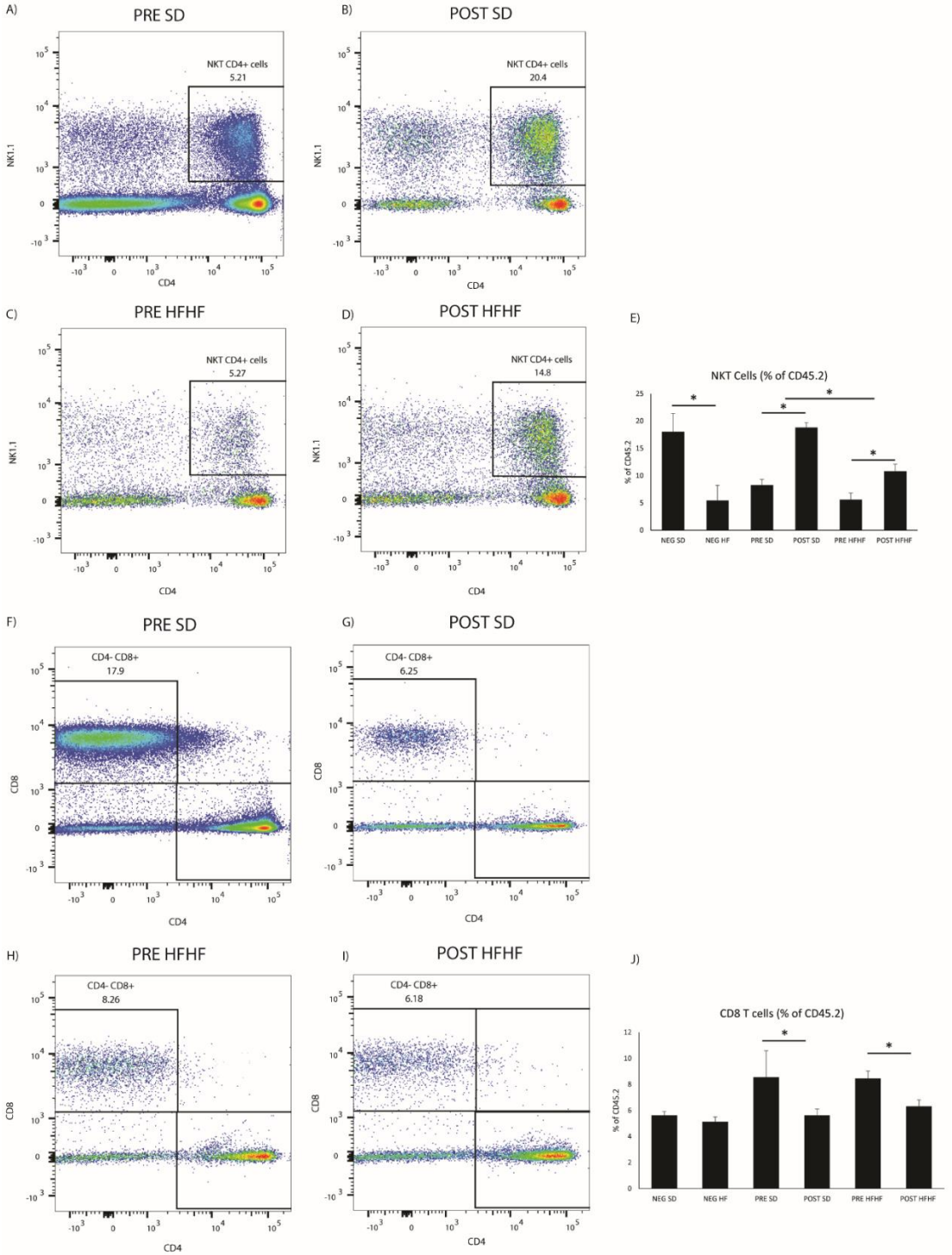

Figure 4-12: The gut microbiome of patients pre-bariatric surgery reduces natural killer T (NKT)-cell and increases CD8+ T-cells in the liver. A-D) Representative flow cytometry plots of CD4 and NK1.1 gated from CD3+B220- population. E) Graph showing percent of NKT cells as a

percentage of CD45.2+ cells across the different groups. F-I) Representative flow cytometry plots of CD4 and CD8 gated from CD3+B220- population. J) Graph showing percent of CD8+ T-cells as a percentage of CD45.2+ cells across the different groups. *Signifies p-value<0.05.

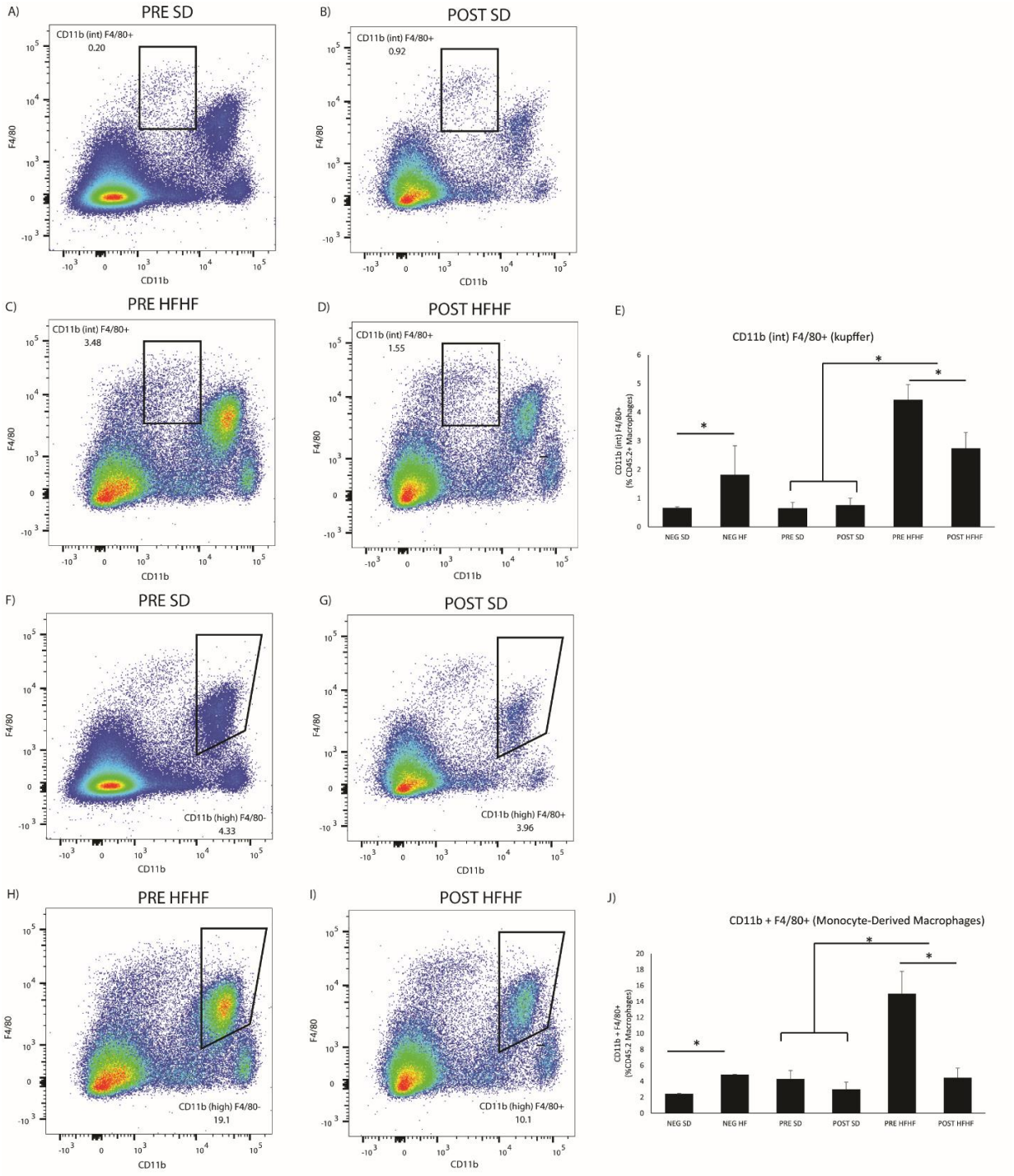

Figure 4-13: The gut microbiome of patients pre-bariatric surgery increases kupffer cells and monocyte-derived macrophages in the liver. A-D) Representative flow cytometry plots of CD11b and F4/80 gated from CD45.2+ Macrophage population. E) Graph showing percent of Kupffer cells as a percentage of CD45.2+ cells across the different groups. F-I) Representative flow

cytometry plots of CD11b and F4/80 gated from CD45.2+ Macrophage population. J) Graph showing percent of monocyte-derived macrophages as a percentage of CD45.2+ cells across the different groups. *Signifies p-value<0.05.

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Chapter 5

Concluding Remarks and Future Directions

In these series of studies, we have shown that the gut microbiome is a highly accurate predictor for several stages of obesity and NAFLD. Many studies before have shown that the gut microbiome of obese individuals is very distinct from those with leaner phenotype.[1-3] In our study we show for the first time how the gut microbiome may also be distinct in patients with specific eating disorders. We show that the gut microbiome is part of a classifier that is highly accurate at predicting food addiction in obese individuals. This particular microbiome profile is characterized by an underrepresentation of *Bacteroides, Akkermansia*, and *Eubacterium*, with an overrepresentation of *Megamonas*. Furthermore, the dysbiosis of the gut microbiome is associated with a significant decrease in a tryptophan-related metabolite, indolepropionate. This metabolite, as mentioned earlier has been implicated as being neuroprotective by potentially reducing antioxidants, DNA damage, and the production of β-amyloid fibrils.[4] These changes noted in the microbiome and fecal metabolite profile was associated to an increased connectivity between two regions of the brain's extended reward network, suggesting that these alterations in the brain-gut-microbiome axis may be important to the sometimes maladaptive eating behaviors of obese individuals.

Furthermore, research has shown that the microbiome and metabolite profile of patients with NAFLD differs at each stage of the disease, from bland steatosis to advanced fibrosis. By using a novel approach that incorporated machine learning, we were able to create and validate a classifier that was highly accurate at identifying NAFLD patients with advanced fibrosis. The main bacteria that was the most important in the classifier was *Prevotella copri*. The prevalence of this inflammatory bacteria in patients with advanced fibrosis suggests that it may play a role in the progression from steatosis to steatohepatitis and fibrosis through the production of reactive oxygen species.

We explored this causal link of the gut microbiome in fatty liver disease by utilizing an antibiotic-treated mouse model of microbial transplantation. In the human data set, we show that bariatric surgery induces long-term changes in the gut microbiome. We also show that the baseline gut microbiome of patients before surgery highly predicts their level of response to bariatric surgery. By taking the microbiome of these patients and transplanting them into antibiotic-treated mice we show a causal relationship between the gut microbiome to obesity and NAFLD. We show that the microbiome of patients before bariatric surgery is able to induce significant weight gain that is accompanied with significant steatosis and inflammation in the liver. We propose that through a combination of increased intestinal permeability and antigen presentation to the portal vein, along with increase inflammatory signaling, the gut microbiome can cause the shift that occurs from NAFL to NASH.

Overall, it is clear that obese individuals have a very distinct microbiome from lean individuals, and this difference is sufficient enough to induce weight, metabolic, and inflammatory changes in the host. Our studies have shown that the microbiome can be used accurately as a noninvasive biomarker for early detection of patients with NAFLD. The data also suggests that the signaling pathways related to the gut microbiome can be used to generate novel therapeutics against NAFLD and potentially fibrosis. Future studies will include the analysis of the portal venous metabolite of the antibiotic-treated animal model as well as serum and adipocyte hormone and cytokine levels. If a metabolite is found that correlates to the findings of NASH in the liver, future studies will involve analyzing its potential effects and signaling pathways.

We are just beginning to understand the role of the gut microbiome in health and disease. Through advances in sequencing technology and better animal modeling, a microbial-based therapy for obesity and NAFLD is potentially on the horizon.

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