Non-Alcoholic Fatty Liver Disease: Investigating the Impact of Bariatric Care and the Role of Immune Function

by

Katherine June Panabaker Schwenger

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Institute of Medical Science
University of Toronto

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Abstract

Non-alcoholic fatty liver disease (NAFLD) ranges from simple steatosis (SS) to nonalcoholic steatohepatitis (NASH) and cirrhosis. It is one of the most common causes of chronic liver disease resulting from obesity. 74-98% of morbidly obese individuals have NAFLD at the time of bariatric surgery. Post-bariatric surgery, NAFLD significantly improves but some patients continue to have persistent disease, particularly fibrosis. The pathogenesis of NAFLD is complex. In addition to insulin resistance, recent research suggests a potential role for liver immune function and intestinal microbiota (IM). The objectives of this research were 1) to prospectively evaluate the impact of bariatric care on metabolic and liver histology parameters by studying two bariatric care interventions: the routinely prescribed pre-operative very-low calorie diet (VLCD) and the Roux-en-Y gastric bypass (RYGB); and 2) to explore in a cross-sectional study whether there are differences in liver immune cells between NAFLD and healthy subjects and whether there are associations with intestinal microbiota (IM). Specifically, the plan was to assess: 1) liver histology post-VLCD and factors associated with NAFLD; 2) the effects of RYGB on liver histology and the factors associated with persistent NAFLD at 12-months post-RYGB; 3) liver immune cell counts in NAFLD compared to controls and the association between these cell counts and IM. We found that at the time of surgery (post-VLCD), the prevalence of NAFLD was low, likely due to the effect of the VLCD. Those with NASH had
higher insulin resistance and presence of diabetes prior to the diet. At 12-months post-RYGB there were further improvements in anthropometry, metabolic measurements and liver histology. However, those with persistent NAFLD had less improvement in waist circumference and glycemic control. The cross-sectional study showed that specific liver immune cells, namely CD163, CD20 and CD45 were significantly increased in NAFLD versus controls and that immune cell counts were significantly correlated with specific bacterial taxa. In conclusion, both VLCD and RYGB have beneficial effects on metabolic and histological parameters but those with persistent NAFLD are more metabolically impaired despite similar interventions. Other potential mechanisms, such as a potential interplay between IM and liver immune cells, require further investigation.
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A very special thank you to my officemates and many dedicated volunteers, especially Dr. Bianca Arendt, Anastasia Teterina, Hannah Da Silva and Carrie Li who have helped with all aspects of my study and PhD. This study would not have been possible without your support and guidance.

My PhD would not have been possible without the unwavering support of my family and friends. I would like to thank Jonathan Yach, my fiancé, for helping me throughout my thesis by editing, reading or simply listening to presentations. His support has been invaluable. I would also like to thank my parents and brother for their constant encouragement and support, especially in these last couple of months. I am grateful to my friends Richard, Shanna, Swapna, Fadl and Craig for keeping me laughing through all of our crazy adventures together. Finally, thank you to my grandmother, Frances Schwenger, who has edited every paper I have written since my first year in undergrad at the University of Guelph.
**Contributions**

I, Katherine Schwenger, am the sole author of this thesis. I was involved in all aspects of this work, including patient recruitment, data input, data analysis and manuscript preparation of all original research. From these studies, I wrote one literature review and three manuscripts, which are either published, in press or under review:


**Original Research:** Under Review at Surgery for Obesity and Related Diseases: Schwenger, K. J. P., Fischer, S., Jackson, T., Okrainec, A. and Allard, J. P. Persistent NAFLD at 12 months post-Roux-en-Y Gastric Bypass Surgery is associated with lower improvements in waist circumference and glycemic control.

Published studies not related to thesis:


and omega-3 polyunsaturated fatty acids in the progression of atherosclerosis in people living with HIV.

I would like to formally acknowledge the following contributions that were made by other individuals:

Dr. Johane Allard (Supervisor): Mentorship and guidance with regards to conducting clinical studies. Clinical and statistical expertise and assisted in data analysis as well as manuscript and dissertation preparation.

Anastasia Teterina (Statistician) Provided mentorship and guidance with regards to statistical analysis and interpretation.

Dr. Sandra Fischer (Pathologist): Provided histological analysis for liver samples used in this manuscript.

Dr. Timothy Jackson (Thesis Advisory Committee member): Mentorship and bariatric surgery expertise, as well as assisted with manuscript and thesis preparation.

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Dr. Mohammed Shafiee (Thesis Advisory Committee member): Mentorship and clinical medicine expertise, as well as assisted with manuscript and thesis preparation.

Dr. Jordan Feld (Thesis Advisory Committee member): Mentorship and NAFLD expertise, as well as assisted with manuscript and thesis preparation.

Dr. Bianca Arndt: Mentorship and guidance on how to conduct clinical research in a hospital setting. Also assisted with patient recruitment and visits for Study C. Assisted in initiating all studies through the Research Ethics Board.

Dr. Amel Taibi: Completed the DNA extraction and qPCR analysis for the intestinal microbiome data in Study C.

Hannah Da Silva: Helped with patient recruitment and database upkeep for Study C.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AASLD</td>
<td>American Association for the Study of Liver Disease</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>BAFF</td>
<td>B-cell activating factor</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSx</td>
<td>Bariatric surgery</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Damage-associated molecular patterns</td>
</tr>
<tr>
<td>DM2</td>
<td>Type 2 Diabetes</td>
</tr>
<tr>
<td>EBWL</td>
<td>Excess body weight loss</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>FFAR3</td>
<td>Free fatty acid receptor 3</td>
</tr>
<tr>
<td>FXR</td>
<td>Farnesoid X receptor</td>
</tr>
<tr>
<td>GF</td>
<td>Germ free</td>
</tr>
<tr>
<td>GGT</td>
<td>y-glutamyltransferase</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>GLP-2</td>
<td>Glucagon-like peptide-2</td>
</tr>
<tr>
<td>HC</td>
<td>Healthy controls</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HOMA</td>
<td>Homeostatic model assessment</td>
</tr>
<tr>
<td>HSC</td>
<td>Hepatic stellate cell</td>
</tr>
<tr>
<td>IBW</td>
<td>Ideal body weight</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IM</td>
<td>Intestinal microbiota</td>
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KC  Kupffer cells
IR  Insulin resistance
LDL  Low-density lipoprotein
LPS  Lipopolysaccharides
MUC2  Secreted mucin 2
NAFLD  Non-alcoholic fatty liver disease
NAS  NAFLD activity score
NASH  Non-alcoholic steatohepatitis
NFKB  Nuclear Factor kappa beta
NK  Natural killer cells
NKT  Natural killer T cells
NL  Normal liver
NLRP  Nucleotide-binding domains
PAMPS  Pathogen-associated molecular patterns
PEMT  Phosphatidylethanolamine methyl transferase
PPAR-γ  Peroxisomal proliferator activated receptor-γ
PUFA  Polyunsaturated fatty acids
PYY  Peptide YY
qPCR  Quantitative polymerase chain reaction
ROS  Reactive oxygen species
RYGB  Roux-en-Y gastric bypass
SCFA  Short chain fatty acids
SREBP-1c  Sterol regulatory element-binding protein-1c
SS  Simple steatosis
TLR-4  Toll-like-receptor-4
TNF-α  Tumor necrosis factor alpha
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>TWH</td>
<td>Toronto Western Hospital</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinediones</td>
</tr>
<tr>
<td>UDCA</td>
<td>Urodeoxycholic acid</td>
</tr>
<tr>
<td>UHN</td>
<td>University Health Network</td>
</tr>
<tr>
<td>VLCD</td>
<td>Very-low calorie diet</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very-low-density lipoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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Organization of the Dissertation

In Chapter 1, I have inserted my review paper on NAFLD describing the epidemiology, pathogenesis, diagnosis, staging and management of the disease. Next, I review two new areas that are related to the pathophysiology of NAFLD; the IM and immune function. I then discuss the risk factors and related comorbidities of obesity, including NAFLD. Finally, I summarize the effects of weight loss on obesity related comorbidities, with a focus on bariatric surgery.

In Chapter 2, I state the thesis objectives and hypothesis as well as provide an overview of the methodological approaches used throughout this dissertation.

Chapters 3, 4 and 5 contain publications or submitted manuscripts of 3 original research studies.

Chapter 6 presents a summary of the main findings and general discussion.

Finally, Chapter 7 describes future directions.
Chapter 1:

Introduction

Preface

Obesity is prevalent among Canadians with 20.2% reporting themselves as obese (defined as a body mass index (BMI) over 30 kg/m$^2$) in 2014 (1). Obesity can be caused by many factors, including diet, lifestyle, drugs, endocrine disorders and genetics. Obesity is associated with multiple co-morbidities including type 2 diabetes (DM2), cardiovascular disease, sleep apnea and non-alcoholic fatty liver disease (NAFLD) (2). Treatments for obesity include dietary changes, increased exercise and activity, behavioural changes, medication and weight loss surgery.

Non-alcoholic fatty liver disease (NAFLD) is one of the most common causes of liver disease worldwide, affecting 15 to 30% of the general population, with higher prevalence in obese individuals (3-6). NAFLD ranges from simple fat deposition in the liver (simple steatosis (SS)) to inflammation (nonalcoholic steatohepatitis or NASH), to fibrosis and cirrhosis (7). Although SS is a benign condition and only a small fraction progress to NASH, 15-20% of those with NASH progress to cirrhosis, which eventually leads to liver failure requiring transplantation (8).

NAFLD is a frequent co-morbidity of the metabolic syndrome and obesity (9). The prevalence of NAFLD increases to 58% in overweight individuals and can be as high as 98% in non-diabetic obese individuals (10). In morbidly obese individuals undergoing bariatric surgery,
the prevalence for NAFLD is high, overall between 74 and 98% (11-13) with mean prevalence of NASH being 37% (10). However, it is not clear from the literature whether these figures were reported after a frequently prescribed pre-operative very-low calorie diet (VLCD) or not. In addition, while bariatric surgery improves weight, co-morbidities and NAFLD (14-16), it is not clear why some patients have persistent liver abnormalities while others become normal after surgery.

Several factors may contribute to NAFLD with insulin resistance (IR) playing a major role (17). More recently, emerging research suggests that intestinal microbiota (IM) may contribute to NAFLD (18-21) as well as to obesity (22) and DM2 (23). A role for hepatic immune function is also suggested (24-26). Our group was the first to detect differences in IM associated with NAFLD in adults, using qPCR (27) and then using 16s rRNA sequencing (28). However, no one has looked at whether there is a link between IM and hepatic immune cells.

With this proposal, I plan first to investigate the prevalence of NAFLD in morbidly obese patients undergoing a VLCD prior to laparoscopic Roux-en-Y gastric bypass (RYGB) and compare results to the current literature. In addition, I will assess the metabolic response to VLCD and contributors to NAFLD. Secondly, I will evaluate liver histology, anthropometric and metabolic parameters 12 months post-RYGB as well as the contributors to persistent NAFLD post-surgery. Finally, I will explore potential relationships between IM and the hepatic immune cells.
1.1 Review Article: Non-Alcoholic Fatty Liver Disease: General Overview

by

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This chapter is modified from the following: Schwenger & Allard. World Journal of Gastroenterology. 2014: 20(7): 1712-1723
Non-alcoholic fatty liver disease (NAFLD) is the most common cause of liver disease worldwide, with a prevalence of 15-30% in Western populations (4-6, 29). The prevalence increases to 58% in overweight individuals and can be as high as 98% in non-diabetic obese individuals (10). NAFLD ranges from simple steatosis to nonalcoholic steatohepatitis (NASH) and potentially cirrhosis (7). NASH is the picture of hepatocellular injury and inflammation of the liver (30). Cirrhosis, which occurs in 25% of patients with NASH, can result in liver failure, portal hypertension, and hepatocellular carcinoma and patients with cirrhosis are at a high risk for developing cardiovascular disease (31). Not only the presence of excess weight and obesity, but the location of fat storage also plays a role in NAFLD pathogenesis. Visceral fat stores increase the risk for NAFLD in both obese and non-obese individuals (32). NAFLD is associated with metabolic syndrome and obesity (9). The diagnosis of NAFLD is made when there is evidence of liver steatosis on imaging modalities and this is associated with features of the metabolic syndrome in the context of a patient who does not have other causes of liver disease (33) and where alcohol consumption is less than 21 drinks and 14 drinks per week for men and women, respectively (34, 35). Diagnosis for NASH is confirmed when a liver biopsy shows the presence of perilobular inflammation, or the presence of hepatocyte ballooning, Mallory hyaline and acidophil bodies with or without fibrosis. Non-invasive tests such as liver enzymes, medical imaging, Fatty Liver Index, NAFLD fibrosis score, FibroMeter and Fibroscan (36) may suggest the presence of NASH by detecting fibrosis. Research is on-going to assess surrogate markers for NASH such as CK18, but this remains experimental (37). Therefore, for a definite diagnosis of NASH, patients still need a liver biopsy.
1.1.2 NAFLD Pathogenesis

In NAFLD, the accumulation of fat in the liver (38) is a result of increased delivery of free fatty acid (FFA) to the liver, increased synthesis, decreased triglyceride export through very-low density lipoprotein (VLDL) and reduced beta-oxidation (39). Universally, patients with NAFLD have insulin resistance (IR) which increases lipolysis from the adipose tissue (17). The resulting FFA are taken up by the liver and can cause lipid peroxidation (17). Lipid peroxidation can increase the production of pro-inflammatory cytokines (17). The increase in FFA can also exceed mitochondrial beta-oxidation, further increasing the oxidative stress (40) and inflammation (40).

Liver de novo lipogenesis (41) also contributes to the steatosis. De novo lipogenesis is due to the hyperinsulinemia associated with IR, which stimulates the enzymes in the de novo lipogenesis pathway, increasing the production and storage of triglycerides. In NAFLD, lipogenesis contributes to 26% of hepatic triglyceride accumulation while it accounts for <5% in healthy individuals (41). Hyperinsulinemia can also cause a reduction in VLDL secretion (42), leading to triglyceride accumulation in the liver.

The presence of inflammation or steatohepatitis depends on a number of factors such as the presence of FFA, inflammatory cytokines and adipokines, oxidative stress and mitochondrial dysfunction (39). Proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) are elevated and generally produced by the liver and adipose tissue, from NF—KB activation (43) (e.g. from lipid peroxidation or activation of Toll-like receptors). Increased TNF-α and IL-6 are also associated with IR by interfering with insulin signaling (30, 44). On the other hand, adiponectin (30, 44) is produced by the
adipose tissue and is an anti-inflammatory adipokine that can increase insulin sensitivity (45). In NAFLD adiponectin is reduced which decreases fatty acid oxidation and hepatic gluconeogenesis (46). The role of other adipokines, like visfatin, leptin and resistin are still equivocal (47-49). Potential pathways are promotion of IR, oxidative stress and inflammation as well as fibrogenesis (50).

Diet is an important contributor to NAFLD, mainly because excessive energy intake leads to obesity, which in turn increases the risk for NAFLD. However, the amount of energy and also the quality of the diet likely play an important role for the development and progression of NAFLD. Diets rich in saturated fat and cholesterol, and low in polyunsaturated fat, fiber and antioxidant vitamin C and E (51) are associated with NASH. High saturated fat diets are associated with IR and hepatic inflammation (51). Other research has also demonstrated a relationship between increased dietary fat consumption and NAFLD (52, 53). However, a study investigating pre-surgical bariatric patients in the United States reported that increased carbohydrate intake can also be associated with hepatic inflammation (54). Among carbohydrates, fructose specifically may contribute to NAFLD progression. Fructose intake has been linked to increasing hepatic fat, inflammation and possibly fibrosis (55). Fructose has also been associated with both an increase in visceral adipose tissue (56) and plasma triglycerides (57).

Recently, new evidence has linked intestinal microbiota to NAFLD pathogenesis. Intestinal microbiota (IM) may play a role in the development of NAFLD, however very few human studies have been conducted and most were cross-sectional (27, 58-60). Our group reported an association between low percentage of fecal Bacteroidetes and the presence of
NASH, independent of diet and body mass index (27). This was followed by a recent study showing that in NAFLD, 8 operational taxonomic units, 6 genera, 6 families and 2 phyla (Bacteroidetes, Firmicutes) were less abundant and; 1 genus (*Lactobacillus*) and 1 family (Lactobacillaceae) were more abundant compared to HC (28). Lower abundance in both NASH and SS patients compared to HC were confirmed by qPCR for *Ruminococcus*, *Faecalibacterium prausnitzii* and *Coprococcus*. no difference was found between NASH and SS (28). This lower abundance in NAFLD (NASH+SS) was independent of BMI and IR (28). In addition, NAFLD patients had higher concentrations of fecal propionate and isobutyric acid and serum 2-hydroxybutyrate and L-lactic acid (28). These findings suggest a potential role for a specific IM community and functional profile in the pathogenesis of NAFLD(28).

Other studies showed an increased abundance of *Escherichia coli* (*E. coli*) associated with higher blood alcohol levels in pediatric patients with NAFLD (60) or differences in IM associated with differences in volatile organic compounds (59). Development of fatty liver on a choline deficient diet was also associated with IM at baseline and single nucleotide polymorphism in the phosphatidylethanolamine methyl transferase (PEMT) gene region (58). IM can be altered by the type of diet consumed and may contribute to NAFLD through several mechanisms. These include salvaging energy from food, contributing to inflammation via cytokines by increasing intestinal permeability leading to endotoxemia, modulating the innate immune system such as activation of Toll-like receptors and inflammasomes, regulating bile acid, metabolizing dietary choline and increasing endogenous ethanol by bacteria (58, 61).
1.1.3 NAFLD Diagnosis

NAFLD should be suspected in individuals who are either obese, diabetic or have metabolic syndrome (61). However, the majority of patients with NAFLD are asymptomatic and the disease may be detected via routine blood tests showing elevated liver enzymes or when an ultrasound is performed for various reasons and detects liver steatosis (refer to Figure 1-1 for summary). However, secondary causes of hepatic steatosis or elevated liver enzymes should be excluded by reviewing patients’ histories and a proper investigation (61, 62). These causes include excess alcohol consumption, medications, toxins, lipodystrophy, autoimmune and inflammatory diseases, nutritional issues (malnutrition, total parenteral nutrition, severe weight loss, and refeeding syndrome), viral hepatitis and metabolic liver disease.

Although it is still not possible to diagnose NAFLD based solely on blood work, elevated transaminases can be used as a first step (63). Elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in the absence of other liver diseases may support NAFLD (62, 64), and have been found in up to approximately 50% of simple steatosis patients and 80% of NASH patients (63). An AST:ALT ratio less than 1 is also exhibited in NAFLD (65) and supports NASH. However, it is important to note that patients with normal transaminases and liver steatosis on imaging may also have NASH (66).

Ultrasonography is a non-invasive tool that is used in the detection of liver steatosis (61, 67). Other imaging techniques such as computed tomography and nuclear magnetic resonance imaging can also detect liver steatosis, but neither of these more expensive techniques provide more information than ultrasonography (67, 68) except for fat
A review conducted by Fetsi et al. concluded that ultrasonography should be used as a first-line diagnostic tool because of its evaluation of liver steatosis and other abdominal organs (67).

The Fatty Liver Index is an algorithm based on four markers; body mass index (BMI), waist circumference, triglyceride and y-glutamyltransferase (GGT) (26), which is confirmed to accurately identify NAFLD (70, 71). This index has been used in population studies (61) and has an accuracy of 0.84 in detecting fatty liver (72). The Fatty Liver Index provides a score out of 100, indicating that a score < 30 can rule out and a score ≥60 to rule in hepatic steatosis (72). The formula for the Fatty Liver Index is 

\[(\frac{e^{0.953\log_e(\text{triglycerides})} + 0.139\times\text{BMI} + 0.718\log_e(\text{GGT}) + 0.053\times\text{waist circumference} - 15.745}{1 + e^{0.953\log_e(\text{triglycerides})} + 0.139\times\text{BMI} + 0.718\log_e(\text{GGT}) + 0.053\times\text{waist circumference} - 15.745})\times 100\]  

Liver biopsy is currently the gold standard for diagnosing NASH (62), as it also establishes the stage of NASH (73). This invasive procedure is used to analyze the degree of hepatocyte injury and level of fibrosis and inflammation (67). However, it is used after imaging techniques, laboratory abnormalities and/or non-invasive methods suggest the presence and severity of NASH (67, 73).
Figure 1-1: Diagnosis and staging of NAFLD

1.1.4 NAFLD Staging

Recent advances have allowed for non-invasive techniques to be used to diagnose the level of inflammation/fibrosis (see Figure 1-1 for summary) (61).

The NAFLD fibrosis score evaluates six variables; age, hyperglycemia, body mass index, platelet count, albumin and AST/ALT ratio (61, 74). The NAFLD fibrosis score formula is:

\[ -1.675 + 0.037 \times \text{age (years)} + 0.094 \times \text{BMI (kg/m2)} + 1.13 \times \text{IFG/diabetes (yes =1, no =0)} + 0.99 \times \text{AST/ALT ratio} - 0.013 \times \text{platelets (} \times 10^9/L) - 0.66 \times \text{albumin (gr/dl)}} \]

This equation is used to classify the probability of fibrosis as < -1.5 for low probability and > -1.5 to < 0.67 for intermediate probability and > 0.67 for high probability (75). Angulo et al. validated the NAFLD fibrosis score using liver biopsies and found that this accurately classifies NAFLD patients with and without advanced fibrosis (74). Furthermore, a study investigating NAFLD in a morbidly obese population undergoing bariatric surgery found that...
the NAFLD fibrosis score is accurate at excluding advanced fibrosis within this population (76). Overall, this tool is widely used in practice to exclude advanced fibrosis.

Another tool used in clinical practice is the FibroMeter. This tool uses age, weight, fasting glucose, AST, ALT, ferritin and platelet count to diagnose significant fibrosis (67, 77, 78). The formula for the FibroMeter is \(-0.007 \times \text{platelets (g/L)} - 0.049 \times \text{prothrombin time (%)} + 0.012 \times \text{AST (UL/L)} + 0.005 \times a2 \text{macroglobulin (g/L)} + 0.021 \times \text{hyaluronate (ng/ml)} - 0.270 \times \text{urea (mmol/L)} + 0.270 \times \text{age (years)} + 3.718\) (79). The FibroMeter provides the probability of significant fibrosis and the percentage of hepatic fibrosis (79). Cales et al. compared the FibroMeter to the NAFLD fibrosis score and found that the FibroMeter provides a more reliable diagnosis for significant fibrosis (77). This tool can be used to confirm or disconfirm advanced fibrosis in NAFLD patients (78).

FibroScan, also known as transient elastography, is another non-invasive method to assess liver fibrosis (80). This method measures liver stiffness, which was originally designed for the hepatitis C population (81), but is now being used in the NAFLD population (82). The FibroScan sends a pulse through the skin, which is circulated through the liver. The velocity of the wave, which is correlated with liver stiffness, is measured by ultrasound. The stiffer the liver the greater the degree of fibrosis (83). The liver stiffness measurement is used to assess the current stage of liver fibrosis. The cutoffs are 4.85, 7.38, 9.28, 13.33 and 25.34 kPa which represent stages, 0 (no steatosis), 1 (perivenular and/or perisinusoidal fibrosis), 2 (combined pericellular portal fibrosis), 3 (septal fibrosis) and 4 (cirrhosis) (80). Yoneda et al. investigated the usefulness of transient elastography in NAFLD patients (80). They found that there is a significant correlation between liver stiffness and fibrosis stage, which was
confirmed by liver biopsy (80). Therefore, this measurement can be used in the NAFLD population to determine the stage of fibrosis. However, special consideration is needed for overweight and obese patients. Studies have shown that obesity, (BMI > 30 kg/m²) provides inaccurate liver stiffness measurements but the use of a FibroScan XL probe has been shown to provide more reliable liver stiffness measurement (84, 85).

Overall, these noninvasive measurements to assess NAFLD/NASH should be used prior to a liver biopsy as they pose minimal risk to the patient. However, liver biopsy should be considered in patients when these noninvasive tests suggesting fibrosis are inconclusive (61), or the patients have risk factors associated with advanced fibrosis, such as age > 50y, presence of diabetes, morbid obesity or metabolic syndrome (86).

1.1.5 NAFLD Medical Management

The goal of managing NAFLD is to improve steatosis and prevent fibrosis. Treating risk factors such as obesity and IR remains the focus of managing NAFLD. Currently, lifestyle interventions, medical treatments, alternative therapies and surgery are being used to treat risk factors associated with NAFLD. In addition, new compounds are being used to treat fibrosis.

1.1.5.1 Lifestyle Interventions

Obesity and NAFLD are associated with poor diet choices (87), sedentary lifestyle (88) and increased energy intake when compared to healthy subjects (87, 88). Weight management through improvements in diet and increased physical activity has been shown to improve liver histology and reduce risks of disease progression. Table 1-1 summarizes lifestyle intervention studies (89).
Table 1-1: Summary of lifestyle intervention studies: Diet and/or physical activity

<table>
<thead>
<tr>
<th>Author, Year</th>
<th>Population, Study Design</th>
<th>Intervention</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>(90)</td>
<td>n=96, 12-month intervention on adults with hepatic steatosis and type 2 diabetes</td>
<td>Combination of moderate caloric restriction (1,200 to 1,800 kcal/day) and increased moderate physical activity (175 min/week)</td>
<td>Significant decreases in BMI, weight, waist circumference, % body fat and HbA1C</td>
</tr>
<tr>
<td>(91)</td>
<td>n=50, longitudinal study with lifestyle intervention in NAFLD adults</td>
<td>10 counselling sessions with a dietitian, and moderate intensity activity 3 hr/week</td>
<td>Significant decreases in body fat and liver fat and improved fitness. NAFLD at baseline resolved in 20 participants.</td>
</tr>
<tr>
<td>(92)</td>
<td>n=28, randomized control trial adults with elevated ALT or AST, BMI of 25-40 Kg/m²</td>
<td>Combination of diet (1000-1500 kcal/day), exercise (10,000 steps per day and 200 min/week of moderate physical activity) and behavior modification</td>
<td>Weight in intervention group decreased by 9.3%, significant improvement of NASH. &gt; 7% weight loss significantly improved steatosis</td>
</tr>
<tr>
<td>(93)</td>
<td>n=152, randomized intervention of adults with elevated liver enzymes, central obesity and metabolic risk factors</td>
<td>Randomized to moderate (6 sessions/10weeks) or low-intensity exercise (3 sessions/4 weeks) or control. Physical activity 150 min/week and low saturated fat and process food diet (1700-2400 kcal/day)</td>
<td>Moderate intensity: improvement in all risk factors, greater reduction in liver enzymes and weight loss than low-intensity exercise</td>
</tr>
<tr>
<td>(94)</td>
<td>n=19, 8 week exercise intervention in NAFLD adults</td>
<td>8 weeks (3x/week) of resistance exercise (n=11) versus control (n=8).</td>
<td>13% reduction in liver lipid. Lipid oxidation, glucose and IR improved. No effect on weight or body fat.</td>
</tr>
</tbody>
</table>
BMI: body mass index, NAFLD: non-alcoholic fatty liver disease, ALT: alanine aminotransferase, AST: aspartate aminotransferase, NASH: nonalcoholic steatohepatitis, IR: insulin resistance

Several studies demonstrated weight loss, physical activity and behavior modification are successful in improving liver enzymes, insulin sensitivity, reducing inflammation and liver histology (90-92, 95, 96). A randomized controlled trial conducted by Promrat et al. used a combination of diet, physical activity and behavior modification to trigger 7%-10% weight loss in obese NASH patients (92). Those who achieved a minimum of 7% weight loss had improvements in their liver histology (92). Another study in NAFLD patients with elevated liver enzymes and central obesity randomized patients to either low intensity (3 sessions/four weeks) or moderate intensity (6 sessions/10 weeks) physical activity, compared to a control group (93). The intervention also included dietary counselling and behavior modification (93). Results showed a reduction in liver enzymes, which was greater in the moderate-intensity lifestyle intervention group versus the control group (93). A 2017 meta-analysis evaluated lifestyle modifications in 20 randomized controlled trials that included a total 1073 NAFLD patients. They found that interventions combining diet and exercise significantly decreased ALT and improved NAFLD activity score (97). With regards to diet, they found that both moderate carbohydrate diets and low/moderate-fat diets yielded similar beneficial effects on liver enzymes. When evaluating the effects of exercise alone compared to standard care, they found that exercise significantly improved ALT and AST levels. Additionally, they found that exercise improved intrahepatic fat, regardless of weight change (97).

Physical activity alone has been found to reduce hepatic steatosis, independent of weight loss. A study on sedentary NAFLD patients examined the effects of resistance
exercises on liver lipid levels (94): 8 weeks (3 times per week lasting 45-60 minutes) of resistance based exercise resulted in a reduction of liver lipids, and improvements of lipid oxidation, glucose control and insulin resistance. A review conducted by Thoma et al., analyzed 23 studies using diet modification, physical activity, or a combination of both (95). The authors arrived at the same conclusion as the meta-analysis (97) and found that lifestyle modifications that led to weight reduction and/or increased physical activity greatly reduced liver fat and improved insulin sensitivity (95).

Overall, lifestyle modification (diet and exercise) resulting in weight loss or increased physical activity alone can reduce liver enzymes and improve liver histology, glucose control, insulin sensitivity and lipid oxidation. Therefore, when developing a treatment plan for NAFLD patients, lifestyle modification should be used as a first step in clinical settings.

1.1.5.2 Medical and Other Non-Surgical Treatments

Lifestyle interventions may not be effective often due to lack of compliance, and so other approaches may be considered in the management of NAFLD. Table 1-2 provides a summary of medical treatment studies. Pharmacological treatment mainly focuses on insulin-sensitizing agents. Three insulin-sensitizing agents, metformin, thiazolidinediones (TZD) and liraglutide, have been investigated in this population, however there are conflicting results.

Table 1-2: Summary of medication intervention studies

<table>
<thead>
<tr>
<th>Author, Year</th>
<th>Population, Study Design</th>
<th>Intervention</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>(98)</td>
<td>n=15, open label study with histologically confirmed NAFLD adults</td>
<td>All patients received 20mg/kg/day of metformin for 48 weeks</td>
<td>In the initial 3 months, there was improvement in ALT and AST levels and insulin sensitivity. After 3 months no further improvement noted.</td>
</tr>
<tr>
<td>Study Number</td>
<td>Study Design and Participants</td>
<td>Intervention</td>
<td>Outcome(s)</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------</td>
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</tr>
<tr>
<td>(99)</td>
<td>n= 57 24-month observational study with NAFLD or NASH overweight and obese children</td>
<td>Metformin was progressively titrated from 250 to 500 mg TID at weekly intervals and patients were given a hypocaloric or isocaloric diet and recommended to engage in 45 min/day of physical activity (n=57) compared to control group (n=30) with the same diet and physical activity recommendations</td>
<td>ALT significantly improved with decreasing body weight. NAS score decreased in both groups, no significant changes in fibrosis.</td>
</tr>
<tr>
<td>(100)</td>
<td>n=63, randomized, double-blind placebo – controlled in NASH adults</td>
<td>32 patients were given rosiglitazone (4 mg/day for 1 month then 8 mg/day for 11 months) versus placebo (n=31)</td>
<td>Improved steatosis and normalized transaminase, but only half of patients responded. Improvement of insulin sensitivity.</td>
</tr>
<tr>
<td>(101)</td>
<td>n=47, randomized controlled study in adults with impaired glucose tolerance or type 2 diabetes with NASH</td>
<td>6 months of hypocaloric diet and 45 mg (n=26) of pioglitazone versus 6 months of hypocaloric diet (n=21)</td>
<td>Diet and pioglitazone improved glucose tolerance and normalized ALT. Histologic features of NASH improved, no significant reduction in fibrosis.</td>
</tr>
<tr>
<td>(102)</td>
<td>n=13 patient cohort with NASH adults</td>
<td>All were treated with 30mg/day of pioglitazone for 48 weeks, then followed up 48 weeks after stopping pioglitazone.</td>
<td>Stopping pioglitazone increased ALT, decreased adiponectin, worsened insulin sensitivity and increased hepatic fat, no change in fibrosis.</td>
</tr>
<tr>
<td>(103)</td>
<td>n=52, randomized, double blind, placebo controlled with NASH patients</td>
<td>26 patients were provided liraglutide and 26 a placebo for 48 weeks.</td>
<td>Liraglutide resolved NASH in 9 patients, whereas only 2 in the placebo arm resolved.</td>
</tr>
<tr>
<td>(104)</td>
<td>n=247, randomization of adults with NASH without diabetes</td>
<td>96 weeks of either 30 mg pioglitazone (n=80), vitamin E (800 IU/day) (n=84) or placebo (n=83)</td>
<td>Vitamin E significantly improved NASH. AST and ALT significantly improved in vitamin E and pioglitazone groups, and reduction in hepatic steatosis with no...</td>
</tr>
</tbody>
</table>
n=45 prospective, double-blind randomized, placebo controlled trial in NASH adults

Received vitamin E and C (1000 IU and 1000mg) (n=23) or placebo for 6 months (n=22); additionally patients received weight loss counselling and encouraged to follow a low-fat diet.

Vitamin treatment significantly improved fibrosis score.

NAFLD: non-alcoholic fatty liver disease, ALT: alanine aminotransferase, AST: aspartate aminotransferase, TID: three times a day, NASH: nonalcoholic steatohepatitis

Metformin is used in the treatment of DM2. It lowers blood glucose by decreasing gluconeogenesis in the liver and intestinal glucose absorption, stimulating glucose uptake in muscle and increasing fatty acid oxidation (106, 107). It also improves insulin sensitivity (108). Clinical studies have investigated the use of metformin in the treatment of NAFLD, specifically looking at liver histology and aminotransferases. Nair et al. conducted a pilot study to investigate the efficiency and safety of metformin in NAFLD patients (98). Patients were prescribed 20mg/kg/day of metformin for one year, comparing liver histology pre and post treatment regimen. Three months into the treatment, aminotransferase decreased, which was related to an improvement in insulin sensitivity. However, this improvement was not sustained for the duration of the treatment; therefore Nair et al. concluded that metformin should not be used for the treatment of NAFLD (98). More recently, a study conducted on children with NAFLD used lifestyle interventions and metformin (1.5g/day for 24 months) to determine the effect on liver enzymes (99). Metformin was no more effective than lifestyle interventions in improving liver enzymes or histology (99). Additionally, other studies have also failed to prove benefits of using metformin to improve liver histology (6, 109). In
Conclusion, metformin should not be used in the treatment of NAFLD, as research has shown that it is ineffective.

TZD’s are peroxisomal proliferator activated receptor-γ (PPAR-γ) agonists that are used primarily in the DM2 population to help improve insulin sensitivity within the liver, muscle and adipose tissue, promote hepatic fatty acid oxidation and decrease hepatic lipogenesis (110, 111). TZD use in NAFLD patients, specifically pioglitazone and rosiglitazone, has been shown to decrease hepatic fat and decrease cellular injury. However, these medications have also been shown to cause weight gain (112). Ratziu et al. studied the treatment and safety of rosiglitazone in NASH patients (100). The treatment group received 4mg/day for the first month, and then 8mg/day for eleven months. They found that rosiglitazone improved steatosis and transaminase levels, and resulted in weight gain (mean gain of 1.5 kg) (100). Belfort et al. studied the effects of a hypocaloric diet (500 kcal reduction) and 45 mg of pioglitazone per day on 55 NASH patients with impaired glucose tolerance or DM2 (101). The results indicated that the diet and pioglitazone improved glycemic control, glucose tolerance, improved liver enzymes and increased hepatic insulin sensitivity (101). Conversely, Lutchman et al. found that discontinuing TZD therapy resulted in NASH recurrence, indicating that long-term use is necessary for successful treatment (102). However, long-term use of TZDs can result in medical complications such as edema, congestive heart failure, osteoporosis and weight gain (102, 113). Overall, pioglitazone is used in the medical community as a treatment for NASH, however, careful consideration is needed when prescribing this pharmacological treatment to patients.
Liraglutide is a glucagon-like peptide-1 (GLP-1) derivative that is used in patients with DM2 to help improve insulin sensitivity. It works by binding to receptors that stimulate insulin secretion, slow gastric emptying and reduce postprandial glucagon (114). In murine models GLP-1 analogues have been shown to improve liver histology and reduce oxidative stress and liver enzymes (115-117). However, human studies are limited. A 2016 multicenter, double-blind, randomized, placebo-controlled phase 2 study investigated the use of liraglutide in 52 patients with NASH (103). Patients were randomized to either receiving a placebo or liraglutide for 48 weeks. Based on a liver biopsy that occurred at the end of the trial, nine (39%) patients had NASH resolved in the liraglutide arm compared to two (9%) in the placebo arm (103). Overall, longer-term studies are needed to fully evaluate the use of liraglutide in the treatment of NASH patients (103).

In addition, vitamin E, an antioxidant, has been used in the treatment of NAFLD, as it inhibits oxidative stress and reduces the promotion of hepatic fibrosis (118). Several studies have been conducted to further analyze the benefits of administering high doses of vitamin E to NASH patients. One notable study is the PIVENS clinical trial, which administered high doses of vitamin E (800 IU/day for 96 days) in non-diabetic patients (104). In this trial, Sanyal et al. found a reduction in hepatocellular inflammation, hepatic steatosis and improvements in liver function tests were noted (104). They concluded that vitamin E is an effective treatment for NASH patients without diabetes (104). Harrison et al. also investigated the effects of a combination of vitamin E (1000 IU/day) and vitamin C (1000 mg/day) on liver histology in 45 diagnosed NASH patients over a 6-month period (105). Their findings were that vitamins E & C were effective in improving fibrosis scores, though no improvements in inflammation or liver function tests were noted (105). Caution needs to
be taken when prescribing vitamin E as studies have shown that there is a potential harm for patients. A meta-analysis of 135,967 people taking over 400 IU/day of vitamin E found that there is an increase of all-cause mortality and therefore its use over 400 IU/day should be avoided (119). In addition, a study conducted by Klein et al. assessed the long-term effects of vitamin E (400 IU/day) (120). The study found that vitamin E supplementation significantly increases the risk of developing prostate cancer in healthy men (120). Overall, caution needs to be taken when prescribing vitamin E.

Therefore, TZDs and vitamin E medical treatment need to be carefully considered when developing a treatment plan for NAFLD/NASH patients.

Due to the rise in NAFLD cases, as well as other compounding diseases, additional therapies have been investigated and used in clinical practice, such as urodeoxycholic acid (UDCA), omega-3 polyunsaturated fatty acids (n-3 PUFA), statins, obeticholic acid and pre- and probiotics. These therapies target risk factors of NAFLD, such as obesity, dyslipidemia, cardiovascular disease, insulin resistance and intestinal microbiota. In addition, a new class of medication targeting bile acids is being developed as an anti-fibrotic agent.

UDCA has been studied in clinical trials to determine its effectiveness on the NAFLD population. UDCA is a naturally occurring fatty acid that has been used in clinical trials to determine its effectiveness for treatment of patients with NAFLD/NASH (121). A randomized double-blind study investigated using UDCA (10mg/kg/day) in obese NAFLD patients over a 3-month period (122). The results showed that UDCA was able to reduce liver enzymes, though there was no effect on liver fat content (122). Lindor et al. conducted a
large randomized trial using UDCA (receiving between 13-15 mg/kg/day) on NASH and found that there were no significant differences between the placebo and UDCA groups (121). Therefore, UDCA is not recommended for the treatment of NAFLD.

N-3 PUFAs have been used in the treatment of hyperlipidemia and cardiovascular disease, and more recently in the treatment of NAFLD (123). Studies have highlighted the correlation between insulin resistance and changes in fatty acids, specifically a deficiency in n-3 PUFA (124). As a result, Capanni et al. investigated the effects of n-3 PUFA supplementation (1 g/day for 12 months) in 56 NAFLD patients (124). Their results indicated that n-3 PUFA improves biochemical aspects of NAFLD as well as liver steatosis (124). Similarly, a literature review conducted by Masterton et al. found that in animal studies n-3 PUFA reduced hepatic steatosis, and improved insulin sensitivity and biochemical markers of inflammation. Human studies yielded similar results (123). Masterton et al. and Capanni et al. concluded that n-3 PUFA is a promising therapeutic approach for NAFLD (123, 124). Additionally, a 2015 double-blind, randomized, placebo-controlled trial investigated the effects of n-3 PUFA on the metabolic and histological parameters related to NASH (125). 17 individuals received 3000 mg/day of n-3 PUFA for an entire year while 17 were given a placebo (125). They found that the n-3 PUFA group had significant fat reduction, regardless of weight loss or gain, based on paired analysis of image–assisted fat morphometry (125). A 2012 meta-analysis also found that n-3 PUFA supplementation reduced liver fat. Benefits could be seen with ≥0.83 g/day of n-3 PUFA, however, the optimal dose is currently unknown (126).
Statins are used in the medical field to manage dyslipidemia and are typically used in patients with cardiovascular disease. NAFLD patients often have dyslipidemia along with other features of metabolic syndrome (127). Several studies have shown that statin use in NAFLD patients with dyslipidemia can improve liver function tests (127-129) as well as steatosis (130). In addition, these studies have determined that the use of statins producing liver injury is rare (127), and that statins are safe to use in NAFLD/NASH patients with dyslipidemia (128, 131). However, there is a lack of evidence to use statins to treat NASH patients without dyslipidemia (34, 35). Therefore, statin use should be considered for NAFLD/NASH patients with dyslipidemia, but at this time, should not be used for the specific treatment of NAFLD/NASH.

Obeticholic acid is a bile acid analogue that is a synthetic farnesoid X receptor agonist, which has been shown to improve IR and DM2 through the activation of farnesoid X receptor (132, 133). A proof-of concept study published in 2013 found that a 6-week treatment of 25 and 50 mg/day of obeticholic acid increased insulin sensitivity in patients with NAFLD who have DM2 and decreased serum liver markers (134). A 2014 multicenter, randomized, placebo-controlled trial assessed the efficacy of obeticholic acid in NASH patients (135). 141 patients received 25 mg/day of the drug for 72 weeks, while 142 received a placebo (135). They found that more of those on obeticholic acid improved liver histology based on a ≥ 2-point improvement in NAFLD activity score than those on the placebo (135). It is important to note that this trial ended early as interim analysis showed that obeticholic acid was superior to the placebo based on histological outcomes (135). Furthermore, treatment was discontinued after finding out that patients receiving obeticholic acid had elevated cholesterol, although the clinical significance of this was not stated (135). A future
phase 3 trial of 25 mg/d of obeticholic acid is planned to evaluate the long-term safety and efficacy of the drug. Overall, obeticholic acid is shown to be a promising treatment for NAFLD, however further studies are needed before it is used for clinical purposes.

Intestinal microbiota (IM) has been shown to be disrupted in obesity and metabolic syndrome (22) and has been implicated in the regulation of energy homeostasis and ectopic fat deposition (136). Recent works (18-21) have also documented dysbiosis in NAFLD compared to controls. All of this supports a potential role for pre- and probiotics in the management of metabolic syndrome and NAFLD. Prebiotics are non-digestible carbohydrates that stimulate growth and activity in the colon (137). There have been a limited number of human clinical trials (137, 138) related to metabolic syndrome and NAFLD. Parnell and Reimer conducted a randomized double-blind, placebo-controlled trial to examine the effects of oligofructose (21g/day for 12 weeks) in 48 overweight and obese adults (137). The results found that oligofructose promoted weight loss and improved glucose regulation (137). Daubioul et al. also used oligofructose (16g/day for 8 weeks) in a randomized double-blind crossover study (138) and investigated the effects of oligofructose on glucose and lipid metabolism in 7 NASH patients. Compared to the placebo, AST and ALT decreased after 8 weeks and insulin levels after 4 weeks, suggesting a beneficial effect of prebiotics in NASH (138).

Probiotics (live microorganisms) have been found to improve liver enzymes and liver histology in NAFLD patients (139). An open pilot study conducted by Loguercio et al. used probiotic VSL#3 (containing 450 billion bacteria in different strains) for 3 months (140). This study had 78 participants, 22 of whom had biopsy-proven NAFLD. In the NAFLD
group, plasma levels and lipid peroxidation markers (malondialdehyde and 4-hydroxynonenal) improved (140). Another study using the same probiotic found that VSL#3 had no beneficial effect on liver disease (141). Solga et al. studied the effect of VSL#3 on 4 NAFLD adult subjects in an open pilot study over a 4-month period (141). All 4 subjects had a significant increase in liver fat, and no significant differences in biochemical or clinical parameters. The small sample size was an important limitation of this study (141). Another study using a randomized double blind clinical trial evaluated the effects of a different probiotic (142). This study evaluated the effects of *Lactobacillus bulgarius* and *Streptococcus thermophillus* (1 tablet/day) in 28 NAFLD patients over a 3-month period (142). The results were that ALT, AST and gamma-glutamyl transferase levels decreased.

Therefore, there is limited data on the effect of pre- and probiotics in NAFLD. Larger longitudinal clinical trials are needed to be able to determine the effect, optimal dose and composition of pre- and probiotics on NAFLD.

### 1.1.6 NAFLD and Bariatric Surgery

NAFLD is very common in the morbidly obese population; in fact, the prevalence of NAFLD in this population is between 75%-100% (143). Bariatric surgery induces weight loss by reducing the size of a patient’s stomach by either removing a portion of the stomach, using a gastric band, or by gastric bypass (144). It is considered in patients who have a body mass index (BMI) greater than 40 or with a BMI of 35 who have obesity related comorbidities (145). Both prospective and retrospective studies have found that bariatric surgery improved insulin resistance, steatosis and inflammation (146). For example, Moschen et al. prospectively found that weight loss after bariatric surgery improved insulin resistance, liver function tests and histology in 18 NAFLD patients (16). Similarly, the
A prospective study by Furuya et al. found that significant weight loss two years post-bariatric surgery significantly improved steatosis and fibrosis in 18 patients with NAFLD (147). However, a recent Cochrane review concluded that there is insufficient data due to a lack of well-designed randomized control study trials to determine if bariatric surgery is an effective treatment for NAFLD (148). Overall, the usefulness of bariatric surgery as a treatment for NAFLD is promising but its effect on inflammation and fibrosis is not clear. Future well-designed studies need to be conducted.

1.1.7 Summary

The increase in NAFLD is a burden to the health care system as it increases the risk of cirrhosis and liver failure which can require liver transplantation. In addition, it is associated with obesity, IR and metabolic syndrome. Currently, its epidemiology is well understood and guidelines for proper care are constantly changing as new information emerges (Figure 1-2 provides an outline of current recommendations). The development of non-invasive measures to assess inflammation and fibrosis are commonly used in practice, with liver biopsy being used only in specific cases or within research protocols. Addressing the risk factors associated with NAFLD, such as IR, weight loss and lipid levels remain the primary way to improve NAFLD. Bariatric surgery, insulin sensitizing agents, antioxidants and fish oil, may also be considered for treatment. There is now emerging research supporting a role for IM which may impact future NAFLD management with the use of pre- and probiotics.
1.2 Role of Intestinal Microbiota in NAFLD Pathogenesis

The pathogenesis of NAFLD is complex and recently there has been substantial research assessing the role of IM in obesity and how it can contribute to NAFLD by inducing inflammation and activating the innate and adaptive immune systems.
1.2.1 Intestinal Microbiota

The human microbiome is a complex environment that encompasses microbes, such as bacteria, Archaea and Eukaryotic microorganisms and viruses, their genomes and their interactions within the gastrointestinal tract (149, 150). The human IM is comprised of approximately $10^{14}$ microorganisms (20) with 1000 known species of bacteria (151). Approximately 90% of the bacteria in the human digestive tract are comprised of two dominant phyla, Bacteroidetes and Firmicutes (152-154). The human IM plays a role in immunity, digestion and metabolism, inflammation and cell proliferation (20, 21, 155). Research suggests that altered IM, known as dysbiosis, has been associated with disease states, including obesity and NAFLD (18-21, 156, 157).

Research studies have shown that there is a relationship between IM composition and obesity. Obese mice have a higher ratio of Firmicutes to Bacteroidetes in comparison to their lean counterparts (158). Another study reported similar findings, with obese mice having fewer Bacteroidetes and greater Firmicutes (159). Several studies were also performed in humans. Different Firmicutes to Bacteroidetes ratios were found in obese subjects when compared to lean controls. Two studies found a higher ratio of Firmicutes to Bacteroidetes in obese humans (160, 161). However, another study investigating the IM in overweight and obese humans found that there was a higher ratio of Bacteroidetes to Firmicutes (162). In addition to BMI, changes in diet and weight can affect IM. A human study found that obese subjects consuming a fat or carbohydrate restricted low-calorie diet resulted in IM changes. Specifically Bacteroidetes increased while Firmicutes decreased, which correlated with weight loss (161). Another study using human subjects found a similar relationship (160). However, Schwieritz et al. found the opposite (163). Overweight and obese subjects had
higher Bacteroidetes than Firmicutes in comparison to lean controls (163). These discrepancies could be due to changes at a lower taxonomical level, therefore, recent studies have investigated changes in bacterial genera and species (156, 157). One study found that there were differences in the abundance of the IM genera and species between lean and obese individuals (157). They found that obese individuals had a significantly higher abundance of *Ruminococcus, Lactobacillus, Fusobacterium ulcerans* and *Fusobacterium varium*, whereas, lean controls had a significantly higher abundance of *Alistipes putredinis, Alistipes shahii, Bacteroides ovatus, Clostridium scindens* and *Faecalibacterium prausnitzii* (157). A 2018 study investigated the IM as well as correlations between IM and clinical variables in 192 individuals of which 145 were obese, 22 were overweight and 25 were health controls (156). The obese group had significantly decreased *Bifidobacterium, Faecalibacterium, and Ruminococcaceae* whereas *Bacillus, Fusobacterium* and *E. Shigella* were significantly increased compared to healthy controls. Furthermore, they found that *Faecalibacterium* was significantly negatively correlated with BMI, waist-to-hip ratio (WHR) and fasting blood glucose, and *Ruminococcaceae_UCG-002* was significantly negatively correlated with BMI and WHR (156). A prospective follow-up study found that children who became overweight by age 7 had lower Bifidobacteria and higher *Staphylococcus aureus* in their infancy when compared to children who remained lean (22). Taken together, it is clear that different IM composition, specifically at the genus and species level, is associated with obesity, which in turn increases the risk of developing NAFLD.

There is also evidence to support the relationship between IM and the liver, due to the liver receiving approximately 75% of its blood supply from the intestine to the portal vein (164). IM may contribute to NAFLD pathogenesis through increased intestinal permeability.
and energy salvaging from the diet (18, 19). Intestinal permeability allows for the translocation of bacterial endotoxins into the bloodstream. This in turn can activate inflammatory cytokines, which triggers systemic inflammation (18). It is important to note that limited human studies have investigated IM’s role in NAFLD pathogenesis (27, 58-60). The following section will discuss IM’s ability to increase energy uptake, intestinal permeability and inflammation, which may contribute to NAFLD pathogenesis.

1.2.1.1 Energy Uptake

Increased energy uptake and storage is one of the IM mechanisms contributing to NAFLD pathogenesis. In 2004 Backhed et al. investigated the introduction of gut microbiota from conventionally raised mice into germ free (GF) mice (165). After 14 days his transfer of microbiota resulted in a 57% increase in total body fat content and development of insulin resistance in these recipient mice, despite a reduction in food intake (165). The proposed mechanisms are that the IM promotes monosaccharide absorption and energy extraction from non-digestible carbohydrates (165). This increases *de novo* synthesis of fatty acids and suppresses fasting-induced adipocyte factor in intestinal cells (165), inhibiting lipoprotein lipase in the adipose tissues. When suppressed, therefore, there is an increase in triglyceride deposition in adipose tissue, which contributes to the development of obesity (165). Other studies also support a role for IM in energy extraction (166, 167). GF mice colonized with ‘obese microbiota’ resulted in a significant increase in total body weight when compared to those colonized with ‘lean microbiota’ (166) and without gut microbiota, mice fed a high fat diet resist the development of obesity and diabetes (167). Another proposed mechanism is through production of short chain fatty acids (SCFA), specifically butyrate. IM breaks down non-digestible carbohydrates releasing SCFA in the human gut (168). Butyrate is metabolized in the epithelium and is formed by Gram-positive anaerobic IM, primarily
within Clostridium genus (169). Greater than 90% of butyrate producers belong to the *Eubacterium* or *Roseburia* genera from clostridium cluster XIVa or are *Faecalibacterium praunitzii* from clostridium cluster IV (169). Butyrate is known to increase satiety, decreasing food intake and delaying gastric emptying through activation of free fatty acid receptor 3 (170) that upregulates gut hormone production of peptide YY (PYY) and glucagon-like peptide-1 (GLP1). These are involved in appetite regulation and are secreted in response to food intake (170). Together these studies not only suggest that IM influences satiety but can also increase energy harvesting, as well as that it can be independently responsible for the development of diet induced obesity and diabetes.

### 1.2.1.2 Intestinal Permeability

Intestinal permeability is another mechanism that could contribute to NAFLD pathogenesis. Intestinal permeability can be affected by the IM as well as bacterial products and metabolites.

The IM can change the intestinal barrier through altering epithelial tight junctions either by enhancing the degradation of the mucus layer or inhibiting the production of mucus (171). Endotoxins, also known as lipopolysaccharides (LPS) are found in the outer membrane of gram-negative bacteria (172). LPS have been found to cause an increase in intestinal tight junction permeability (173). In a 2013 study, it was found that LPS increased the permeability of the tight junctions in both *in vitro* and *in vivo* models through the increase of TLR-4 enterocyte expression (173). Specific bacteria types have also been implicated in intestinal permeability. One study found that *Helicobacter pylori* disrupts the tight junction protein zonula occludens-1 in human gastric epithelial cells, which ultimately increases
intestinal permeability (174). In addition, one study evaluated *H. pylori* infection on intestinal permeability using the oral sucrose tolerance test and found *H. pylori* infection increases mucosal permeability of both the stomach and the intestine (175).

SCFAs have also been implicated in intestinal permeability. Specifically, butyrate has been shown to improve the gut barrier through the modulation and induction of mucins and tight junction proteins. Mucin 2 (MUC2) is the main secreted mucin in the colon (176) thus providing MUC2 provides a protective barrier for the intestinal epithelium (176). A study investigated the effects of butyrate on the production of MUC2 in the human colon cancer cell line, LS174T (177). They found that butyrate concentrations (1-2mM) stimulated MUC2 production (177). Other studies have had similar results with specifically butyrate increasing MUC2 gene expression in cell lines (176, 178, 179). Several studies have also suggested that butyrate can enhance the barrier function through the modulation of specific genes. Wang et al. (2012) found that sodium butyrate enhanced the tight junction protein claudin-1 expression in an in vitro cdx2-IEC cell line (180). A review of butyrate concluded that butyrate improved tight junction structure through increased expression of claudin, occludin, cingulin and zonula occludens proteins (181).

GLP-2 (glucagon-like peptide-2), a gastrointestinal peptide, has also been implicated in the intestinal barrier as it improves tight junctions (182). In a 2009 study using a murine model, it was found that prebiotic treatment specifically increased intestinal GLP-2 production (182). Furthermore, they found that this was associated with tight junction improvement and a reduction in intestinal permeability in both obese and diabetic mice (182). Another study found that the probiotic *Bifidobacterium animalis* subsp. *lactis*
significantly increased GLP-2, as well as significantly elevated mRNA tight junction protein 1 expression in obese rats (183).

Increased intestinal permeability due to the widening of the tight junctions can lead to the translocation of bacterial fragments and endotoxemia, which is the presence of endotoxins in the blood. Animal (184-186) and human studies (187-189) have found that endotoxemia is elevated in NAFLD. One study found that endotoxin concentration was significantly higher in those with NAFLD compared to HC and that there was significant correlation between IR and serum endotoxins (187), suggesting that endotoxins may promote IR. In a 2012 human study, it was found that those with NAFLD had higher endotoxins and increased intestinal permeability than those who were HC (189). One study found that plasminogen activator inhibitor-1, which is known to be increased in NAFLD, was increased in plasma and was positively correlated with plasma endotoxins (188). In addition, hepatic TLR4 expression, which can be induced by endotoxemia and lead to IR and inflammation, was significantly higher in NAFLD versus HC (188). Alisi et al. in 2010 found similar findings (190) in children with NAFLD where higher serum concentrations of endotoxin and plasminogen activator inhibitor-1 compared to HC (190). Animal studies also support a role for endotoxins leading to inflammation in NASH (172). One study found that LPS injections in mice resulted in significant increases in hepatic TNF-a production (172).

There are other mechanisms by which endotoxemia can contribute to NAFLD development. Two recent studies have investigated the relationship between LPS and sterol regulatory element-binding protein (SREBP)-1c. The first study found that LPS treated mice induced hepatic SREBP-1c activation and the expression of SREBP-1c genes, resulting in
hepatic lipid accumulation (191). Another study found that mice treated with LPS had decreased plasma adiponectin levels, increased plasma leptin levels, and greater expression of SREBP-1c in the liver compared to the control group that were associated with the development of NAFLD (192).

In conclusion, IM can contribute to lower expression of tight junction proteins, resulting in an increase in intestinal permeability, bacterial translocation and serum endotoxins. These endotoxins in turn can promote inflammation, through increased pro-inflammatory cytokines, IR and increased hepatic lipid accumulation. These studies further illustrate the complex role that IM plays in the development of NAFLD.

1.2.1.3 Chronic Inflammation

There are several mechanisms in which the IM can contribute to chronic systemic inflammation which in turn can contribute to obesity, IR and NAFLD.

The translocation of bacterial components into the circulatory system can trigger an inflammatory response by activating Toll-like-receptor-4 (TLR-4) (193). TLR-4 activates proinflammatory cytokines such as tumour necrosis factor-a (TNF-a) and interleukin-6 (IL-6) (194). Studies have found that TLR-4 activation triggers an inflammatory cascade including the nuclear factor-kB pathway (195), which induces TNF-a. Increased levels of TNF-a have been related to IR and NASH (196) and are essential for hepatic fat deposition and NASH development (197-199). In addition, TLR-4 enhances transforming growth factor–B signaling, resulting in fibrogenesis (200, 201).
Another IM mechanism contributing to inflammation involves inflammasomes. Inflammasomes are composed of leucine-rich-repeat containing proteins and nucleotide-binding domains (NLRPs) which govern the cleavage of pro-inflammatory cytokines. NLRPs are also sensors for pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern molecules (DAMPs) of which DAMPs are known to induce reactive oxygen species (ROS) production which can activate the NLRP3 inflammasome (202, 203). Henao-Majia et al. investigated the relationship between inflammasome-mediated dysbiosis and NAFLD progression (204). They demonstrated that changes in gut microbiota were associated with NLRP 3 and 6 inflammasome deficiency, worsening NASH and increasing TNF-a expression due to the influx of TLR4 and TLR9 antagonists into the portal circulation (204). This was also demonstrated in another murine study where methionine-choline deficient and NLRP3 deficient mice had an increased tendency for NAFLD and liver damage when compared to wild-type mice (205).

Food intake can also contribute to inflammation by its interaction with IM. Fructose in particular can contribute to inflammation and has been implicated in the pathogenesis of NAFLD. Excess consumption of fructose upregulates de novo lipogenesis and inhibits fatty-acid beta-oxidation (206, 207). Recently, a study using mice with fructose-induced NAFLD found that fructose significantly decreased Bifidobacterium and Lactobacillus and tended to increase LPS (208, 209). Another study also found that the development of fructose-induced NAFLD was associated with increased TLR expression (210). It is clear that fructose consumption can alter the IM as well as contribute to systemic inflammation, thus contributing to the pathogenesis of NAFLD.
In conclusion, IM can contribute to inflammation by the production of endotoxins, inflammasome dysfunction and interaction with dietary components such as fructose. This leads to activation of TLR-4 and production of inflammatory cytokines which can recruit and activate hepatic immune cells. Emerging research suggests a role for immune function in NAFLD that could be influenced by the IM.

1.2.2 Immune Function

The recruitment and activation of hepatic immune cells can be caused either by local signals or signals from sources such as the aforementioned IM (211). The immune system is divided into the innate and adaptive immune systems. The innate immune system defends against microorganisms and toxins, whereas the adaptive immune system is antigen specific and requires self-non-self-recognition (212). Together, both immune systems protect against disease.

1.2.2.1 Innate Immune System

The innate immune response is triggered by pattern recognition receptors (213). The innate immune system includes leukocytes such as macrophages, natural killer (NK) cells and natural killer T (NKT) cells (214).

1.2.2.1.1 Macrophages

Kupffer cells (KC) represent the largest group of fixed macrophages in the body and account for about 20-25% of non-parenchymal cells in the liver (215). KCs are critical components of the innate immune system, residing within the sinusoidal vascular space (215). KCs can be activated by various endogenous and exogenous stimuli including LPS (215). Activation of KC in the liver triggers the production of inflammatory cytokines, such as TNF-α, and ROS (215). These cytokines in turn play a key role in regulating the
phenotype and function of neighboring parenchymal and non-parenchymal cells (215). Consequently, modified KCs phenotype and function are essential in the development of various chronic and acute liver diseases. In recent years, animal studies have identified a role for KCs in the pathogenesis of NAFLD, with the number of KCs reported to be increased in the liver of rats with NAFLD (216) and KCs being recruited and activated in mice with high fat diet induced NASH (217). In addition, KC inactivation has been shown to prevent the development of NAFLD and inflammation (218). Depletion of KCs in donor animals was also observed to prevent fatty livers after liver transplantation (219, 220). In human studies, KCs are also involved in the development of NASH (220, 221). One study found that CD163+ is expressed in macrophages, including KCs (222). In a pediatric study, CD163 was significantly higher in those with a NAS ≥5 compared to those with a NAS score <5 (26). However, there are conflicting results as others reported no differences in CD163 between adults with a fatty liver versus HCs (223). Overall, there are very few studies assessing CD163 in human adults with NAFLD.

1.2.2.1.2 NK and NKT Cells

NK and NKT cells may also play a role in the pathogenesis of NAFLD. NK cells in the liver play a role in linking the innate and adaptive immune response (224). Studies show conflicting results in regard to NAFLD. Activated NK cells were found to have anti-fibrotic effects, by releasing interferon-γ (IFN-γ) which induces hepatic stellate cell (HSC) cycle arrest and apoptosis (225). However, IFN-γ also results in hepatocyte apoptosis and thus causes hepatic injury (225). NKT cells, which can be expressed by hepatocytes and antigen presenting cells, share properties of both T cells and NK cells (226). NKT cells can secrete cytokines and therefore play a critical role in directing the immune system (226). They are able to do this through the NKT cells ability to produce T helper 1 cells, which are
proinflammatory, and T helper 2 cells, which are anti-inflammatory (226). In a murine study, it was found that the severity of steatosis was negatively correlated with NKT cell numbers (227). Furthermore, in another murine study, which used the methionine and choline deficient diet to induce NAFLD, mice fed this diet had a significant reduction in the number of NKT cells (228). However, in humans the research is conflicting. One study in adults found that those with NAFLD had fewer NKT cells when compared to HC (229), whereas another found that the percentage of NKT cells (CD3+/CD56+ expression) increased with disease severity in adults with NAFLD (230). It has been hypothesized that NKT cells seem to be depleted during steatosis, however increase as the disease progresses, thus contributing to inflammation and fibrosis (231).

1.2.2.2 Adaptive Immune System

The adaptive immune response, which is characterized by immunological memory, is mediated by T and B lymphocytes (232).

1.2.2.2.1 T Lymphocytes

Recently, research has focused on understanding T lymphocytes in the context of NAFLD pathogenesis. As previously stated, T helper cells, which are a subgroup of T lymphocytes, play an important role in hepatic immune function. Specific T cell populations have been studied in NAFLD, including CD3 and CD8+ (211). CD3+, which represents the total hepatic T lymphocyte population appears to remain stable in NASH (212). However, CD8+ cells have been found to dominate the portal tracts of individuals with NASH (25). Furthermore, studies have shown that CD8+ T cells drive adipose tissue inflammation through the recruitment of monocytes and macrophages, thus the downstream effects could contribute to NAFLD pathogenesis (233, 234).
1.2.2.2 B Lymphocytes

B lymphocytes represent 6% of intrahepatic cells (214) and as much as half of the intrahepatic lymphocyte population (235). B lymphocytes may play a role in HSC activation and liver fibrosis (235), however, their role in NAFLD needs further investigation. Animal studies also showed that the B-cell activating factor (BAFF) might contribute to high-fat induced hepatic steatosis by inducing IR, inflammation in adipose tissue (236), and regulating lipid metabolism in the liver (237). In humans with NASH, an increase in serum BAFF was reported. Additionally, serum BAFF levels were found to be correlated with hepatic B-cell content (237). Diet may also play a role. One murine study found that when fed high fructose water, B-cell deficient mice and wild-type mice develop the same level of glucose intolerance and IR (238). Further research is needed to investigate the role of B lymphocytes role in the development and progression of NAFLD.

1.2.3 Summary

In summary, both the IM and hepatic immune response can play a role in the development and progression of NAFLD. Further research is needed to investigate potential associations between hepatic immune cells and IM. If associations exist, underlying mechanisms linking the two will need to be investigated and this may lead to novel treatment options.

1.3 Obesity and Associated Comorbidities as Risk Factors for NAFLD

1.3.1 Epidemiology

The World Health Organization (WHO) states that being overweight and obese are considered the 5th leading risk of death in the world (239). Overweight is defined as a body mass index (BMI) (kilograms of weight divided by height in meters squared), between 25.0
and 29.9 kg/m$^2$ (240), whereas obesity is defined as a BMI greater than 30 kg/m$^2$ (240). Obesity is further categorized by BMI. Class I is a BMI between 30.0 to 34.9 kg/m$^2$, Class II is a BMI between 35.0 to 39.9 kg/m$^2$ and Class III is a BMI of 40.0 kg/m$^2$ or greater (240).

In 2005, the WHO estimated that 1 billion people worldwide were overweight and 300 million were obese (241). In 2014, 20.2% of Canadian adults reported themselves as obese, while in 2011 the Public Health Agency of Canada reported that 2.7% of Canadians were morbidly obese (Class III) (242). The numbers of overweight and obese individuals are on the rise in Canada, and pose a serious public health problem. Obesity-related costs in Canada are estimated to be between $4.6 to $7.1 billion Canadian dollars annually for health care costs and productivity loss (1). Obesity has been established as a risk factor for premature mortality (243-245). In a meta-analysis that included 2.88 million participants, it was found that in comparison to normal weight individuals, being obese was associated with all-cause mortality with a hazard ratio of 1.18 (95% CI 1.12-1.25) (246). Furthermore, obesity is also associated with comorbidities, including DM2, dyslipidemia, hypertension, heart disease and NAFLD (247, 248). Obesity and some of its comorbidities can also contribute to NAFLD.

1.3.2 Pathogenesis of Obesity: Brief Overview

1.3.2.1 Diet and Energy Balance

One of the longest known contributors to obesity occurs when energy intake from food consumption exceeds energy expenditure, resulting in a positive energy balance (249). A consequence of this positive energy balance is the increase in body mass, which is usually 60-80% fat (250). The recent rise in obesity, researchers have theorized, is a result of the societal increase in calorie consumption and the availability of calorically-dense, nutrient-
poor food and a decrease in active lifestyles. However most of these studies are limited to correlational findings (251-253).

In 2009, a study using data from the 2004 Canadian Community Health Survey investigated the association between diet and excess weight in Canadian adults (254). 6,454 individuals completed a 24-hour dietary recall and reported their current height and weight (254). They found that total kilocalories consumed were significantly higher in obese men and women than their non-obese counterparts (254). Diet quality is also associated with obesity. In 2015, a study analyzed data from the 2005-2008 National Health and Nutrition Examination Survey (255). 9,551 adults completed a 24-hour diet recall and the association between dietary energy density, which is the ratio of energy per weight of food, and markers of obesity, including BMI, were examined (255). They found that energy density was positively correlated with obesity in both sexes, and that obese adults had significantly higher dietary energy density than compared to lean adults (p <0.0001) (255). Other studies have also concluded that there is a positive relationship between energy density and BMI (256, 257). In addition to energy density, sugar-sweetened beverages have also been shown to increase the risk of obesity (258-260). A meta-analysis found an association between soft drink intake with increased caloric intake and weight gain (258). Additionally, a diet that is high in red and processed meats, refined carbohydrates, sugar-based drinks and fast food have also been linked to obesity (252, 261). Overall, both caloric consumption and diet composition have a clear influence and are related to body weight as well as to the risk of becoming obese.
Decreased energy output, or physical inactivity, is another potential reason for a positive energy balance. A review article highlighted the current patterns and long-term trends related to physical activity in the United States (262). What they found was that there was an overall trend of declining physical activity, which they attribute to a decline in work-related activity, transportation activity and activity within the home, and an overall increase in sedentary behaviour (262). The study of sedentary activity, specifically television watching, has become a focal point for obesity research. A study in 2008, which analyzed 46,612 respondents from the 2007 Canadian Community Health Survey investigated the prevalence of obesity compared to time engaged in sedentary behaviours in Canadian adults aged 20 to 64 years (263). The sedentary behaviours considered were television watching, computer use and reading (263). They found that reading was not related to obesity. However, they found that television viewing and frequent computer users (11 hours per week or greater) increased the odds of obesity in both sexes (263). Other studies investigating television watching and obesity have found similar associations (264-266).

1.3.2.2 Genes

Genetics have also been found to influence obesity, including the mutations of a single gene, multiple genes (also known as common obesity) and genetic syndromes (267). Single gene mutations are rare and have been discovered in genes that play a role in energy homeostasis and food intake, such as congenital leptin and leptin receptors (268, 269). Multiple gene mutations have been a focus of obesity genetic research. Recently, with the help of the Genome Wide Association Study (270), the association between common genetic variations and obesity has been discovered (267). These genes are involved in metabolism and appetite, however more research is needed to identify all genes involved in obesity (267, 271, 272). Obesity is also a clinical feature of multiple genetic syndromes. These syndromes...
include Prader-Willim, Bardet-Biedl, Cohen, Ayazi and MOMO syndrome (273). Overall, it is estimated that up to 70% of weight is attributed to a genetic cause from obesity (274), likely associated with energy metabolism and appetite regulation.

Newer research has now implicated IM as a contributor to the pathogenesis of obesity and mechanisms have been reviewed in section 1.3.1. In addition, genes from the microbiome may be implicated in energy metabolism (275). Recent technical advances have allowed researchers to determine the genetic information of an entire community of microorganisms using metagenomics shotgun sequencing (276). Using this technique, a recent study compared the fecal microbial diversity of individuals who were obese (BMI >30), overweight (BMI 25-30) and lean. They found that the gut microbiomes of those who are obese were more likely to have low gene counts rather than high (242). Low gene counts have been correlated with higher levels of body fat, IR, dyslipidemia and inflammation (242, 277). They also found that obese individuals had put on more weight in the past 9 years if they had low microbiome gene counts compared to obese individuals with high gene counts (242). Overall, the microbiome has the potential to influence energy metabolism and contribute to NAFLD.

1.3.3 Obesity Related Comorbidities

Obesity greatly affects quality of life and lifespan, mostly through associated comorbidities. These include insulin resistance, Type 2 diabetes, dyslipidemia, metabolic syndrome and non-alcoholic fatty liver disease (NAFLD), with some of these comorbidities contributing to NAFLD. This section will briefly go over the relationship between obesity and these comorbidities.
1.3.3.1 Insulin Resistance and Type 2 Diabetes

Insulin resistance (IR) and type 2 diabetes (DM2) are associated with obesity as well as NAFLD. IR is a term that describes the body’s cell resistance to the effects of the uptake, metabolism and storage of insulin and glucose (278). In IR, the cells are resistant to the insulin, therefore blood glucose remains high (278). Additionally, in response to the high circulating blood glucose levels, the beta cells in the pancreas increase their production of insulin, causing high blood insulin levels or hyperinsulinemia (278). Recent research has suggested that IR might be related to the substances that are secreted by adipocytes, or fat cells (279). In obesity, adipose tissue dysfunction caused by fat cell hypertrophy has been shown to be an important contributor to the pathogenesis of IR and DM2, however the exact mechanisms are not well understood (279, 280).

There are two types of adipose tissue, white and brown (281). The brown adipose tissue primary function is to provide energy expenditure in the form of heat for adapting to the cold (282). In obesity, white adipose tissue is the major component of the body’s adipose tissue (281). One of the primary functions of white adipose tissue is to store excess energy (283). Adipocytes, which is the most abundant cell type, stores this energy as triglycerides, and when the body needs energy, fatty acids are released via lipolysis (283). Additionally, the adipose tissue also produces peptide hormones, cytokines and activated lipids such as TNF-α, IL-6 and leptin which may also contribute to NAFLD (284). When the adipose tissue is dysfunctional, there is an increased production of pro-inflammatory cytokines and, via lipolysis, fatty acids (285). Chronic inflammation in the white adipose tissue is the primary proposed mechanism linking obesity and IR (285). Specifically, this increase in lipolysis and release of fatty acids from the adipose tissue and the increase in pro-inflammatory cytokines
can impair insulin signaling in peripheral tissues through the downregulation of PPAR-y and/or activation of TLRs (286). In addition, this increase in fatty acids/pro-inflammatory cytokines can activate pro-inflammatory pathways such as the IKKβ/NF-κB and JNK pathways in the peripheral tissues (286). Overall, obesity, specifically white adipose tissue, plays an essential role in developing local and systemic IR, as well as contributes to the pathogenesis of NAFLD.

In obesity, IR can lead to DM2 (287). However, the majority of obese individuals with IR do not develop hyperglycemia, a hallmark sign of DM2 (288). DM2 is characterized by hyperglycemia, IR and impaired insulin secretion (289). DM2 is diagnosed as having a fasting blood glucose test of 7.0 mmol/L or greater and/or a HbA1c of 6.5% or greater (289). In Canada, obese adults account for 61% of DM2 cases (290). The association between obesity and IR and DM2 exists when there is a dysfunction in pancreatic islet B-cells (291, 292). This dysfunction causes insulin to be inadequately released, thus causing fasting blood glucose to rise (291). Obesity and IR contribute to B-cell dysfunction through the increased oxidative stress and inflammation by proinflammatory cytokines, as well as inflammation caused by the influx of free fatty acids (292). This inflammation induces B-cell dysfunction which can lead to B-cell death (292). Overall, obesity is associated with IR due to chronic inflammation and this increases the risk of developing DM2 and NAFLD.

1.3.3.2 Dyslipidemia

Dyslipidemia is commonly seen in obese individuals (293). 53.1% to 62.2% of overweight individuals and 62.5% to 68% of obese individuals have dyslipidemia (294). The most common changes seen in obesity include high serum concentrations of total cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides and very-low-density lipoprotein
(VLDL) cholesterol as well as low serum concentrations of high-density lipoprotein (HDL) cholesterol (295, 296). Multiple metabolic pathways are involved in lipid storage, transport and uptake. In obesity, hypertriglyceridemia is generally the cause of dyslipidemia (293). This is the result of an increase in free fatty acids (FFA) being released from the adipose tissue through lipolysis, from IR (293). This is turn increases the delivery of FFA to the liver, which results in hepatic triglyceride accumulation and an increase in hepatic VLDL synthesis (293). In addition, this increase in FFA inhibits lipoprotein lipase in the adipose tissue, thus decreasing triglyceride clearance and promoting hypertriglyceridemia. Moreover, increased hepatic VLDL can inhibit lipolysis of chylomicrons, thus contributing further to hypertriglyceridemia (293). Finally, the formation of small, dense LDL, a type of LDL cholesterol, also occurs (293).

Current treatment for obesity-associated dyslipidemia includes lifestyle interventions, such as weight loss, increased physical activity and consumption of a healthy diet (293). Additionally, if deemed appropriate, pharmaceutical treatment can be considered in addition to lifestyle interventions (297). Overall, dyslipidemia is commonly associated with obesity (295) and increases one’s risk of cardiovascular disease (298) and NAFLD (127).

1.3.3.3 Metabolic Syndrome

Metabolic syndrome is a condition that encompasses a group of risk factors that increase the likelihood of developing chronic diseases such as DM2, cardiovascular disease and cancer (299-301). It is also associated with NAFLD (302). The diagnosis of metabolic syndrome is determined if any three of the following criteria are met: 1) waist circumference $\geq 102$ cm for males/ $\geq 88$ cm for females, 2) triglycerides $\geq 1.7$ mmol/L, 3) blood glucose $\geq 5.6$ mmol/L, 4) HDL cholesterol $< 1.03$ mmol/L for males/ $< 1.30$ mmol/L for females and/or 5) blood pressure $\geq 130/85$ mmHg. If an individual is using any medication for the above
criteria it is considered as meeting that specific criterion (303). Obesity contributes to the development of metabolic syndrome in many ways. As previously discussed, through different mechanisms, obesity is a risk factor for developing IR, DM2 and dyslipidemia, all of which are included in the criteria used to diagnose metabolic syndrome. Hypertension is another criterion used to diagnose metabolic syndrome. Obesity is a known risk factor of hypertension, and weight gain is known to raise blood pressure (304, 305). In obesity, enhanced renal tubular sodium reabsorption is the primary cause for hypertension (306). The final criteria for metabolic syndrome is intra-abdominal fat accumulation, which is directly linked to obesity (303). The prevalence of metabolic syndrome is increased in those who are obese (307). The National Health and Nutrition Examination Survey found that the prevalence of metabolic syndrome was 5% in individuals with a normal BMI, 22% in those who are overweight and 60% in those who are obese (307). Overall, obesity is directly related to the development of numerous comorbidities including metabolic syndrome. When investigating clinical manifestations of obesity, it is essential to consider the interconnected pathophysiology that exists between these metabolic disorders, including NAFLD.

1.3.4 Summary

Overall, obesity and related co-morbidities are on the rise in today’s society, and with that comes an increased burden on the health care system. There is a complex interplay between various mechanisms related to obesity and its comorbidities that increase the risk of NAFLD. Treating obesity is challenging due to compliance challenges regarding lifestyle changes. Weight loss due to diet and exercise has been shown to improve NAFLD and reduce other comorbidities. However, this is generally short lived as people return to their previous habits. A more definitive treatment may be required and over the past decade,
bariatric surgery has been shown to dramatically reduce weight and improve general obesity-related comorbidities, including NAFLD.

1.4 Weight Loss and Obesity Related Comorbidities

Considering that the prevalence of obesity is rising, and is strongly associated with several co-morbidities and increased mortality it is recommended to treat obesity first with a calorie-restricted diet and regular physical activity to achieve clinically important weight loss and a reduction in obesity-related symptoms (308). Despite being the cornerstone of treatment for obesity, achieving long-term success with lifestyle interventions still remains challenging. Therefore, if lifestyle interventions are inadequate, the Canadian Clinical Practice Guidelines suggest that adults with clinically severe obesity (BMI ≥40 kg/m² or ≥35 kg/m² with comorbidities) may be considered for bariatric surgery (308). The number of bariatric surgeries performed each year is increasing in Canada (309), but research is minimal in Canada on how effective the surgery is, particularly related to NAFLD. This chapter will briefly review the effects of diet and exercise on obesity, however the focus will be on protocol, procedure and effects of bariatric surgery in the Canadian context.

1.4.1 Diet and Exercise Regime

Guidelines suggest that a reduction of at least 5% body weight is needed for the treatment of obesity (310, 311). The first line of treatment for obesity is to modify an individual’s lifestyle to promote weight loss through diet and exercise. This causes a negative energy balance by increasing energy expenditure beyond caloric needs (312). There are many studies that have evaluated the effects of diet and exercise individually and combined on obesity. In this section, this research will be briefly explored.
The initial goal is to reduce the amount of calories consumed in a diet, leading to a negative energy balance promoting weight loss (313). Many studies have investigated the various effects of caloric restricted diets on weight loss, but with relevance to this dissertation this discussion will focus on the use of the very-low-calorie diet (VLCD) consisting of <800 kcal/day and low-calorie diets, consisting of 800-1800 kcal/day. Tsai et al., in a 2006 meta-analysis investigated the safety, efficacy and clinical use of both the VLCD and low-calorie diets (314). In the short-term, both the VLCD and low-calorie diet induced significant weight loss with a mean difference of 16.1 ± 1.6% and 9.7 ± 2.4%, respectively (314). It is important to note that the VLCD, compared to the low-calorie diet, induced significantly greater short term weight loss (314). However, in the long-term, ranging from 1 to 5 years after completion of the VLCD, the mean weight loss for VLCD was 6.3 ± 3.2 and for low-calorie diet was 5.0 ± 4.0 of initial weight, with both groups regaining weight initially lost (314). The authors indicated that maintaining a 15% to 25% weight loss of initial weight is unlikely for most patients, and that bariatric surgery is the only reliable method for sustained weight loss of this magnitude (314).

Physical activity is another form of lifestyle intervention and influences total energy expenditure (313). However, there is a debate on whether physical activity alone can significantly affect weight loss (315-317). A 2007 meta-analysis investigated the effect of exercise on weight loss outcomes at 6, 12, 24, 35 and 48 months and found that exercise alone did not result in successful weight loss, but weight gain was also not observed (315). Another meta-analysis found that exercise-only interventions induced moderate weight loss compared to diet-only or combined exercise and diet interventions (318). These studies
support that it is likely preferable to use a combination approach, as exercise alone may or may not induce weight loss. It is likely preferable to use a combined approach.

Many studies have highlighted the importance of combining both physical activity and caloric restriction to maximize weight loss. One meta-analysis, using 18 randomized trials, compared the effectiveness of a combined diet and exercise intervention versus diet alone in overweight and obese subjects (313). It found that the combined intervention caused greater long-term weight loss than diet alone (313). Another meta-analysis compared diet or exercise interventions versus diet and exercise combined programs for weight loss in overweight and obese subjects (319). They found that in the short term (less than 6-months) weight loss was similar between diet only interventions and combined programs (319). However, in the long term (12 to 18 months), weight loss was greater in combined programs versus diet only or physical activity only programs (319). However, even though combined programs are superior for weight loss, these programs are associated with weight regain in the long-term (313).

In conclusion, diet-only, exercise-only and combined interventions have the potential to induce initial weight-loss (313, 318, 319). However, long-term maintenance of the weight-loss achieved during lifestyle interventions is variable (313, 314). Therefore, in the case where lifestyle interventions are deemed inadequate for long-term weight-loss, bariatric surgery could be considered on a case-by-case basis (308).
1.4.2 Bariatric Surgery

1.4.2.1 Bariatric Surgery Protocol

In Canada, according to the Canadian Clinical Practice Guidelines, bariatric surgery is offered to individuals who were unsuccessful in losing weight by lifestyle modifications and have a BMI of 40 kg/m² or greater or have a BMI of 35 kg/m² or greater with at least 1 obesity related comorbidity (308, 320). The comorbidities include coronary heart disease, type II Diabetes mellitus, hypertension, diagnosed sleep apnea and/or gastroesophageal reflux disease (309, 321). Exclusion criteria for bariatric surgery patients include poorly controlled medical conditions and/or complicated surgical histories which could increase the risk of adverse events during the surgical procedure (322). Additionally, exclusion criteria include substance use disorders and failure to cease smoking (322).

In Ontario, patients are referred from their family physician to a bariatric program and go through an orientation session. Afterward, if patients decide to proceed with the program, they go through a multistage preoperative evaluation that includes consultation with a dietitian, a social worker and a registered nurse as well as psychological or psychiatric assessment and surgical consultation before they become eligible for bariatric surgery (320, 322, 323). Patients must clear each stage of this program before moving forward with the evaluation (320). Two types of bariatric surgery are fully covered by Ontario Health Insurance (OHIP) (309). These include the Roux-en-Y gastric bypass procedure, which is the procedure of choice, and the vertical sleeve gastrectomy, which is only paid for by OHIP if deemed medically necessary (309). The only cost to patients is the preoperative very-low calorie diet, Optifast®, which will be discussed in the next section. After surgery, patients have follow-up assessments at the 1, 3, 6 and 12-month mark (309, 322). Following the first
year, follow up assessments occur annually for up to 5 years postoperatively (322). These assessments are with the interdisciplinary bariatric team to ensure that patients are being monitored closely and are reaching/maintaining their postoperative goals (309).

1.4.2.2 Optifast®

A preoperative very-low calorie diet (VLCD) with Optifast®, is routinely used for individuals undergoing bariatric surgery at our center (Toronto Western Hospital: TWH) and elsewhere (324). Optifast® is a 900 kcal/day meal replacement liquid-based diet (325). The patients are provided four shakes a day with each shake consisting of 225 calories, 22.5 grams of protein, 7.5 grams of fat and 16.8 grams of carbohydrates (325). The duration of this diet is prescribed at a rate of 1 week per 100 lbs of weight, according to the TWH bariatric program protocol. In addition to this low carbohydrate, high protein meal replacement, patients are instructed to drink as much water, black tea, black coffee or diet soda as they please. Additionally, patients are allowed to consume sugar-free Jell-O, chicken, beef or vegetable stock that is less than 10kcal/serving, as well as chew sugar-free gum.

In the morbidly obese population, abdominal surgery is challenging due to limited space in the abdominal cavity caused by the large amount of inter-abdominal fat and an enlarged liver due to steatosis (326). The enlarged liver can hinder the division of the gastric pouch gastro-jejunostomy due to visual obstruction caused by the liver (327). Research has shown that the use of a VLCD helps to significantly reduce the fat within the abdominal cavity and reduce the size of the liver in bariatric surgery patients, which helps to facilitate the surgery (328-330). The use of VLCD’s has been shown to significantly reduce liver size as well as BMI (326, 328). One study in 2004 investigated the effects of a 2-week preoperative Optifast® diet on liver size in the bariatric surgery population (328). It was
found that within 2 weeks, there was a significant reduction in liver size, based on CT scans (328). Additionally, there was a significant reduction in the percentage of fat and BMI (326). Another study, using a comparable VLCD found that 4 weeks on a preoperative VLCD significantly reduced weight, BMI, liver volume and liver fat (based on MRI and spectroscopy) in the morbidly obese population (326). Both studies did not report changes in biochemical variables and it is not known how this diet affect liver histology in patients with NAFLD, including NASH. Additionally, these studies did not investigate the role that the preoperative VLCD plays in the postoperative benefits seen in the bariatric surgery population. A 2009 review article investigated what effect preoperative weight loss had on postoperative outcomes (331). It was found that preoperative weight loss resulted in greater postoperative weight loss, and results suggested that preoperative weight loss could identify patients who would be more compliant postoperatively (331). This study did not investigate the role of VLCD’s on liver histology or on postoperative biochemical values.

In conclusion, VLCD is routinely prescribed prior to laparoscopic bariatric surgery. However, the effect of VLCD on liver histology and clinical or biochemical outcomes is not well documented.

1.4.2.3 Roux-en-Y Gastric Bypass Surgical Procedure

Bariatric surgery is a surgical operation for weight loss that is achieved by either malabsorption and/or restriction (332). In bariatric surgery, malabsorption is achieved by either bypassing part of the small intestine or diverting the biliopancreatic secretions (333, 334). Restrictive procedures reduce the size of the stomach, thus reducing food intake (334).
The preferred bariatric surgical procedure in Canada is the laparoscopic Roux-en-Y gastric bypass (RYGB) (309), which is the gold standard. This is because research suggests that the RYGB results in better long-term outcomes than other procedures (335). This procedure is both restrictive and malabsorptive (336). It involves creating a 15-30 mL gastric pouch by separating it from the distal stomach, thus restricting food intake (337). Additionally, the first section of the small intestine, the duodenum and part of the proximal jejunum, is bypassed, and the Roux limb of the jejunum is anastomosed to the gastric pouch (337), thus limiting nutrient absorption (337). The biliopancreatic limb, which contains the remaining stomach and the first section of the small intestine, is connected to the Roux limb, approximately 150 cm distally from the gastrojejunostomy (338). The Roux limb acts as a funnel for consumed food, which is then digested and absorbed once it reaches the common channel (336). The common channel is where gastric acid, pancreatic enzymes, intrinsic factor and bile combine with food (336). In addition to malabsorption and restriction, the RYGB procedure could also induce weight loss by other mechanisms such as altering gut hormones (339). These hormones include ghrelin, glucagon-like peptide-1 (GLP-1) and cholecystokinin, which regulate satiety and appetite (339, 340). Overall, the RYGB procedure induces weight loss by restricting the size of the stomach, reducing caloric intake and inducing nutrient malabsorption. In addition, there is alteration of gut hormones that regulate satiety and appetite. All of this leads to changes in gastrointestinal physiology (341).

1.4.3 Effect of Bariatric Surgery on Obesity and Related Comorbidities

Bariatric surgery leads to sustained weight loss and improvement of medical comorbidities such as DM2, dyslipidemia, metabolic syndrome and NAFLD (342, 343). However, results vary between bariatric surgery procedures. This review will focus on RYGB, as it is the gold standard and the surgery used on our study patients.
1.4.3.1 Obesity

RYGB leads to a reduction of approximately 20-30% of body weight after 3 years and is the most common bariatric surgery procedure worldwide (342, 344). A meta-analysis, using observation studies, investigated BMI changes post-gastric bypass and found that compared to baseline, the mean BMI change was -14.32 (-19.02, -9.62) kg/m\(^2\) (mean (95% confidence interval) 1-year post-gastric bypass (343). Additionally, compared to baseline the mean BMI change was -12.93 (-17.39, -8.47) kg/m\(^2\), -16.78 (-20.57, -12.99) kg/m\(^2\), -17.86 (-22.20, -13.53) kg/m\(^2\) and -15.96 (-20.52, -11.40) kg/m\(^2\) after 2-, 3-, 4- and 5-years post-RYGB respectively (343). In this meta-analysis, however, it was not stated if the pre-operative BMI was collected before or after a VLCD, which is commonly used pre-laparoscopic bariatric surgery. Another study investigated weight changes in 1,533 RYGB patients pre-RYGB and up to 3-years post-RYGB (342). Baseline BMI was taken within 30-days of the procedure and the median BMI was 46.6 (42.4, 51.9) kg/m\(^2\) (median (Q1, Q3)) (342). Significant weight loss was seen 3-years post-RYGB, with the median percent weight change being 31.5% (24.6%, 38.4%) compared to baseline (342). An important observation made by the authors was that most of the weight loss occurred within the first year post-RYGB (342). Similar to the meta-analysis, this study did not indicate whether the baseline BMI was taken before or after a pre-surgery VLCD. In Canada, the Ontario Bariatric Registry has been collecting clinical data on consenting bariatric surgery patients since 2010 (345). One study using the registry data found that on average, individuals lost 44 kg after 1 year and that BMI significantly decreased from a baseline average of 49.7 kg/m\(^2\) to 33.6 kg/m\(^2\) 1 year post-bariatric surgery (345). However, this study included multiple types of bariatric surgery and did not indicate if the baseline data was collected before or after a pre-surgery VLCD. Overall, results indicate that after bariatric surgery, specifically RYGB, a
majority of individuals lose a significant amount of weight but it is not clear whether a pre-surgery VLCD was used and what was the contribution of VLCD on the short-term weight loss and changes in metabolic parameters prior and after surgery. However, it is clear that bariatric surgery leads to sustained weight loss in the long term.

1.4.3.2 Insulin Resistance and Type 2 Diabetes

The current approach that clinicians use to improve DM2 and IR include weight loss through lifestyle modifications and/or the use of prescription medication (346, 347). However, most people with DM2 fail to achieve an HbA1c lower than 7.0% (348). In a 2004 meta-analysis, the impact of bariatric surgery on obesity-related comorbidities was investigated (349). It found that RYGB resolved diabetes in a mean of 83.7% of individuals (95% CI, 77.3 to 90.1%) and significantly improved fasting insulin by a mean of -121.26 pmol/L (95% CI, -137.31 to -105.20) (349). In a more recent meta-analysis investigating the long-term effects of RYGB on DM2, they found that diabetes was improved or in remission in 89.2% of individuals (350). Furthermore, they found that the mean HbA1c and fasting blood glucose decreased significantly by 1.8% (95% CI, -2.5 to -0.9) and 60.4 mg/dl (95% CI, -75.0 to -45.8), respectively (350).

It is evident that RYGB improves and/or resolves both IR and DM2, however, the mechanisms by which this occurs is multifactorial. Three proposed mechanisms are both weight dependent and independent and include the effect on insulin sensitivity, the reduction of lipotoxicity and the alterations of gut hormones (351). Research has found that insulin sensitivity improves after RYGB in those who have lost significant weight (352). What has been observed is that adiponectin, an insulin sensitizing hormone, and insulin-receptor concentrations in the muscle increase (352). Furthermore, there is a decrease in intramuscular
and intrahepatic lipids (352). Interestingly, research has found that DM2 resolution and normal insulin sensitivity can be seen before major weight loss has been achieved, however the exact mechanism is not clear (353, 354). Many studies have proposed mechanisms as to why DM2 resolution occurs post-RYGB independent of weight loss (355, 356). These include the increase of post-prandial peptide YY, incretin hormones GLP-1 and GIP as well as impaired ghrelin secretion (355, 356). Additionally, research has investigated the possibility of how excluding the proximal small intestine could downregulate anti-incretin factors, which are currently unidentified (357). Another hypothesis is the effect of RYGB on bile acids. It has been found that circulating levels of bile acids have been correlated with measures of insulin sensitivity (358). One study investigated the enterohepatic recirculation of bile acids that occurs following RYGB (359). They found that the concentration of total serum bile acids was significantly higher in individuals 2-4 years post-RYGB compared to obese individuals who did not undergo RYGB surgery (359). They also found that bile acids were positively correlated with adiponectin and peak GLP-1 and negatively correlated with thyrotrophic hormone, fasting triglycerides and 2-hour postprandial serum glucose (359). This research demonstrates the possibility that the change in anatomy caused by RYGB could alter bile acids and thus improve glucose and lipid metabolism.

Overall, RYGB improves, if not resolving DM2 and IR, through various mechanisms that require more research. As DM2 and IR are associated with NAFLD, the degree of metabolic improvement seen following the bariatric care process may be of benefit for liver histology.
1.4.3.3 Dyslipidemia

Current recommendations for the improvement of lipid profiles include lifestyle measures and, if required, pharmaceutical therapy (297). Bariatric surgery, specifically RYGB, has been shown to improve lipid profile independent of weight loss (360). One study found that 1 year post-RYGB, total cholesterol was reduced by a mean of 16%, triglycerides by a mean of 63%, LDL cholesterol by 31% and VLDL cholesterol by 74%, whereas HDL cholesterol increased 39% (361). Another study found that even 5-years post-RYGB, triglycerides, LDL and total cholesterol were still significantly lower than prior to RYGB, and that HDL was significantly higher (362).

Even though RYGB significantly improves the lipid profile, the exact mechanism(s) in which RYGB improves the lipid profile is not clear. One proposed mechanism is the role of bile acids post bariatric surgery (363). In human studies, it has been found that fasting serum bile acid levels post-RYGB were elevated compared to lower preoperative levels (359, 364). Bile acid metabolism is associated with cholesterol homeostasis, and it has been shown that serum bile acid levels are negatively correlated with triglyceride levels, post-RYGB (359). Additionally, loss in fat mass is another mechanism which improves lipid control. Studies have found that post-RYGB, greater the loss of fat mass leads to greater improvement in lipid parameters (360, 361, 365). Food intake can also affect lipid levels (294, 366). RYGB affects the quality and quantity of foods consumed and has been shown to alter gut hormones, which may affect micro- and macronutrient digestion and metabolism (367). These alterations can also contribute to reducing lipid levels. Other mechanisms have also been reported to influence lipid levels post-RYGB such as affecting transfer or transport proteins, changes in HDL and LDL receptors as well as alterations in the IM (367).
These improvements in lipid profile reported post-RYGB may also contribute to improving NAFLD but it is not clear from the literature what is the contribution of diet, including the pre-bariatric VLCD.

1.4.3.4 Metabolic Syndrome

The cornerstone treatment for improving metabolic syndrome includes lifestyle modifications that target the underlying causes, such as excess adipose tissue (368). Regarding RYGB, a longitudinal study reported that 53.4% of patients had metabolic syndrome prior to surgery and that 12 months post-surgery, metabolic syndrome was present in only 12.5% of patients with improvements noted especially in glycemic control (369). In another comparable study, metabolic syndrome significantly improved 12-months post-RYGB, with only 11.5% of patients fulfilling the diagnostic criteria (370). Again, improvement in postoperative glycemic homeostasis parameters and IR was noted (370). Therefore, improvement in glycemic control and IR are important factors contributing to the resolution of the metabolic syndrome post bariatric surgery (371). Since metabolic syndrome is also associated with NAFLD it is likely that improvement in these parameters contribute to improvement of NAFLD post-RYGB.

1.4.3.5 Non-Alcoholic Fatty Liver Disease

When managing NAFLD, the overall goal is to improve histological markers by treating known risk factors such as IR and obesity (372). Currently, lifestyle interventions and medical treatment of the metabolic syndrome are the first line of treatment. However, bariatric surgery has emerged as a potential treatment for NAFLD (372).
Studies have found that improvements in steatosis, inflammation and fibrosis were seen post-RYGB. However, other studies found that some patients had new or worsening fibrosis (147, 373-376). A 5-year prospective study evaluating the effects of bariatric surgery on liver histology in 211 patients, found that the percentage of patients with steatosis decreased from a mean of 37.4% to 16% with ballooning score decreasing from a mean of 0.2 to 0.1 (377). Between baseline and 5-years post bariatric surgery the number of patients with probable or definite NASH significantly decreased from 99 (27.4%) to 30 (14.2%) individuals (377).

Mechanisms by which bariatric surgery improves NAFLD include improvement in IR, DM2, lipid profile and BMI as well as reduction in inflammatory parameters associated with these conditions (378). Also, in addition to the decrease in IR associated with weight loss, decreases in visceral adipose tissue and ghrelin as well as increases in GLP-1, adiponectin and bile acids post-RYGB may play a role (377). The increase in GLP-1, bile acids and adiponectin also improves lipid metabolism (379). By improving both IR and lipid metabolism, liver histology improves because there is a reduction in peripheral lipolysis and hepatic de novo lipogenesis (375).

Chronic inflammation associated with obesity has also been shown to improve post-RYGB. Weight loss secondary to RYGB has been found to be associated with significant reductions in markers of hepatic inflammation, macrophage chemoattractant protein 1 and IL-8 expression (380). Additionally, one study found that the significant weight loss achieved 12-months post-RYGB significantly reduces serum pro-inflammatory cytokines IL-18 and TNFR2 and significantly reduces serum C reactive protein, which is a marker for
inflammation (381). The IM may also play a role. RYGB induces changes in IM that are associated with improvement in various metabolic parameters (382). One study found that Firmicutes decrease post-bariatric surgery (383). Another study found that obese patients and controls had higher levels of *Faecalibacterium prausnitzii* (anti-inflammatory) when compared to obese patients with DM2 before surgery (384). Post-bariatric surgery, *F. prausnitzii* increased in all patients, which was negatively correlated with changes in inflammatory parameters, independent of DM2 and caloric intake (384). However, the data on this population in regard to IM and NAFLD is limited.

Therefore, RYGB, as a treatment of obesity, improves several obesity-related comorbidities through various mechanisms related to glucose and lipid metabolism, inflammation and IM, all of which may impact NAFLD. However, very few studies assessed separately, the effect of VLCD and RYGB on liver histology as well as metabolic and anthropometric parameters. Since not every patient may respond similarly to these interventions, it is of interest to assess these factors and determine what influence they may have on the response.

1.4.4 Summary

Current evidence supports the benefits of RYGB in morbidly obese individuals, with significant improvements in weight, IR, DM2, lipid metabolism and NAFLD. However, these studies do not consider the effect of VLCD as a potential contributor to this improvement and factors associated with better response are not very well studied. Therefore, part of the plan for this thesis is to add to the current body of knowledge by evaluating the effect of VLCD and RYGB on NAFLD and assess some of the factors that may contribute to the improvement in liver histology.
Chapter 2:

Thesis Objectives, Hypotheses & Methodological Approach

Preface

In this Chapter, I present the aims and hypotheses of my thesis followed by an outline of the study designs for the three studies which are entitled A) Non-Alcoholic Fatty Livers Disease in Morbidly Obese Individuals Undergoing Bariatric Surgery: Prevalence and Effect of Pre-Bariatric Very Low-Calorie Diet; B) Persistent NAFLD at 12-Months Post-Roux-en-Y Gastric Bypass Surgery is Associated with Lower Improvements in Waist Circumference and Glycemic Control; and C) Markers of Activated Inflammatory Cells are Associated with Disease Severity and Intestinal Microbiota in Adults with Non-Alcoholic Fatty Liver Disease. This is followed by Chapters 3, 4 and 5 which are the publications or the submitted manuscripts corresponding to the 3 studies. Each of these Chapters includes an introduction, detailed methodology, results and discussion. Chapter 6 presents a summary of the main findings and a general discussion and it is followed by Chapter 7 describing future directions.
2.1 Thesis Objectives and Hypotheses

Study A:

Aim: The aim of this study is to determine 1) the prevalence of NAFLD (SS and NASH) in a population sample of morbidly obese individuals undergoing RYGB post VLCD; 2) the effects of VLCD on metabolic and anthropometric parameters and; 3) the factors associated with NAFLD.

Main Hypothesis: Post-VLCD, the prevalence of NAFLD (SS and NASH) will be comparable to other reports in the literature.

Secondary Hypothesis: Metabolic and anthropometric parameters will improve after VLCD.

Tertiary hypothesis: IR will be associated with the presence of NAFLD post-VLCD.

Study B:

Aim: The aim of this study is to evaluate the effect of RYGB on NAFLD at 12-months post-surgery and determine the metabolic and anthropometric factors associated with persistent NAFLD.

Main Hypothesis: RYGB surgery will significantly improve all liver histological parameters, including steatosis, inflammation and fibrosis.

Secondary Hypothesis: Patients with NAFLD at 12-months post-RYGB will have higher IR compared to those who do not have NAFLD.
Study C:

**Aim:** The aim of this study is to compare hepatic immune cell counts in patients with biopsy confirmed NAFLD versus healthy controls and determine if there is an association between hepatic immune cell counts and the IM.

**Main Hypothesis:** Patients with NAFLD will have higher hepatic immune cell counts compared to healthy controls, namely CD163 positive cells.

**Secondary Hypothesis:** Specific beneficial bacterial taxa, especially *F. prausnitzii*, will be negatively correlated with hepatic immune cell counts.
2.2 Study Design

2.2.1 Part A:

This was a prospective cohort study of morbidly obese patients undergoing a pre-surgical very-low calorie diet (VLCD) followed by a Roux-en-Y gastric bypass (RYGB) where liver biopsies were taken at the time of surgery and at 12 months post-operatively. The prevalence of NAFLD at the time of surgery was a cross-sectional study.

Morbidly obese patients from the TWH Bariatric Program who were undergoing bariatric surgery were approached for participation in this research study. Study visit 1 occurred during their preadmission visit, prior to starting the pre-surgical VLCD. During this visit, anthropometric measurements and fasting blood work were collected. Study visit 2 occurred on the day of bariatric surgery after the VLCD. During this visit anthropometrics and fasting blood work were collected. An intraoperative wedge biopsy was performed and sent to pathology for histological analysis.

Patients were included if they had fulfilled the NIH criteria for bariatric surgery (385) and were found suitable for laparoscopic RYGB by the multidisciplinary TWH bariatric team. Other criteria were age $\geq$18 years and alcohol consumption $<$20g/day. If known to have hyperlipidemia or DM2, patients needed to be on a stable drug regime for $\geq$3 months prior to study entry. Patients were excluded if they had liver disease of other etiology; medication known to precipitate steatohepatitis 6 months prior to study entry; regular intake of non-steroidal anti-inflammatory drugs, pre, pro or antibiotics, urodeoxycholic acid or any experimental drug within the past 3 months prior to study entry; type 1 diabetes; chronic GI
diseases; previous GI surgery modifying the anatomy; smoking, pregnancy or breastfeeding, not tolerating Optifast® (standard weight loss VLCD prior to bariatric surgery), having uncontrolled DM2 or not on a stable drug regime for at least 3 months prior to study entry.

2.2.2 Part B:

This was a prospective cohort study investigating changes in liver histology in post-bariatric surgery patients. Patients included in this study were individuals who underwent Roux-en-Y gastric bypass (RYGB) surgery from Part A and agreed to participate in Part B. In addition to the measurements taken in Part A, a final study visit occurred at 12-months post-RYGB where fasting blood work and anthropometrics were taken. In addition, a second liver biopsy was taken, using the ultrasound guided needle technique as per standard medical protocol (386). Liver samples were sent to pathology for histological analysis. Inclusion and exclusion criteria were the same as Part A. In addition, patients needed to agree to comply with post-bariatric protocol for 1-year.

2.2.3 Part C:

This was a cross-sectional study comparing hepatic immune cells between adult patients with biopsy-proven NAFLD and healthy controls. In addition, the association between hepatic immune cells and specific bacterial taxa was evaluated.

Subjects were recruited from the hepatology department at the University Health Network (UHN), Toronto, Ontario. Patients suspected to have NAFLD due to persistent liver enzymes and obesity were evaluated by a hepatologist as per standard practice. Patients were given a general weight loss diet and physical activity advices. Those who had persistently elevated liver enzymes at 6-months follow-up were subsequently booked for a liver biopsy to
confirm NAFLD and assess disease severity. At that time patients were recruited for the study and provided a consent form. Once consent was given, subjects were provided detailed instructions on how to collect their stool sample. On the day of the ultrasound guided liver biopsy, fasting bloodwork and anthropometric data was collected.

Healthy controls were recruited during their first screening visit at the Living Liver Donor Clinic at the Toronto General Hospital (part of UHN). After consent, the same instructions were provided for the stool collection. One week prior to liver donation, healthy controls returned to the hospital for a study visit during which anthropometric measurements and fasting blood work were taken as well as the stool sample for IM. Approximately one week later, a wedge liver biopsy was obtained during their donation surgery.

Inclusion criteria included individuals 18 years or older with confirmation of NAFLD or a healthy liver based on liver biopsy. Exclusion criteria included NAFLD diagnosis of other etiology, liver transplant expected within one year, significant liver complications or any other complications for a liver biopsy, >20g of alcohol intake per day, pregnant or lactating, presence of gastrointestinal diseases, use of medications known to cause steatohepatitis, insulin, NSAIDS, anti/pre/pro biotics or experimental drugs within the last 3 months.

2.3 Statistical Analysis

2.3.1 Data Analysis

All statistical analyses were conducted using standard statistical software packages, primarily SAS version 9.3 (SAS Institute Inc., Cary, NC). The specific statistics used in each
study will be discussed in greater detail in each study’s methodology section. Overall, measured parameters were compared between groups using paired t-test, Kruskal-Wallis test followed by Wilcoxon ranked sum, or chi-square and Fisher’s exact test as necessary. Correlations were assessed using Spearman’s correlation coefficient. All tests were performed at the 5% significance (alpha) level. Bonferroni’s correction method was used to account for multiple comparisons between diagnostic groups.

2.3.2 Sample Size Calculations

Calculations were performed using standard statistical software packages, SAS version 9.3 (SAS Institute Inc., Cary, NC).

Part A:

The sample size calculation for the main outcome of the cross-sectional study, the prevalence of NAFLD (defined as at least 5% steatosis of hepatocytes) in the morbidly obese population, was based on the mean prevalence of steatosis in obese patients undergoing bariatric surgery being 91% which was obtained from a paper by Machado et al., 2006 (10). The formula used to calculate sample size was $n = (1.96)^2 \frac{\rho(1 - \rho)}{d^2}$ where 1.96 represents a 95% confidence interval, $\rho$ represented the prevalence for the outcome, which is 91% (0.91) of the population and $d$ represents a margin of error, which is 0.05 (387). Therefore, the sample size required is 126.

The sample size calculation for the main outcome of the prospective cohort study, the change in HOMA-IR before and after VLCD, was based on a study by Wahlroos et al., 2007 that reported the percentage change in HOMA-IR score after 6 weeks of a VLCD being 24.9 with a standard deviation of 39.6 (388). The sample size formula used was $n = 2 + \ldots$
C(\frac{s}{d})^2 \text{ where } C \text{ is the constant of 7.85 which represents a 5\% level of significance and 80\% power, } s \text{ is the standard deviation and } d \text{ is the difference of the mean (389). Therefore, the sample size required is 23 to achieve a power of 80\% and a level of significance of 5\% (two sided), for detecting a mean percentage of the differences of 24.9 between matched pairs, assuming the standard deviation of the differences to be 39.6\%.}

Part B:

The sample size calculation for the main outcome of this prospective cohort study, the change in NAS post-RYGB, was based on a study by Froylich et al., 2016 that reported a mean change in NAS score as 3 with a standard deviation of 2 after 1.7 years post-RYGB (390). The sample size formula used was \( n = 2 + C(\frac{s}{d})^2 \) where C is the constant of 7.85 which represents a 5\% level of significance and 80\% power, s is the standard deviation and d is the difference of the mean (389). Therefore, the study will need at least a sample size of 7 to achieve a power of 80\% and a level of significance of 5\% (two sided), for detecting a mean of the differences of 3 between matched pairs, assuming the standard deviation of the differences to be 2.

Part C:

This was an exploratory study; thus, no sample size calculation was computed.

### 2.4 Materials and Methods

#### 2.4.1 Liver Biopsy

##### 2.4.1.1 Liver Histology

The liver tissue was preserved in formalin within 15 minutes of the liver biopsy and stored at 4°C in a refrigerator and later embedded in paraffin. For the purposes of our study, liver disease was scored using the Brunt system (391) for all liver biopsies and the NAFLD
activity score (NAS) was also calculated (392). Details of liver scoring can be found in Appendix 2-1.

2.4.1.2 Hepatic Immune Cells

In the cross-sectional study (Study C), hepatic immune cells were assessed. Immunostaining of these formalin-fixed paraffin-embedded liver biopsies was performed using primary monoclonal mouse antibodies raised against cell markers CD45, CD3, CD163, and CD20. CD45, known as common leukocyte antigen, is one of the most abundant leukocyte cell surface glycoproteins and its expression is restricted to hematopoietic cells (393). CD3 antigen represents the most specific as well as the most sensitive T cell lineage marker, including NK and NKT (393). CD163 is a member of the cysteine scavenger receptor superfamily that is expressed in the cells of monocyte/macrophage origin, including Kupffer cells (222). CD20 is a marker for B-cells (394). Staining was performed at the UHN Laboratory Medicine Program. The density of cells within the portal tract and liver lobule which stain positive to those monoclonal antibodies was determined by counting the number of positive cells in ten random portal tracts and in ten areas of lobular region at a magnification of x200 under light microscopy.

2.4.2 Stool Analysis

For the cross-sectional study (Study C), stool analysis was performed to assess the IM as part of another study (28). The protocol is briefly described.

2.4.2.1 Specimen Collection

Subjects were provided a stool collection kit, which included a plastic collection/storage container with a lid, an insulated bag and cooling elements. Subjects were provided instruction on how to collect, store and transport the stool sample. Samples were collected within 24 hours of the study appointment, and kept frozen in the patient’s home.
freezer. On the day of the appointment the patient transported the sample in the insulated bag with cooling agents to the hospital, where it was immediately stored at -80°C until analysis. This stool collection method has been used in a study published in Nature to establish a human gut microbial gene catalogue (395).

2.4.2.2 Intestinal Microbiota Measurement

The concentration of specific microorganisms and groups was measured by quantitative real-time polymerase chain reactions (qPCR) based on taxa identified from sequencing analysis (28). The following description provides the detailed procedure of DNA extraction and quantitative real-time PCR (27, 28, 396).

DNA Extraction:

1. The thawed stool sample was immediately homogenized with a 400-masticator blender for 60 seconds. The pH and dry weight was measured.
2. 0.1 grams was used for DNA extraction using the E.Z.N.A.™ Stool DNA Isolation Kit (Omega Bio-Tek, Doraville, GA, USA).
3. The purity and concentration of DNA was measured using ThermoScientific Nanodrop 1000 Spectrophotometer (ThermoScientific Rockford, IL).
4. The DNA was stored at -20°C.

Quantitative Real-Time PCR:

1. The extracted fecal DNA was analyzed by qPCR, TaqMan Gene Expression Master Mix and specific TaqMan primers- Minor Groove Binder (MGB) probe set (Applied Biosystems, Forest City, CA).
2. These primers are designed to amplify 16S rDNA for specific groups/genera of gut microorganisms as well as total counts. Primers and probe sets are adapted from the literature (383, 397, 398).

3. A 384 wells block 7900HT thermocycler ran the assays in triplicate (Applied Biosystems, Forest City, CA).

4. The PCR reaction mix was prepared by pipetting 20 µL into a 1.5 mL micro-centrifuge tube. The tube was inverted until reaction compounds were mixed, then briefly centrifuged.

5. 20 µL of PCR reaction was transferred to the reaction plate, sealed and centrifuged. The plate was then loaded into the instrument using default parameters.

6. The number of microorganism cells in fecal samples was calculated by interpolation of standard curves (obtained from ten-fold serial dilutions of known quantities of bacterial DNA (399)) and expressed as g of wet feces weight.

2.4.3 Blood Work

For all three studies, the following blood work was performed.

2.4.3.1 Routine Blood Biochemistry and Hematology

Blood work was collected in the morning after the subject had been fasting for 12 hours. The certified UHN Laboratory Medicine Program analyzed samples for HbA1c, fasting insulin, glucose, liver enzymes, lipid profile, albumin and platelets using standard protocol.

Hemoglobin A1C in plasma was measured by ion exchange high performance liquid chromatograph (Variant II analyzer, Bio-Rad Laboratories). The homeostasis model was used for assessment [glucose (mmol/L) X insulin (mU/L / 22.5)] as an indirect measure of
insulin resistance (400). Serum fasting insulin was determined by semi-automated immunoassay (Abbott IMX Architect i2000 system), Abbott Laboratories. Fasting blood glucose was measured by the enzymatic hexokinase method on an Architect c8000 system (Abbott Laboratories). Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in plasma were measured using an automated method on the Architect c8000 system (Abbott Laboratories). Triglycerides, total cholesterol and high-density lipoprotein (HDL) were measured using an enzymatic reaction on the Architect C16000 system (Abbott Laboratories). Low-density lipoprotein (LDL) was calculated by subtracting HDL from total cholesterol. Platelet aggregation was measured using a manual platelet aggregometer.

2.4.4 Anthropometrics

2.4.4.1 Body Mass Index

For the bariatric patients, body weight and height were measured using the bariatric clinic’s calibrated hospital-grade standing weight scale for bariatric subjects and a stadiometer. BMI was calculated by dividing weight (kg) by height (m) squared. For the cross-sectional study, body weight and height were measured by a trained research professional for HC and NAFLD patients. Height was measured using a stadiometer and weight was measured using a calibrated hospital-grade chair scale.

2.4.4.2 Waist Circumference

Waist circumference was measured in the two prospective cohort studies. Waist circumference was measured by placing the measuring tape horizontally at the umbilicus (due to subjects being morbidly obese) (401). For this measurement subjects stood erect with arms by their side. After locating the umbilicus, the waist circumference was measured to the nearest millimeter. During this process subjects were asked to breath normally in order to
keep their abdomen relaxed. This measurement was taken three times, with an average to the nearest millimeter calculated.

2.4.5 Blood Pressure

In the two bariatric studies a Registered Nurse took the subject’s blood pressure using an automatic blood pressure monitor.
Chapter 3: Non-Alcoholic Fatty Liver Disease in Morbidly Obese Individuals Undergoing Bariatric Surgery: Prevalence and Effect of Pre-Bariatric Very Low Caloric Diet

by

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This chapter is modified from the following: Schwenger, Fischer, Jackson, Okrainec and Allard. Obesity Surgery 2018.
3.1 Abstract

**Background:** Non-alcoholic fatty liver disease (NAFLD) affects 75 to 100% of patients undergoing bariatric surgery (BSx), with non-alcoholic steatohepatitis (NASH) being present in 24% to 98% of patients. We don't know whether these rates were before or after a very low-calorie diet (VLCD) often prescribed before laparoscopic BSx and what is the prevalence of NAFLD post-VLCD.

**Purpose:** To determine the prevalence of simple steatosis (SS) and NASH in obese individuals undergoing BSx post-VLCD and assess biochemical markers pre- and post-VLCD in a subgroup of patients.

**Methods:** 139 patients undergoing BSx at a single Canadian bariatric program had biochemical and clinical variables collected pre-VLCD. In 21 patients, biochemical measurements were repeated post-VLCD. During BSx, a wedged liver biopsy was performed in all patients and histology was reported as normal liver (NL), SS or NASH.

**Results:** NAFLD was diagnosed in 76.3% of BSx patients with 61.9% having SS and 14.4% having NASH; 23.7% had NL. Those with NASH had significantly higher (P<0.05) pre-VLCD ALT, AST, insulin resistance and proportion of individuals with diabetes compared to those with NL. Overall, VLCD resulted in significant decreases in BMI, ALP, fasting glucose and insulin, HbA1c, total cholesterol, HDL and LDL cholesterol and significant increases in AST and ALT. Changes were similar between groups.

**Conclusions:** Post-VLCD, the prevalence of NAFLD and NASH were lower compared to published reports, with almost 25% of patients having a NL. With VLCD, metabolic and clinical changes were similar between the 3 groups suggesting that pre-VLCD factors may affect liver histology.
3.2 Introduction

Obesity in Canada is on the rise and is a grave public health issue. The estimated cost for obesity in Canada ranges between $4.6 billion to $7.1 billion Canadian dollars annually for health care costs and lost productivity (1). In 2013, 18.8% of adults in Canada reported themselves as obese (defined as a body mass index (BMI) over 30 kg/m$^2$), which increased to 20.2% in 2014 (242). In addition, the Public Health Agency of Canada reported that in 201, 2.7% of Canadians were morbidly obese (defined as a BMI over 40 kg/m$^2$). Obesity is associated with diabetes mellitus type 2 (seen in up to 42% of morbidly obese individuals (342, 402, 403)), insulin resistance (402), cardiovascular disease (403) and non-alcoholic fatty liver disease (NAFLD) (404, 405).

NAFLD ranges from simple steatosis (SS) to nonalcoholic steatohepatitis (NASH), which can progress to cirrhosis (14) and hepatocellular carcinoma (406). The prevalence of NAFLD is 15-30% of the general population (4-6, 29), which increases to 58% in overweight individuals and to 75-100% in obese individuals undergoing bariatric surgery (10). Liver biopsies have documented NASH in 24% to 98% of those undergoing bariatric surgery (10). However, it is not clear if these rates were documented before or after a very low-calorie diet (VLCD), which is now routinely prescribed prior to laparoscopic bariatric surgery.

Research has suggested that bariatric surgery is an effective tool for long-term weight loss and improvement of comorbidities, including NAFLD (14-16). However, the prevalence and features of NAFLD in obese Canadians undergoing bariatric surgery have not yet been reported. The purpose of this study was to describe the clinical characteristics and prevalence
of NAFLD in a population sample of obese individuals undergoing bariatric surgery at a single Canadian Centre after a routinely prescribed VLCD and, in a subgroup of patients, to assess the changes in biochemical markers pre and post-VLCD in relation to liver histology.

3.3 Methods and Materials

This cross-sectional and prospective cohort study was conducted from September 2013 to May 2016 and included bariatric surgery patients living in the Greater Toronto Area who were enrolled at the University Health Network (UHN) Bariatric Program. Patients were recruited after the surgeon confirmed the plan for bariatric surgery. Patient data was collected and a VLCD diet was prescribed for a duration of 1 week per 100 pounds’ body weight. This VLCD is routinely prescribed as part of the pre-bariatric protocol, to reduce the size of the liver (328) in order to facilitate surgical access for performing the laparoscopic Roux-en-Y gastric bypass. It is a 900 kcal/day meal replacement liquid-based diet (Optifast ®) (325). The patients are provided four shakes a day with each shake consisting of 225 calories, 22.5 grams of protein, 7.5 grams of fat and 16.8 grams of carbohydrates (325). In addition to this low carbohydrate, high protein meal replacement, patients are instructed to drink as much water, black tea, black coffee or diet soda as they please. Additionally, patients are allowed to consume sugar-free Jell-O, chicken, beef or vegetable stock that is less than 10 calories per serving, as well as chew sugar-free gum.

Patients were included in the study if they fulfilled the National Institutes of Health criteria for bariatric surgery (385) and had been assessed by the multidisciplinary UHN bariatric team as suitable for bariatric surgery. Study criteria included age ≥18 years and alcohol consumption <20g/day. If known to have hyperlipidemia or DM2, patients needed to
be on a stable drug regimen for $\geq 3$ months prior to study entry. Patients were excluded from the study if they had liver disease of other etiology; medication known to precipitate steatohepatitis 6 months prior to entry; regular intake of non-steroidal anti-inflammatory drugs within the past 3 months prior to study entry; type 1 diabetes; or were smoking, pregnant or breastfeeding.

Anthropometrics and blood samples were collected at baseline before VLCD and, in a subgroup of patients, these measurements were repeated after VLCD. At the time of bariatric surgery, a wedged liver biopsy was performed in all patients. Ethics for this study were approved by the UHN Research Ethics Board (REB # 13-6115-A).

**Anthropometrics**

Body mass index (BMI), waist circumference, blood pressure and medication history were taken by a registered nurse. BMI was calculated by dividing weight (kg) by height (m) squared. Waist circumference was measured by placing the measuring tape horizontally at the umbilicus (401). Blood pressure was taken using an automatic blood pressure monitor.

**Blood Samples**

Plasma and serum were collected after 12 hours of fasting. The UHN Laboratory Medicine Program analyzed samples for HbA1c, insulin, glucose, liver enzymes, lipid profile and platelets using standard laboratory tests. The homeostasis model for insulin resistance was also calculated using fasting serum glucose and insulin (400).

**Liver Biopsy**
Liver samples were obtained during bariatric surgery after the VLCD. The site of the liver biopsy was consistent across all subjects and occurred in the anterior border of the left lobe in segment 3. A piece of liver tissue was obtained by laparoscopic wedge biopsy and was preserved in 10% formalin within 15 minutes of collection for a minimum of 24 hours. Paraffin embedded tissue sections were stained using hematoxylin-eosin and Masson trichrome protocols. A pathologist blinded to the study analyzed the liver biopsy prospectively. For the purposes of our study, liver disease was scored using the Brunt system (391). The Brunt system scores steatosis, inflammation, fibrosis and ballooning of hepatocytes. SS was diagnosed if the patient had >5% fat in their liver. NASH was confirmed when ballooning of hepatocytes was present.

Statistics

Statistical calculations were performed by the SAS program. Measured parameters were compared between groups using paired t-test, Kruskal-Wallis test followed by Wilcoxon ranked sum, or chi-square and Fisher’s exact test as necessary, with Bonferroni correction for multiple comparisons between diagnostic groups. Data was considered to be statistically significant at a p-value of less than 0.05.

3.4 Results

General Patient Characteristics

The sample consisted of 139 bariatric surgery patients with a liver biopsy. 102 (73.4%) were female. The median age was 44 ranging from 21 to 65 years. Table 3-1 shows the overall characteristics and Figure 3-1 shows the medication use of patients.
Table 3-1: Overall characteristics of bariatric surgery patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n</th>
<th>Median (Minimum, Maximum) or n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>139</td>
<td>44 (21, 65)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>139</td>
<td>47.06 (35.22, 72.87)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>139</td>
<td>132.10 (91.73, 247.50)</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>124</td>
<td>130 (107.95, 192)</td>
</tr>
<tr>
<td>Gender (% female)</td>
<td>139</td>
<td>102 (73.38%)</td>
</tr>
<tr>
<td>Country of Origin (Canada)</td>
<td>117</td>
<td>77 (65.81%)</td>
</tr>
<tr>
<td>Presence of Metabolic Syndrome (%)*</td>
<td>121</td>
<td>61 (68.42%)</td>
</tr>
<tr>
<td>Diagnosed Diabetes (%)</td>
<td>134</td>
<td>36 (26.87%)</td>
</tr>
<tr>
<td>Impaired Fasting Glucose **</td>
<td>88</td>
<td>32 (36.36%)</td>
</tr>
</tbody>
</table>

* Metabolic syndrome is determined if any of the 3 following criteria are met; waist circumference ≥ 102 cm for males/ ≥88 cm for females, triglycerides ≥ 1.7 mmol/L, blood glucose ≥ 5.6 mmol/L, HDL cholesterol < 1.03 mmol/L for males/< 1.30 mmol/L for females and/or blood pressure ≥ 130/85 mmHg. If an individual is using any medication for the above criteria it is considered as meeting that specific criterion(303).  
** Those using hyperglycemic medication were excluded from this measurement.

![Figure 3-1: Medication use of 133 bariatric surgery patients pre-VLCD](image_url)

Characteristics Based on Liver Biopsy
NAFLD was diagnosed in 106 (76.3%) patients while 33 (23.7%) had normal liver (NL) histology based on liver biopsies performed at the time of surgery. Those with normal liver histology were classified as normal liver (NL). Of those diagnosed with NAFLD, 86 (81.1%) had simple steatosis (SS) and 20 (18.9%) had NASH. Therefore, for all patients undergoing bariatric surgery, 61.9% had SS and 14.4% had NASH.

The median for clinical and biochemical variables assessed at baseline, prior to the VLCD leading to surgery, can be seen in Table 3-2 for those with NL, SS and NASH. Age, gender, BMI, waist circumference, country of origin, metabolic syndrome, impaired fasting glucose, blood pressure, lipid-lowering and antidepressant medication usage did not significantly differ between the three groups but those with NASH had the highest proportion of type 2 diabetes, insulin resistance (IR) (based on the homeostatic model assessment (HOMA)) and usage of hyperglycemic medication. Additionally, before VLCD, HbA1c, AST and ALT were highest and, platelets and total cholesterol were lowest in the NASH population.

Table 3-2: Differences of clinical and biochemical measures between groups before VLCD

<table>
<thead>
<tr>
<th>Variable</th>
<th>NL (n=33)</th>
<th>SS (n=86)</th>
<th>NASH (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>44 (21, 63)</td>
<td>44.5 (21, 65)</td>
<td>48 (32, 62)</td>
</tr>
<tr>
<td>Gender n (% female)</td>
<td>22 (66.67%)</td>
<td>65 (75.58%)</td>
<td>15 (75.00%)</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>128.6 (108.0, 166.0)</td>
<td>130.4 (109.0, 192.0)</td>
<td>135.9 (114.8, 157.0)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>46.74 (35.65, 63.79)</td>
<td>47.00 (35.22, 72.87)</td>
<td>48.92 (43.37, 61.71)</td>
</tr>
<tr>
<td>Diagnosed Diabetes n (% of patients)</td>
<td>4 (12.12%) of 33\textsuperscript{A}</td>
<td>23 (28.40%) of 81</td>
<td>9 (45%) of 20\textsuperscript{A}</td>
</tr>
<tr>
<td>Hyperglycemic Medication Use n (%)</td>
<td>2 (6.45%) of 31\textsuperscript{AB}</td>
<td>23 (28.05%) of 82\textsuperscript{A}</td>
<td>9 (45%) of 20\textsuperscript{B}</td>
</tr>
</tbody>
</table>
### Insulin Resistance* n (% of patients)
(based on HOMA IR)

<table>
<thead>
<tr>
<th></th>
<th>15 (75.0%) of 20</th>
<th>57 (93.4%) of 61</th>
<th>15 (100%) of 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Biochemical Characteristics

#### Liver Enzymes

<table>
<thead>
<tr>
<th>Enzyme (U/L)</th>
<th>AB</th>
<th>AC</th>
<th>BC</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST</td>
<td>18 (14, 42)</td>
<td>24 (12, 530)</td>
<td>34 (18, 120)</td>
</tr>
<tr>
<td>ALT</td>
<td>18 (9, 36)</td>
<td>27 (1.5, 85)</td>
<td>44 (20, 138)</td>
</tr>
<tr>
<td>ALP</td>
<td>76.5 (46, 125)</td>
<td>80 (37, 142)</td>
<td>70.5 (44, 123)</td>
</tr>
</tbody>
</table>

#### Blood Glucose Control

<table>
<thead>
<tr>
<th>Measurement</th>
<th>AB</th>
<th>AC</th>
<th>BC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/L) *</td>
<td>5.3 (4.5, 9.5)</td>
<td>5.4 (3.8, 8.3)</td>
<td>5.95 (5.10, 10.6)</td>
</tr>
<tr>
<td>Insulin (pmol/L) *</td>
<td>123 (61, 511)</td>
<td>132 (50, 1352)</td>
<td>130 (92, 668)</td>
</tr>
<tr>
<td>HbA1c*</td>
<td>0.06 (0.04, 0.08)</td>
<td>0.06 (0.05, 0.07)</td>
<td>0.06 (0.05, 0.12)</td>
</tr>
</tbody>
</table>

#### Lipid Profile

<table>
<thead>
<tr>
<th>Lipid (mmol/L)</th>
<th>AB</th>
<th>AC</th>
<th>BC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol **</td>
<td>5.08 (3.37, 6.36)</td>
<td>4.74 (2.34, 7.68)</td>
<td>4.14 (2.15, 5.47)</td>
</tr>
<tr>
<td>Triglycerides **</td>
<td>1.38 (0.70, 3.17)</td>
<td>1.36 (0.59, 4.09)</td>
<td>1.49 (1.24, 2.49)</td>
</tr>
<tr>
<td>LDL Cholesterol **</td>
<td>3.40 (1.15, 4.92)</td>
<td>2.89 (1.17, 5.01)</td>
<td>2.48 (1.17, 5.01)</td>
</tr>
<tr>
<td>HDL Cholesterol **</td>
<td>1.17 (0.79, 2.19)</td>
<td>1.16 (0.68, 1.94)</td>
<td>1.08 (0.78, 1.48)</td>
</tr>
</tbody>
</table>

#### Complete Blood Count

<table>
<thead>
<tr>
<th>Variable</th>
<th>AB</th>
<th>AC</th>
<th>BC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets (x 10⁹/L)</td>
<td>281 (164, 466)</td>
<td>266.5 (33, 485)</td>
<td>213.5 (130, 366)</td>
</tr>
</tbody>
</table>

* Those using hyperglycemic medication were excluded from this measurement.
** Those using lipid lowering medication were excluded from this measurement.
HOMA-IR: homeostasis model assessment estimated insulin resistance

### Effect of the Very Low-Calorie Diet

Table 3-3 shows the mean differences between pre and post VLCD of clinical and biochemical measurements. Overall, the mean duration of the VLCD was 2.60 ± 0.66 (SD) weeks with a median of 3 weeks (minimum: 0.29; maximum: 4 weeks). Weight loss per week of VLCD was 3.69 kg ± 2.58. The VLCD significantly decreased BMI, blood glucose control measurements, ALP, total, LDL and HDL cholesterol and platelets. However, VLCD significantly increased AST and ALT levels. When changes between the 3 groups were compared (see Table 3-4), weight loss and changes in biochemical values during VLCD did
not significantly differ between NL, SS and NASH. There was also no difference in the duration of the VLCD between the 3 groups.

Table 3-3: Difference of clinical and biochemical measures pre and post VLCD

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean Difference (95% Confidence Interval)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical Characteristics (n=81)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>-2.25 (-3.00, -1.51)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Liver Enzymes (n=21)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>8.71 (3.85, 13.58)</td>
<td>0.0013</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>10.00 (1.20, 18.80)</td>
<td>0.0276</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>-15.75 (-22.66, -8.84)</td>
<td>0.0001</td>
</tr>
<tr>
<td><strong>Blood Glucose Control Measurements (n=19)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>-1.13 (-1.89, -0.38)</td>
<td>0.0053</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>-148.8 (-210.9, -86.62)</td>
<td>0.0002</td>
</tr>
<tr>
<td>HbA1c</td>
<td>-0.0026 (-0.0042, -0.0011)</td>
<td>0.0018</td>
</tr>
<tr>
<td><strong>Lipid Profile (n=19)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>-0.89 (-1.17, -0.61)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>-0.39 (-0.84, 0.07)</td>
<td>NS</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/L)</td>
<td>-0.53 (-0.72, -0.33)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/L)</td>
<td>-0.19 (-0.29, -0.09)</td>
<td>0.0006</td>
</tr>
<tr>
<td><strong>Complete Blood Count (n=19)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelets (x 10⁹/L)</td>
<td>-24.50 (-42.56, -6.44)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 3-4: Changes between clinical and biochemical measures between groups pre and post VLCD

<table>
<thead>
<tr>
<th>Variable</th>
<th>NL</th>
<th>SS</th>
<th>NASH</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight Loss on VLCD (kg)</td>
<td>-6.35 (-19.10, -3.09)</td>
<td>-7.18 (-30.87, -0.66)</td>
<td>-7.25 (-19.07, -4.53)</td>
<td>0.2722</td>
</tr>
<tr>
<td>BMI (kg/m²) after VLCD</td>
<td>-2.40 (-5.94, -1.24)</td>
<td>-2.52 (-11.48, -0.25)</td>
<td>-2.46 (-7.27, -2.04)</td>
<td>0.2416</td>
</tr>
<tr>
<td>% Excess Body Weight Loss</td>
<td>8.4% (-8.0, 24.4)</td>
<td>9.6% (0.0, 29.8)</td>
<td>9.2% (6.9, 25.3)</td>
<td>0.4052</td>
</tr>
<tr>
<td>Days on VLCD</td>
<td>17.5 (7, 28)</td>
<td>21 (2, 28)</td>
<td>21 (10, 28)</td>
<td>0.4911</td>
</tr>
<tr>
<td><strong>Laboratory Measurements</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change in Blood Glucose Control Measurements</td>
<td>n=6</td>
<td>n=13</td>
<td>n=5</td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>-0.5 (-4.1, 0.2)</td>
<td>-0.5 (-3.8, 50.0)</td>
<td>-0.80 (-3.70, 0.20)</td>
<td>0.5708</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------</td>
<td>------------------</td>
<td>---------------------</td>
<td>--------</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>-156 (-241, -90)</td>
<td>-151 (-580.0, 0.0)</td>
<td>-74 (-331, 15)</td>
<td>0.8169</td>
</tr>
<tr>
<td>HbA1c</td>
<td>-0.00 (-0.01, 0.00)</td>
<td>-0.00 (-0.01, 0.00)</td>
<td>-0.00 (-0.01, -0.00)</td>
<td>0.9991</td>
</tr>
<tr>
<td>Insulin Resistance (based on HOMA IR)</td>
<td>2 (33.3%)</td>
<td>6 (46.2%)</td>
<td>3 (60%)</td>
<td>0.7721</td>
</tr>
<tr>
<td><strong>Change in Liver Enzymes</strong></td>
<td>n=5</td>
<td>n=11</td>
<td>n=5</td>
<td></td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>8.0 (0.0, 38.0)</td>
<td>10.0 (0.0, 25.0)</td>
<td>1.0 (-10.0, 21.0)</td>
<td>0.3287</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>11.0 (-3.0, 70.0)</td>
<td>6.0 (0.0, 58.0)</td>
<td>-1.5 (-38.0, 10)</td>
<td>0.1157</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>-19.0 (-31.0, -8.0)</td>
<td>-9.5 (-26.0, 0.0)</td>
<td>-10.0 (-57.0, 1.0)</td>
<td>0.4800</td>
</tr>
</tbody>
</table>

*Variables expressed as: Median (minimum, maximum) or n (percentage of patients) as appropriate*

### 3.5 Discussion

This study shows that, in this homogenous population of obese patients, the prevalence of NAFLD is lower than what is reported in the literature. This could be due to the routinely prescribed VLCD pre-bariatric surgery. Despite similar metabolic and anthropometric changes induced by VLCD, liver histology was variable among subjects, with those with NASH, being more likely to have severe IR (based on HOMA) and/or diabetes at baseline compared to those with NL.

This study population sample is comparable to the general bariatric surgery population of Ontario and Canada. A recent study of 5007 bariatric surgery patients in Ontario cited the mean age as 44.6 years, with 81.9% being female and 29.6% having diabetes (407). The Ontario Bariatric Registry cited the average BMI in Ontario bariatric surgery patients as 48.6 kg/m² (408). In the 2014 Bariatric Surgery in Canada report, it was cited that 80% of Canadian bariatric surgery patients are women, the average age is 45 years.
and 13% have type 2 diabetes (408). Therefore, our results could be generalizable to the Canadian population undergoing bariatric surgery.

The prevalence of NAFLD was lower than published reports from the US, South America and Europe where the prevalence of NAFLD ranges from 84% to 96% (11), with SS being from 85% to 98% and NASH from 24% to 98% in morbidly obese patients (10). Variations in the reported prevalence may reflect differences in patient populations, assessment techniques or histological criteria (10). For those who were assessed by liver biopsy during bariatric surgery (10), none of these studies reported on the use or not of pre-bariatric VLCD. Therefore, it is possible that our lower prevalence rate is due to the routinely prescribed VLCD. The purpose of VLCD prior to laparoscopic bariatric surgery is to reduce liver size (328, 409) due to steatosis which can cause a visual obstruction, increasing the risk of conversion to open surgery (410). VLCD has been shown to significantly reduce liver steatosis which can explain the lower proportion of SS in this study (411) and the increased presence of normal liver found in biopsies. However, VLCD has not been reported to improve NASH, such as reducing hepatocyte ballooning which is a diagnostic hallmark for NASH. Therefore, the lower proportion of NASH found in our patients compared to the literature may be due to other baseline factors known to be associated with NASH, such as age or presence of diabetes (412, 413) that could be different between study populations.

Noninvasive clinical and biochemical markers have been associated with NAFLD severity including age, BMI, waist circumference, diabetes, liver enzymes, fasting glucose and insulin (414). Contrary to other studies on NAFLD in the general populations (62), our
study did not find significant differences between groups with regards to age, BMI and waist circumference. This could be because our study targeted a relatively homogenous bariatric population undergoing similar pre-bariatric protocol with VLCD. Other factors may have been involved in the difference in liver histology, such as the presence of insulin resistance and diabetes (17). Indeed, our results show that at baseline before VLCD, patients with NASH had a significantly higher proportion of diabetes, usage of hyperglycemic medication, higher HbA1c and IR (based on HOMA). High fasting glucose was also strongly associated with NASH. After VLCD, patients were found to have NASH despite a reduction in IR, HbA1c, fasting glucose, insulin and improvements in anthropometric and biochemical parameters, suggesting that having more severe IR or diabetes predisposed these patients to have persistent NASH (415) despite a weight reducing intervention such as VLCD.

Those with NL had a response to VLCD that was similar to the other 2 groups (NASH, SS) in terms of metabolic and anthropometric parameters but the severity of IR and presence of diabetes at baseline was lower. Other mechanisms may have contributed to the normal histology in this patient group. For example, fat distribution may have been different. It is known that abdominal fat distribution, independent of body weight or body fat, is a predictor of hepatic steatosis (416, 417). Genetics is another factor that could explain why patients had NL post VLCD, while others still had SS or NASH. For example, genetic variations in lipid metabolism genes have been associated with fat accumulation in the liver (418). Intestinal microbiota is another factor that could explain this phenomenon. The intestinal microbiota is determined by multiple factors which include diet (419-421), environmental, geographical and host genetic factors (422). Recently, new evidence has linked intestinal microbiota to NAFLD pathogenesis (58).
The results of this study found that SS and NASH patients had significantly higher AST and ALT when compared to NL pre VLCD. When comparing the biochemical values to normal levels, median AST and ALT values were within the normal range, except for ALT in NASH which was elevated. In our study, most individuals with NASH had normal liver enzymes which was also reported by others (66). Although enzymes elevation, changes in ratio of AST:ALT and other non-invasive indicators (44-49) can suggest NASH, liver biopsy is the gold standard for diagnosis (62). It is interesting to note that with VLCD, there was an elevation in both AST and ALT in all groups while there was a decrease in ALP. The metabolic and anthropometric changes we observed with VLCD are consistent with published reports (388, 411) including AST and ALT elevations (95, 423, 424) which may not be associated with liver histological changes (425). Andersen et al. suggested that these findings could represent a hepatotoxic factor due to rapid mobilization of fat stores (425).

Another finding was that serum total cholesterol and LDL were significantly lower in NASH when compared to SS and NL pre VLCD. There was no difference in lipid lowering agents or diet (data not shown) between groups. It is conceivable that a difference in lipid metabolism could explain this as reductions in HDL, LDL and total cholesterol have been reported to be positively correlating with liver disease severity (426). Changes in lipid metabolism such as a decrease in lipoprotein synthesis and degradation of the lipoprotein complex within the liver have been reported (427).

Our study has limitations. This was a single center study but when compared to the bariatric population of Ontario and Canada, our patient characteristics were similar to the
larger population. The prevalence of NAFLD, especially SS, is likely underestimated, due to the VLCD Optifast® regimen, although the prevalence of NASH should be similar. The majority of blood work was collected prior to VLCD. Only a small proportion of patients had bloodwork post VLCD, at the time of bariatric surgery and liver biopsy. There were logistic issues with the operating rooms that prevented us from taking blood from a larger number of subjects on the day of surgery. It is possible that the small sample size prevented us from detecting differences between groups during VLCD with regards to changes in laboratory parameters. However, considering that the VLCD duration and changes in anthropometric and clinical parameters were similar between groups, this is less likely.

In conclusion, this study is the first to document in Canada the prevalence of NAFLD, post VLCD, in obese individuals undergoing laparoscopic bariatric surgery. Results show that the prevalence of NAFLD and NASH is lower compared to published reports and this may be partly the result of the usage of VLCD prior to surgery. Despite similarities in patients’ demography, VLCD intervention and clinical response to VLCD, we found differences in liver histology post VLCD from normal to SS to NASH. Differences in response may be due to baseline factors such as IR severity, presence of diabetes or other mechanisms such as genetics and intestinal microbiome. Future studies are needed to investigate the long-term effect of VLCD as a potential treatment intervention for NAFLD.

Conflict of Interest

Authors 1-3 and 5 have no conflict of interest to declare. Author 4 has relevant financial activities outside of the submitted work. He is provided an honorarium for speaking and teaching from Ethicon and Medtronic.
Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent

Informed consent was obtained from all individual participants included in the study.
Chapter 4: Persistent NAFLD at 12 Months Post-Roux-en-Y Gastric Bypass Surgery is Associated with Lower Improvements in Waist Circumference and Glycemic Control

by

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This chapter is modified from a submitted paper under review at Surgery for Obesity and Related Diseases.
4.1 Abstract

**Background/Objectives:** In obese individuals undergoing Roux-en-Y Gastric Bypass (RYGB), non-alcoholic fatty liver disease (NAFLD) is seen in 75 to 100% of cases. This improves post-surgery but some patients continue to have persistent NAFLD. The purpose of this study was to determine the factors associated with persistent NAFLD at 12-months post-RYGB.

**Subjects/Methods:** This is a prospective cohort study of 42 patients who underwent RYGB. Liver biopsy, biochemical and clinical parameters were collected pre- and 12-months post-RYGB. Based on histology at 12-months, patients were separated in 2 groups: normal liver (NL) and persistent NAFLD.

**Results:** At baseline NAFLD was diagnosed in 85.7% of patients and at 12-months post-RYGB, NAFLD was present in 19.1% of patients. Patients who had a NL at baseline, remained with a NL. RYGB resulted in significant decreases in body mass index (BMI), waist circumference, blood pressure, AST, ALT, fasting glucose and insulin, HbA1c and triglycerides, and significant increases in HDL cholesterol. Changes were similar in both groups except for waist circumference, which showed lower changes in those with persistent NAFLD. These patients also had significantly higher (P<0.05) fasting glucose and insulin with a higher proportion of patients having insulin resistance compared to those with NL.

**Conclusions:** RYGB resulted in significant improvements in liver histology, biochemical and clinical parameters. However, despite similar weight loss, persistent NAFLD was associated with less improvement in waist circumference and worse glycemic control.
4.2 Introduction

Obesity is a significant health problem and is on the rise in today’s society. As obesity rises, so do associated comorbidities, which include non-alcoholic fatty liver disease (NAFLD). Studies have found that as body mass index (BMI) increases, so does the prevalence of NAFLD (9). In obese individuals undergoing bariatric surgery, NAFLD is seen in 75-100% of cases (10). NAFLD includes a spectrum of liver abnormalities, which ranges from benign simple steatosis (SS) to non-alcoholic steatohepatitis (NASH), which in some cases progresses to hepatic cirrhosis (428). Current management of NAFLD includes weight loss through lifestyle modifications, as well as pharmacological treatment that targets NAFLD pathogenesis (372). However, in those who are obese, weight loss through lifestyle modification may be ineffective and thus other weight loss strategies may be considered.

Current research suggests that bariatric surgery is effective at improving obesity-related comorbidities as well as sustained long-term weight loss (343). However, the effect on liver histology, specifically NASH and fibrosis has shown conflicting results (429). Also, for those with persistent NAFLD or NASH post-surgery, it is unclear what factors are associated with persistent histological abnormalities. The purpose of this study was to assess the impact of Roux-en-Y gastric bypass (RYGB) on NAFLD and related parameters after 12-months post-surgery and identify the factors associated with persistent histological abnormalities.

4.3 Methods and Materials

This is a prospective cohort study that was conducted from September 2013 to June 2017. It includes bariatric surgery patients from a University Hospital in Canada. The study protocol was approved by appropriate Research Ethics Boards and conformed to the ethical
guidelines of the 1975 Declaration of Helsinki. All participants gave their informed written consent.

Subjects:

Patients were approached after the surgeons confirmed that the patients were suitable for bariatric surgery according to the criteria stated by the National Institutes of Health (385). Study criteria also included age \( \geq 18 \) years and alcohol consumption \(<20\text{g/day}\). If known to have hyperlipidemia or DM2, patients needed to be on a stable drug regimen for \( \geq 3 \) months prior to study entry. Clinical, nutritional and biochemical data were collected at baseline, prior to surgery, and at 12-months post-RYGB surgery. A wedged liver biopsy was performed intraoperatively and an ultrasound guided needle biopsy was performed at 12-months post-RYGB.

Patients were excluded from the study if they had: liver disease of other etiology; medication known to precipitate steatohepatitis 6 months prior to study enrollment; regular intake of non-steroidal anti-inflammatory drugs within the past 3 months prior to study entry; type 1 diabetes; or were smoking, pregnant or breastfeeding.

Biochemical and Clinical Data:

Patient’s anthropometrics, blood pressure and medication history were taken by a registered nurse. Percent excess body weight loss (EBWL) was then calculated (% EBWL = 100 \times \frac{\text{weight loss since RYGB/preoperative excess body weight}}{\text{preoperative excess body weight}}) using the Hamwi method (430).
Plasma and serum were collected after a 12-hour fast and analyzed by the hospital Laboratory Medicine Program using standardized methods. The homeostasis model (HOMA) for insulin resistance (IR) was also calculated using fasting serum glucose and insulin (400).

**Histology:**

The liver biopsies were preserved in 10% formalin within 15 minutes of collection, and later, were embedded in paraffin. A pathologist blinded to the study assessed the liver histology for the presence of steatosis, inflammation, ballooning of hepatocytes and fibrosis using the Brunt system (391). Additionally, the NAFLD Activity Score (NAS) was used to evaluate disease severity (431). SS was diagnosed if the liver had >5% steatosis and NASH was confirmed when ballooning of hepatocytes was present.

**Statistical Analysis:**

Statistical calculations were performed using the SAS 9.4 program. Parameters of interest were compared between groups using paired t-test, Kruskal-Wallis test, Wilcoxon ranked sum, or chi-square and Fisher’s exact test as necessary. Spearman correlation coefficients and partial Spearman correlation coefficients were used to examine the relationship between change in liver histology and clinical/biochemical variables. Statistical significance was considered when p-value was less than 0.05.

**4.4 Results**

Forty-two patients completed the study, 32 (76.2%) were female and the median age was 47.2 (42, 55 (1st quartile, 3rd quartile)) years. Before bariatric surgery, 85.7% were diagnosed with NAFLD and 14.3% were considered to have a normal liver (NL). At 12-months post-RYGB, 8 (22.2%) out of the 36 individuals at baseline continued to have
NAFLD, whereas NAFLD resolved in 28 (77.8%) of individuals. The 6 subjects who had NL at baseline remained with NL. Table 4-1 shows the clinical and biochemical measurements before and 12-months after RYGB, as well as the changes in parameters. RYGB significantly improved weight, BMI, waist circumference, presence of metabolic syndrome, IR, blood pressure, AST, ALT, glucose, insulin, HbA1c and triglycerides and significantly increased HDL cholesterol. RYGB did not have a significant effect on the number of individuals with diagnosed diabetes or levels of ALP, total cholesterol and LDL cholesterol.

Table 4-1: Clinical and biochemical values and changes in parameters between baseline and 12-months post-RYG

<table>
<thead>
<tr>
<th>Variable</th>
<th>Before Surgery</th>
<th>12-Months After Surgery</th>
<th>Changes</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>133.31 (113.85, 151.60)</td>
<td>95.74 (84.37, 107.73)</td>
<td>-37.56 (-48.97, -26.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>48.15 (42.53, 53.53)</td>
<td>34.53 (30.15, 38.54)</td>
<td>-13.6 (-15.62, -8.96)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>% EBWL</td>
<td></td>
<td>51.7 (40.6, 63.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>133.19 (120, 145)</td>
<td>106.76 (97, 114)</td>
<td>-27.84 (-32.11, -23.15)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Presence of Metabolic Syndrome n (% of patients)</td>
<td>18 (48.7%)</td>
<td>9 (24.3%)</td>
<td></td>
<td>0.0352</td>
</tr>
<tr>
<td>Diagnosed Diabetes n (% of patients)</td>
<td>14 (34.2%)</td>
<td>9 (22.0%)</td>
<td></td>
<td>0.1250</td>
</tr>
<tr>
<td>Insulin Resistance n (% of patients)</td>
<td>26 (92.9%)</td>
<td>7 (25%)</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(based on HOMA IR)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic BP mm Hg</td>
<td>126.00 (118, 133)</td>
<td>120.41 (108, 130)</td>
<td>-8.51 (-18, 2)</td>
<td>0.0008</td>
</tr>
<tr>
<td>Diastolic BP mm Hg</td>
<td>81.77 (79, 86)</td>
<td>77.41 (71, 85)</td>
<td>-5.3 (-9, 0)</td>
<td>0.0009</td>
</tr>
<tr>
<td><strong>Biochemical Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>28.42 (19, 34)</td>
<td>23.03 (17, 26)</td>
<td>-8.57 (-9, 5)</td>
<td>0.038</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>29.31 (18, 33)</td>
<td>23.26 (16, 28)</td>
<td>-12.3 (-18, 0.5)</td>
<td>0.007</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>80.56 (68, 91)</td>
<td>81.87 (65, 96)</td>
<td>1.61 (-7.5, 7.5)</td>
<td>0.59</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.79 (5.2, 6.2)</td>
<td>5.30 (4.7, 5.3)</td>
<td>-0.87 (-1.4, 0)</td>
<td>0.005</td>
</tr>
</tbody>
</table>
All histological measurements significantly improved after surgery. Overall, there was a significant reduction in NAS, from baseline of 2.07 ± 1.53 to a 12-months post-RYGB of 0.33 ± 0.78. Of those who had NAFLD at baseline, only 2 individuals had no change in NAS and 1 worsened, while NAS improved in 33 individuals. Of the individuals who had persistent NAFLD, 3 of them had NASH at baseline whereas 5 had SS. A summary of histological values taken at baseline and 12-months post-RYGB can be seen in Table 4-2.

Table 4-2: Histological values at baseline and 12-months post-RYGB

<table>
<thead>
<tr>
<th>Variable</th>
<th>Before Surgery</th>
<th>12-Months After Surgery</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical Diagnosis</td>
<td>n (% patients)</td>
<td>n (% patients)</td>
<td></td>
</tr>
<tr>
<td>NL</td>
<td>6 (14.3%)</td>
<td>34 (80.9%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SS</td>
<td>27 (64.3%)</td>
<td>7 (16.7%)</td>
<td></td>
</tr>
<tr>
<td>NASH</td>
<td>9 (21.4%)</td>
<td>1 (2.4%)</td>
<td></td>
</tr>
<tr>
<td>Steatosis Grade</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>0</td>
<td>6 (14.3%)</td>
<td>34 (80.9%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>25 (59.5%)</td>
<td>8 (19.0%)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>11 (26.2%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
</tbody>
</table>
We then classified patients into 2 groups, based on liver histology assessed at 12 months post-RYGB: 8 patients with persistent NAFLD and 34 with NL. All those who had NL at baseline remained with NL at 12 months and were included in the NL group. The mean clinical and biochemical variables at 12-months post-RYGB as well as the changes in parameters (calculated between 12-months post-RYGB and baseline) were compared between the 2 groups. Table 4-3 shows the parameters that were significantly different between the 2 groups in addition to other non-significant parameters relevant to NAFLD. Those with persistent NAFLD at 12-months post-RYGB had significantly higher glucose, insulin and insulin resistance at 12-months post-RYGB compared to those with NL while all the other parameters, including BMI, remained similar between groups. Changes in parameters between baseline and 12-months post-RYGB were also compared between the 2 groups (Table 4-4). Those with persistent NAFLD had significantly less change in waist circumference compared to those with NL.
Table 4-3: Parameters known to be associated with NAFLD: comparison between those with persistent NAFLD versus NL at 12-month post-RYGB

<table>
<thead>
<tr>
<th>Variable</th>
<th>NL (n=34)</th>
<th>NAFLD (n=8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>34.47 (30.40, 38.40)</td>
<td>34.30 (29.20, 38.12)</td>
<td>0.9489</td>
</tr>
<tr>
<td>% EBWL</td>
<td>52.2 (41.5, 61.3)</td>
<td>49.6 (37.8, 66.1)</td>
<td>0.5861</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>104.3 (97.3, 111.8)</td>
<td>115.4 (97.0, 124.90)</td>
<td>0.1212</td>
</tr>
<tr>
<td>Presence of Metabolic Syndrome n (% of patients)</td>
<td>6 (18.18%)</td>
<td>4 (50.0%)</td>
<td>0.0821</td>
</tr>
<tr>
<td>Diagnosed Diabetes n (% of patients)</td>
<td>6 (17.65%)</td>
<td>3 (37.50%)</td>
<td>0.3364</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>22.68 (17.0, 26.0)</td>
<td>22.88 (19.0, 26.0)</td>
<td>0.5421</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>22.26 (16.0, 28.0)</td>
<td>25.75 (18.0, 31.0)</td>
<td>0.3353</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>82.29 (67.0, 96.0)</td>
<td>73.00 (63.0, 78.5)</td>
<td>0.2977</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.85 (4.2, 9.8)</td>
<td>5.35 (4.40, 10.5)</td>
<td>0.0428</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>46 (22, 636)</td>
<td>65 (38, 341)</td>
<td>0.0401</td>
</tr>
<tr>
<td>HOMA IR Score</td>
<td>1.78 (0.75, 46.14)</td>
<td>4.05 (1.52, 11.11)</td>
<td>0.0311</td>
</tr>
<tr>
<td>Presence of Insulin Resistance n (% of patients) (based on HOMA IR)</td>
<td>5 (16.13%)</td>
<td>5 (62.5%)</td>
<td>0.0164</td>
</tr>
<tr>
<td>HbA1c</td>
<td>0.05 (0.05, 0.06)</td>
<td>0.06 (0.05, 0.07)</td>
<td>0.3767</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.08 (0.80, 1.21)</td>
<td>1.37 (0.77, 2.00)</td>
<td>0.5534</td>
</tr>
</tbody>
</table>

Variables expressed as: Mean (1st quartile, 3rd quartile) or n (percentage of patients) as appropriate

BMI, body mass index; % EBWL, percent excess body weight loss; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; HOMA-IR, homeostatic model assessment- insulin resistance.

Table 4-4: Changes in parameters known to be associated with NAFLD between baseline and 12-months post-RYGB: comparison between those with persistent NAFLD versus NL

<table>
<thead>
<tr>
<th>Variable</th>
<th>NL (n=34)</th>
<th>NAFLD (n=8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change Score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>-14.09 (-15.88, -9.94)</td>
<td>-11.1 (-13.23, -8.04)</td>
<td>0.1322</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>-30.02 (-39.00, -29.62)</td>
<td>-20.03 (-27.2, -18.62)</td>
<td>0.0340</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>-5.83 (-7.0, 6.0)</td>
<td>-24.60 (-19.0, -5.0)</td>
<td>0.1050</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>-10.27 (-14.0, 5.0)</td>
<td>-24.17 (-36.0, -4.0)</td>
<td>0.1254</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>2.57 (-7.0, 7.0)</td>
<td>-4.0 (-11.0, 14.0)</td>
<td>1.0000</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>-0.81 (-1.4, -0.1)</td>
<td>-0.27 (-1.4, 0.2)</td>
<td>0.7029</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>-140.87 (-159.0, -45.0)</td>
<td>-70.0 (-187, 3.0)</td>
<td>0.7062</td>
</tr>
<tr>
<td>HOMA IR Score</td>
<td>-7.67 (-6.38, -2.45)</td>
<td>-4.52 (-7.46, 0.13)</td>
<td>0.8666</td>
</tr>
</tbody>
</table>

98
<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean Change Score (1st quartile, 3rd quartile)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c</td>
<td>-0.01 (-0.02, -0.01)</td>
<td>0.0659</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>-0.28 (-0.66, 0.07)</td>
<td>0.7419</td>
</tr>
</tbody>
</table>

Variables expressed as: Mean Change Score (1st quartile, 3rd quartile)

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; HOMA-IR, homeostatic model assessment- insulin resistance.

We then looked at specific histological parameters to determine if there were any correlations between histological changes and metabolic parameters. Overall there were no correlations except a weak but significant correlation between a higher percentage of EBWL and improvements in total inflammation ($r=0.396$, $p=0.0094$). There was also a near significant correlation between EBWL and improvement in NAS score ($r=0.299$, $p=0.0548$) and lobular inflammation ($r=0.284$, $p=0.068$). As expected, there was also a correlation between the improvement in ALT and improvements in NAS score ($r=0.372$, $p=0.0168$). Other correlations between metabolic and anthropometric parameters and changes in histology were not significant.

### 4.5 Discussion

In this prospective cohort study, RYGB significantly improved NAFLD, including NASH and fibrosis, based on liver biopsies at 12 months post-surgery. This was associated with improvements in various anthropometric and metabolic parameters, including % EBWL. Those who had persistent NAFLD at 12 months had significantly higher glucose, insulin and IR (based on HOMA) and less improvements in waist circumference over 12 months than those with NL. This suggests that post-RYGB, glycemic control and visceral fat loss may play a role in liver recovery from NAFLD.
Overall, we found significant improvement in liver steatosis, lobular inflammation and fibrosis at 12-months post-RYGB. These results are consistent with a recent systematic review (429). There were also significant reductions in AST and ALT (429) similar to our study. In our study, fibrosis worsened in four (9.5%) individuals. Speculative causes as to why worsening fibrosis occurs include having a higher BMI and/or worse IR compared to those with improved fibrosis after bariatric surgery. Additional factors may also play a role (432).

NAFLD severity is associated with clinical and biochemical markers which include older age, obesity, increased waist circumference, dyslipidemia, IR, type two diabetes and elevated liver enzymes (34). Unlike other published studies (62), we did not find a significant difference in age, BMI, dyslipidemia, type two diabetes and liver enzymes between those with NAFLD and those with NL at 12-months post-RYGB (61). This could be explained by the fact that our population was quite homogenous because of the selection process for patients undergoing RYGB according to the NIH criteria (145). However, we found differences between the two groups related to markers of diabetes, with significantly higher glucose, insulin, HOMA-IR and presences of IR in those with persistent NAFLD. Additionally, we saw less improvement in waist circumference in those with persistent NAFLD, which may suggest underlying problems with glycemic control and lipid metabolism associated with greater visceral adipose tissue. IR is strongly associated with NAFLD, as it increases lipolysis from the adipose tissue, and thus the resulting fatty acids are taken up by the liver, which can cause lipid peroxidation (433) contributing to inflammation and fibrosis. A recent study investigating NAFLD 12-months post-bariatric surgery in 82 patients found that those with persistent NASH after the bariatric procedure had more
frequent refractory IR (66.7% versus 21.1%) and less weight loss (12). Another study investigating the effects of bariatric surgery on NAFLD found that refractory IR profile is an independent criterion of steatosis and ballooning 1-year post-RYGB (377). These findings are similar to our study despite including multiple bariatric surgical procedures. However, it is not clear why some individuals have improvements in IR post bariatric surgery while others do not. Potential mechanisms include genetics (434), the intestinal microbiome (435), poor adherence to post-bariatric regimen (436) and presence of visceral fat (437). Recently, the role of the intestinal microbiome in the pathogenesis of chronic diseases has been highlighted (438). The intestinal microbiome may contribute to IR by increasing intestinal permeability which leads to the translocation of bacteria (435). This in turn can increase the production of pro-inflammatory cytokines, contributing to an increased inflammatory state, which plays a role in IR (435).

We found that those with persistent NAFLD 12-months post-RYGB had significantly less reduction in waist circumference and increased IR than those with NL despite having similar BMI and weight loss. There was also some association between % EBWL and improvements in liver histology markers. Central obesity has been shown to be associated with NAFLD, with visceral fat being correlated with increased free fatty acids, gluconeogenesis and IR (439). As adipose tissue expands during obesity, there is an increase in pro-inflammatory adipokines and a decrease in anti-inflammatory adipokines, which results in local and peripheral inflammation further contributing to IR and NAFLD (440). In that regard, visceral fat has been found to be more harmful than subcutaneous fat (441) as it increases proinflammatory cytokines and adipokines (442). Another study also found that excessive weight loss post-laparoscopic adjustable gastric band decreases IL-6 and TNF-a in
most individuals as well as increases adiponectin and its receptors in the adipose tissue and improved insulin sensitivity (443). Therefore, taken together, greater visceral fat and waist circumference plays a role in chronic inflammation and IR. This could explain the persistence of NAFLD post-RYGB.

The strengths of this study include the use of a liver biopsy at both time points to determine liver histology. Additionally, all the subjects were well characterized and underwent the same bariatric procedure (RYGB) and had paired liver biopsies. The limitations of this study include the small sample size due to the invasiveness of the second liver biopsy. However, similar studies had comparable sizes (390) but included mixed bariatric surgical procedures.

In summary, we found that RYGB surgery significantly improved multiple biochemical and clinical variables as well as all liver histological parameters. However, persistent NAFLD at 12-months post-RYGB was associated with less improvement in glycemic control and waist circumferences, which likely contribute to the persistent disease. Other factors such as genetics and the intestinal microbiome may also play a role.

Acknowledgements: The authors acknowledge the important contributions made by the clinical staff from the Toronto Western Hospital Bariatric Program. This study was funded by the Canadian Institutes for Health Research, Operating Grant MOP-126139.

Conflict of Interest: No conflict of interest to declare.
Chapter 5: Markers of Activated Inflammatory Cells are Associated with Non-Alcoholic Fatty Liver Disease and Intestinal Microbiota

by

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This chapter is modified from a submitted paper in press at the International Journal of Molecular Medicine.
5.1 Abstract

**Aim:** Several mechanisms contribute to the pathogenesis of non-alcoholic fatty liver disease (NAFLD). The intestinal microbiota (IM) and liver immune function cells have been implicated in NAFLD, but data on their potential associations have been scarce. The aim of this study was to investigate whether there are differences in hepatic inflammatory cell markers between NAFLD and healthy controls (HC), and in an exploratory sub-study to determine whether these markers are associated with certain IM, like *Faecalibacterium prausnitzii*.

**Methods:** This was a prospective, cross-sectional study of adults with biopsy-confirmed NAFLD and HC. Clinical and laboratory data were collected. Fecal IM were assessed by qPCR and immune cells by immunohistochemistry. NAFLD activity score (NAS) was used for disease severity.

**Results:** 42 subjects were studied: 8 HC and 34 NAFLD. Hematopoietic cell marker CD45+ and Kupffer cell marker CD163+ were higher in NAFLD compared to HC, and those with a NAS ≥5 had higher levels of CD20+ cells, a marker of B cells versus a NAS of 0 or 1-4. In 39 patients (5 HC, 34 NAFLD) IM was measured: *Faecalibacterium prausnitzii* was negatively correlated with CD45+ (r=-0.394, p=0.015) and CD163+ (r=-0.371, p=0.022) cells in the portal tract and *Prevotella* was negatively correlated with CD20+ (r=-0.353, p=0.028) cells in the liver lobule.

**Conclusions:** Hepatic immune cell counts are increased in NAFLD versus HC and associated with disease severity. Specific immune cells in portal or lobular areas correlated with specific fecal IM, like *F Prausnitzii*, suggesting a potential role for IM in hepatic inflammation.
5.2 Introduction

There are several mechanisms involved in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) (428), including a potential role for the intestinal microbiota (IM) (27, 58-60, 444) and the hepatic immune system (24-26). Elevated hepatic immune cell markers such as CD45, CD163, CD20 and CD3 have been detected in NAFLD, mostly in pediatric studies (24-26). CD45 is a marker of hematopoietic cells (393), and an increase of CD45 indicates activation of one or more inflammatory cell types (26). CD163 is another immune cell marker which is expressed on Kupffer cells (KCs). When activated, KCs produce cytokines and chemokines, contributing to hepatic injury (211). In addition, markers of B cells such as CD20 (25, 394) and CD3, a marker for T-cell activation (393), have been reported to be elevated in the liver of patients with NAFLD, however the results are conflicting (24-26).

Significant differences in IM have also been reported in NAFLD compared to controls (27, 58-60, 444). IM can contribute to NAFLD by several mechanisms including increases in intestinal permeability and bacterial translocation (445) inducing pro-inflammatory cytokines that can activate hepatic immune cells (172). To our knowledge, no studies assessed both IM and hepatic immune cell markers in the same patients to determine if there are associations between specific IM and immune cell markers.

The aim of this study was to investigate whether there are differences in specific hepatic inflammatory cell markers between NAFLD and healthy controls (HC), using the antigens CD45, CD163, CD20 and CD3, and to explore whether these markers are associated with specific IM.
5.3 Methods and Materials

This was a prospective, cross-sectional study, which was conducted at the University Health Network (UHN), Toronto, Canada. The study protocol was approved by the UHN and University of Toronto Research Ethics Boards and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. All participants gave their informed written consent.

Subjects

Adults with persistently elevated alanine aminotransferase (ALT) levels were assessed by a hepatologist in order to rule out other causes of liver disease. Patients with biopsy-proven NAFLD were eligible for recruitment. Healthy adults undergoing assessment for live liver donation at the Living Liver Donor Transplant Program at UHN were eligible for recruitment as HC. One stool sample, fasting blood work and anthropometric measurements were collected from participants prior to the liver biopsy.

Participants were excluded if they had: liver disease other than NAFLD; end-stage liver disease; anticipated need for a liver transplant within 12 months; chronic gastrointestinal diseases; previous surgery of the gastrointestinal tract that modifies the anatomy; use of medications associated with steatosis/steatohepatitis (e.g. corticosteroids); use of pre- or probiotics within the past 6 months; use of vitamin E or fish oil supplements; consumption of more than 20 g of alcohol per day; pregnancy or lactating state.

Biochemical and Clinical Data
Participants’ anthropometrics, smoking status, alcohol consumption habits, medication usage and samples were collected prior to the liver biopsy. The UHN Laboratory Medicine Program analyzed fasting blood samples for HbA1c, insulin, glucose, liver enzymes, lipid profile and platelets using standard laboratory tests. The homeostasis model for insulin resistance was calculated using fasting serum glucose and insulin (400).

Histology

Liver Histology Assessment:

Liver samples for NAFLD patients were taken by percutaneous needle biopsy, and an intraoperative wedge biopsy was obtained for HC. The biopsy was preserved in 10% formalin within 15 minutes of collection and later was embedded in paraffin. Standard stains were used for the diagnosis of NAFLD and morphologic evaluation and to rule out iron loading. A single pathologist assessed the liver histology for the presence of steatosis, inflammation and fibrosis using the validated and reproducible Brunt system (391). Additionally, the NAFLD Activity Score (NAS) was used to evaluate disease severity (431).

Hepatic Immune Cells:

The formalin-fixed paraffin-embedded liver tissue underwent immunostaining using primary monoclonal mouse antibodies raised against cell markers CD45, CD163, CD20 and CD3. Staining was performed at the UHN Laboratory Medicine Program. For this, 4µm formalin-fixed paraffin-embedded sections were dewaxed in 5 changes of xylene and brought down to water through graded alcohols. Antigen retrieval or unmasking procedures were applied. Endogenous peroxidase was blocked with 3% hydrogen peroxide. The detection systems used were dependent on the marker. Two detections kits were used: ImmPress anti-
Rabbit Kit (Vector Laboratories, Burlingame, CA) for CD3 and Mach 4 (Inter-Medico, Markham, ON) for CD20, CD45 and CD163. After following the kit instructions, color development was performed with freshly prepared DAB (Dako, Carpinteria CA). Finally, sections were counterstained lightly with Mayer’s Hematoxylin, dehydrated in alcohols, cleared in xylene and mounted with Permount mounting medium (Fisher Scientific) (see Appendix 5-1). A pathologist blinded to the study groups determined the density of the cells that stained positive for the aforementioned monoclonal antibodies in both the portal tract and liver lobule. This was determined by counting the number of positive cells in ten random portal tracts and in ten areas of lobular region at a magnification of x200 under light microscopy.

Stool Sample Collection and Measurements

Participants provided a stool sample according to previously published methods (446). Stools samples were stored at -80°C until analyses. Total DNA from fecal samples was extracted as previously described (27). qPCR was then performed in a 7900HT thermocycler (Applied Biosystems, Forest City, CA), using 50 ng of DNA and 16S rRNA-based qPCR assays to quantify total bacteria and selected groups/genera of gut microorganisms. These assays were adapted from the literature to target total bacteria, Bacteroidetes, Prevotella, Alistipes, Coprococcus, Ruminococcus, Clostridium leptum, C. coccoides, Lactobacillus, Faecalibacterium prausnitzii, Bifidobacterium and Escherichia coli (see Appendix 5-2) (397, 398). These bacteria were chosen based on previous literature and the work of our group, suggesting an association with NAFLD (27, 28, 58-60, 444). The number of microbial cells in the fecal samples was calculated by interpolation with standard curves and expressed as cells per gram (cells/g) of wet feces and normalized to total counts.
(relative abundance), as previously described (27). The IM was compared between the three disease groups for total bacteria and presence of bacterial taxa of interest as a percentage of total bacteria.

**Statistical Analysis**

Measured parameters were compared between groups using the Kruskal-Wallis test followed by Wilcoxon ranked sum, or chi-square and Fisher’s exact test as necessary, with Bonferroni correction for multiple comparisons between diagnostic groups. Data was considered to be statistically significant with a p-value of less than 0.05. Spearman correlation coefficients and partial Spearman correlation coefficients were used to examine the relationship between the bacterial relative abundances and immunohistochemistry. Analysis was performed using tools SAS 9.4 and R 3.2.5.

### 5.4 Results

**Demographic and Laboratory Results:**

A total of 42 participants were included in this study: 12 with simple steatosis (SS), 22 with non-alcoholic steatohepatitis (NASH) and 8 with HC. **Table 5-1** summarizes demographic and laboratory results. Those with SS and NASH were older and had higher AST and ALT levels compared to healthy controls (HC). Additionally, those with NASH had higher BMI, fasting insulin, HOMA-IR score and triglycerides compared to HC.

<table>
<thead>
<tr>
<th>Variables</th>
<th>HC (n=8)</th>
<th>SS (n=12)</th>
<th>NASH (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (% male)</td>
<td>62.5</td>
<td>58.33</td>
<td>45.45</td>
</tr>
<tr>
<td></td>
<td>Median (Min, Max)</td>
<td>Median (Min, Max)</td>
<td>Median (Min, Max)</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>35.50 (23.00, 48.00)</td>
<td>50.50 (33.00, 68.00)</td>
<td>45.50 (29.00, 61.00)</td>
</tr>
<tr>
<td><strong>BMI (kg/m^2)</strong></td>
<td>26.75 (23.76, 35.27)</td>
<td>26.60 (23.54, 37.15)</td>
<td>31.80 (24.17, 49.53)</td>
</tr>
<tr>
<td>Waist-to-Hip Ratio</td>
<td>0.90 (0.77, 1.03)</td>
<td>0.93 (0.85, 1.04)</td>
<td>0.98 (0.81, 1.05)</td>
</tr>
<tr>
<td><strong>AST (U/L)</strong></td>
<td>17.50 (14.00, 20.00)</td>
<td>26.00 (19.00, 112.00)</td>
<td>44.00 (18.00, 114.00)</td>
</tr>
<tr>
<td><strong>ALT (U/L)</strong></td>
<td>15.00 (11.00, 21.00)</td>
<td>45.50 (21.00, 168.00)</td>
<td>68.00 (22.00, 141.00)</td>
</tr>
<tr>
<td><strong>ALP (U/L)</strong></td>
<td>85.00 (64.00, 105.00)</td>
<td>64.50 (40.00, 105.00)</td>
<td>72.50 (37.00, 107.00)</td>
</tr>
<tr>
<td><strong>Glucose (mmol/L)</strong></td>
<td>5.00 (4.40, 6.00)</td>
<td>5.80 (4.70, 11.40)</td>
<td>5.60 (4.20, 7.60)</td>
</tr>
<tr>
<td><strong>Insulin (pmol/L)</strong></td>
<td>49.00 (20.00, 85.00)</td>
<td>33.00 (15.00, 437.00)</td>
<td>110.00 (36.00, 720.00)</td>
</tr>
<tr>
<td><strong>HOMA IR</strong></td>
<td>1.73 (0.68, 3.78)</td>
<td>2.99 (0.54, 21.04)</td>
<td>4.89 (1.21, 40.00)</td>
</tr>
<tr>
<td><strong>HbA1c</strong></td>
<td>0.05 (0.05, 0.06)</td>
<td>0.06 (0.05, 0.09)</td>
<td>0.06 (0.05, 0.07)</td>
</tr>
<tr>
<td><strong>Triglycerides (mmol/L)</strong></td>
<td>1.01 (0.57, 1.38)</td>
<td>1.02 (0.62, 3.97)</td>
<td>1.52 (0.28, 5.90)</td>
</tr>
<tr>
<td><strong>Total cholesterol (mmol/L)</strong></td>
<td>5.13 (4.85, 6.35)</td>
<td>4.80 (3.88, 6.88)</td>
<td>4.64 (2.63, 9.83)</td>
</tr>
<tr>
<td><strong>High density lipoprotein (HDL) (mmol/L)</strong></td>
<td>1.33 (0.83, 2.07)</td>
<td>1.28 (0.95, 1.72)</td>
<td>1.12 (0.34, 1.60)</td>
</tr>
<tr>
<td><strong>Low density lipoprotein (LDL) (mmol/L)</strong></td>
<td>3.44 (3.07, 3.88)</td>
<td>2.73 (2.18, 5.06)</td>
<td>2.80 (0.73, 4.94)</td>
</tr>
</tbody>
</table>

BMI, body mass index; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; HOMA-IR, homeostatic model assessment-insulin resistance. Values are express as median (minimum, maximum) or %. For each comparison, identical letters indicate the statistically significant differences between the groups.

**Immunohistochemistry and Liver Histology:**

As seen in Table 5-2 and Figure 5-1, those with SS and NASH had a significantly higher number of CD45^+ cells in liver lobule and more CD163^+ cells in the portal tract compared to HC. Additionally, those with NASH had a significantly higher CD45^+ cells in the portal tract compared to HC.

We also assessed the patients according to NAS (Table 5-3). Those with a NAS ≥ 5 had a significantly higher number of CD45^+ and CD163^+ cells in the portal tract compared to...
those with a NAS of 0. Additionally, those with a NAS ≥ 5 had significantly higher CD20+ cells in the liver lobule compared to those with a NAS of 0 or a NAS of 1-4. Those with a NAS of 1-4 also had significantly higher CD163+ cells in the portal tract compared to those with a NAS of 0.

Table 5-2: Immunohistochemistry by diagnosis of liver disease

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HC (n=8)</th>
<th>SS (n=12)</th>
<th>NASH (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45P</td>
<td>21.05 (6.60, 42.70)a</td>
<td>53.81 (10.80, 94.90)</td>
<td>44.95 (12.10, 189.00)a</td>
</tr>
<tr>
<td>CD45L</td>
<td>122.05 (45.33, 155.00)ab</td>
<td>148.70 (78.33, 196.40)a</td>
<td>139.65 (102.90, 221.80)b</td>
</tr>
<tr>
<td>CD3P</td>
<td>25.75 (7.90, 43.50)</td>
<td>38.34 (7.40, 95.80)</td>
<td>32.55 (8.80, 144.00)</td>
</tr>
<tr>
<td>CD3L</td>
<td>53.20 (30.00, 86.60)</td>
<td>67.75 (30.300, 112.30)</td>
<td>62.15 (21.70, 119.00)</td>
</tr>
<tr>
<td>CD20P</td>
<td>1.50 (0.30, 7.90)</td>
<td>5.30 (0.20, 19.20)</td>
<td>5.05 (0.13, 63.90)</td>
</tr>
<tr>
<td>CD20L</td>
<td>5.20 (1.67, 8.20)</td>
<td>4.45 (1.67, 10.70)</td>
<td>6.05 (2.25, 23.00)</td>
</tr>
<tr>
<td>CD163P</td>
<td>1.35 (0.00, 4.00)ab</td>
<td>5.80 (0.00, 28.70)a</td>
<td>5.70 (1.30, 24.40)b</td>
</tr>
<tr>
<td>CD163L</td>
<td>149.60 (37.00, 226.30)</td>
<td>132.15 (43.33, 226.30)</td>
<td>137.85 (65.50, 264.13)</td>
</tr>
</tbody>
</table>

L, cell counts of liver lobule; P, cell counts of portal tract.
Values are expressed as median (minimum, maximum). For each comparison, identical letters indicate the statistically significant differences between the groups.

Figure 5-1: Immunohistochemical labeling for CD163 and CD45 in liver biopsies of healthy controls (HC) and patients with non-alcoholic steatohepatitis (NASH). Portal tracts (arrows) and lobules in HC show a lower number of CD163+/CD45+ cells compared to samples with NASH.
Table 5-3: Immunohistochemistry by NAS

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NAS=0 (n=8)</th>
<th>NAS 1-4 (n=22)</th>
<th>NAS ≥ 5 (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45P</td>
<td>21.05 (6.60, 42.70) a</td>
<td>46.51 (10.80, 102.80)</td>
<td>47.30 (30.70, 189.00) a</td>
</tr>
<tr>
<td>CD45L</td>
<td>122.05(45.33, 155.00)</td>
<td>144.35 (78.33, 196.40)</td>
<td>133.40(111.50, 221.80)</td>
</tr>
<tr>
<td>CD3P</td>
<td>25.75 (7.90, 43.50)</td>
<td>27.40(7.40, 106.40)</td>
<td>37.80 (25.20, 144.00)</td>
</tr>
<tr>
<td>CD3L</td>
<td>53.20 (30.00, 86.60)</td>
<td>65.55(24.75, 119.00)</td>
<td>68.90 (21.70, 112.30)</td>
</tr>
<tr>
<td>CD20P</td>
<td>1.50 (0.30, 7.90)</td>
<td>4.15(0.13, 30.30)</td>
<td>5.50 (2.40, 63.90)</td>
</tr>
<tr>
<td>CD20L</td>
<td>5.20 (1.67, 8.20) a</td>
<td>4.90 (1.67, 11.20) b</td>
<td>7.40 (3.80, 23.00) ab</td>
</tr>
<tr>
<td>CD163P</td>
<td>1.35 (0.00, 4.00) ab</td>
<td>5.15 (0.00, 28.70) a</td>
<td>6.30 (2.60, 19.50) b</td>
</tr>
<tr>
<td>CD163L</td>
<td>149.60 (37.00, 226.30)</td>
<td>159.45 (43.33, 264.13)</td>
<td>121.40 (65.50, 157.70)</td>
</tr>
</tbody>
</table>

L, cell counts of liver lobule; NAS, non-alcoholic fatty liver disease score P, cell counts of portal tract. Values are expressed as median (minimum, maximum). For each comparison, identical letters indicate the statistically significant differences between the groups.

Immunohistochemistry, Intestinal Microbiota and Liver Histology:

A subset of 39 patients, 12 with SS, 22 with NASH and 5 HC provided stool samples, for IM. Correlations between CD cell counts and specific bacteria taxa are shown in Table 5-4. *F. prausnitzii* was negatively correlated with CD45+ (Figure 5-2) and CD163+ (Figure 5-3) cells in the portal tract. For the rest of the IM, *Prevotella* was negatively correlated (Figure 5-4) with the CD20+ cell count in the liver lobule. Other taxa did not correlate.
Table 5-4: Correlation between immunohistochemistry and relative abundances of microbial taxa

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Prevotella</th>
<th>Alistipes</th>
<th>Coprococcus</th>
<th>Ruminococcus</th>
<th>Clostridium leptum</th>
<th>C. coccoides</th>
<th>Lactobacillus</th>
<th>F. prausnitzii</th>
<th>Bifidobacterium</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45P</td>
<td>-0.041</td>
<td>0.042</td>
<td>-0.194</td>
<td>0.138</td>
<td>-0.291</td>
<td>0.220</td>
<td>0.037</td>
<td>-0.394</td>
<td>-0.173</td>
<td>0.064</td>
</tr>
<tr>
<td></td>
<td>(0.804)</td>
<td>(0.801)</td>
<td>(0.244)</td>
<td>(0.403)</td>
<td>(0.072)</td>
<td>(0.178)</td>
<td>(0.825)</td>
<td>(0.015)</td>
<td>(0.300)</td>
<td>(0.698)</td>
</tr>
<tr>
<td>CD45L</td>
<td>-0.086</td>
<td>-0.162</td>
<td>-0.158</td>
<td>-0.122</td>
<td>-0.126</td>
<td>-0.181</td>
<td>-0.123</td>
<td>-0.192</td>
<td>-0.074</td>
<td>0.077</td>
</tr>
<tr>
<td></td>
<td>(0.602)</td>
<td>(0.331)</td>
<td>(0.343)</td>
<td>(0.459)</td>
<td>(0.443)</td>
<td>(0.269)</td>
<td>(0.455)</td>
<td>(0.248)</td>
<td>(0.659)</td>
<td>(0.639)</td>
</tr>
<tr>
<td>CD3P</td>
<td>-0.069</td>
<td>0.051</td>
<td>-0.044</td>
<td>0.186</td>
<td>-0.196</td>
<td>0.220</td>
<td>0.027</td>
<td>-0.233</td>
<td>-0.194</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>(0.675)</td>
<td>(0.762)</td>
<td>(0.793)</td>
<td>(0.256)</td>
<td>(0.233)</td>
<td>(0.178)</td>
<td>(0.870)</td>
<td>(0.160)</td>
<td>(0.244)</td>
<td>(0.889)</td>
</tr>
<tr>
<td>CD3L</td>
<td>-0.155</td>
<td>-0.083</td>
<td>0.102</td>
<td>0.151</td>
<td>0.076 (0.647)</td>
<td>-0.150</td>
<td>-0.121</td>
<td>-0.195</td>
<td>0.239</td>
<td>0.128</td>
</tr>
<tr>
<td></td>
<td>(0.348)</td>
<td>(0.620)</td>
<td>(0.544)</td>
<td>(0.360)</td>
<td>(0.463)</td>
<td>(0.361)</td>
<td>(0.463)</td>
<td>(0.240)</td>
<td>(0.083)</td>
<td>(0.436)</td>
</tr>
<tr>
<td>CD20P</td>
<td>-0.132</td>
<td>-0.019</td>
<td>-0.053</td>
<td>0.133</td>
<td>-0.019</td>
<td>0.180</td>
<td>-0.025</td>
<td>-0.259</td>
<td>-0.133</td>
<td>-0.085</td>
</tr>
<tr>
<td></td>
<td>(0.424)</td>
<td>(0.910)</td>
<td>(0.750)</td>
<td>(0.421)</td>
<td>(0.230)</td>
<td>(0.274)</td>
<td>(0.880)</td>
<td>(0.116)</td>
<td>(0.426)</td>
<td>(0.608)</td>
</tr>
<tr>
<td>CD20L</td>
<td>-0.353</td>
<td>0.079</td>
<td>0.216</td>
<td>0.063</td>
<td>-0.048</td>
<td>-0.067</td>
<td>-0.053</td>
<td>0.055</td>
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<td>-0.092</td>
</tr>
<tr>
<td></td>
<td>(0.028)</td>
<td>(0.637)</td>
<td>(0.192)</td>
<td>(0.705)</td>
<td>(0.774)</td>
<td>(0.682)</td>
<td>(0.748)</td>
<td>(0.745)</td>
<td>(0.897)</td>
<td>(0.576)</td>
</tr>
<tr>
<td>CD163P</td>
<td>-0.008</td>
<td>-0.081</td>
<td>-0.202</td>
<td>-0.057</td>
<td>-0.181</td>
<td>0.140</td>
<td>0.054</td>
<td>-0.371</td>
<td>-0.208</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>(0.962)</td>
<td>(0.628)</td>
<td>(0.225)</td>
<td>(0.732)</td>
<td>(0.269)</td>
<td>(0.394)</td>
<td>(0.742)</td>
<td>(0.022)</td>
<td>(0.211)</td>
<td>(0.934)</td>
</tr>
<tr>
<td>CD163L</td>
<td>0.070</td>
<td>-0.101</td>
<td>0.005</td>
<td>-0.165</td>
<td>0.016 (0.921)</td>
<td>-0.126</td>
<td>-0.197</td>
<td>-0.043</td>
<td>0.022</td>
<td>0.069</td>
</tr>
<tr>
<td></td>
<td>(0.674)</td>
<td>(0.547)</td>
<td>(0.974)</td>
<td>(0.315)</td>
<td>(0.445)</td>
<td>(0.229)</td>
<td>(0.797)</td>
<td>(0.797)</td>
<td>(0.894)</td>
<td>(0.675)</td>
</tr>
</tbody>
</table>

L, cell counts of liver lobule; P, cell counts of portal tract. Values are expressed as Correlation Coefficient (p-value)
Figure 5-2: *F. prausnitzii* negatively correlated with CD45P+

Figure 5-3: *F. prausnitzii* negatively correlated with CD163P⁺
Next, we investigated the correlation of IM and immunohistochemistry based on histological diagnosis (see Table 5-5). We found that *F. prausnitzii* was negatively correlated with CD163P in those with SS, however it was not significant in those with NASH or HC. Finally, it was found that *Bifidobacterium* was negatively correlated with CD20\(^+\) and CD163\(^+\) cell counts in the portal tract in those with SS.

Table 5-5: Correlation between immunohistochemistry and relative abundances of microbial taxa based on NAFLD diagnosis

<table>
<thead>
<tr>
<th>Taxa</th>
<th>CD Cell</th>
<th>Diagnosis</th>
<th>Correlation Coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. prausnitzii</em></td>
<td>CD45P</td>
<td>HC</td>
<td>-0.700</td>
<td>0.188</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SS</td>
<td>-0.399</td>
<td>0.199</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NASH</td>
<td>-0.309</td>
<td>0.174</td>
</tr>
<tr>
<td></td>
<td>CD163P</td>
<td>HC</td>
<td>-0.300</td>
<td>0.624</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SS</td>
<td>-0.594</td>
<td>0.042</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>NASH</td>
<td>CD20P HC</td>
<td>SS</td>
<td>NASH</td>
</tr>
<tr>
<td>-----------------</td>
<td>------</td>
<td>----------</td>
<td>----</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-0.144</td>
<td>0.535</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

L, cell counts of liver lobule; P, cell counts of portal tract.

5.5 Discussion

Our results demonstrate that patients with NAFLD have elevated CD cell markers that are associated with disease severity. In addition, some of these CD cell markers are associated with the relative abundance of specific bacterial taxa in feces, particularly *F. Prausnitzii* as well as *Prevotella* and *Bifidobacterium*. To our knowledge, this finding has never been reported before and requires further investigation. Very few studies, mostly pediatric, (24-26) showed elevation of specific CD markers in NAFLD as well as associations with disease severity. Others demonstrated differences in IM between NAFLD and HC (59, 60, 444, 445) and associations with disease severity (447). However, none assessed both parameters in the same patients to determine if there are potential associations between hepatic immune cells and specific IM.

Recent evidence supports a role for the immune system in the progression of NAFLD (448). Like others, (24, 26) we found that those with NAFLD, specifically NASH, had significantly higher numbers of total immune cell population (CD45+) in both the lobule and the portal tract when compared to controls. However, the number of KC (CD163+) was higher only in the portal tract, and there was no significant difference in the numbers of T cells or B cells in either portal or lobular areas between HC and SS/NASH. Our data also
demonstrated that those with higher NAS had altered numbers of total immune cell population (CD45⁺) and KC (CD163⁺), but only in the portal tract. Although NAFLD is histologically assessed based on lobular features of inflammation, portal inflammatory infiltrate was recently studied by Gadd et al. (25) because of its role in the development of portal fibrosis. In that study, the presence of portal inflammation was strongly correlated with fibrosis stage (25). Additionally, using immunostaining for broad leukocyte subset markers (CD68, CD3, CD8, CD4, CD20 and neutrophil elastase) and selected inflammatory markers, they found that cells expressing all markers examined were identified throughout the liver lobules and in portal tracts. Portal tracts were more densely populated by these cells, and the population was dominated by CD68 macrophages and CD8 lymphocytes at all stages of disease (25). Like in that study, we also found that the numbers of B cells (CD20⁺) was higher in the liver lobules of those with higher NAS scores, but there was no difference in T cell (CD3⁺) counts in either the lobular or the portal area. CD20⁺ and CD3⁺ cells levels were also not significantly different between NAFLD and HC. In contrast, one pediatric study found lower CD3⁺ in children with NASH (26). Of interest, two other human studies found that the intrahepatic ratio of CD3⁺/CD56⁻ cells was increased in NAFLD patients with more severe liver disease (230, 449). One study, using flow cytometric analysis, found that as NAS increased so did the amount of intrahepatic CD3⁺/CD56⁻ cells (449), whereas the other study found that percentage of intrahepatic CD3⁺/CD56⁻ cells was significantly higher in patients with moderate to severe steatosis when compared to those with mild and no steatosis (230). Therefore, one of the reasons we did not observe a significant difference in CD3⁺ cells is because the differences are seen in a cell subgroup.

In regard to CD45⁺ and CD163⁺, our results were also comparable to others. Pediatric studies (24, 26) showed increased numbers of CD45⁺ and CD163⁺ cells in children with more
disease severity, and another study in adults showed that activated KCs (CD163+) contributed to the development of NASH (221). However, a recent study of 45 individuals with NAFLD undergoing bariatric surgery found no difference in the intrahepatic numbers of CD163+ between those with and without NASH (450) suggesting that perhaps patients with morbid obesity are different. In our study, we found higher CD163+ cells in NAFLD versus HC, and we did see an association with disease severity as previously reported in pediatric patients (26).

Taken together, these results suggest that in NAFLD there is an infiltration of inflammatory cells which is associated with disease severity, suggesting a possible role in the pathogenesis of the disease. Research suggests that altered IM, which has been shown in NAFLD (27, 59, 60, 444, 445, 447), may play a role in the pathogenesis through bacterial translocation leading to systemic chronic inflammation (18), which has the potential to activate hepatic inflammatory cells through the production of cytokines. Using qPCR to evaluate several bacterial taxa, we found that *F. prausnitzii*, *Prevotella* and *Bifidobacterium* were negatively correlated with specific CD cell markers. *F. prausnitzii* is the most abundant bacterium in the healthy IM, and as hypothesized was found to be negatively correlated with hepatic immune cells (451). In animal models, *F. prausnitzii* has been shown to have anti-inflammatory effects (452, 453) and is also reported to be low in other human inflammatory conditions such as inflammatory bowel disease (453). Therefore, lower *F. prausnitzii* could play a role in the higher hepatic inflammatory cell infiltrate. *Prevotella*, which produces high levels of short-chain fatty acids (SCFAs), has been found to be in lower abundance in individuals with NASH (445, 447) and SCFAs have been reported to have anti-inflammatory effect (454), supporting a role for *Prevotella. Bifidobacterium* is another SCFA producer that
has been reported to be reduced in NASH (60). SCFAs have been shown to protect against gut inflammation (455).

The strengths of this study are the defined characterization of the study subjects with liver histology and the quantification of specific bacterial taxa reported to be associated with NAFLD. Another is that the liver immunostaining allowed for the assessment of relationships between IM and CD markers. The limitations are the cross-sectional design, which does not prove causality and the small sample size due to the invasiveness of the liver biopsy. However, similar studies assessing immunohistochemistry in NAFLD had comparable samples sizes (25, 26) but did not include IM assessment. The relationship we report between immune cell infiltrate and IM is based on associations and does not prove causality. Additional studies are required to determine the role of specific bacterial taxa in the immunopathogenesis of NAFLD.

In summary, we found that B cells and Kupffer cells but not T cells were associated with NAFLD and disease severity, and specific immune cells in portal or lobular areas correlated with specific gut microbial taxa, particularly *Faecalibacterium prausnitzii*. Future research should investigate the specific immune cell subgroups, inflammatory mediators and IM during intervention studies to further explore their roles in NAFLD pathogenesis.

**Acknowledgements**

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Tract and Disease) Centre funded by the Canadian Foundation for Innovation and Ontario Research Fund, project number 19442 and 30961.
Chapter 6:
Summary of Main Findings & General Discussion

Preface

NAFLD is highly prevalent in obese patients undergoing bariatric surgery. However, it is not clear in published reports whether or not this prevalence was identified after a VLCD which can be routinely prescribed pre-bariatric surgery. Furthermore, it is not clear how a VLCD impacts anthropometric and metabolic parameters prior to surgery. Bariatric surgery is recognized as a beneficial treatment for obesity, but it has not yet been approved for treating NAFLD specifically, despite the growing body of literature showing significant improvements in liver histology. Part of the reason for this is that the long-term effects of bariatric surgery on the liver are not fully known, nor is why some patients may have persistent NASH and fibrosis post-surgery or potentially worsening of liver histology. In this dissertation, the focus has been to: 1) determine the prevalence of NAFLD (SS and NASH) in a population sample of morbidly obese individuals undergoing RYGB post-VLCD, and to determine the effects of the VLCD on metabolic and anthropometric parameters and to assess pre-VLCD factors associated with NAFLD; 2) evaluate the effect of RYGB on NAFLD at 12-months post-surgery and determine the metabolic and anthropometric factors associated with persistent NAFLD; and 3) compare hepatic immune cell counts in patients with biopsy confirmed NAFLD versus healthy controls and determine if there is an association between hepatic immune cell counts and IM. If there is an association, this exploratory study would lead us to assess immune cell counts in the liver biopsies of our cohort of bariatric surgery patients to determine if there is a relationship between
IM from stool collection, immune cell counts and persistent NAFLD. In the following sections I will summarize the main results as well as discuss the overall findings and their implications.

6.1 Summary of Findings

The first study focused on the prevalence of NAFLD (SS and NASH) in a population sample of 139 morbidly obese adults undergoing bariatric surgery. In a sub-group of 21 patients it also evaluated the effects of the pre-surgical VLCD on anthropometric and metabolic parameters. It was hypothesized that the prevalence of NAFLD (SS and NASH) would be comparable to other reports. However, our prevalence was lower compared to the existing literature. NAFLD was diagnosed in 76.3% of individuals of which 61.9% had SS and 14.4% had NASH. This is less than other published reports which found a prevalence of between 85% - 98% for NAFLD and 24% - 98% NASH (10). The secondary hypothesis was confirmed as VLCD significantly improved anthropometric and metabolic parameters. This included statistically significant reductions in BMI, IR, fasting glucose and insulin, HbA1c, total cholesterol, HDL and LDL cholesterol. However, there were significant increases in AST and ALT post-VLCD. The tertiary hypothesis was also confirmed as pre-VLCD IR was associated with the presence of NAFLD. It was found that among those with SS and NASH, there was a significantly higher proportion of individuals who had IR pre-VLCD. Furthermore, those with NASH were also found to have significantly higher pre-VLCD ALT, AST and a higher proportion of individuals with diabetes compared to those with NL. This is the only study that clarifies the use of VLCD when reporting the prevalence of NAFLD in bariatric surgery patients and to our knowledge is the only study that reported the prevalence.
of NAFLD in Canadian bariatric patients. The results demonstrate that disease severity is associated with more metabolic impairments such as higher IR and the presence of diabetes.

Building on the results from the first study, we then followed a subgroup of 42 patients for 12-months post-RYGB and assessed again anthropometric and metabolic parameters as well as repeated the liver biopsy at 12-months. As hypothesized, RYGB significantly improved anthropometric and metabolic measurements as well as all histological parameters. At the time of RYGB, 36 of the 42 individuals were diagnosed with NAFLD, whereas at 12-months post-RYGB only 8 continued to have persistent NAFLD. The secondary hypothesis was also confirmed as there were a greater proportion of individuals with persistent NAFLD at 12-months who had IR compared to those who did not have NAFLD. It was also found that the change in waist circumference over 12 months was significantly less in those with persistent NAFLD compared to those without NAFLD. Therefore, the overall results indicate that NAFLD significantly improves post-RYGB along with anthropometric and metabolic parameters, but those with persistent disease are more metabolically impaired.

The third study was a cross-sectional study comparing a different group of NAFLD patients (non-bariatric) to HC. This was an exploratory study that compared hepatic immune cells between groups and investigated potential relationships between hepatic immune function and specific bacterial taxa identified from a previous study (28). As hypothesized, we found higher hepatic immune cell counts in NAFLD patients compared to HC, with significantly higher CD45P, CD45L, and CD163P. Those with more severe NAFLD (a NAS ≥ 5) also had significantly higher CD20L than those with a NAS of 0 or between 1-4. The
secondary hypothesis was also supported by the findings that specific taxa were significantly correlated with hepatic immune cells, particularly *F. prausnitzii* and *Prevotella*. This is of interest considering that these taxa were previously reported to be associated with metabolic abnormalities (27, 58-60, 444). Though this last study was exploratory, results suggest a possible link between specific IM and hepatic immune cells which could contribute to NAFLD pathogenesis. Further research is planned to assess the effect of RYGB on the hepatic immune cells from the liver biopsies collected in our cohort of bariatric patients and to investigate the relationship with changes in IM from stool being collected for that purpose. These results could contribute further to our understanding of NAFLD pathogenesis.

### 6.2 General Discussion

The prevalence of NAFLD and NASH at the time of bariatric surgery was lower when compared to published reports (11). This is likely due to the effect of VLCD on anthropometric and metabolic parameters prior to surgery. According to the 2017 AASLD Guidelines the management of NAFLD should include lifestyle modifications, which consist of diet, exercise and weight loss (35). These guidelines state that weight loss of at least 3-5% of body weight appears necessary to improve steatosis and a greater weight loss of 7-10% is needed to improve the histopathological features of NASH (35). In our cohort, the mean duration of the VLCD was 2.60 ± 0.66 (SD) weeks with a mean weight loss per week of 3.69 kg ± 2.58. This resulted in a median percentage excess body weight loss of 9.3%, which according to AASLD guidelines should improve the majority of histopathological features of NASH, including fibrosis (35).
In the bariatric surgery community, the VLCD is routinely prescribed as it helps facilitate the surgery (328-330), and has been found to significantly reduce liver steatosis (411). Prior to bariatric surgeries being performed laparoscopically, VLCD wasn’t routinely prescribed as it was not needed (323). When comparing the histology of liver biopsies taken during an open surgery where VLCD wasn’t prescribed the prevalence of NAFLD was 85% and NASH was 33%, which is higher than the prevalence of 76.3% with NAFLD and 14.4% with NASH seen in our study (Chapter 3), suggesting a potential effect from VLCD (456). However, one study compared the histological findings of bariatric surgery patients who consumed a 6-week preoperative VLCD to those who did not (457) and found no difference between the groups with regards to the prevalence of NASH. Changes in metabolic and anthropometric measurements pre- and post-VLCD were not reported (457). Studies reporting the effects of VLCD in the bariatric surgery population primarily reported on changes in liver size and body composition, but no liver biopsies were taken at the time of surgery (326, 328, 411). Others showed some changes in metabolic parameters, such as significant decreases in HOMA-IR score, and total and HDL cholesterol pre- and post-VLCD (327, 388). However, both studies also did not have a liver biopsy taken at the time of bariatric surgery and both had a small sample size, of 14 (327, 388). One study investigated the effects of bariatric surgery on NAFLD 12-months post-surgery and found that bariatric surgery significantly improved NAFLD (based on liver function tests) rapidly after surgery. However, at baseline they took blood work and a liver biopsy on the day of surgery, which occurred after 2 weeks of a VLCD. Therefore, the impact of the pre-surgical VLCD on post-surgical NAFLD improvement was not considered, which was stated as a study limitation (458). It is important to note that even though the VLCD is routinely prescribed prior to laparoscopic bariatric surgery, it is rarely mentioned in studies, and its effect on
anthropometric and metabolic parameters prior to surgery are not reported. Based on our study, it is likely that VLCD contributes to the short-term beneficial effect of bariatric surgery on glucose and lipid parameters as well as contributing to the improvement in liver histology post-bariatric surgery in those with NAFLD.

The lower prevalence of NASH in our study may also be explained by the use of more stringent criteria to diagnose NASH, which was defined as active ballooning of hepatocytes. Other studies used a broader definition, such as the NAFLD Activity Score (30). For NASH, it is possible that the prevalence we report is lower than other studies because we used liver biopsies as opposed to imaging techniques that are used to detect NAFLD. Imaging techniques such as ultrasound and the FibroScan, or non-invasive methods such as the Fatty Liver Index can detect steatosis in the liver (67, 73) but they are unable to assess inflammation, fibrosis and the degree of hepatocyte injury (67). A recent study investigated the accuracy of non-invasive measures of NAFLD compared to an intraoperative biopsy in obese individuals undergoing bariatric study (459). They found that the single use of either preoperative liver enzymes, liver ultrasound or a visual assessment done intraoperatively demonstrated low sensitivity, specificity and accuracy in detecting steatosis, steatohepatitis or fibrosis (459).

Finally, the lower prevalence of NAFLD/NASH in our sample could also be due to our sample of Canadians undergoing bariatric surgery representing a different population than the ones previously reported. A 2010 article compared population health in the United States to Canada (460). They used the data from the Joint Canada/United States Survey of Health from 2002/2003. They found that both obesity and the prevalence of chronic
conditions, including diabetes, heart disease and hypertension, are higher in the United States than in Canada. Another study compared the prevalence of diabetes in adults aged 20 to 79 years across nations and found that Canada had the third highest at a rate of 9.2%; however, the highest was the United States at a rate greater than 10% (461). Dietary composition also differs between nations. In a 2014 analysis of intake and sources of sugars in the Canadian diet, it was found that in absolute amounts Canadian adults on average consume 52 grams of added sugar per day versus the US population which consumes 77 g/day (462, 463). Furthermore, a study investigating high fructose corn syrup found that Canadians consume on average 9.13 kg/year per capita versus the US which consumes 24.78kg/year per capita (464). As fructose has been reported to be associated with increased hepatic fat, inflammation and possibly fibrosis (55), it is possible that the US has higher rates of NAFLD due to dietary consumption of fructose. In conclusion, it is possible that our prevalence of NAFLD and NASH is lower because we have a less unhealthy population that consume less dietary sugars, specifically fructose.

Besides bariatric surgery, another use of the VLCD is to facilitate long term weight loss, however its long-term effects for this purpose are debatable. The VLCD is a short-term prescribed diet, which means that the reintegration of regular food is necessary. Two studies investigated the long-term effects after the short-term VLCD. 5-years after the VLCD, between 45% and 121% weight regain was seen (465, 466). These extreme starvation diets are rarely used to treat obesity, instead they are used when patients are closely monitored and receive behavioral and dietary counseling to help maintain weight loss (314). Even though we saw significant weight loss and improvement in metabolic parameters, post-VLCD, it is
unlikely to be sustained. Therefore, at this time it would not be recommended as a lifestyle modification for NAFLD improvement.

Contrary to VLCD, bariatric surgery does have long-term benefits with sustained weight loss and improvement in comorbidities. RYGB surgery is known to improve liver histology in the majority of individuals. In the second study, we investigated the effect of RYGB surgery on NAFLD. We found that the prevalence of NASH at the time of RYGB was 21.4%, whereas 12-months post-RYGB the prevalence of NASH was reduced to 2.4%. Fibrosis also significantly improved in most individuals, but worsened in 3 individuals. Studies have found that fibrosis may worsen in some patients due to the rapid weight loss, which could aggravate pre-existing hepatic lesions (467, 468).

A limitation to using the liver biopsy for NAFLD diagnosis is sampling error. One study found that when comparing two liver biopsy samples, taken at the same time with a minimum length of 15 mm, in 51 NAFLD patients that the fibrosis stage was different in 41% of paired samples (469). However, we did attempt to prevent sampling errors by collecting 2 liver core samples with a sufficient length of 15-16mm, which is necessary to accurately evaluate fibrosis (470). When comparing the improvement in liver histology post-RYGB our results are comparable to published reports. Studies listed in Table 6-1 also found significant improvement in all histological criteria post-RYGB. Most of these studies, with the exception of one (471), also had small sample sizes due to the invasive nature of doing a liver biopsy. The larger study (471) had a sample size of 261 and reported improvements in severe steatosis and NAS greater than or equal to 3. However, this study did not report changes in detailed histological criteria, such as lobular inflammation or fibrosis. Therefore,
in general, our sample size is similar to most studies but we also report more on detailed histological criteria.

Table 6-1: Summary of the effects of RYGB on liver histology

<table>
<thead>
<tr>
<th>Patients (Human Adults)</th>
<th>Follow up Period</th>
<th>Effects on Liver Histology Post-RYGB</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>1.7 +/- .7 years</td>
<td>Significant improvements in steatosis, lobular inflammation, hepatocyte ballooning, fibrosis and NAS score.</td>
<td>(390)</td>
</tr>
<tr>
<td>18</td>
<td>2 years</td>
<td>Significant improvements in steatosis and fibrosis</td>
<td>(147)</td>
</tr>
<tr>
<td>21</td>
<td>1 year</td>
<td>Significant improvements in steatosis, lobular inflammation, hepatocyte ballooning, fibrosis and NAS score</td>
<td>(472)</td>
</tr>
<tr>
<td>19</td>
<td>21 months</td>
<td>Significant improvements in steatosis, lobular inflammation, hepatocyte ballooning, portal and lobular fibrosis</td>
<td>(473)</td>
</tr>
<tr>
<td>261</td>
<td>1 year</td>
<td>NAS score and percentage steatosis significantly improved</td>
<td>(471)</td>
</tr>
<tr>
<td>16</td>
<td>305 +/- 131 days</td>
<td>Significant improvements in steatosis, inflammation, hepatocyte ballooning, portal and perisinusoidal fibrosis</td>
<td>(474)</td>
</tr>
</tbody>
</table>

Our study specifically assessed the effect of one type of surgery, the RYGB. Other studies and reviews have combined multiple surgery types together to demonstrate the benefits of bariatric surgery. A systematic review and meta-analysis found that steatosis, steatohepatitis and fibrosis improve or completely resolve in the majority of individuals post-bariatric surgery, however this analysis included seven different types of bariatric surgery (405). Another study found that hepatic inflammation did not improve at 1 and 5 years post-bariatric surgery, but it included various types of surgery such as bilio-intestinal bypass, gastric bypass and gastric band surgery (377). Each bariatric surgery method yields drastically different anatomical changes, which can influence weight loss and metabolic parameters, therefore influencing the degree of NAFLD improvement. These anatomical
changes can restrict food consumption, alter gut hormones and/or cause changes to the IM that may influence glucose and lipid metabolism. For example, a study investigating the post prandial gut hormones between gastric bypass and sleeve gastrectomy found that those with a sleeve gastrectomy had lower acyl ghrelin and des-acyl ghrelin levels and greater concentrations of resistin than the gastric bypass group (475). Ghrelin is a peptide hormone that is produced and secreted in the stomach and duodenum and plays a role in regulating appetite (476). In obesity, ghrelin levels are low and are also inversely related to IR and weight gain (477). Resistin, a macrophage-derived peptide, is involved in IR and inflammation (478, 479). Studies have found that resistin levels are related to liver histology. Specifically, resistin has been found to be negatively correlated with steatosis grade (480), and it is higher in patients with NASH versus those with simple steatosis (481). Therefore, when evaluating the benefits of bariatric surgery, it is important to analyze each type of surgery independently, as the mechanisms for metabolic improvement may be different thus yielding variable results between types of surgery.

We also analyzed the metabolic and anthropometric factors associated with persistent NAFLD post-RYGB. Contrary to other studies that only reported the histological changes pre- and post-RYGB without stating the factors associated with persistent NAFLD (390, 471-474), we found that those with persistent NAFLD had significantly worse glycemic control and significantly less change in waist circumference than those who did not have NAFLD, despite similar BMI. Less change in waist circumference indicates that the location of fat might contribute to the pathogenesis of NAFLD. Central obesity has been found to be correlated with an increase in FFA, gluconeogenesis and IR (439, 482, 483). Furthermore, recent research suggests that alterations in adipokines, which are hormones released from the
adipose tissue that are involved in insulin sensitivity and inflammation, could contribute to NAFLD pathogenesis, specifically adiponectin, resistin and leptin (480, 484, 485). We also found that subjects with persistent NAFLD had worse glycemic control and significantly higher fasting glucose and insulin, and a higher proportion of those patients had IR compared to those with a NL at 12-months post-RYGB. These findings confirm what is known about the role of IR and NAFLD pathogenesis, however it is unclear why RYGB improves IR in some individuals but not in others. One could postulate that there might be other mechanisms contributing to this phenomenon, such as the IM or genetics. Genetic polymorphisms have been shown to contribute to both the development and severity of liver damage in patients with NAFLD. One example is PNPLA3, which has a 27-fold increase of developing NAFLD, regardless of other metabolic or clinical factors (486). Other genetic variants, such as ectoenzyme nucleotide pyrophosphate phosphodiesterase 1 and insulin receptor substrate-1, have been found to be associated with NAFLD as these polymorphisms affect insulin receptor activity thus predisposing individuals to liver damage (487). It is possible that individuals with persistent NAFLD and/or IR 12-months post-RYGB carry these polymorphisms.

Recently, evidence has shown that hepatic immune cells may also play a role in NAFLD pathogenesis by interacting with hepatocytes, stellate cells and sinusoidal endothelial cells, and during physiological processes and pathological conditions secreting pro-inflammatory cytokines (488). Studies have found that elevated Kupffer cells (24, 26) and B cells (25) were associated with NAFLD and disease severity. However, the studies investigating the Kupffer cells were in the pediatric population (24, 26) and the article by Gadd et al. focused only on broad leukocyte markers (25). In our study, we found that those
with NAFLD had significantly higher CD163, a marker for Kupffer cells, compared to HC. Interestingly, studies have found that both activated Kupffer cells and B cells are associated with insulin resistance and DM2 (236, 450, 488), however the exact mechanism remains unclear. In a recent study, it was found that a marker for Kupffer cells, soluble CD163, was significantly higher in morbidly obese individuals with a NAS ≥ 5 compared to those with a NAS < 5 (450). Using a multivariate analysis, they also found that changes in soluble CD163 reduction post-bariatric surgery were independently associated with corresponding changes in liver enzymes and HOMA-IR, and not with a decrease in BMI (450). Thus, this suggests that soluble CD163 reduction is associated with improved insulin sensitivity and not weight loss (450).

We also found that CD20, a marker for B lymphocytes, was increased in individuals with a NAS score ≥ 5 compared to those with a NAS score of 1-4 or 0. Hepatic B cell activation in relation to NAFLD pathogenesis is not well understood, however it could be a major factor in disease progression. In a murine model, B-cell activating factor (BAFF) has been found to be expressed in adipocytes and affects both insulin sensitivity and lipogenesis (489). A recent human study found that serum BAFF was significantly higher in those with NASH compared to HC. Furthermore, they found that serum BAFF was significantly correlated with HOMA-IR in patients with NASH (490). Thus, this study shows that BAFF is involved in IR progression in those with NASH. Though we did not specifically investigate the relationship between B-cell activation and insulin sensitivity, we saw that those with NASH had a significantly higher percentage of patients with IR. It would be of interest to determine how systemic and hepatic IR are related to both hepatic Kupffer cell and B-cell activation and NAFLD progression.
In our study, we found that specific bacteria taxa, *F. prausnitzii, Prevotella* and *Bifidobacterium* were correlated with specific CD cell markers. As previously stated, the immune system is the first line of defence against invading pathogens. The liver receives approximately 75% of its blood supply from the intestine to the portal vein (164), which makes bacterial translocation a critical component of hepatic pathophysiology. This bacterial translocation can activate the hepatic immune cells through pattern recognition receptors, thus contributing to the pathogenesis of NAFLD (491).

*F. prausnitzii* was found to be significantly negatively correlated with CD45 and CD163 in our study and is a bacterial species from the Firmicutes phylum that is abundant in the healthy IM (451). It is a known SCFA producer and has been suggested to have anti-inflammatory properties (452, 453). In the bariatric surgery population, it was found that obese patients without DM2 and lean controls had increased levels of *F. prausnitzii* compared to obese patients with DM2 (384). Furthermore, it was found that post-RYGB surgery, *F. prausnitzii* numbers increased, which was negatively correlated with changes in inflammatory parameters (384). Our correlation findings of *F. prausnitzii*, though moderate, align with what is known in the literature.

It was also found that CD20 (marker for B-cells) and *Prevotella* had a moderate inverse relationship. *Prevotella* is a bacterial genus from the Bacteroides phylum, which is a SCFA producer (445, 447). The results of *Prevotella’s* involvement in NAFLD are conflicting. One study reported that *Prevotella* was associated with IR in a non-diabetic cohort (492) and has been linked to obesity (60, 493) and NAFLD (60, 494). However,
*Prevotella* has also been found to be in lower abundance in those with NASH (445, 447). These conflicting results could be the result of differences in the *Prevotella* species, however future research is needed to better understand the role of *Prevotella* in the pathogenesis of NAFLD.

We also found that *Bifidobacterium* was strongly negatively correlated with CD20 and CD163 in those with SS. *Bifidobacterium*, which belongs to the *Actinobacteria* phylum and is also abundant in healthy IM (60). It is another SCFA producer that has been reported to be reduced in NASH (60). Lower levels of *Bifidobacterium* has also been associated with obesity (22). Our results, though only seen in the SS diagnosis, support what is known about this genus.

*F. prausnitzii*, *Prevotella* and *Bifidobacterium* are all SCFA producers. Research has predominantly focused on the SCFA butyrate, suggesting that butyrate could reduce oxidative stress and inflammation while improving colonic barrier function and satiety (168). This indicates that butyrate could play a role in influencing some of the mechanisms involved with NAFLD pathogenesis (168). The majority of research investigating butyrate and NAFLD used probiotics and focused on risk factors associated with NAFLD. Specifically, researchers have investigated the use of a butyrate-producing probiotic VSL#3 and MIYAIRI 588. VSL#3 intervention prevented intestinal inflammation and epithelial barrier dysfunction through significant increases in PPAR-γ expression and reversed IR, which protected against the development of NASH in mice (495). A human open pilot study investigated VSL#3 use in 78 participants, in which 22 had biopsy-proven NAFLD (140). After three months, the NAFLD participants had significantly improved plasma lipid peroxidation markers and liver
damage, which was assessed by biochemical liver tests (140). MIYARI 588 supplementation has improved risk factors associated with NAFLD. Mice fed a high-fat diet + MIYARI 588 had decreased lipid droplets in the liver, increased hepatic cholesterol catabolism enzymes and excretion transporters and an increase in hepatic PPAR-γ compared to mice without probiotic (496). VSL#3 and MIYAIRI 588 may improve NAFLD and influence related mechanisms, however butyrate was not directly measured in these studies. Overall, there is limited data exploring the relationship between SCFA and NAFLD. Future studies should use metagenomic analysis as it would allow for a better characterization of the IM and the function of SCFA.

The results of this exploratory study are hypothesis-generating. In a second step, we plan to assess the effect of RYGB on these immune cells by examining the liver biopsies that we have pre- and post- RYGB and determine if there is an association between changes in liver histology, IM and immune cells.

6.3 Strengths

These studies exhibit several strengths. NAFLD was diagnosed by liver biopsy and liver histology and was assessed in detail following well-validated criteria. This was performed by a pathologist blinded to the study protocol. The subjects were well-characterized as having NAFLD or HC and their liver histology was evaluated for presence of steatosis, inflammation, and fibrosis using the Brunt system which is both validated and reproducible (391, 497, 498). The NAFLD Activity Score was used to evaluate disease severity (431).
In addition, the study population was relatively homogeneous as all individuals went through a screening process for bariatric surgery and underwent the same pre-bariatric surgery protocol. They consumed the same prescribed VLCD and underwent the same bariatric procedure, followed by the same post-operative protocol. Also, this is the first study assessing NAFLD in a Canadian bariatric population, establishing data in the Canadian context for better relevance to patients in this population, which differs from that of the United States. Furthermore, few studies have reported the prevalence of NAFLD at the time of surgery and at 12-months post-operatively and there is a lack of data of the effects of the routinely prescribed pre-operative VLCD on metabolic and anthropometric parameters. Lastly, all the participants in the second study (Chapter 4) had paired liver biopsies 12-months post-RYGB.

The third study was exploratory. Very few studies have investigated the hepatic immune function in humans with NAFLD (24, 26), and even fewer have investigated it in the adult population (223, 450). In addition, the methods used in this study, particularly the quantification of specific bacterial taxa and the hepatic immunostaining, allowed for the assessment of the relationship between CD cell markers and IM, which has never been done before. Thus, though exploratory, this study expands the literature on this topic in novel ways.
6.4 Limitations

There are several limitations to these studies that should be taken into account when interpreting the results.

In all three studies, there was a relatively small sample size, however similar studies had comparable sample sizes (25, 26, 390, 499). The sample size is smaller due to the invasiveness of the liver biopsy as well as the stool sample needed for the study presented in Chapter 5. Also, the small sample size of the VLCD cohort was due to a small proportion of individuals being able to have blood work taken on the day of surgery, post-VLCD. Logistical issues regarding patient-flow in the pre-operative care unit, as well as within the operating room, prevented us from acquiring blood samples. There was also low sample size for the HC subjects as we were only able to assess their liver if the patient underwent a liver donation. However, no studies had liver biopsies from HC which constitutes a strength. For SS, the liver biopsy was rarely deemed medically necessary as SS is a benign asymptomatic disease, and often non-invasive measures were used to assess liver status, such as ultrasound or Fibroscan.

The design of each of the three studies was another limitation. All three studies were observational studies that used either a cross-sectional design or a prospective cohort design. Therefore, causal inferences cannot be made. All three studies were conducted at a single site, therefore making the results difficult to generalize. However, these results provide a foundation for future multi-center studies. In the bariatric studies, one of the limitations was that the patients were pre-screened by the UHN Bariatric Program prior to being enrolled,
thus creating a sample bias. However, this is according to standard of care where NIH criteria are followed to assess patients. Another study design limitation was the timing of the liver biopsy in Chapter 3. The liver biopsy was taken after the pre-surgical VLCD. This VLCD has been shown to significantly improve liver histology (411) and therefore affects the prevalence of NAFLD. Due to ethics and logistics it was not possible to obtain a liver biopsy before and after the VLCD. In order to determine the effect of the VLCD we used non-invasive biochemical markers pre- and post-VLCD. Another limitation specific to the last study was the use of correlation to establish a relationship between IM and immune function. This study was exploratory and results will be used to assess immune cells in the prospective cohort study going through bariatric surgery to determine the relationship between changes in IM and immune cells.

Another limitation regarding liver biopsies was that two different biopsy techniques were used for paired biopsies in Chapter 4. Wedge biopsies allow for shallow tissue sampling, which may not represent the inner parenchyma, whereas a needle biopsy allows for deeper tissue sampling, which avoids subcapsular fibrosis found in wedge biopsies (386). With the limitations presented in the operating room in both patient populations, such as surgical tools and timing, we were limited to a wedge biopsy at the time of bariatric surgery.

Patient compliance was another limitation seen in the bariatric population. Both before and after bariatric surgery there are several factors that patients must adhere to. First of all, patients are placed on a strict VLCD. If the patients do not follow this diet, changes could be seen in both biochemical and histological samples. Second of all, patients must also adhere to a rigorous post-RYGB diet plan. This plan includes specific micro and
macronutrient consumption as well as consumption of vitamin and mineral capsules. In order to ensure compliance in these studies, we asked patients to record what they ate pre- and post- VLCD and RYGB and to adhere to the dietary plan put forth by the UHN Bariatric Clinic. Deviations from this protocol were noted by the research assistant and considered when analyzing the data.

6.5 Conclusion

In conclusion, VLCD and RYGB significantly improve liver histology. This was associated with significant improvements in all metabolic parameters in addition to anthropometric measurements. However, for those who had persistent NAFLD, there was more impairment in metabolic parameters associated with glucose metabolism as well as less improvement in waist circumference. Considering that all individuals underwent similar interventions, it is possible that in those individuals with persistent NAFLD, factors other than obesity contributed to this finding such as genetics and IM. In addition, there could be an association between IM and hepatic immune cells that require further study in our cohort of bariatric patients.

This dissertation contributes to the growing evidence known about the complexity of NAFLD pathogenesis. We were able to demonstrate the impact of the VLCD on metabolic and anthropometric measurements as well as the prevalence of NAFLD at the time of bariatric surgery in morbidly obese Canadians. We were also able to report on the hepatic benefits seen in the Canadian population after RYGB surgery. In addition, we were also able to show that the hepatic immune system is associated with NAFLD severity and that there
could be a potential relationship between the hepatic immune system and IM. Future studies are needed to further investigate the role the hepatic immune system and IM play in NAFLD pathogenesis, as well as the potential therapeutic opportunities that each of these could provide for NAFLD treatment and prevention.
Chapter 7:

Future Directions

Based on the studies presented in the previous chapters, there are several areas that future research should explore in NAFLD. This includes the relationship between NAFLD and the IM through the bariatric care process, the role of hepatic immune cell function in NAFLD pathogenesis and how IM influences hepatic immune cells.

The future plan is to assess the changes in IM and bacterial products between pre- and post-bariatric surgery and determine whether there is an association with histology and metabolic parameters. I also plan to perform metagenomics analysis to assess bacterial genes and function that may contribute to improvement in liver histology and metabolic parameters. It would be of interest to determine if IM plays a role in the metabolic and histological response to VLCD and RYGB as stool is already being collected at four different time points. Very few studies (18-21) investigated IM in NAFLD and most were conducted in the non-bariatric obese population. Although some studies (383, 384) assessed the changes in IM and metabolic parameters pre- and post-RYGB, none assess NAFLD during bariatric surgery in relation to IM. In addition, diet may play a role in the liver response. For example, an intervention study found that subjects consuming a choline deficient diet resulted in the development of a fatty liver (58). In that study, specific IM compositions prior to the intervention were associated with higher liver fat accumulation resulting from the choline deficient diet. This suggests an interaction between IM and diet in the development of liver steatosis which was assessed by magnetic resonance imaging (58). However, no liver biopsies were performed to determine the presence of NASH (58).
Several bacterial products may also play a role in the development of NAFLD and should be further investigated; specifically, short-chain fatty acids (SCFA) and other ester volatile compounds, ethanol, and endotoxins (59, 500, 501). Only two studies specifically investigated metabolic products produced by IM in NAFLD patients. The first study found that specific patterns of fecal ester volatile organic compounds were associated with differences in IM when NAFLD patients, diagnosed on ultrasound, were compared to controls (59). This study did not have liver biopsies to assess the presence of NASH and the groups were not matched for BMI. The second study found that in a pediatric population there was an increased abundance of *E. coli* in patients with NASH compared to healthy controls, which was associated with higher blood alcohol levels (60).

Therefore, it would be of interest to assess bacterial products or perform metabolomics studies throughout the bariatric care process to determine if some products are associated with specific changes in liver histology or metabolic parameters. These studies on IM and bacterial products can lead to animal studies where specific IM or products can be further studied to confirm their role in NAFLD.

Furthermore, studies should also investigate the underlying mechanisms linking the IM with the hepatic immune system in those with NAFLD pre- and post-bariatric surgery. This would allow us to improve our understanding of the mechanisms behind immune cell activation, specifically to determine if the IM is contributing to the activation of these hepatic immune cells. To better comprehend this relationship, plasma endotoxins and bacterial products such as SCFA and ethanol could be measured. This would allow us to determine if
the IM and bacterial products are translocating across the mucosal lining and entering the peripheral blood stream and liver. It would also be of interest to determine the presence and identify bacterial DNA within the liver tissue by using 16S rRNA sequencing that may play a role in the activation of the immune cells. Being able to better understand the influence of the IM on hepatic immune cell activation would help us to gain knowledge on the pathogenesis of NAFLD, and could lead to the development of new treatment options.

Immune cells such as T lymphocytes, NK and NKT cells can also be further investigated in NAFLD as there have been very few studies on this. One study found that in obese mice and human NASH patients, peripheral CD4 T cells migrate quicker to chemokine CXCL12 when compared to HC, thus indicating the potential role that dysfunctional chemotaxis plays (502). Furthermore, as previously stated, studies have shown that CD8+ T cells drive adipose tissue inflammation; thus, the downstream effects could contribute to NAFLD pathogenesis (233, 234). NK cells, which were discussed earlier, are another area of interest as activated NK cells release interferon-γ (IFN-γ) which induces hepatic stellate cell (HSC) cycle arrest and apoptosis (225). As HSC activation is crucial for fibrosis, NK cells have anti-fibrotic effects. Conversely, IFN-γ also results in hepatocyte apoptosis and thus causes hepatic injury (225). In NAFLD, some studies found NKT cell numbers decreased compared to healthy controls (229), while in others the percentage of NKT cells increased with the severity of NAFLD (230). It would also be of interest to study these cell markers throughout the bariatric care process and determine the changes between liver biopsies taken during bariatric surgery and 12-months post-RYGB and assess the associations with changes in histology and IM. As well, I plan to assess bacterial fragments in liver biopsies to determine if this could be associated with immune cells.
After completion of the PhD, I plan to complete a post-doctoral fellowship under Dr. Allard’s supervision. It will focus on the relationship between the IM and bacterial products in relationship to NAFLD in morbidly obese individuals both before and after RYGB surgery, as well as the role of the hepatic immune system. In addition, it will investigate potential confounding variables such as diet intake and environmental factors. Stool samples, liver biopsies, blood work, dietary and physical activity logs and environmental questionnaires have already been collected in this patient population. Obesity is correlated with NAFLD and both are rising in today’s society. NAFLD could progress to cirrhosis and even liver transplant, furthering obesity’s burden on the healthcare system. This proposed study will provide more information regarding the role of IM and potential mechanisms contributing to NAFLD in the morbidly obese, which could lead to the development of targeted treatment options.
References


394. Caldeira PC, Oliveira e Silva KR, Vidigal PV, Grossmann Sde M, do Carmo MA. Inflammatory cells in minor salivary glands of patients with chronic hepatitis C.


421. Payne AN, Chassard C, Lacroix C. Gut microbial adaptation to dietary consumption of fructose, artificial sweeteners and sugar alcohols: implications for host-microbe interactions


Appendix 2.1: Assessment of NAFLD

**Brunt System** (391)

**Grading of Steatosis:**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Percentage of Hepatocytes Involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>1</td>
<td>5-33%</td>
</tr>
<tr>
<td>2</td>
<td>&gt;33-66%</td>
</tr>
<tr>
<td>3</td>
<td>&gt;66%</td>
</tr>
</tbody>
</table>

**Grading of NASH:**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Steatosis</th>
<th>Ballooning</th>
<th>Lobular Inflammation</th>
<th>Portal Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mild (Predominantly macrovesicular, involved &lt;33% up to 66% of the lobules)</td>
<td>Occasionally observed; zone 3 hepatocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Scattered and mild acute inflammation and occasional chronic inflammation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>None or mild</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Moderate (Any degree and usually mixed macro and microvesicular)</td>
<td>Obvious &amp; present in zone 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Polymorphs may be noted associated with ballooned hepatocytes, pericellular fibrosis, mild chronic inflammation may be seen</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mild to moderate</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Severe (&gt;66% (panacinar); commonly mixed steatosis)</td>
<td>Predominantly zone 3; marked</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Scattered acute and chronic inflammation; polymorphs may appear concentrated in zone 3 areas of ballooning and perisinusoidal fibrosis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mild or moderate</td>
<td></td>
</tr>
</tbody>
</table>

**Staging of Fibrosis:**

<table>
<thead>
<tr>
<th>Fibrosis Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No fibrosis</td>
</tr>
<tr>
<td>1</td>
<td>Zone 3 perisinusoidal fibrosis/pericellular fibrosis, focal or extensive</td>
</tr>
<tr>
<td>2</td>
<td>Same as Stage 1 with focal or extensive periportal fibrosis</td>
</tr>
<tr>
<td>3</td>
<td>Bridging fibrosis, focal or extensive</td>
</tr>
<tr>
<td>4</td>
<td>Cirrhosis</td>
</tr>
</tbody>
</table>
**NAFLD Activity Score (NAS)**

<table>
<thead>
<tr>
<th>Item</th>
<th>Score</th>
<th>Extent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steatosis</td>
<td>0</td>
<td>&lt;5%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5-33%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&gt;33-66%</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&gt;66%</td>
</tr>
<tr>
<td>Hepatocyte Ballooning</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Few balloon cells</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Many cells/prominent ballooning</td>
</tr>
<tr>
<td>Lobular Inflammation</td>
<td>0</td>
<td>No foci</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>&lt;2 foci/200x field</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2-4 foci/200x field</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&gt;4 foci/200x field</td>
</tr>
</tbody>
</table>

**Interpretation:**
Scores 0-2 are not diagnostic of NASH, scores between 3-4 are borderline diagnostic of NASH and scores between 5-8 are diagnostic of NASH.
### Appendix.5-1: Materials and Methods for Immunohistochemical Staining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Catalogue Number</th>
<th>Pretreatment</th>
<th>Dilution (Incubation)</th>
<th>Detection Kit used</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>Dako</td>
<td>A0452</td>
<td>Tris-EDTA 9.0</td>
<td>1/300 1hr</td>
<td>Impress Rabbit</td>
</tr>
<tr>
<td>CD20</td>
<td>Dako</td>
<td>M0755</td>
<td>Citrate 6.0</td>
<td>1/500 1hr</td>
<td>MACH 4 kit</td>
</tr>
<tr>
<td>CD45</td>
<td>Dako</td>
<td>M0701</td>
<td>Citrate 6.0</td>
<td>1/1000 1hr</td>
<td>MACH 4 kit</td>
</tr>
<tr>
<td>CD163</td>
<td>Leica</td>
<td>NCL-L-CD163</td>
<td>Citrate 6.0</td>
<td>1/200 1hr</td>
<td>MACH 4 kit</td>
</tr>
</tbody>
</table>
### Appendix.5-2: List of primers and probes used in this study

<table>
<thead>
<tr>
<th>Target organism</th>
<th>Sequence (5' to 3')</th>
<th>Annealing temp (°C)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| All Bacteria            | Forward: CGGTGAATACGTTCCCGG  
                          Reverse: TACGGCTACCTTGTTACGACTT  
                          Probe: CTTGTACACACGGCGGTTC  | 60  | (397)    |
| C. coccoides group      | Forward: GACGCCCGTGGAAGGA  
                          Reverse: AGCCCCAGCCTTTACATC  
                          Probe: CGGTACCTGACTAAGAAG  | 60  | (397)    |
| C. leptum group         | Forward: CCTTCCGTGCCGCAGTTA  
                          Reverse: GAATTAACCATCATTCCACCT  
                          Probe: CACAATAAGTAATCCACC  | 60  | (397)    |
| Bacteroidetes phylum    | Forward: CCTTCGATGGATAGGGGT  
                          Reverse: CACGCTACTTCGCTTGCAG  
                          Probe: AAGGTCGCCACATTTG  | 60  | (397)    |
| Bifidobacterium genus   | Forward: CGGGTGAGTAATGCGTGACCA  
                          Reverse: TGATAGGACGGGACCCCA  
                          Probe: CTCCTGGAAACGGGTG  | 60  | (397)    |
| Lactobacillus genus     | Forward: TGGATGCCTGGCAGTTAGGA  
                          Reverse: AAATCTCCGGATCAATACCAT  
                          Probe: TAATAGTTCGCTTACCATC  | 60  | (503)    |
| Ruminococcus genus      | Forward: GAGTGAATGGTAGAAGCGGAATTC  
                          Reverse: GCCGCTACTCCCAAGGTGG  | 60  | (504)    |
| Prevotella genus         | Forward: GGTTCGAGAGGAAGGGTTGCCC  
                          Reverse: TCCTGCACGGCTACGGCTG  | 60  | (505)    |
| Coprococcus genus       | Forward: CATCTGATGACGGTTTCTTAACC  
                          Reverse: GTTGCGGGACTTAACCAACC  | 55  | This study |
| Alistipes genus         | Forward: ATGGGCATGCGTTGATAGGC  
                          Reverse: CTTGTACGTACAGTTGAGG  | 55  | This study |
| F. prausnitzii          | Forward: TGTTAACCTCGTTGTTGAGGAAGATAA  
                          Reverse: GCCGCTCCCTTTACACCCA  
                          Probe: CAAGGAAGTGCACGGCTAATACGTGCAAG  | 60  | (506)    |
| E. coli                 | Forward: CATGCCGCGTGTATGAAAGAA  
                          Reverse: CGGGTAACCGTCAATGAGCAA  | 60  | (506)    |