NOVEL BIOMARKERS FOR NON-ALCOHOLIC FATTY LIVER DISEASE AND ASSOCIATED SYMPTOMS

by

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DEDICATION

I dedicate this dissertation to my loving husband, Eddie, to whom I am extremely grateful for his unwavering support throughout my years in graduate school.

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First and foremost I would like to express my sincere gratitude to Dr. Ancha Baranova for taking me on as her student, for her ongoing encouragement, and for all her support, academically and otherwise during the long process of earning this Ph.D. Dr. Baranova is an accomplished researcher who is passionate and productive in the pursuit of biomedical studies; yet she is also as dedicated to her students as she is to her research, and for that I feel very fortunate to have become a member of her laboratory. I also would like thank other members of her laboratory, namely Dr. Aybike Birerdinc and Dr. Mike Estep, for their professional assistance and personal friendship during my time in the Biosciences Ph.D. program. They too have been a constant source of encouragement and help, and I am extremely grateful to both of them.

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LIST OF ABBREVIATIONS

Abbreviation	**Full form**
ALT	alanine aminotransferase
AST	aspartate aminotransferase
AUC	area under the curve
BMI	body mass index
CI	confidence interval
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
ROC	receiver operating characteristic
SD	standard deviation

ABSTRACT

NOVEL BIOMARKER PANELS FOR NON-ALCOHOLIC FATTY LIVER DISEASE AND OTHER OBESITY-RELATED DISORDERS

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George Mason University, 2011

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Obesity is on the rise in populations across the world, and represents a major health concern. It is a component of Metabolic Syndrome, a collection of risk factors that predispose to diabetes and cardiovascular disease. Metabolic Syndrome is often accompanied by non-alcoholic fatty liver disease (NAFLD), a spectrum of liver disease ranging from simple steatosis, to non-alcoholic steatohepatitis (NASH) and liver fibrosis. Currently, the gold standard for NASH and liver fibrosis diagnostics is liver biopsy; thus, a non-invasive procedure for detecting and staging NAFLD is greatly needed.

The research presented herein involves evaluating various kinds of soluble biomarkers and the development of a novel, serum-based biomarker panel for NASH and NASH-related fibrosis. The biomarker panel comprises proteins that reflect the disease process of NASH and NASH-related fibrosis, including hormones derived from adipose tissue (adipokines) and proteins involved in fibrogenesis and cell death. While the sample size in this study was small at 79 patients, it is anticipated that subsequent testing of the

panel on larger populations of NAFLD patients will ultimately support its use in clinical settings.

A second study was conducted with the goal of discovering novel, as-of-yet untested biomarkers of NASH and NASH-related fibrosis that may be tied to the deregulation of cell signaling pathways in adipose tissue. A previous study used a phosphoproteomic approach to discover that several kinase-driven pathways were deregulated in the adipose tissue of patients with NASH and NASH-related fibrosis; enrichment analysis showed that these pathways were linked to the regulation of cell functions by insulin, as well as signal transduction by AKT and PIP3. Subsequent pathway analyses were then conducted to identify a set of secreted, soluble proteins associated with these pathways. From this set two promising candidates were selected based on extensive literature searches; these were the chemokine CCL-2/MCP-1, and soluble Fas ligand. These candidates were then tested on a small cohort of patients with NASH and NASH-related fibrosis to determine if they had the potential to be diagnostically predictive, and it was discovered that both worked reasonably well as biomarkers of fibrosis. Consequently, these molecules may be released at abnormal levels by adipose tissue in patients with NAFLD and may in turn play a role in fibrogenesis associated with NASH; they would therefore be good candidates to test in future development of biomarker panels for NASH-related fibrosis.

A third study was undertaken to evaluate the association between levels of various soluble molecules and fatigue in patients with NAFLD or hepatitis C. Specifically, I correlated self-reported assessments of fatigue dissecting this condition into fatigue associated with physical activity (peripheral fatigue) or more global lack of energy and

motivation (central fatigue) with measures of inflammation, or with abnormalities of glucose and lipid metabolism. The study demonstrated that a substantial majority of patients with chronic liver disease report significant peripheral fatigue. This type of fatigue was linked to elevated serum levels of IL-6 and IL-8, linking it to an inflammatory component, which is not the case for central fatigue.

OVERVIEW

Obesity has reached epidemic proportions, both in the U.S. and worldwide. The World Health Organization reports that in 2005, approximately 1.6 billion adults and 20 million children under the age of 5 were overweight, and an additional 400 million adults were obese (World Health Organization, 2011). Overweight and obesity are expected to increase, such that by 2015, the projected numbers of overweight and obese adults is 2.3 billion and 700 million, respectively. Excessive weight, as determined by body mass index, is a significant health concern because it is a risk factor for several chronic diseases including cardiovascular disease, diabetes, musculoskeletal disorders (*e.g.* osteoarthritis), and some cancers (*e.g.* breast and colon). Obesity also predisposes to Metabolic Syndrome (MS), a suite of metabolic changes that increase the risk of Type II Diabetes (T2D) and coronary heart disease. The guidelines for defining Metabolic Syndrome vary by source, but typically include insulin resistance, obesity, elevated levels of triglycerides and glucose and decreased levels of HDL-cholesterol in the blood, and high blood pressure (Scaglione *et al.*, 2010). T2D may be considered a component of Metabolic Syndrome as well (Scaglione *et al.*, 2010).

Nonalcoholic fatty liver disease (NAFLD) is considered the hepatic manifestation of Metabolic Syndrome (Marchesini *et al.*, 2001), and is the most common form of chronic liver disease in the U.S. and worldwide (Lazo and Clark, 2008). Thus, NAFLD is

strongly correlated with T2D (Li *et al.*, 2002), and obesity (Junior *et al.*, 2006), and appears to be inextricably linked to insulin resistance regardless of weight (Chitturi *et al.*, 2002). Although the disease was initially observed predominantly in females who were obese and diabetic (*e.g.* see Itoh *et al.*, 1987), it is now recognized to span multiple demographic groups, affecting children and adults, men and women, and various ethnicities (Browning *et al.*, 2004; Lavine and Schwimmer, 2004). The prevalence of NAFLD has been estimated as high as 30% in the U.S. adult population, 20% in the non-U.S. adult population, and 2.6% in the pediatric population (Lazo and Clark, 2008; Tominaga *et al.*, 1995). Individuals who are obese appear most prone to contracting NAFLD; 60-95% of patients with NAFLD are obese, with the morbidly obese having a 95% prevalence of the disease (*reviewed in:* Collantes *et al.*, 2004). Likewise, approximately 23-53% of obese children have NAFLD.

NAFLD represents a spectrum of liver disease that can lead to the development of cirrhosis and hepatocellular carcinoma, and thus is potentially lethal (El-Zayadi, 2008; Matteoni *et al.*, 1999; Mishra and Younossi, 2007). Patients with late stages of NAFLD, known as NASH and NASH-related fibrosis, are at much higher risk for progression to more advanced liver disease than those with the benign form, steatosis (Ekstedt *et al.*, 2006; Matteoni *et al.*, 1999; Rafiq *et al.*, 2009). In fact, it is estimated that 32%-37% of patients with NASH will progress to fibrosis, 10-20% will develop cirrhosis, and 8-12% will die of liver-related conditions (El-Zayadi, 2008; Matteoni *et al.*, 1999; Mishra and Younossi, 2007). Thus, diagnosis and management of NAFLD even at its earliest stages is of utmost importance. Approximately 3-5% of Americans have NASH, and up to one-

third have steatosis (Collantes *et al.*, 2004, Browning *et al.*, 2004). Thus, diagnosis and management of NAFLD even at its earliest stages is of utmost importance.

The research presented herein aims to identify novel serum-based biomarkers for NAFLD and for the fatigue associated with chronic liver disease. Chapter 1 presents a detailed description of NAFLD including its diagnosis, epidemiology, and pathology. Chapter 1 also reviews the biomarkers and biomarker panels for NAFLD and for liver fibrosis that have been published to date. Chapter 2 presents a discussion of the methods used in this research together with the study aims for the three sets of experiments that were conducted. Chapter 3 contains the results of the first set of experiments, in which a novel, serum-based biomarker panel for the identification of NASH and NASH-related fibrosis was developed, tested and validated. In Chapter 4, the results of experiments are presented in which the potential contribution of adipose tissue to NAFLD is investigated, with the specific goal of testing two adipose-derived serum proteins as candidate biomarkers of NASH and NASH-related fibrosis. Chapter 5 summarizes an investigation into whether a targeted collection of cytokines and hormones, measured in sera, could be used as representative biomarkers of fatigue related to chronic liver disease. Finally, general conclusions based on the results of all experiments are presented in Chapter 6.

1: INTRODUCTION

A. Non-Alcoholic Fatty Liver Disease.

Definition. Non-alcoholic fatty liver disease (NAFLD) is a disease characterized by two predominant histological features of the liver: over-accumulation of lipids within hepatocytes (known as *steatosis*), and inflammation together with steatosis and evidence of cell death, known as *steatohepatitis*. NAFLD is diagnosed in cases where other causes of liver disease, such as viral hepatitis and alcohol abuse, have been ruled out. By definition, NAFLD is limited to patients with fatty liver whose intake of alcohol does not exceed 30-40 grams per day in men or 20 grams per day in women (Junior *et al.*, 2006), although the threshold for "non-alcoholic" varies by study. One study, for example, cited a criterion of <40 grams per week (Feldstein *et al.*, 2003) while another used a criterion of no more than 20 grams per week (Puljiz *et al.*, 2010).

Pathology. NAFLD encompasses a range of pathologies including (in increasing order of increasing severity) steatosis alone (simple steatosis), steatosis with non-specific inflammation, non-alcoholic steatohepatitis (NASH), NASH-related fibrosis, and cirrhosis (Falck-Ytter *et al.*, 2001; Matteoni *et al.*, 1999; Younossi *et al.*, 2002). All stages of NAFLD are defined histologically; Figure 1 illustrates the features that are characteristic of each of these stages. Steatosis is diagnosed when 5% or greater of liver

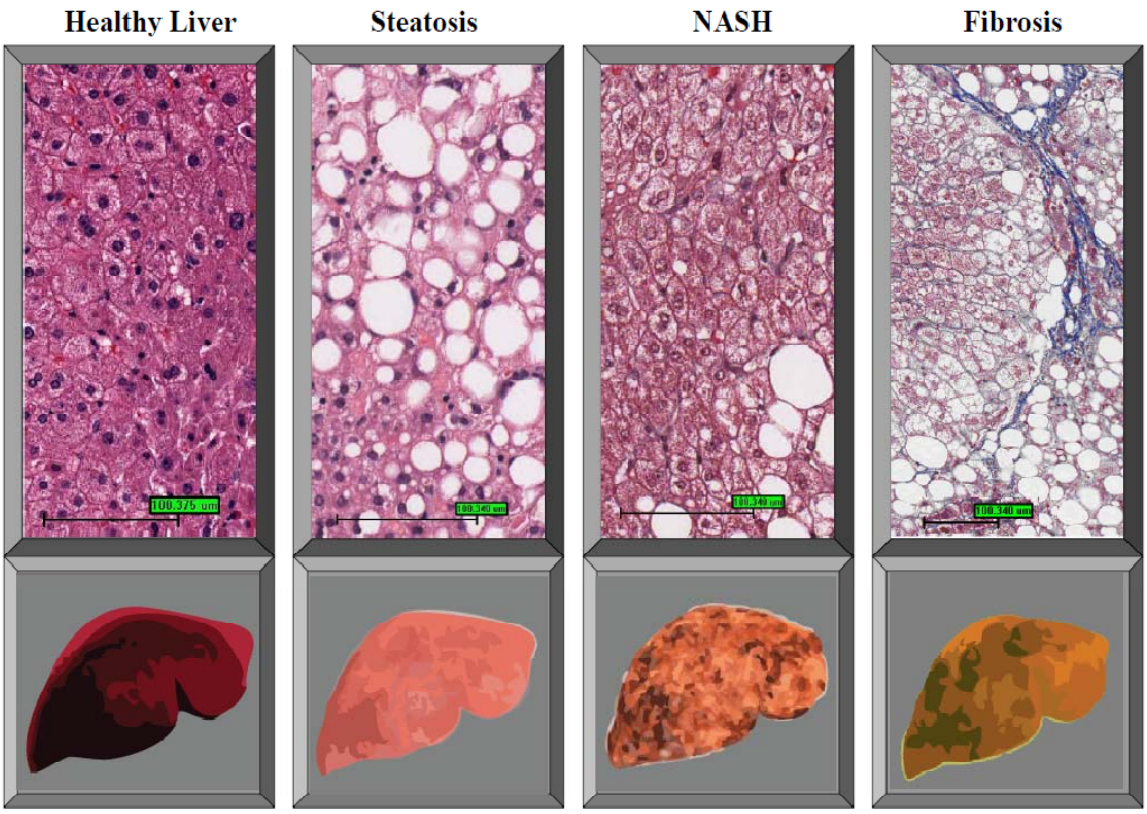

Figure 1. Histological images and corresponding superficial views of the liver showing (left to right) progression from a healthy state to increasing severity of NAFLD. While healthy hepatocytes appear uniform in color and shape, they enlarge as lipids over-accumulate (steatosis) and nuclei are pushed to the periphery of the cells. NASH is characterized by evidence of cell damage and death coupled with steatosis and inflammation. Fibrosis develops with the progression of NASH, and can be portal, perisinusoidal, or pericellular in its distribution. *Reprinted with permission of Dr. Birerdinc (Baranova et al., 2010).*

weight is filled by lipids, whereas NASH is diagnosed when steatosis is accompanied by hepatocellular injury and inflammation either with or without fibrosis (Preiss and Sattar, 2008). In hepatocytes, lipids accumlate in vesicles that displace the cytoplasm; in steatosis, the vesicles frequently become so enlarged that they push the nucleus to the periphery of the cell; this is known as macrovesicular steatosis. Macrovesicular steatosis is graded on a scale of 0 to 4, with 0 representing not present and 4 representing its presence in over 2/3rd of the liver. NASH is associated with a number of histological features but not all need to be present for the diagnosis of NASH, since combinations of histological features are often patient-specific (Sanyal, 2002). Definitions of NASH always include steatosis, and then may be based on a combination of (1) ballooning degeneration; (2) lobular neutrophilic inflammation; (3) Mallory-Denk bodies; and (4) portal, perisinusoidal or pericellular fibrosis. In combination the first three features represent necroinflammatory activity in the liver. NAFLD is *graded* from 1 to 3, with 1 representing mild necroinflammatory activity and 3 representing severe necroinflammatory activity. NAFLD is *staged* according to the type and extent of fibrosis. Stages 1 through 3 represent varying extents of perisinusoidal and pericellular fibrosis with or without portal fibrosis, whereas stage 4 represents cirrhosis. Beyond this overview, diagnosis of NASH can be somewhat specific to the experience of a given histopathologist, and depending on the histological features in question the characterization of NAFLD can be relatively consistent or inconsistent among histopathologists (Younossi *et al.*, 1998). The lack of standardization has been considered

problematic for some time, and some have argued for the development of a standardized approach to the diagnosis of NAFLD, and NASH in particular (Bondini *et al.*, 2007). Recently, the NAFLD activity score (NAS) has been in use; NAS a scoring system based on histological features of NAFLD (excluding fibrosis) designed by the Nonalcoholic Steatohepatitis Clinical Research Network (CRN) to help assess, in a standardized manner, histological changes in the liver during clinical trials (Kleiner *et al.*, 2005). It was validated in a cohort of NASH patients outside the CRN (Hjelkrem *et al.*, 2011) however some have critiqued unintended uses of the NAS, specifically finding inconsistencies when using NAS thresholds for the diagnosis of NASH in lieu of histologic diagnoses (Brunt *et al.*, 2011).

Distinguishing NASH from steatosis is crucial since NASH can progress to end stage liver disease, whereas steatosis often remains benign (Matteoni *et al.*, 1999; Teli *et al.*, 1995). For example, in a prospective study of NAFLD patients, Matteoni *et al.* (1999) found that those patients with NASH were more likely to progress to cirrhosis or die of liver-related disease than those with milder forms of NAFLD (*i.e.* steatosis and steatosis with non-specific inflammation). Likewise, NASH has been heavily implicated in the etiology of cryptic cirrhosis and may lead to hepatocellular carcinoma arising from cirrhosis (*reviewed in:* Starley *et al.*, 2010; Smedile and Bugianesi, 2005). Curiously, with the onset of cirrhosis, steatosis often disappears and other histological features of NAFLD (*e.g.* Mallory bodies, perisinusoidal fibrosis) become non-detectable, hence the reason many diagnoses of cirrhosis are necessarily labeled "cryptogenic" even though NAFLD may be suspected (Sanyal, 2002).

Histologically, alcoholic steatohepatitis and NASH look alike; for example, both involve steatosis, fibrosis, and Mallory bodies, and neither is associated with any histological feature that is unique to that disease (Itoh *et al.*, 1987). However, it has been reported that alcoholic steatohepatitis involves greater degrees of inflammation, hepatocellular injury, Mallory bodies, and perisinusoidal fibrosis, whereas in NASH there is a greater number of glycogen nuclei (Sanyal, 2002).

NAFLD is thought to arise and progress in two major pathological steps, known as the "two hits" (Day and James, 1998). The disease begins with the net accumulation of lipids in hepatocytes, usually in the form of triglycerides, possibly in reaction to a pre-existing state of insulin resistance or hyperinsulinemia (Collantes *et al.*, 2004; El-Zayadi, 2008). Progression to NASH results from a second "hit" to the liver, involving a combination of oxidative stress generated by the oxidation of fatty acids, and cytokine signaling, particularly by tumor necrosis factor alpha, which contributes both to oxidative stress and insulin resistance (Collantes *et al.*, 2004). The second hit is then succeeded by a number of events resulting in hepatocyte injury, inflammation, and fibrosis (Chitturi and Farrell, 2001). Apoptosis is an important component of NASH and also is mechanistically linked to the progression of fibrosis, therefore forming another dimension of the second hit.

Beyond the "two-hit hypothesis," the pathogenesis of the disease is not well understood for several reasons. First, definitive diagnosis of NAFLD is based on liver biopsy, an invasive procedure that precludes the ability to conduct population-based studies (Browning and Horton, 2004). Cohort and serial biopsy studies are typically

limited in scope due to short-term follow-up and selection bias during biopsy (Day, 2005). The subjectivity of diagnosis is also problematic. Typically, NAFLD is diagnosed and staged based on the histological features of a biopsy specimen, as interpreted by a hepatopathologist. Although the histological features associated with steatosis and NASH are well defined, the minimum diagnostic criteria used by pathologists to differentiate NASH from steatosis (*e.g.* the minimal amount of fat necessary to diagnose NASH; the presence or absence of hepatocyte ballooning) are not standardized, despite attempts to prioritize such criteria (Hubscher, 2006; Neuschwander-Tetri and Caldwell, 2003; Ratziu *et al.*, 2009). Moreover, there is variability in the interpretation of histological features among pathologists. Younossi *et al.* (1998) measured intraobserver and interobserver variability among histopathologists using 19 parameters corresponding to the histological spectrum of NAFLD, and found that there was inter- and intraobserver concordance within only eight parameters. The parameters in agreement included ballooning degeneration, glycogen nuclei, the extent and location of steatosis, and perivenular fibrosis, whereas parameters of inflammation were not consistently scored. Thus, Younossi *et al.* concluded that only some histological features of NAFLD are uniformly interpreted by pathologists. Unfortunately, non-histological diagnostic markers that might be used in concordance with a biopsy-based diagnosis do not exist, which is particularly problematic when the liver exhibits unspecific or ambiguous looking lesions (Ratziu *et al.*, 2009).

Challenges of Non-Invasive Diagnosis. One of the most challenging aspects of diagnosing NAFLD is that it is typically asymptomatic despite the fact that about half of all patients exhibit hepatomegaly, or enlarged liver (Collantes *et al.*, 2004; Sanyal 2002). The few complaints that are associated with NAFLD include fatigue, general malaise, and upper right quadrant abdominal pain. Only advanced stages of the disease produce more telltale signs of liver dysfunction, such as jaundice and ascites. If a patient has risk factors for NAFLD, serum levels of the aminotransferases, aspartate- and alanine aminotransferases (AST and ALT, respectively) may be assessed. These enzymes are not unique to hepatocytes, however when serum levels of AST and ALT exceed normal ranges they may be considered indicative of NAFLD, assuming other sources of liver disease (*e.g.* hepatitis B or C infection, excessive intake of alcohol) have been ruled out (Clark *et al.*, 2003). Nonetheless, aminotransferases cannot be used as definitive biomarkers of NAFLD because (1) they do not correlate well with the progression of liver disease (Kallei *et al.*, 1964); (2) many NAFLD patients have normal levels of aminotransferases (Browning *et al.*, 2004; Junior *et al.*, 2006; Mofrad *et al.*, 2003); and (3) even when aminotransferases are elevated in NAFLD patients, the levels are typically mild to moderate (twofold to threefold) and only rarely are as high as 10 to 15 times normal (Collantes *et al.*, 2004). Other blood-based indicators of liver function, such as bilirubin, do not reach abnormal levels until the onset of cirrhosis or liver failure (Sanyal, 2002). In patients who are hospitalized, alcoholic steatohepatitis is easily distinguished from NASH because it leads to higher bilirubin levels and an AST/ALT ratio of >2,

compared to <1 for those with NAFLD; however, for those with modest alcohol consumption AST/ALT ratios are similar, making the diseases difficult to distinguish.

Currently, liver biopsy is the only means by which NAFLD is definitively diagnosed and staged, particularly with regard to distinguishing simple steatosis from NASH (Duvnjak *et al.*, 2007). However, liver biopsy poses a small but serious health risk to patients, is costly, and is subject to sampling error (Bondini *et al.*, 2007; Cadranel *et al.*, 2000; Ratziu *et al.*, 2005), making it the "imperfect gold standard." In rare instances, (<0.1% of patients), liver biopsy can be lethal (McGill *et al.*, 1990; Tobkes and Nord, 1995). Approximately 5% of patients undergoing liver biopsy require immediate hospitalization for biopsy-related complications, which are more likely to occur in patients with cirrhosis or hepatitis (Perrault *et al.*, 1978). About 1% of patients experience post-biopsy hemorrhaging; in some cases severe enough to warrant a blood transfusion (Mahal *et al.*, 1981). Hemorrhaging is more likely in patients with histologically defined liver disease. This observation is primarily explained by associated clotting abnormalities rather than by structural problems with the liver. Pain and hypotension also are typical side effects of liver biopsy that can require hospitalization (Janes and Lindor, 1993). Thus, a liver biopsy is typically not conducted until there is further evidence of advanced disease, such as chronically high aminotransferase levels despite significant lifestyle changes, or the presence of obesity, diabetes, or other risk factors for NAFLD (Collantes *et al.*, 2004).

Non-invasive imaging methods such as ultrasonography, computerized tomography (CT) scan, and magnetic resonance imaging (MRI) can be used to detect certain features

of NASH. Altogether, imaging methods are better suited to diagnose liver fibrosis as compared to liver inflammation or steatosis. In fibrosis, deposition of the ECM leads to alterations in liver microstructure that are reflected by an increase in the liver stiffness and changes in the blood flow. These changes can be quantified using Transient Ultrasound Elastography (Fibroscan), an ultrasound-based technology for quantitatively assessing hepatic stiffness that has been introduced in the last several years both in Europe and in other parts of the world. Fibroscan measures the stiffness (or elasticity) of the hepatic parenchyma using both ultrasound (5 MHz) and low-frequency (50 Hz) elastic waves produced by a specialized ultrasound vibrator applied to the body wall and coupled with 1D ultrasound imaging that measures the propagation speed of a wave using a pulse-echo ultrasound. Since fibrotic tissue is harder than healthy liver tissue, the shear wave measurement provides immediate quantitative assessment of the "degree of stiffness." FibroScan was reported to be of value in the diagnosis of fibrosis accompanying various liver diseases including hepatitis B and C, alcoholic liver disease, and non alcoholic fatty liver disease (NAFLD) (Laharie *et al.*, 2010). Meta-analysis of the existing literature, however, indicates that the diagnostic performance of transient ultrasound elastography is diminished in patients with early-stage hepatic fibrosis, increased fatty infiltration of the liver on biopsy, or high body mass index ($\geq$ 28 kg/m^2) (Talwalkar, 2010).

When used to detect steatosis, non-invasive, imaging methods such as ultrasonography, CT scan, and MRI lose sensitivity; for example, ultrasonography and MRI are insensitive to degrees of steatosis less than 30% (Junior *et al.*, 2006; Sanyal,

2002). Furthermore, these methods cannot be used to diagnose NASH or diffuse fibrosis, and they are difficult to standardize. Differences in sensitivities and specificities can arise due to differences in protocol; for example, using a CT scan to detect steatosis, sensitivity can range from 54% to 93% depending on the length of time following intravenous contrast injection (*reviewed in:* Sanyal, 2002). Likewise, inter- and intravariability in absolute liver attenuation numbers are high among multiple CT scans due to a variety of factors including differences in type and calibration of scanners and different regions targeted during scans. Sanyal (2002) reviewed the relative performances of these imaging techniques and found that while ultrasonography is most sensitive to lipid accumulation, when the accumulation is patchy or focal, CT scan and MRI perform better. CT imaging is the best method for semi-quantitation and comparative studies.

Treatment. There is no curative treatment for NAFLD; as of yet, pharmacotherapies are used only in clinical trials and many are known to generate undesirable side effects (Adams *et al.*, 2005; Siebler and Galle, 2006). To slow the progression of NAFLD, current treatments involve two strategies: (1) promote weight loss by diet modification and exercise, and in some cases also with bariatric surgery or administration of Orlistat, an enteric lipase inhibitor; and (2) address insulin resistance with the use of insulin sensitizing agents (*e.g.* metformin, thiazolidinediones) (Adams *et al.*, 2005; Junior *et al.*, 2006; Mishra and Younossi, 2007). Other medications such as the hepatoprotective agent, ursodeoxycholic acid and anti-oxidants (vitamins C and E) have been tested for their

ability to restore plasma levels of liver enzymes or alleviate other aspects of NAFLD (*e.g.* steatosis), but their therapeutic value remains unproven.

A drawback to these treatment strategies is that they are lengthy, and the duration of treatment is uncertain. For example, improvements in biochemical and histological indicators of NAFLD were seen from 3 to 23 months after beginning a diet and exercise type of intervention, and after 6 months when weight loss was surgically mediated (Mishra and Younossi, 2007). Likewise, biochemical and histological indicators of NAFLD improved when patients were given drugs to control metabolic abnormalities, such as insulin sensitizers and lipid lowering medications, but these improvements were seen after one month to one year depending on the drug. The positive effects of vitamins C and E, which are thought to protect against cell damage from free radicals, were seen after 6 to 12 months in some studies, and were not seen at all in other studies (Collantes *et al.*, 2004; Mishra and Younossi, 2007).

There are limited surgical options for treating NAFLD. Liver transplantation is conducted in cases where the disease has progressed to cirrhosis combined with liver failure or carcinoma (Adams *et al.*, 2005), however due to the metabolic and systemic nature of the disease, steatosis and steatohepatitis have high rates of recurrence following transplantation (Contos *et al.*, 2001). Bariatric surgery appears to reduce or even reverse the clinical symptoms of NAFLD; in one study, 82% of patients who underwent bariatric surgery showed dramatic improvement in histological features associated with NAFLD, including necroinflammatory changes and fibrosis (Dixon *et al.*, 2004). The efficacy of these surgeries in improving NAFLD has been attributed to the substantial weight loss

experienced by patients. Nonetheless, bariatric surgery is reserved for individuals who are morbidly obese (NIH, 1991) and is performed with the primary goal of reducing the patient's weight; thus improvement in liver disease following surgery is a favorable outcome but not an implicit goal.

Necessity for the development of NAFLD biomarkers. Given the high prevalence of risk factors for NAFLD in the general population and the silent nature of the disease, it is not unreasonable to suspect that many cases are undiagnosed. For example, 21% of men and 27% of women are obese (Mokdad *et al.*, 2001), and it is estimated that ~75% of those with obesity may have NAFLD and up to 20% may have NASH (Lazo and Clark, 2008). Likewise, over 7% of adults have diabetes mellitus, and weight gain increases the odds for developing T2D, regardless of race or gender (Cohen *et al.*, 2009; Mokdad *et al.*, 2001). The current estimate for the prevalence of NAFLD among diabetics is 30-50% (Bellentani *et al.*, 2010). One study found that adults who have been recently diagnosed with diabetes mellitus are at increased risk for the development of advanced liver disease in the form of cirrhosis, liver failure, or other ailment requiring liver transplant, which they attributed to longstanding cases of NAFLD that had gone undetected (Porepa *et al.*, 2010).

Another reason that NAFLD might be undiagnosed in many individuals is the fact that insulin resistance (IR) may be overlooked. IR is the main common feature among T2D, Metabolic Syndrome, and NAFLD, and is considered the primary driver of NAFLD (Bugianesi *et al.*, 2005). In fact, the association between NAFLD and IR is essentially

universal: it exists even in lean, non-diabetic patients with NAFLD, in both mild and severe cases of NASH, and has been observed across ethnic groups (Chitturi *et al.*, 2002; Kruger *et al.*, 2010; Marchesini *et al.*, 1999). However, in clinical settings IR may be overlooked, particularly if other, more obvious risk factors for NAFLD (*e.g.* obesity, elevated AST/ALT levels) are lacking. For example, in one study, 16% of individuals falling into the most insulin-resistant study group were of normal weight (McLaughlin *et al.*, 2004). Likewise, age may predispose to insulin imbalances and in turn, NAFLD. Petersen *et al.* (2003) tested glucose tolerance in healthy elderly and young subjects matched for fat mass and lean body mass, and found that the elderly subjects responded with slightly higher and significantly higher plasma glucose and insulin concentrations, respectively, compared to young subjects. Furthermore, the rate of glucose metabolism was 40% lower in the elderly group. Although insulin resistance was not detected in the livers of study participants, the elderly group had a 225% higher intrahepatic triglyceride content. Thus, is it conceivable that many cases of NAFLD are missed because individuals lacking more obvious risk factors for NAFLD are not tested for insulin levels.

A **biomarker**, or **biological marker**, is a quantifiable biomolecule that could be reliably used as an indicator of a certain biological state; *e.g.* a state of disease. An ideal biomarker would detect the presence of disease with high accuracy and be sensitive and specific for the disease in question; furthermore, it would be based on measurements from tissues collected in a non-invasive manner and it would be sufficiently cost effective to be put into routine clinical practice. A serum-based biomarker or panel of biomarkers meets these criteria. Serum is processed from blood; it represents the acellular constituent

of blood and as such, is easily collected by nurses during patient visits and also easily assessed for a wide variety of proteins by immunoassay. Many manufacturers make easy-to-use immunoassay kits that can be used to measure protein levels in up to 80 samples at a time, making patient screening somewhat high-throughput. In assessing patients for NAFLD, a biomarker panel incorporating a number of proteins is more likely to be successful than a single-endpoint biomarker due to the complexity of NAFLD and the fact that NAFLD involves physiological processes not unique to the liver, such as inflammation and fibrosis. As outlined in section C of this chapter, a number of efforts using one or multiple serum-based proteins as candidate biomarkers of NAFLD have been undertaken, with moderate success. It should be noted that targeting proteins rather than other types of serum-based molecules offers several advantages, primarily that many have already been associated with NAFLD, and there are a wide variety of commercially kits available to detect proteins in serum.

An ideal setting for a biomarker or biomarker panel for NAFLD would be a community health center, where routing testing on large numbers of individuals could take place. In these centers, sample collection and laboratory testing could be performed by family practitioners or nurses on site. Patients would benefit not only from non-invasive and accurate assessment of NAFLD status but also from repeated testing to help them track disease progress. It is likely that patients with frequent, up-to-date information on their disease status will be more motivated to make and maintain the changes in life style necessary to prevent the progression of NAFLD than they would be if only presented with the prospect of having NAFLD based on liver enzyme measurements or

other risk factors. Furthermore, implementation of a NAFLD biomarker panel in such settings would more than likely lead to widespread diagnosing of NAFLD, in turn enhancing our understanding of the epidemiology of NAFLD.

B. How NAFLD and Obesity are Related

Recently, obesity has become recognized as a state of chronic, systemic inflammation characterized in part by elevated serum levels of pro-inflammatory cytokines (*e.g.* tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6)) and other inflammatory factors (*e.g.* C-reactive protein) and decreased levels of anti-inflammatory factors (*e.g.* adiponectin, interleukin-10 (IL-10)) (O'Rourke, 2009; Karalis *et al.*, 2009; Nathan, 2008). Upregulation of these secreted factors is due to activation of several inflammatory signaling pathways, some of which involve components that also contribute to insulin resistance (*e.g.* Jun N-terminal kinase; *reviewed in:* Karalis *et al.*, 2009). Exactly how obesity, insulin resistance and inflammation are causally linked remains unknown, although some clues have been identified. For example, free fatty acids can bind to innate immune receptors (*e.g.* Toll-like Receptor 4) in adipocytes, initiating the release of pro-inflammatory cytokines (de Luca and Olefsky, 2008; Song *et al.*, 2006). Additionally, the release of pro-inflammatory cytokines and adipokines by adipocytes may be a reaction to hypoxic conditions caused by hypertrophy and hyperplasia of visceral adipose tissue (O'Rourke, 2009; de Luca and Olefsky, 2008). Cultured adipocytes exposed to hypoxic conditions increase their secretion of inflammatory adipokines such as IL-6, leptin, and monocyte migration inhibitory factor (MIF), while the secretion of adiponectin, an anti-

inflammatory adipokine, decreases (Wang *et al.*, 2007). Average adipocyte size plays a role as well; Skurk *et al.* (2007) reported that the secretion of pro-inflammatory cytokines interleukin-6 (IL-6) and interleukin-8 (IL-8) are significantly higher in hypertrophic adipocytes even after correction for cell surface, whereas the secretion of the anti-inflammatory factors interleukin-10 (IL-10) and adiponectin are significantly lower or had no relationship to adipocyte size, respectively. White blood cells likely contribute to the inflammatory process as well. Although macrophages normally occur in adipose tissue, the extent of their infiltration is directly proportional to the degree of adiposity (Weisberg *et al.*, 2003). Macrophages are thought to be responsible for most of the secretion of TNF-α and for some of the secretion of other inflammatory factors from adipose tissue (Fain *et al.*, 2008; Fain, 2006; Weisberg *et al.*, 2003). These, and other mechanisms allowing expanded adipose tissue to release inflammatory factors have significant health consequences; circulating inflammatory factors cause inflammation in distant organs and tissues (*e.g.* liver, bronchial lining and arterial wall), leading to progressing conditions such as insulin resistance and atherosclerosis (Nathan, 2008).

Adipose Tissue and NAFLD. An important aspect to the pathology of NAFLD is the role of visceral adipose tissue. Adipose tissue is increasingly regarded as an active endocrine organ that secretes molecules with paracrine and endocrine function, in addition to providing a mechanism for the storage of lipids. The molecules released by adipose tissue include adipocyte-derived hormones as well as cytokines, which are released by both adipocytes and other cells of the adipose tissue, such as macrophages (Kershaw and Flier,

2004). Many of these adipose-derived molecules, collectively known as adipokines (Fain *et al.*, 2004), play a role in energy homeostasis or immune response, but are also implicated in the development of obesity and insulin resistance (*reviewed in:* Kershaw and Flier, 2004). For example, leptin is secreted by adipocytes in proportion to adipose mass and signals energetic sufficiency, thereby suppressing additional energy intake. However, in cases of obesity, circulating leptin reaches excess levels and leptin resistance occurs. Likewise, interleukin-6 (IL-6) is a pro-inflammatory cytokine released by adipocytes, particularly from visceral adipose tissue, that correlates positively with obesity, decreased glucose tolerance, and insulin resistance. In fact, a number of pro-inflammatory cytokines are released by adipocytes and other cellular and matrix components of adipose tissue (Fain, 2006; Kershaw and Flier, 2004), and may contribute to the chronic inflammatory state characteristic of obesity (Karalis *et al.*, 2009; Nathan, 2008; O'Rourke, 2009). It has been proposed that the increased circulating levels of pro-inflammatory cytokines associated with obesity may alter hepatic gene expression affecting fatty acid synthesis and metabolism, leading to impaired fatty acid oxidation and other metabolic changes that promote steatohepatitis (Delgado, 2008).

Previous work in our laboratory has provided strong evidence for the role of pro-inflammatory adipokines in the progression of NAFLD from simple steatosis to NASH (Baranova and Younossi, 2007; Jarrar *et al.*, 2008) as well as the utility of selected pro-inflammatory cytokines, markers of apoptosis, and adipose-derived hormones in predicting NASH (Jarrar, 2008; Younossi et al., 2008). Gene expression profiling in obese patients with and without NASH revealed that the adipose tissue of patients with

NASH expressed a number of molecules in the TNFα network and soluble serum-circulating proteins (*e.g.* CCL26, IL-18, cathepsin B) (Baranova *et al.*, 2007). Gene expression analysis also showed that the liver tissue of obese patients without liver disease has decreased expression of CCL7/MCP-3, which attracts macrophages during inflammation (Baranova *et al.*, 2007).

C. **Previously Described Biomarkers of NAFLD and NASH.**

Introduction. For the past ten years or so a large number of studies have been undertaken to identify a reliable biomarker or set of biomarkers that could accurately identify NAFLD and stages of NAFLD. These biomarkers have been based on a wide variety of variables ranging from mRNA and protein expression in tissues, to serum molecules, to measures of oxidative stress and to demographic and clinical variables. Miller *et al.* (2011) recently provided a thorough review of these studies, dividing biomarker studies into three types of general concept: those using non-targeted approaches, studies of association, and algorithm tests. Non-targeted approaches involve techniques such as gene or protein microarrays validated by rtPCR and genome-wide association studies, with the advantages of novel biomarker discovery and a relative lack of bias; disadvantages are that these techniques can be costly and therefore typically involve only small study cohorts. In contrast, studies of association compare levels of targeted markers between diseased and non-diseased cohorts. These markers have ranged from clinical measurements such as circulating levels of liver enzymes (*e.g.* AST, ALT, and γ-glutamyltransferase (GGT), to adipokines (*e.g.* adiponectin, resistin, and leptin), to

inflammatory markers (*e.g.* TNFα, IL-6), to markers of oxidative stress (*e.g.* oxidized LDL). Markers of fibrosis (hyaluronic acid, HA) and apoptosis (cytokeratin-18) also have been tested in this context. Algorithm tests involve the development of predictive mathematical models for the diagnosis and staging of NAFLD, which are then evaluated for performance using area under the curve receiver operating characteristic (AUROC) analysis. These tests can be further categorized by diagnosis; specifically targeting steatosis, NASH, or fibrosis. Several of these have been tested on relatively large cohorts of patients with NAFLD. Miller *et al.* provide a detailed comparison of 13 of such tests which shows AUC values ranging from 0.763 to 0.936 in the training sets. In total, Miller *et al.* reviewed 50 studies reporting reliable biomarkers for NAFLD, excluding studies based on imaging techniques and in the pediatric population. A subset of these biomarker tests along with their strengths and weaknesses are discussed in more detail below.

Biomarker Panels for NAFLD. Previous efforts to discover and validate a serum-based biomarker specific to NAFLD have particularly focused on differentiating NASH or liver fibrosis from steatosis. In some cases the biomarker was represented by a single biomolecule endpoint, and in others a panel of biomarkers was developed. Those using a single endpoint have targeted various aspects of the pathology of NASH, including hormones released by adipose tissue (*e.g.* adiponectin), markers of oxidative stress (lipid peroxidation products), apoptosis (*e.g.* cytokeratin-18 fragments), hepatic inflammation (*e.g.* tumor necrosis factor α), and fibrosis (components of extracellular matrix; *reviewed in:* Bambha and Yee, 2008). However, the drawbacks of single-endpoint biomarkers is

that they are either not representative of the liver exclusively, or of NASH to the exclusion of other liver diseases. For example, cytokeratin-18 fragments are elevated in the serum of patients with other chronic liver diseases, as well as those with non-liver specific malignancies (Olofsson *et al.*, 2007; Yagmur *et al.*, 2007).

It has been suggested that a panel of biomarkers of NAFLD, combining various aspects of the disease process, might prove more reliable than those based on single endpoints (Bambha and Yee, 2008). This approach was taken recently by our laboratory, in which a preliminary biomarker panel distinguishing NASH from steatosis was established (Younossi et al., 2008). The panel comprised a suite of serum proteins released from the cells during apoptosis and necrosis, both of which occur in NASH, as well as pro-inflammatory cytokines and adipokines associated with the pathogenesis of NAFLD. The model was successful in predicting NASH, with a sensitivity and specificity of 95% and 70%, respectively, and an area under the ROC curve (AUC) value of 0.908, and it performed better than previously reported serum-based biomarkers for NASH, which consist of single-endpoint measurements of cytokeratin 18-derived antigens (Wieckowska *et al.*, 2006; Yilmaz *et al.*, 2007). However, the limitation of this model is that it did not include markers of fibrosis. Although NASH can occur without fibrosis, the onset of fibrosis is a stronger indicator of progressive disease that can lead to cirrhosis or liver-related death (Matteoni *et al.*, 1999).

Interestingly, several biomarker panels for liver fibrosis have been developed and validated in the context of NAFLD. An early predecessor is one that came to be known as the Original European Liver Fibrosis Panel (OELF), and was based on the serum factors

hyaluronic acid (HA), tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), and aminoterminal peptide of pro-collagen III (P3NP), as well as age (Rosenberg *et al.*, 2004). This panel was successful at distinguishing the presence of fibrosis vs. no fibrosis in patients with alcoholic liver disease and NAFLD; however, the NAFLD cohort constituted only 61 of the total 912 patients enrolled in the study. A recent study tested the OELF panel in 192 NAFLD patients and showed that the removal of age did not compromise model performance, resulting in the Enhanced Liver Fibrosis panel (ELF) (Guha *et al.*, 2008). The ELF further improved on the OELF by delineating 3 distinct stages of fibrosis: severe, moderate, and none. Similarly, a very large cohort (N = 733) of biopsy-proven NAFLD patients was used to design and validate a fibrosis index, named the NAFLD Fibrosis Score, based solely on parameters routinely collected in clinical settings (Angulo *et al.*, 2007). These parameters included: age, body mass index (BMI), AST/ALT ratio, platelet count, and hyperglycemia, and successfully distinguished NAFLD patients with and without advanced fibrosis.

Although these panels have greatly improved the detection of NAFLD, they all suffer from the same shortcoming: modest predictive power. For the NAFLD Fibrosis Score, depending on the upper and lower thresholds used, the sensitivities ranged from 51-82% and 43-77% for the groups of patients used to build or validate the index, respectively. Furthermore, the index failed to categorize 25% of the total study cohort. The results for the ELF were similar: when the thresholds for sensitivity and specificity were set to 90%, the ELF failed to classify 14%, 38%, and 52% of NAFLD patients with severe, moderate, or any fibrosis, respectively. When the ELF was combined with “simple markers”

constituting the NAFLD Fibrosis Score, the results improved slightly, increasing AUC values by 2, 3, and 5 percentage points to 0.84, 0.93, and 0.98 for the models predicting any fibrosis, moderate fibrosis, and severe fibrosis, respectively (Guha *et al.*, 2008).

Two biomarker panels designed to detect fibrosis and cirrhosis in other chronic liver diseases have been previously tested in the context of NAFLD. One, known as the the AST to platelet ratio index (APRI), was designed to detect fibrosis and cirrhosis in patients with chronic hepatitis C (CHC) (Wai *et al.*, 2003). For a given patient, the APRI is simply calculated as: [(AST (in IU/L)/ULN)*100/platelet (109 L)], where ULN = upper limit of normal (Wai *et al.*, 2003). In CHC patients, the APRI correlates significantly with the stage of fibrosis, and distinguishes fibrosis from cirrhosis. However, the performance of the APRI is inferior to other biomarker panels (discussed below) when applied to NAFLD patients (Cales *et al.*, 2009). Another biomarker panel called the FibroMeter was designed to predict the stage and extent of fibrosis in viral and alcoholic chronic liver diseases (Cales *et al.*, 2005). While testing the FibroMeter as a diagnostic tool for NAFLD patients, the panel was refined and renamed the FibroMeter NAFLD, and included the parameters: glucose, AST, ferritin, platelet, ALT, body weight, and age (Cales *et al.*, 2009). As demonstrated in 235 NAFLD patients from two medical centers, the FibroMeter NAFLD greatly outperformed the NAFLD Fibrosis Score and the APRI in predicting significant (mid-stage) fibrosis, with an overall accuracy of 91% (AUC = 0.941) vs. 86% for the NAFLD Fibrosis Score (AUC = 0.884, $p = 0.008$) and 84% for the APRI (AUC = 0.866, $p < 0.001$) (Cales *et al.*, 2009). However, all three tests performed similarly in cases of severe fibrosis and cirrhosis. The authors concluded that

both the stage of liver fibrosis and the relative prevalence of each stage in a given population greatly affect test performance.

D. Development of a Biomarker Panel for NASH and NASH-related Fibrosis

As described previously, NAFLD is a widespread liver disease that represents the manifestation of Metabolic Syndrome, and encompasses a histological spectrum beginning with steatosis with or without inflammation, and progressing to NASH and NASH-related hepatic fibrosis. In a subset of patients with NASH and accompanying fibrosis, chronic liver disease (*e.g.*, cirrhosis, hepatocarcinoma) may develop, potentially leading to liver failure (Matteoni *et al.*, 1999). Liver biopsy, the only definitive means of diagnosing and staging NAFLD, is an invasive procedure with inherent health risks and other drawbacks (*e.g.*, cost); consequently it is only conducted in cases where evidence of progressive liver disease is sufficient to warrant its use. A non-invasive, serum-based biomarker panel for the diagnosis and staging of NAFLD would be a practical alternative to liver biopsy that could be conducted safely and repeatedly on patients with, or at risk of NAFLD. None are currently in clinical use but several studies have reported prototype biomarkers or biomarker panels which fall into two general categories: those for the prediction of NASH (*e.g.*, NASH Diagnostics™ (Younossi et al., 2008); Apoptosis Biomarker (Wieckowska *et al.*, 2006)) and those for the prediction of hepatic fibrosis caused by NAFLD or other diseases (*e.g.*, OELF and ELF (Rosenberg *et al.*, 2004); NAFLD Fibrosis Score (Angulo *et al.*, 2007); APRI model (Cales *et al.*, 2009; Wai *et al.*, 2003)). The primary drawbacks to these models, when applied to patients with NAFLD,

are (1) modest predictive power; (2) failure to encompass more than one stage of NAFLD or to target NAFLD specifically; and (3) among tests for fibrosis, a tendency to favor the detection of advanced fibrosis over milder forms. Because NASH and NASH-related fibrosis are the stages of NAFLD that predispose to progressive liver disease, it would be desirable to have a set of non-invasive tools that could identify both of these disease states. For such a tool to be clinically useful, it also should be easily collected, disease-specific, and not cost-prohibitive for widespread use.

In this study, an attempt was made to develop a serum-based, non-invasive biomarker panel for NASH and NASH-related fibrosis that would not only would reliably detect these two stages of NAFLD, but would do so at a high level of performance. We chose to develop a panel of biomarkers rather than select single-endpoint markers to improve disease specificity, and we analyzed demographic and clinical data in combination with laboratory (biomarker) measurements in model development. Our goal was to develop the biomarker panel for three possible clinical endpoints: NASH, and NASH-related fibrosis, and advanced fibrosis. We also strived to comprehensively measure the disease process by including markers reflecting adipose-derived signaling, apoptosis, and fibrogenesis. The markers of apoptosis and fibrogenesis chosen for incorporation into the biomarker panel were previously successful in other studies; however, the adipose-derived signaling molecules selected for study were relatively novel candidate biomarkers (Younossi *et al.*, 2008). All candidate markers were quantified in serum by ELISA or EIA, such that if the panel succeeded in distinguishing NASH and NASH-related fibrosis from benign forms of liver disease, it could be theoretically put into

practice at reasonable medical cost, and will be acceptable for community clinical settings.

E. Novel Biomarkers of NAFLD Reflecting the Role of Adipose Tissue

There is a strong association between obesity and NAFLD; in fact, the occurrence of NAFLD is strongly correlated with anthropometric indices of body size and visceral body fat including BMI, waist-to-hip ratio, and waist-to-height ratio (Jiang *et al.*, 2010). The biological underpinnings of this relationship are unknown, although immune factors as well as imbalances in adipokine secretion (Krawczyk *et al.*, 2010) are widely implicated.

A recent study used a proteomic approach to examine whether cell signaling events in adipose tissue were differentially regulated in patients with and without NAFLD, and specifically whether phosphorylated forms of signaling molecules (indicating activated pathways) were useful in predictive models for NASH and NASH-related fibrosis (Younossi *et al.*, 2010). This study found that factors in the insulin signaling pathway, specifically AKT1 and IRS1, were significant predictors of NASH when integrated into a model that also included clinical and demographic data. NASH-related fibrosis was not as well predicted by phosphoproteomic data regardless of whether or not protein phosphorylation levels were combined with demographic and clinical data in the modeling effort; however of the models developed, the most predictive model for advanced fibrosis included GSK3 and cyclic AMP-regulated protein kinase A (PKA). Thus, phosphoproteomic markers are useful as predictors of NASH and NASH-related fibrosis in the context of multiple regression modeling. For clinical purposes, however, it

is of interest to know (1) whether differentially phosphorylated proteins, indicating de-regulated cell signaling in the adipose tissue of patients with NASH and fibrosis, are associated with secreted molecules; (2) whether a selected subset of the pool of secreted molecules may be easily measured in human serum and are present at different levels in patients with and without NASH and NASH-related fibrosis; and (3) whether such serum-based molecules are useful in statistical analyses aimed at distinguishing diagnostic patient groups or at developing predictive models for NASH and NASH-related fibrosis. If molecules meeting these criteria are identified, they may serve as useful targets for future studies of biomarkers of NAFLD that may one day be used in clinical practice. The current study was designed to investigate this topic.

F. Biomarkers of Central and Peripheral Fatigue in Patients with Chronic Liver Disease

Fatigue is a symptom common to a broad spectrum of chronic diseases including diseases of the intestine, kidney, liver, and lungs; it is a predominant feature of chronic fatigue syndrome and is also associated with cancer, metabolic and neurological diseases, and vector-borne diseases such as Lyme's disease. Disease-related fatigue can manifest directly from the disease process, or it can be related to indirect factors such as age, sex, medications, and the duration of symptoms (*reviewed in:* Carneiro *et al.*, 2011). Chronic fatigue is particularly debilitating in that its effects can be physical, cognitive and even social in nature; it is associated with pain, stress, sleep deprivation, anemia, and

infections, and can lead to emotional difficulties that reduce quality of life, including depression and diminished self-esteem.

A number of liver diseases are associated with fatigue including NAFLD (Raszeja-Wyszomirska *et al.*, 2008), alcoholic hepatitis (Sass and Shaikh, 2006) hepatitis A, B, C and E (Wilkins *et al.*, 2010; Jacobson *et al.*, 2010; Terzic *et al.*, 2009; Lee *et al.*, 2008), and primary biliary cirrhosis (Abbas *et al.*, 2010). Ongoing study and treatment of patients by the Center for Liver Diseases (Fairfax INOVA Hospital, Annandale, VA) has allowed insight into the nature of fatigue associated with chronic liver diseases. In chronic hepatitis C (HCV), for example, patients often present with listlessness, lack of motivation, sleep and mood disorders, and a global (overall) sense of loss of energy. Fatigue may persist after treatment for the virus has been completed and liver tests and viral load have improved or normalized. This has led to the belief that fatigue is not necessarily a result of severity of liver involvement as measured by abnormal enzymes, nor is it a correlate of viral load. No adequate explanations for HCV-related fatigue have been established, but some reports suggest there are abnormalities of the neuroendocrine pathways associated with regulation of cortisol and the stress response (Swain, 2000; Swain and Maric, 1995). Alterations in circulating cytokine levels also have been implicated in HCV-related fatigue, and the adipokine leptin as well as its secretogogue TNFα have been associated with the severity of fatigue in chronic HCV (Piche *et al.*, 2002). In a recent study, measures of fatigue based on the Fatigue Impact Scale questionnaire were significantly correlated with plasma leptin levels even after adjusting for fat mass (Anty *et al.*, 2011). The same study also found that serum levels of total and

free L-carnitine, an endogenous compound involved in lipid metabolism, were inversely correlated with the severity of fatigue, suggesting that L-carnitine supplementation could alleviate fatigue in patients with chronic liver disease.

Metabolic abnormalities, liver disease, and fatigue certainly appear to be linked. Many patients with metabolic syndrome and NAFLD suffer from fatigue (Newton, 2010; Raszeja-Wyszomirska *et al.*, 2008). A 2010 study investigating the relationship between metabolic syndrome and chronic fatigue syndrome (CFS) found that Americans with CFS are twice as likely to have metabolic syndrome and furthermore that there is a graded relationship between the two diseases, such that the addition of each factor contributing to metabolic syndrome is associated with a 37% increase in the likelihood of having CFS (Maloney *et al.*, 2010). The number of factors contributing to metabolic syndrome also correlates with the severity of fatigue in CFS patients. In NAFLD, fatigue is reported to be a significant problem that is associated with daytime sleepiness and autonomic dysfunction (Newton, 2010). About half of patients with NAFLD have mild cognitive impairment while almost the remaining half have more severe cognitive impairment, characterized by problems with memory and concentration. NAFLD patients also have reduced physical activity (Newton, 2008). Interestingly, NAFLD-related fatigue does not correlate with the degree of liver damage or with insulin resistence, nor is it related to hepatic encephalopathy (Newton, 2010). At the Center for Liver Diseases, fatigue in patients with NASH is often reported as difficulty performing physical activity and in sustaining daily routines. NASH is thought to be the result of persistent fatty infiltration in the liver that progresses to liver fibrosis as a result of inflammation secondary to the

presence of fat. Metabolic problems lead to abnormalities of energy production and are associated with fatty infiltration of mitochondria, relative insulin resistance, glucose intolerance, dyslipidemia and an abnormal inflammatory profile (*e.g.* C-reactive protein, pro-inflammatory cytokines). Thus, the abnormalities of the metabolic and inflammatory profiles may contribute to the fatigue associated with NASH.

Despite fatigue's significance to patients, its mechanisms or biological characteristics have not been fully understood. Studies exploring the quality, intensity and characteristics of fatigue use a variety of objective and self-report measures, whose validation and sensitivity are not fully established. As a result, there is often conflicting data reported about associations with, and causes of fatigue. There has been discussion and increasing acceptance in the literature that fatigue can be defined as central or peripheral. Central fatigue occurs when physical or mental activities are difficult to initiate or sustain; whereas peripheral fatigue describes reduced muscle function resulting from organ or system, but not cerebral, over-activity (Gerber, 2010). Treatments for each type of fatigue differ, therefore it is important to be able to properly distinguish the features of fatigue reported by patients. The discovery of an accurate and objective biomarker panel that could distinguish central and peripheral fatigue and be standardized for use in clinical settings has the potential to greatly improve patients' quality of life, in that fatigue and associated systemic symptoms may be alleviated by targeted interventions (Newton, 2010).

This study investigates the use of serum biomarkers in a population of patients with two common forms of chronic liver disease, NASH and HCV, who frequently have

disabling fatigue. The definitions we propose to use are that central fatigue is the failure to initiate and/or sustain attention intensive tasks and activities requiring self-motivation; and that peripheral fatigue is a decline in physical performance resulting from inadequate cardiorespiratory, muscle metabolic and physiological function. The biomarker panel to be constructed will be multi-dimensional and include both objective and self-report information. Objective parameters will include serum levels of (1) serotonin, a neurotransmitter with many functions including the regulation of mood, sleep, and muscle contraction; (2) pro-inflammatory cytokines tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), and interleukin-8 (IL-8), which are controlled through the hypothalamic-pituitary-adrenal (HPA) axis and are altered in expression in relation to viral infection and chronic fatigue syndrome (Cho *et al.*, 2006; Swain and Maric, 1995); (3) C-peptide insulin, a cleaved portion of proinsulin that remains stable in the bloodstream; (4) AST and ALT, serum markers of liver function; and (5) glucose; and (6) lipids (*e.g.* total cholesterol, HDL). Measures of physical fitness (*e.g.* walk distances) as well as measures of metabolic output based on answers to standardized questionnaires will also contribute to objective measures of fatigue in this study.

2: METHODS AND MATERIALS

A. NASH and NASH-related Fibrosis Biomarker Panel

Study Aim and Resources. The primary goal of this study was to develop a non-invasive, serum-based, diagnostic panel of biomarkers for NAFLD, differentiating NASH and NASH-related fibrosis from steatosis (with and without inflammation) and livers with minimal histological changes. The fibrosis-, adipose-, and apoptosis-related markers were tested in combination with demographic and clinical data for their combinatorial sensitivity and specificity to discern NASH, fibrosis, and advanced fibrosis of the liver. The panel was validated in an independent cohort of patients with NAFLD and cross-compared in the same cohort of patients with a number of known NAFLD and NASH diagnostic panels previously described in the literature.

The study was conducted in collaboration with the Center for Liver Diseases at Fairfax INOVA Hospital (Annandale, VA), which is conducting an ongoing study of NAFLD that began in 2001. Human serum samples have been collected by the Center from histologically-proven patients with NAFLD, and a repository of specimens has been assembled. This repository has been made accessible for research, and includes liver and adipose tissues as well as fasting serum samples gathered at the time of liver biopsy, all codified to retain the anonymity of the donors and stored at -80^{o}C. A subset of liver tissue from each patient was histologically processed and read by a single hepatopathologist,

who used a standardized approach to stage and grade each sample for steatosis, NASH, and fibrosis (see *histopathology*).

Extensive clinical and demographic data were available for all patients. Based on a pre-defined data collection form, each patient's personal and family histories were obtained along with information on drug and alcohol use, and the presence of diabetes mellitus, hypertension, or hyperlipidemia as defined by clinical diagnosis requiring medical therapy. Each patient underwent a physical examination in which height, weight, hip and waist measurements were obtained. Laboratory tests included fasting glucose, serum aminotransferases (AST and ALT), lipid panel, viral serologies (HbsAg and HCV antibody), ceruloplasmin levels, antinuclear antibodies, and iron studies. The data were collected for each patient after obtaining informed consent. Patients with evidence of excessive alcohol use (≥ 10 g/d), other causes of liver disease (*e.g.*, hepatitis B, hepatitis C, autoimmune liver disease) and those receiving treatment with PPAR-γ agonists were excluded. The study protocol was approved by the Institutional Review Board of Fairfax INOVA Hospital.

Candidate Biomarkers. The candidate biomarkers chosen for testing targeted three aspects of the pathology of NAFLD: adipose tissue signaling, cell death, and fibrosis. The biomarkers representing adipose tissue signaling included adiponectin and resistin. Although these proteins have not been extensively tested as biomarkers of NAFLD, they performed well as components of a biomarker panel for NASH alone (Younossi *et al.*, 2008), and it was of interest to see if they would perform equally well in a new cohort of

patients with NAFLD. Given the strong link between obesity and NAFLD and the fact that adipose tissue acts as an endocrine organ, particularly in cases of adiposity, it is reasonable to suspect that signaling from adipose tissue in obese individuals plays a mechanistic role in the development of NAFLD.

Two markers of cell death were included in this study; these were total CK-18 (M65 antigen), reflecting total cell death, and caspase-cleaved CK-18 (M30 antigen), a marker of apoptosis. Both have been successfully used as biomarkers of apoptosis in other studies, including those of liver disease (Olofsson *et al.*, 2007; Yagmur *et al.*, 2007; Younossi *et al.*, 2008). Since subtraction of the serum levels of M30 antigen from serum levels of M65 antigen indicates cell death by necrosis, this subtraction was also included in the development of predictive models for NASH and NASH-related fibrosis. Finally, we included biomarkers of fibrosis; these were specifically, hyaluronic acid (HA), amino-terminal propeptide of type III collagen (P3NP), and TIMP-1 (Rosenberg *et al.*, 2004).

Approach. A number of steps were required to develop, test, and validate a biomarker panel that could be used to differentiate NAFLD patients with NASH and NASH-related fibrosis *vs.* a control group. First, serum from a cohort of NAFLD patients was assessed for concentrations of seven candidate biomarkers. These measurements were then paired with clinical data for the same patient cohort and the entire dataset was subjected to multiple regression analysis in order to develop diagnostic models representing: (1) NASH; (2) any hepatic fibrosis; and (3) advanced hepatic fibrosis. The models were assessed for predictive power by calculating each model's sensitivity, specificity, positive

and negative predictive values, and receiver operating characteristic (ROC) area under the curve (AUC). Finally, the three models were validated by a 10-fold cross-validation procedure, and compared to other published biomarker panels for NAFLD. These methods are outlined in more detail below.

Patient Cohort. A total of 79 patients were included in this study. All but eight patients were morbidly obese (BMI $\geq$ 40); of the remaining eight, seven were obese (BMI $\geq$ 30) and one was overweight (BMI = 26.7). For each of these patients the following demographic and clinical information was available: gender, race, age, height, weight, BMI, presence of Diabetes mellitus type II (henceforce abbreviated as "diabetes"), and blood levels of triglycerides, total cholesterol, glucose, AST, ALT, and the ratio of AST to ALT. Additionally, the diagnosis and stage of NAFLD was available for each patient based on liver biopsy. Table 1 shows the numbers of patients falling into each NAFLD category (defined in *Histopathology*). This table displays a matrix rather than a linear series of counts because there is overlap among patients falling into the NASH and NASH-related fibrosis groups. This overlap is due to the fact that NASH may be diagnosed with or without the presence of fibrosis; and conversely, in one case fibrosis occurred in the absence of NASH but that patient exhibited steatosis and, therefore, remained in the study. Due to the nature of the statistical analysis (specifically, the regression component), outcomes such as NASH or fibrosis must be considered independently; thus, while a patient may be included in the NASH group, that patient also may be included in the fibrosis group if fibrosis was present.

Table 1. Patient cohort for the NASH and NASH-related fibrosis biomarker panel study. Disease categories are defined in *Histopathology*. Numbers in italics show the extent to which disease categories overlapped.

	Total in diseased group		***Total in control group***	
NASH	40	*38 – fibrosis (any)* *2 – no fibrosis*	39	*38 – controls* *1 – fibrosis (mild)*
Any Fibrosis	39	*38 – NASH* *1 – no NASH*	40	*38 – controls* *2 – NASH*
Advanced Fibrosis	16	*all NASH*	63	*38 – controls* *22 – NASH & mild fibrosis* *2 – NASH & no fibrosis* *1 – mild fibrosis & no NASH*

The control group (N = 38) for this study included patients with varying degrees of steatosis with or without inflammation (see *Histopathology*) as well as several who had "minimal non-specific changes in the liver." The control group did not include any individuals with entirely normal liver histology, simply because none were available: the vast majority of patients who donated their tissues to the Center for Liver Disease were obese or morbidly obese and had compelling medical reasons to suspect liver disease, and thus did not exhibit normal liver function.

Two patients were diagnosed with NASH but not fibrosis and were included in the control group for comparisons involving fibrosis; however, it is conceivable that these patients had undetected fibrosis due to sampling error during liver biopsy. Therefore, as a quality control step, I plotted the serum levels of all seven candidate biomarkers grouped by control, NASH only, and any form of fibrosis (Figure 2). The resulting scatterplots

demonstrated that for each of the seven biomarkers, it was fair to put the two NASH-only patients in the control group when testing for fibrosis because their biomarker values fell within the range exhibited by the control group. However, the scatterplots also show that for each biomarker, the range of values exhibited by the fibrosis group partially overlaps the ranges of the NASH and control groups. That observation, together with how few patients presented with NASH alone underscore the importance of fibrosis as a component of NASH and demonstrate that, at least from a biomarker standpoint, NASH and fibrosis are essentially inseparable. Consequently, it is imperative to include markers of fibrosis in the development of biomarker panels aimed at detecting NASH.

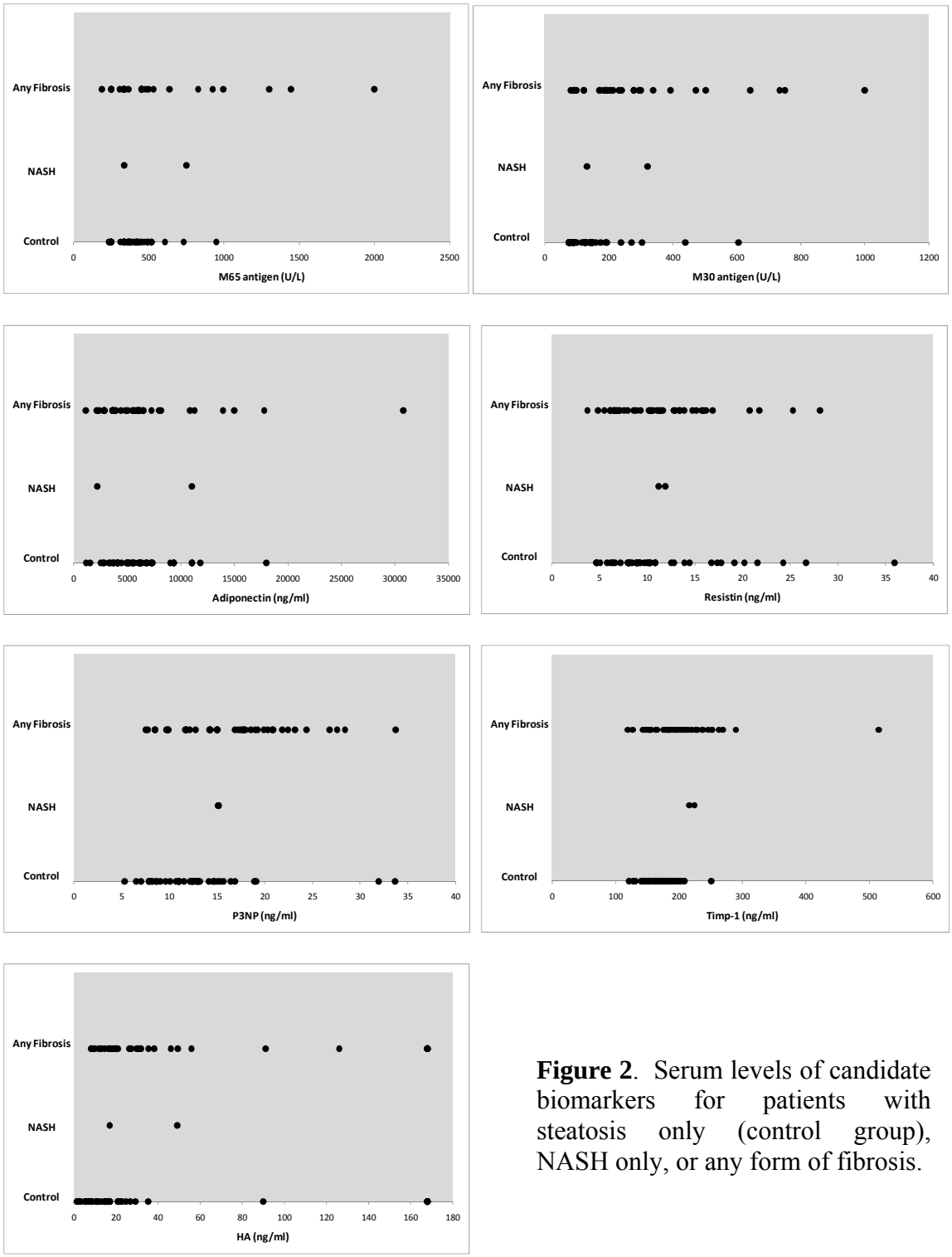

Figure 2. Serum levels of candidate biomarkers for patients with steatosis only (control group), NASH only, or any form of fibrosis.

Histopathology. Each liver biopsy specimen was fixed in formalin, routinely processed for histology, sectioned, and stained with hematoxylin-eosin (H & E) and Masson trichrome. All histological slides were read by a single hepatopathologist, Dr. Zachary Goodman of the Armed Forces Institutes of Pathology (Washington, D.C.), who was blinded to all clinical and demographic data. The slides were reviewed following a predetermined histologic grading system. H & E stained slides were used to determine the extent of steatosis, which was graded as an estimate of the percentage of tissue occupied by fat vacuoles as follows: 0 = none, 1 = <5%, 2 = 6-33%, 3 = 34-66%, 4 = >66%. Other histological features evaluated in H & E sections included portal inflammation, lymphoplasmacytic lobular inflammation, polymorphonuclear lobular inflammation, Kupffer cell hypertrophy, apoptotic bodies, focal parenchymal necrosis, glycogen nuclei, hepatocellular ballooning, and Mallory-Denk bodies. Patients who had hepatic steatosis (with or without non-specific inflammation) or NASH were considered to have NAFLD. NASH was defined as steatosis, lobular inflammation, and ballooning degeneration with or without Mallory Denk bodies, and with or without fibrosis (Bondini, 2007).

Fibrosis was assessed using Masson trichrome stained slides. Portal fibrosis and interlobular pericellular fibrosis were staged as follows: 0 = none, 1 = mild, 2 = moderate, 3 = marked. When present, bridging fibrosis was noted as many or few bridges. Cirrhosis was identified by parenchymal nodules surrounded by fibrous tissue, and was categorized as incomplete or established depending on the degree of loss of acinar architecture. Fibrosis was categorized into two groups: (1) none to minimal

fibrosis, and (2) advanced fibrosis. The "none to minimal fibrosis" group had no or only mild portal or pericellular fibrosis. The "advanced fibrosis" group had at least moderate portal or pericellular fibrosis, bridging fibrosis, or cirrhosis.

Measurement of Serum-based Analytes. Fasting serum glucose levels were measured by glucose oxidase-based kits (Sigma-Aldrich, MO, USA) according to the manufacturer's protocol. Serum levels of selected markers were measured by Enzyme Linked Immunosorbent Assay (ELISA) or Enzyme Immunoassays (EIA), following the manufacturer's protocol in all cases. The overriding principle of each kit was based on sandwich ELISA. The kits were purchased from various manufacturers as follows: adiponectin, resistin and TIMP-1 were measured using "Quantikine" kits from R & D Systems (Minneapolis, MN, USA); HA and P3NP were measured with kits from USCN Life (Wuhan, China); and M65 and M30 antigens were measured with kits from Peviva (Bromma, Sweden).

All samples were codified and aliquoted prior to use in assays, and some were diluted for use in a particular assay when recommended by the corresponding protocol. Dilutions were two-fold, ten-fold, or 100-fold and these were accounted for in the final calculation of analyte concentration. Due to the size of the study cohort (N = 79), two assays were required per analyte since each assay, conducted on a 96-well plate, could be used to assess a maximum of 40 patients. All measurements of samples, standards, controls, and blanks were performed in duplicate. Each plate included a standard curve and a blank,

consisting of the diluent used to dilute the standards (and samples, when diluted). Positive controls were used for quality assurance when provided by the manufacturer.

The absorbance values were measured with a plate reader (ELx800) at 450 nm, and again at 630 nm; absorbance values measured at 630 nm were then subtracted from those at 450 nm to account for absorbance of the plate itself, producing a "wavelength correction." Prior to the wavelength correction, the average blank absorbance value was subtracted from all other absorbance values for both the 450- and 630 nm readings. (Reversing the order of subtractions and performing the wavelength correction first followed by subtracting the average blank value produced identical results.) Calibration (standard) curves were constructed by plotting the net average absorbances of the standards on Y-axis and the concentrations on X-axis; axes were log transformed and curve fitting was (usually but not always) conducted using a 4-parameter or spline formula, as specified by the manufacturer's protocol. Concentrations of analyte in each sample were calculated from the standard curve using Gen5 software. If samples were diluted, the concentrations were multiplied by the dilution factor to calculate the final concentrations. The average concentration was calculated for each duplicate pair of readings and was used in subsequent analyses. The standard deviation and percent coefficient of variation (% CV) also were calculated for each duplicate pair of readings and it was observed that for the vast majority of duplicate readings (~36 of every 40 pairs) in each assay, the % CV was below 10%.

Data Analysis. The entire study cohort was divided into sub-cohorts according to the following diagnostic comparisons: (1) those with NASH were compared to those without NASH; (2) those with any hepatic fibrosis were compared to those with no fibrosis; and (3) those with advanced hepatic fibrosis were compared to those with minimal to no fibrosis. Each of the comparisons was further analyzed separately. Specifically, for each of the sub-cohorts within each comparison, the demographic, clinical, and laboratory data were analyzed by calculating the means and standard deviations for all continuous variables, and counts and percentages for all categorical variables. Then, two-sample statistical tests were performed for each parameter in each comparison. To choose an appropriate statistical test, all continuous variables were tested for normality by the Shapiro Wilk test; a p-value ≤ 0.05 was considered significant and thus indicative that the data came from a non-normally distributed population. For normally distributed data comparisons between groups were made by two-tailed, two-sample t-test assuming separate (unpooled) variances, and for non-normally distributed data comparisons between groups were made by Mann-Whitney U (Wilcoxon rank sum) test. For categorical variables, group comparisons were made using the Pearson chi-square test for homogeneity except in cases where at least one cell count was < 5; in those cases a Fisher's exact test was performed. A p-value ≤ 0.05 was considered significant in all cases.

In collaboration with the Fairfax INOVA Hospital statistician, Dr. Maria Stepanova, multiple linear regression with stepwise, bidirectional selection was used to develop predictive models for the occurrence of NASH, hepatic fibrosis, and advanced hepatic

fibrosis. This procedure starts with a complete model containing all the predictors potentially associated with a dependent variable, and stops when no more improvement in data fitting is possible with the addition or removal of any predictor. Here, the predictors were the demographic, clinical, and laboratory parameters, and the dependent variable was the diagnostic outcome (*e.g.*, NASH). To meet the applicability criteria for regression modeling, a data transformation for non-normally distributed variables was used. At each step of model design, including variable selection and model training, the resulting regression models were the ones with the highest coefficient of determination (R^2 value). This maximized the portion of variability that could be accounted for by the models and the likelihood that future outcomes would be correctly predicted by the models.

The stability of the variable selection process and model design were verified at each step in a series of tenfold cross-validation (10-CV) experiments. In those experiments, the complete cohort was partitioned into ten subsamples, and of the ten subsamples, a single subsample was retained as the validation data for testing the model, and the remaining nine samples were used as training data. Using the predictors obtained from the model with the highest R^2 value, multiple regression was run on the training set to generate new beta-values, and the betas were then used on the validation set to calculate diagnostic outcomes (*e.g.*, NASH). The outcomes were then compared against the true diagnoses. This process was repeated ten times, with each of the ten subsamples used exactly once as the validation data. The predictive power of the developed models, namely the sensitivity, specificity, positive and negative predictive values, and the area under the

ROC-curve (AUC) with 95% confidence intervals were measured for each resulting model after completion of the 10-CV procedure.

Parametric and non-parametric tests as well as tests of normality were performed using Mystat: a Student Version of Systat (v.12). Chi square- and Fisher's Exact tests were performed using S-Plus (v.8). Multiple regression and the 10-CV experiments were executed with Matlab R2007a (The MathWorks, Natick, MA) software, while ROC curves and measures of predictive power were generated using MedCalc (http://www.medcalc.be/).

Comparison with Previously Reported NASH and Fibrosis Biomarker Panels. For validation purposes, and in collaboration with Dr. Stepanova, the biomarker panel developed in this study was compared to previously reported, non-invasive biomarker panels that have been used to detect NASH or fibrosis from blood samples. This validation step was performed by running the previously developed biomarker panels on our study cohort and comparing their actual, cohort-specific measures of performance (*e.g.*, specificity, sensitivity) with those achieved by our panel. For the prediction of NASH specifically, the panels chosen included NASH Diagnostics®, previously developed in our laboratory (Younossi et al., 2008) and the Apoptosis Biomarker, consisting of the level of cleaved cytokeratin CK-18 (M30 antigen) (Wieckowska *et al.*, 2006). The three different combinations of the β-coefficients and thresholds for distinction between the presence and absence of NASH were selected so that the ruling-in threshold corresponded to a sensitivity of at least 90%, the ruling-out threshold had a

specificity of at least 90%, and the optimal threshold was selected such that the Euclidian distance from the 100% accuracy point on the ROC-curve was minimized. For the prediction of any fibrosis and advanced fibrosis, the following previously reported panels were tested: ELF and OELF (Rosenberg *et al.*, 2004), NAFLD Fibrosis Score (Angulo *et al.*, 2007), the combined ELF + NAFLD Fibrosis Score (Guha *et al.*, 2009), and the APRI model (Cales *et al.*, 2009; Wai *et al.*, 2003). The coefficients for the regression models and the thresholds for ruling-in and ruling-out any fibrosis, advanced fibrosis, and NASH were those provided by the authors of the models. All models were tested on the study cohort by cross validation (as previously described) and p-values were recorded. Sensitivity, specificity, and positive and negative predictive values were calculated for each of the given thresholds. Additionally, ROC curves were plotted and AUC values were estimated for each of the models.

B. Novel Biomarkers of NAFLD Reflecting the Role of Adipose Tissue

Study Aims and Approach. Due to the strong correlation between the occurrence of NAFLD and the amount of visceral body fat (Jiang *et al.*, 2010) it was of interest to investigate the prospect that deregulated cell signaling in adipose tissue could result in imbalances in secreted molecules that in turn contribute to the pathology of NAFLD. If such molecules were identified, they might serve as accurate biomarkers for the occurrence of NASH and NASH-related fibrosis. With those ideas in mind, the specific aims of this study were as follows: (1) to use the phosphoproteomic data set (Younossi *et al.*, 2010) in an enrichment analysis to identify which signaling pathways were most

represented by the collection of phosphoproteins, and specifically which pathways and constituent components were most likely responsible for differential levels of adipocyte signaling between patients with and without NASH and NASH-related fibrosis; (2) to identify a collection of secreted molecules closely associated with the pathways of interest and from those, to choose two novel candidate biomolecules to be tested as biomarkers of NASH and NASH-related fibrosis; (3) to measure serum levels of these two candidate molecules by ELISA in a cohort of NAFLD patients that represent a subset of the cohort used in Younossi *et al.* (2010); and (4) to investigate whether diagnostic groups are significantly different in their levels of the candidate molecules and whether serum levels of the candidate molecules, in combination with demographic and clinical data, are useful in predictive models of NASH and NASH-related fibrosis.

The resources available for this study were as described in Section A for the NASH and NASH-related Biomarker Panel study. Briefly, the study was conducted in collaboration with the Center for Liver Diseases at Fairfax INOVA Hospital (Annandale, VA) and was approved by the Institutional Review Board of Fairfax INOVA Hospital. Study subjects participated with informed consent and donated serum, liver and adipose tissues as part of the ongoing study of NAFLD. These patients had histologically-proven NAFLD as interpreted by a single hepatopathologist and did not exhibit other signs of liver disease or excessive alcohol use, and did not use pharmaceuticals that would interfere with studies of liver disease. Demographic and clinical data were collected for each patient, as outlined in Section A.

Patient Cohort. A total of 39 patients were initially included in the study; however two were discovered to have fibrosis in absence of steatosis or NASH and therefore may not have belonged in the NAFLD cohort, and were omitted from further analysis. Of the remaining 37 patients, most (N = 31) were morbidly obese (BMI ≥ 40), five were obese (BMI ≥ 30), and one was overweight (BMI = 28.2). For this study, detailed histological information was available for each patient including (1) stage of portal and pericellular fibrosis; (2) grade of inflammation including portal inflammation, lobular lymphoplasmocytic inflammation, and lobular Kuppfer cell hypertrophy; and (3) grade of ballooning degeneration. The diagnostic data available for this cohort included the diagnoses of NASH and NASH-related fibrosis. Additionally, I created an "advanced fibrosis" category for instances where either portal or pericellular fibrosis exceeded stage 1. The demographic and clinical data associated with this patient cohort included gender, race, age, BMI, presence of Diabetes mellitus type II (henceforth abbreviated as "diabetes"), hypertension, or hyperlipidemia, platelet and white blood cell counts, and serum levels of AST, ALT, albumin, bilirubin, hemoglobin, glucose, triglycerides, total cholesterol, and high-density lipoprotein (HDL).

Table 2 shows the numbers of subjects in each diagnostic category (for definitions see *Histopathology*, Section A). As in the previous study there was overlap among patients falling into diagnostic groups. All patients with NASH had at least mild fibrosis in the form of portal or pericellular fibrosis, and six patients had both portal and pericellular fibrosis. Ten "control" patients without NASH were diagnosed with fibrosis, however in all cases the fibrosis was portal in nature and arguably not as characteristic of NASH as is

pericellular fibrosis, which is associated with NASH in adults (Hall and Kirsch, 2005). A large proportion (86%) of the study cohort had some form of fibrosis; of those, twelve had pericellular fibrosis and the remainder had portal fibrosis. In the advanced fibrosis group, two patients had stage 2 pericellular fibrosis and four had stage 2 portal fibrosis. Only three of the six members of the advanced fibrosis group had NASH; those without NASH had portal fibrosis. The remaining control group for the study comprised five patients with low grade (1 or 2) steatosis only. All patients in the study cohort had some degree of steatosis, however. Additionally, all but one had some degree of lobular inflammation, and none had bridging fibrosis or cirrhosis.

Table 2. Patient cohort for the study of novel adipose-related biomarkers. Disease categories are defined in *Histopathology* (Section A). Numbers in italics show the extent to which disease categories overlapped.

	Total in diseased group		***Total in control group***	
NASH	22	*22 – fibrosis (any)* *0 – no fibrosis*	15	*10 – fibrosis (any)* *5 – steatosis only*
Any Fibrosis	32	*22 – NASH* *10 – no NASH*	5	*5 – steatosis only* *0 – NASH*
Advanced Fibrosis	6	*3 – NASH* *3 – no NASH*	31	*19 – NASH & mild fibrosis* *7 – mild fibrosis, no NASH* *5 – steatosis only*

Histopathology. The histological examinations of liver specimens as well as diagnoses and staging of NAFLD for this study were as described in Section A, as the samples used

in this study were drawn from the Center for Liver Disease's repository for the ongoing study of NAFLD.

Pathway Analysis. To identify secreted molecules that may be released by adipose tissue and tested as serum-based biomarkers for NASH and NASH-related fibrosis, enrichment analyses coupled with group statistical comparison of phosphoprotein expression in adipose tissue between patients with and without NASH and fibrosis was conducted. Two software packages, MetaCore software (GeneGo, Inc.) and Pathway Studio software (Ariadne Genomics), were used to determine (1) which signaling pathways were most significantly represented by the collection of phosphorylated proteins tested in the publication by Younossi *et al.* (2010); (2) which components of those pathways were most responsible for potential differences in adipose tissue signaling between patients with and without NASH; and (3) to identify a collection of secreted molecules that could be linked to the pathways identified in step 1, with particular regard to the phosphoproteins that were significant predictors in models predicting NASH and NASH-related fibrosis. Because this analysis was feedback-driven and therefore somewhat iterative, details on how the analysis was conducted are given in Chapter 4 along with the corresponding results.

ELISAs. Serum levels of CCL-2 were measured by the Human CCL2/MCP-1 Immunoassay Quantikine ELISA kit, while serum concentrations of sFasL were assessed using the Human Fas Ligand/TNFSF6 Quantikine ELISA kit; both from R & D Systems

(Minneapolis, MN). All measurements were performed in duplicate, and absorbance readings at 450 nm were made using an ELx800 plate reader. No samples were diluted prior to performance of an assay, and the manufacturer's instructions were followed. Other quality control steps (*e.g.* wavelength correction) as well as the calculation of standard curves were as performed as previously described.

Statistical Analyses. The statistical approach for this study followed that used in the previous study; however, cross validation and ROC curve analysis were not performed. Briefly, the entire study cohort was divided into sub-cohorts according to the following diagnostic comparisons: (1) those with NASH were compared to those without NASH; (2) those with any hepatic fibrosis were compared to those with no fibrosis; and (3) those with advanced hepatic fibrosis were compared to with minimal to no fibrosis. Within each sub-cohort, group statistical comparisons were conducted for all demographic, clinical, laboratory, and histological variables. To choose an appropriate statistical test, all continuous variables were tested for normality by the Shapiro Wilk test; a p-value $\leq$ 0.05 was considered significant and thus indicative that the data came from a non-normally distributed population. For normally distributed data comparisons between groups were made by two-tailed, two-sample t-test assuming separate (unpooled) variances, and for non-normally distributed data comparisons between groups were made by Mann-Whitney U (Wilcoxon rank sum) test. For categorical variables, group comparisons were made using the Pearson chi-square test for homogeneity except in

cases where at least one cell count was < 5; in those cases a Fisher's exact test was performed. No statistical test was performed in cases where a cell count was 0.

In addition to group comparisons, multiple linear regression using stepwise bidirectional selection was performed to identify predictive models for the diagnostic outcomes of NASH, any hepatic fibrosis, and advanced hepatic fibrosis. In these analyses, it was not possible to use all 26 variables in the data set as potential predictors in the models because the study cohort consisted of just 37 patients, and testing 26 predictors would have increased the risk of "over-fitting" the models to the data set. Consequently, the number of variables was reduced to 15 (representing less than half the size of the study cohort) and each outcome was tested with the following set of predictors: gender, race, age, BMI, diabetes, hyperlipidemia, AST, ALT, total bilirubin, glucose, total cholesterol, triglycerides, HDL, CCL-2, and FasL. Omitted from the regression analyses were the histological parameters (*e.g.* pericellular- and portal fibrosis, portal- and lobular inflammation, etc.) since the goal is to develop a serum-based biomarker panel; albumin, white blood cell count, platelet count, and hemoglobin since these measures are not specific to liver function or to Metabolic Syndrome; and hypertension, because data were missing for some patients and consequently these patients would have been excluded from the entire regression analysis by the statistical software.

A p-value ≤ 0.05 was considered significant for all tests. Parametric and non-parametric tests as well as tests of normality were performed using Mystat: a Student

Version of Systat (v.12). Chi square- and Fisher's Exact tests, as well as multiple linear regression were performed using S-Plus (v.8).

C. Biomarkers of Chronic and Peripheral Fatigue in Patients with Chronic Liver Disease.

Study Aims. Fatigue is a common symptom of chronic liver diseases, including non-alcoholic fatty liver disease (NAFLD) and chronic hepatitis C (HCV). The aim of this study was to determine whether, in patients with chronic liver disease, there is a correlation between self-reports of physical activity-associated fatigue (peripheral fatigue) or more global lack of energy and motivation (central fatigue) with serum levels of serotonin and markers of inflammation, or with abnormalities of glucose and lipid metabolism.

Patient Cohort. A total of 31 patients participated in the study, with informed consent. All patients had chronic liver disease in the form of biopsy-proven NAFLD or HCV with viremia, and were untreated. Specifically, 10 and 20 patients were diagnosed with NAFLD or HCV, respectively, and an additional patient had both diseases. Ten of the patients were diagnosed with Metabolic Syndrome, not all of whom had NAFLD. The study cohort had the following demographic and clinical characteristics: age 52.5 ±6.8 years, 66.7% male, BMI 32.4 ± 5.5, 26.7% with Diabetes Mellitus, and 0% with cirrhosis. Fasting blood samples were obtained and assessed for levels of selected cytokines and hormones (IL-6, IL-8, TNF- α, serotonin, and C-peptide insulin), liver

enzymes (AST, ALT), glucose, and lipids (triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol, and the non-HDL fraction of cholesterol). Five patients returned for follow-up visits and the serum from those visits were included in study.

Measures of Fatigue. Self-reports of central fatigue included standardized and valid measures of depression (short form of CES-D), vitality/energy (vitality subscale of the SF36; transformed to a scale of 0 to 100) and level of activity (Human Activity Profile). Maximum activity scores (MAS) from the Human Activity Profile are mathematically related to Metabolic Equivalent of Task (MET), a calculation relating physical activity to metabolic rate. For this study, MAS values were converted back to MET values to provide a more objective measure of physical exertion.

For peripheral fatigue, patients were first divided into tertiles by MET value. The middle third was omitted from further analysis; the remaining top third (those with MET >8.8, representing strenuous activity) and bottom third (those with MET <7.5 representing less strenuous activity) became "high MET" (*i.e.* not peripherally fatigued) and "low MET" (peripherally fatigued) groups; respectively. Other variables used as measures of peripheral fatigue included (1) distance covered within six minutes of walking; and (2) Borg scale value. The Borg scale is a standardized, self-report method for rating perceived physical exertion.

A patient was defined as having central fatigue if they had a CES-D score >7 combined with a SF-36 vitality index score <45; patients meeting only one of these criteria or neither were classified as not having central fatigue. The CES-D score is

designed to measure depression and is based on a standardized, self-report form in which symptoms of depression are listed. The vitality scoring system within the SF-36 form is designed to measure perceived, overall (global) fatigue; *e.g.*, feeling "worn out" and "tired."

ELISAs. Serum levels of IL-6, IL-8, and TNF-α were measured by the Human IL-6 Immunoassay Quantikine ELISA kit, the Human CXCL8/IL-8 Immunoassay Quantikine ELISA kit, and the Human TNF- α Immunoassay Quantikine ELISA kit, respectively; all from R & D Systems (Minneapolis, MN). Serum levels of serotonin and C-peptide insulin were quantified using the Serotonin ELISA and C-peptide ELISA kits from Alpco (Salem, NH). Serum lipid profiles (including glucose concentrations) and liver function tests (*i.e.* ALT, AST, and AST:ALT) were conducted using the Cholestech LDX system and associated test cassettes (Inverness Medical, Hayward,CA). In ELISA assays, all measurements were performed in duplicate, and absorbance readings at 450 nm were made using an ELx800 plate reader. No samples were diluted prior to performance of an assay, and the manufacturer's instructions were followed. Other quality control steps (*e.g.* wavelength correction) as well as the calculation of standard curves were as performed as described in the study of the NASH and NASH-related Fibrosis Biomarker Panel.

Statistical Analysis. Tests for normality were conducted on all serum-based variables using the Shapiro Wilk test; all variables were non-normally distributed except for LDL cholesterol. Consequently, for group comparisons involving LDL cholesterol the two-

sample, two-tailed student t-test assuming separate variances was used, and correlations were assessed by Pearson correlation coefficient. For all other variables, group comparisons were made by Mann-Whitney U or Kruskal Wallis test, and correlations were assessed by Spearman Rho. Medians and quartiles, or means and standard deviations are provided for non-normally distributed- and normally distributed variables, respectively. Statistical tests were performed using Mystat: a Student Version of Systat (v.12); however, since this software does not provide p-values for Spearman coefficients, Spearman rho correlations and associated p-values were computed using an on-line calculator (http://faculty.vassar.edu/lowry/VassarStats.html). The criterion for significance for all statistical tests was 0.05.

Results.

Predictors of NASH. Table 3 shows all demographic, clinical, and laboratory data and the outcomes of group statistical comparisons for patients with and without NASH. Not surprsingly, liver fibrosis was overwhelmingly more prevalent in NASH patients than those without NASH; namely, 95% of patients with NASH had some degree of fibrosis and 40% had advanced fibrosis. One patient in the non-NASH group had stage 1 pericellular fibrosis (*i.e.*, mild fibrosis). The clinical attributes of the NASH group were in accordance with known risk factors for NAFLD; The occurrence of diabetes ($p = 0.035$), weight ($p = 0.009$), and AST- and ALT levels ($p = 0.002$ and 0.007, respectively) were significantly higher in the NASH group as compared to non-NASH cohort. Furthermore, NASH patients had slightly higher BMI than non-NASH patients but not significantly ($p = 0.06$). The NASH group predominantly consisted of males ($p = 0.004$). NASH patients also had significanly lower total cholesterol levels ($p = 0.041$) relative to the non-NASH group. The NASH group also was significantly taller ($p = 0.037$), most likely due to male prevalence. Regarding the parameters reflecting adipose tissue signaling and fibrosis, NASH patients had significantly higher serum levels of M30 antigen ($p < 0.001$), TIMP-1 ($p < 0.001$), P3NP ($p < 0.001$), and HA ($p = 0.001$) than non-NASH patients.

Table 3. Demographic, clinical, and laboratory data for patients with and without NASH. Entries are counts for discrete measures (with percentage of group total given in parentheses) or mean ± S.D. for continuous measures. A p-value of ≤ 0.05 was considered significant. Significant results are shown in bold text. Chi = chi square test of homogeneity; FE = Fisher's exact test; MW = Mann-Whitney U test; 2T = two-sample t-test (2-tailed).

	NASH	***no NASH***	***P-value***	***Test***
N	40	39		
Fibrosis	38 (95%)	1 (3%)	**< 0.001**	FE
Advanced fibrosis	16 (40%)	0 (0%)	**< 0.001**	FE
Diabetes	14 (36%)	5 (13%)	**0.035**	Chi
Female	25 (63%)	36 (92%)	**0.004**	Chi
Caucasian	28 (72%)	26 (67%)	0.806	Chi
Age	44 ± 10	41 ± 10	0.137	MW
Height (cm)	170 ± 10	166 ± 8	**0.037**	2T
Weight (kg)	141 ± 31	127 ± 24	**0.009**	MW
BMI	49 ± 9	46 ± 6	0.060	MW
AST (U/L)	33 ± 24	21 ± 9	**0.002**	MW
ALT (U/L)	43 ± 22	30 ± 21	**0.007**	MW
AST/ALT	0.81 ± 0.22	0.88 ± 0.35	0.553	MW
Triglycerides (mg/dL)	174 ± 96	180 ± 88	0.604	MW
Total cholesterol (mg/dL)	188 ± 33	204 ± 36	**0.041**	2T
Glucose (mg/dL)	121 ± 46	106 ± 31	0.388	MW
Adiponectin (ng/mL)	6644 ± 5420	6709 ± 5145	0.538	MW
M30 (U/L)	295 ± 219	174 ± 174	**< 0.001**	MW
M65 (U/L)	555 ± 406	388 ± 145	0.127	MW
M65-M30 (U/L)	272 ± 283	257 ± 164	0.651	MW
Resistin (ng/mL)	11.5 ± 5.4	12.1 ± 6.7	0.988	MW
TIMP-1 (ng/mL)	206 ± 64	168 ± 24.7	**< 0.001**	MW
P3NP (ng/mL)	17.1 ± 6.0	12.9 ± 5.8	**< 0.001**	MW
HA (ng/mL)	76 ± 68	41 ± 58	**0.001**	MW

By multiple regression the following predictive ($p = 9.1e^{-06}$) model for NASH was identified: probability of NASH = -0.0298 + (0.3103)[diabetes] + (-0.3224)[gender] + (0.0120)[BMI] + (-0.0012)[triglycerides] + (0.0004)[M30] + (0.0007)[M65-M30].

Diabetes and gender are categorical variables and entered as follows: diabetes, 1 = yes, 0 = no; gender, 1 = female, 0 = male. The standard deviation and p-value associated with each beta value are shown in Table 4.

Table 4. Model for the prediction of NASH.

	β-value	**± *S.D.***	***p-value***
(Intercept)	-0.0298	0.3567	0.9337
Diabetes	0.3103	0.1157	0.0093
Gender	-0.3224	0.1187	0.0084
BMI	0.0120	0.0063	0.0613
Triglycerides (mg/dL)	-0.0012	0.0006	0.0508
M30 antigen (U/L)	0.0004	0.0002	0.0835
M65 – M30 (U/L)	0.0007	0.0003	0.0096

Using an optimized threshold (*i.e.*, the one maximizing sensitivity and specificity) of >0.3641, this model has a sensitivity of 79% (*95% C.I.* 62 – 91%), specificity of 74% (*95% C.I.* 57 – 87%) positive predictive value (PPV) of 73% (*95% C.I.* 56 – 86%), and negative predictive value (NPV) of 80% (*95% C.I.* 63 – 92%). The AUC value for the associated ROC curve is 0.809 (*95% C.I.* 0.699 – 0.892). The ROC curve is shown in Figure 3.

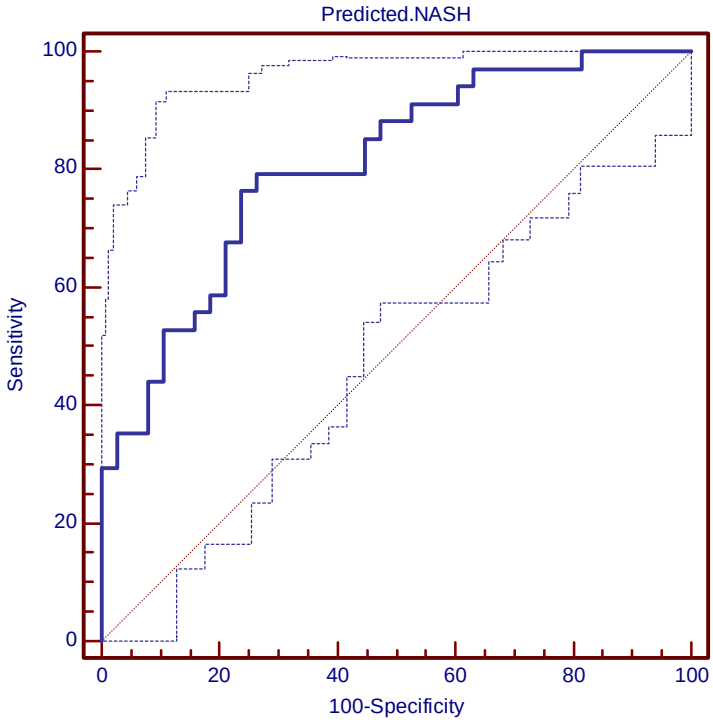

Figure 3. ROC curve for the model predicting NASH. The solid line indicates the collection of thresholds and their associated sensitivities and specificities, dashed lines represent 95% confidence intervals, and the linear, dotted line represents what would be observed at random (*i.e.* if the model had no predictive power).

To illustrate the importance of including serum proteins in the predictive model of NASH, multiple linear regression was repeated using only the clinical variables available in this study. A predictive model for NASH emerged as follows: NASH = –2.674 + 0.4312[diabetes] + 0.0169[height] + 0.0122[BMI] + 0.0065[AST] – 0.0028[Total cholesterol]. Although this model was significant (p = 0.0005) its overall performance when validated on an independent cohort of NASH patients was poor, with a sensitivity

of 43%, specificity of 79%, PPV and NPV of 75% and 48%, respectively, and an AUC of 0.541. The ROC curve for this model is shown in the Figure 4.

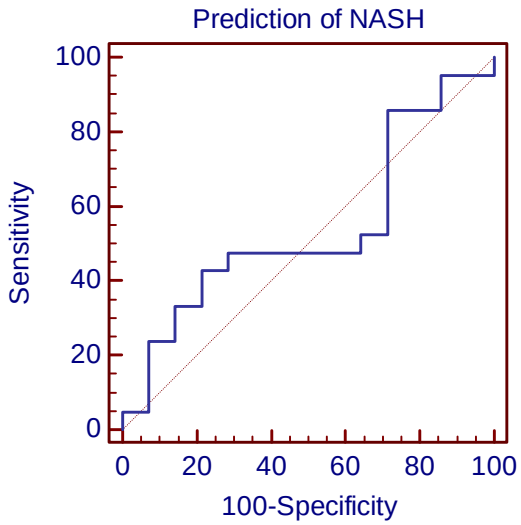

Figure 4. ROC curve for the model predicting NASH based on clinical variables alone.

Predictors of Fibrosis. Table 5 shows all demographic, clinical, and laboratory data and the outcomes of groupwise statistical comparisons for groups of patients with and without any hepatic fibrosis. The vast majority (97%) of patients with liver fibrosis also were diagnosed with NASH. Forty-one percent of patients with fibrosis had advanced fibrosis. As in the NASH comparison, the fibrosis group was more likely to be male ($p = 0.013$) and had significantly lower total cholesterol levels than the no fibrosis group ($p = 0.009$). In terms of risk factors for NAFLD, the fibrosis group had significantly higher weight ($p = 0.018$), AST- and ALT serum levels ($p = 0.005$ and 0.006, respectively), and were nearly significantly higher in BMI ($p = 0.051$). In terms of adipose signaling and markers

of fibrosis, patients with fibrosis had significantly higher levels of M30 antigen ($p < 0.001$), TIMP-1 ($p = 0.002$), P3NP ($p = 0.002$), and HA ($p = 0.001$).

Table 5. Demographic, clinical, and laboratory data for groups of patients with and without any hepatic fibrosis. Entries are counts for discrete measures (with percentage of group total given in parentheses) or mean ± S.D. for continuous measures. A p-value of ≤ 0.05 was considered significant. Significant results are shown in bold text. Chi = chi square test of homogeneity; FE = Fisher's exact test; MW = Mann-Whitney U test; 2T = two-sample t-test (2-tailed).

	Fibrosis	***no Fibrosis***	***P-value***	***Test***
N	39	40		
NASH	38 (97%)	2 (5%)	**< 0.001**	FE
Advanced fibrosis	16 (41%)	0 (0%)		
Diabetes	13 (34%)	6 (15%)	0.087	Chi
Female	25 (64%)	36 (90%)	**0.013**	Chi
Caucasian	27 (71%)	27 (68%)	0.925	Chi
Age	44 ± 10	41 ± 10	0.124	MW
Height (cm)	170 ± 10	170 ± 8	0.069	2T
Weight (kg)	141 ± 31	128 ± 24	**0.018**	MW
BMI	49 ± 9	46 ± 7	0.051	MW
AST (U/L)	32 ± 25	22 ± 10	**0.005**	MW
ALT (U/L)	44 ± 32	29 ± 21	**0.006**	MW
AST/ALT	0.79 ± 0.21	0.90 ± 0.35	0.242	MW
Triglycerides (mg/dL)	171 ± 97	183 ± 88	0.299	MW
Total cholesterol (mg/dL)	186 ± 31	206 ± 36	**0.009**	2T
Glucose (mg/dL)	120 ± 47	108 ± 30	0.744	MW
Adiponectin (ng/mL)	6646 ± 5470	6703 ± 5110	0.578	MW
M30 (U/L)	299 ± 225	176 ± 171	**< 0.001**	MW
M65 (U/L)	556 ± 417	396 ± 152	0.181	MW
M65-M30 (U/L)	269 ± 281	261 ± 176	0.610	MW
Resistin (ng/mL)	11.6 ± 5.5	12.0 ± 6.6	0.957	MW
TIMP-1 (ng/mL)	205 ± 65	170 ± 27	**0.002**	MW
P3NP (ng/mL)	17.0 ± 6.2	13.1 ± 5.7	**0.002**	MW
HA (ng/mL)	77 ± 69	41 ± 57	**0.001**	MW

Analysis of the data set by multiple linear regression revealed that the following model was significantly (p = 0.00013) predictive of any hepatic fibrosis: probability of Fibrosis = -0.0417 + (0.2593)[diabetes] + (-0.2714)[gender] + (0.0123)[BMI] + (-0.0014)[triglycerides] + (0.0004)[M30 antigen] + (0.0006)[M65 – M30]. Diabetes and gender are categorical variables and entered as follows: diabetes, 1 = yes, 0 = no; gender, 1 = female, 0 = male. The standard deviation and p-value associated with each beta value are shown in Table 6.

Table 6. Model for the prediction of any hepatic fibrosis.

	β-value	***± S.D.***	***p-value***
(Intercept)	-0.0417	0.3728	0.9113
Diabetes	0.2593	0.1209	0.0357
Gender	-0.2714	0.1240	0.0323
BMI	0.0123	0.0066	0.0662
Triglycerides (mg/dL)	-0.0014	0.0006	0.0347
M30 antigen (U/L)	0.0004	0.0002	0.0918
M65 – M30 (U/L)	0.0006	0.0003	0.0257

Using an optimized threshold of >0.5689, this model has a sensitivity of 52% (*95% C.I.* 34 – 69%), specificity of 90% (*95% C.I.* 76 – 97%) positive predictive value of 81% (*95% C.I.* 58 – 94%) and negative predictive value of 69% (*95% C.I.* 54 – 81%). The AUC value for the associated ROC curve is 0.796 (*95% C.I.* 0.676 – 0.883). The ROC curve is shown in Figure 5.

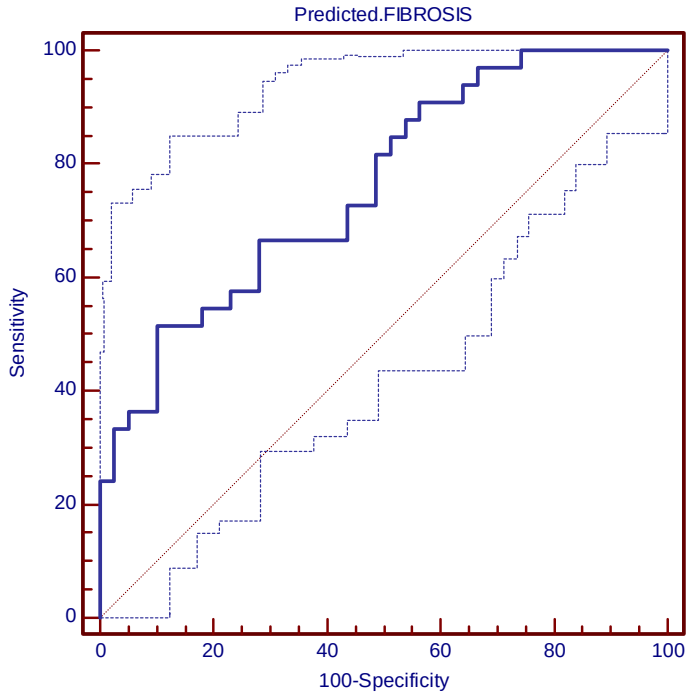

Figure 5. ROC curve for the model predicting any hepatic fibrosis. The solid line indicates the collection of thresholds and their associated sensitivities and specificities, dashed lines represent 95% confidence intervals, and the linear, dotted line represents what would be observed at random (*i.e.*, if the model had no predictive power).

To illustrate the importance of including serum proteins in the predictive model of fibrosis, multiple linear regression was repeated using only the clinical variables available in this study. A predictive model for fibrosis emerged as follows: Fibrosis = 0.1757 + 0.3182[diabetes] + 0.0056[weight] + 0.0061[AST] – 0.0037[Total cholesterol]. Although this model was significant (p = 0.0009) its overall performance when validated on an independent cohort of NASH patients was modest, with a sensitivity of 74%, specificity

of 80%, PPV and NPV of 96% and 33%, respectively, and an AUC of 0.703. The ROC curve for this model is shown in Figure 6.

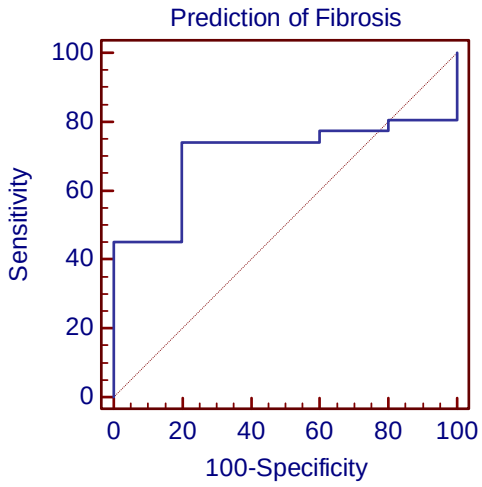

Figure 6. ROC curve for the model predicting fibrosis based on clinical variables alone.

Predictors of Advanced Fibrosis. Table 7 shows all demographic, clinical, and laboratory data and the outcomes of group statistical comparisons for patients with and without advanced fibrosis. All of the patients with advanced fibrosis had NASH, whereas only 38% of the patients with mild or no fibrosis were diagnosed with NASH, a significant difference between the groups ($p < 0.001$). No demographic factors were significantly different between patients with advanced fibrosis and those without it; however, AST levels were significantly higher in patients with advanced fibrosis ($p = 0.008$), and these patients were relatively higher in BMI ($p = 0.075$). In terms of secreted molecules, M30

antigen and TIMP-1 were significantly higher in the advanced fibrosis group (p = 0.003 and 0.001, respectively).

Table 7. Demographic, clinical, and laboratory data for patients with and without advanced fibrosis. Entries are counts for discrete measures (with percentage of group total given in parentheses) or mean ± S.D. for continuous measures. A p-value of ≤ 0.05 was considered significant. Significant results are shown in bold text. Chi = chi square test of homogeneity; MW = Mann-Whitney U test; 2T = two-sample t-test (2-tailed).

	Advanced Fibrosis	**mild or *no* *Fibrosis***	***P-value***	***Test***
N	16	63		
NASH	16 (100%)	24 (38%)	**< 0.001**	Chi
Fibrosis	16 (100%)	23 (37%)		
Diabetes	6 (38%)	13 (21%)	0.295	Chi
Female	11 (69%)	50 (79%)	0.569	Chi
Caucasian	14 (88%)	40 (65%)	0.141	Chi
Age	44 ± 12	42 ± 10	0.541	MW
Height (cm)	169 ± 8	167 ± 9	0.609	2T
Weight (kg)	141 ± 26	132 ± 29	0.142	MW
BMI	51 ± 9	47 ± 8	0.075	MW
AST (U/L)	39 ± 31	24 ± 14	**0.008**	MW
ALT (U/L)	47 ± 37	34 ± 25	0.102	MW
AST/ALT	0.88 ± 25	0.83 ± 0.3	0.423	MW
Triglycerides (mg/dL)	139 ± 36	187 ± 99	0.086	MW
Total cholesterol (mg/dL)	197 ± 35	196 ± 35	0.957	2T
Glucose (mg/dL)	109 ± 40	114 ± 39	0.315	MW
Adiponectin (ng/mL)	8101 ± 7414	6302 ± 4528	0.611	MW
M30 (U/L)	319 ± 225	210 ± 196	**0.003**	MW
M65 (U/L)	685 ± 567	421 ± 203	0.356	MW
M65-M30 (U/L)	305 ± 337	256 ± 204	0.946	MW
Resistin (ng/mL)	12.6 ± 5.4	11.6 ± 6.2	0.297	MW
TIMP-1 (ng/mL)	227 ± 84	177 ± 35	**0.001**	MW
P3NP (ng/mL)	16.0 ± 5.2	14.8 ± 6.5	0.235	MW
HA (ng/mL)	78 ± 73	54 ± 63	0.155	MW

By multiple regression the following significant ($p = 6.2e^{-05}$) model for advanced fibrosis was identified: probability of Advanced Fibrosis = -0.1760 + (0.2189)[diabetes] + (-0.0014)[triglycerides] + (0.0065)[AST] + (0.0022)[TIMP-1]. Diabetes is a categorical variable and entered as follows: 1 = yes, 0 = no. The standard deviation and p-value associated with each beta value are shown in Table 8.

Table 8. Model for the prediction of advanced fibrosis.

	β-value	***± S.D.***	***p-value***
(Intercept)	-0.1760	0.1765	0.3225
Diabetes	0.2189	0.0996	0.0316
Triglycerides	-0.0014	0.0005	0.0084
AST	0.0065	0.0027	0.0177
TIMP-1	0.0022	0.0009	0.016

Using an optimized threshold of >0.2442, this model has a sensitivity of 87% (*95% C.I.* 60 – 98%), specificity of 70% (*95% C.I.* 56 – 82%) positive predictive value of 45% (*95% C.I.* 27 – 64%), and negative predictive value of 95% (*95% C.I.* 83 – 99%). The AUC value for the associated ROC curve is 0.807 (*95% C.I.* 0.695 – 0.892). The ROC curve is shown in Figure 7.

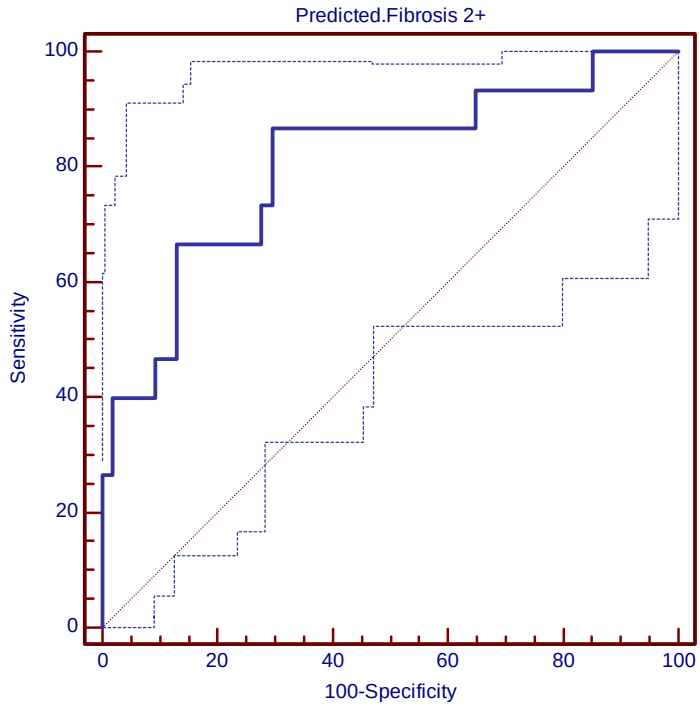

Figure 7. ROC curve for the model predicting advanced fibrosis. The solid line indicates the collection of thresholds and their associated sensitivities and specificities, dashed lines represent 95% confidence intervals, and the linear, dotted line represents what would be observed at random (*i.e.*, if the model had no predictive power).

To illustrate the importance of including serum proteins in the predictive model of advanced fibrosis, multiple linear regression was repeated using only the clinical variables available in this study. A predictive model for fibrosis emerged as follows: Advanced Fibrosis = 0.4831 + 0.3681[diabetes] – 0.2473[gender] + 0.1564[race] + 0.018[AST] – 0.0087[ALT] – 0.001[Triglycerides] – 0.0025[Glucose]. Although this model was significant (p = 0.00007) its overall performance when validated on an

independent cohort of NASH patients was modest, with a sensitivity of 100%, specificity of 32%, PPV and NPV of 22% and 100%, respectively, and an AUC of 0.661. The ROC curve for this model is shown in Figure 8.

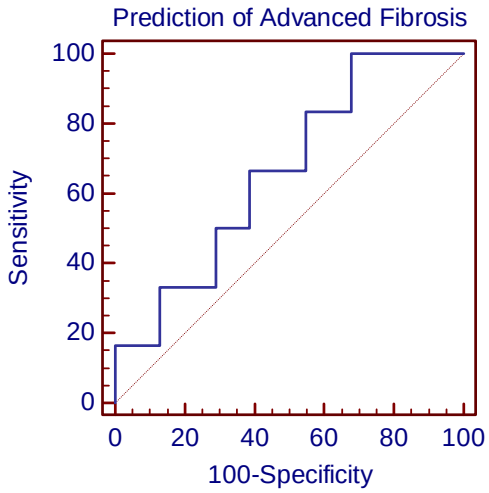

Figure 8. ROC curve for the model predicting advanced fibrosis based on clinical variables alone.

Model comparisons. For the three models predicting NASH (this study, NASH Diagnostics (Younossi et al., 2008), and M30 antigen (Wieckowska *et al.*, 2006)), our model performed best at predicting NASH in our study cohort (Table 9). Specifically, our model had an AUC value of 0.81 (*95% C.I.* = 0.70 – 0.89) and a p-value of 9.07e $^{-06}$ compared to an AUC of 0.71 (*95% C.I.* = 0.60 – 0.81) and p-value of 0.0081 for M30 antigen, and an AUC of 0.643 (*95% C.I.* = 0.52 – 0.75) and p-value of 0.0041 for NASH Diagnostics.

Table 9. Comparison of predictive models for the diagnosis of NASH.

	Threshold	*Sensitivity*	*Specificity*	*PPV (%)*	*NPV (%)*	*AUC*	*p-value*
Our model	0.221	91.18	47.37	60.8	85.7	0.809	9.07E-06
	0.3641	79.41	73.68	73.0	80.0		
	0.6183	44.12	92.11	83.3	64.8		
NASH Diagnostics	0.311	81.58	16.67	50.8	46.2	0.643	0.00415
	0.4408	65.79	66.67	67.6	64.9		
	0.5757	28.95	91.67	78.6	55.0		
M30 antigen	201	90.0	33.33	58.1	76.5	0.714	0.0081
	273	72.5	64.10	67.4	69.4		
	395	45.0	82.05	72.0	59.3		
	537	27.5	87.18	68.7	54.0		

A total of six models were tested for predicting any degree of fibrosis (Table 10). Of these, ours performed best with an AUC of 0.80 (*95% C.I.* = 0.68 – 0.88) and p-value = 0.00013. The next best performing models were the ELF and OELF, each with an AUC of 0.75 (*95% C.I.* = 0.63 – 0.84) and p-values of 0.0002 and $9.5e^{-05}$, respectively, and the combined ELF + NAFLD Fibrosis Score model with an AUC of 0.78 (*95% C.I.* = 0.66 – 0.87) and p-value = 0.0013. The NAFLD Fibrosis Score alone had modest predictive power in our study cohort (AUC = 0.62, *95% C.I.* = 0.50 – 0.73, p-value = 0.0031), as well as the APRI model with an AUC = 0.70 (*95% C.I.* = 0.58 – 0.80) and a p-value = 0.007.

Table 10. Comparison of predictive models for the diagnosis of fibrosis. "Combined" refers to the combined ELF + NAFLD Fibrosis Score model.

	Threshold	*Sensitivity*	*Specificity*	*PPV (%)*	*NPV (%)*	*AUC*	*p-value*
Our model	0.2188	90.91	43.59	57.7	85.0	0.796	0.00013
	0.4242	60.61	71.79	64.5	68.3		
	0.5689	51.52	89.74	81.0	68.6		
OELF	0.2806	94.44	38.46	58.6	88.2	0.747	9.5E-05
	0.4755	69.44	71.79	69.4	71.8		
	0.686	38.89	92.31	82.4	62.1		
ELF	0.2391	91.67	33.33	55.9	81.2	0.751	0.00018
	0.3776	80.56	64.10	67.4	78.1		
	0.6519	41.67	92.31	83.3	63.2		
NAFLD	0.2841	90.91	26.32	51.7	76.9	0.621	0.00308
Fibrosis	0.4282	63.64	57.89	56.8	64.7		
Score	0.6303	24.24	81.58	53.3	55.4		
Combined	0.1836	96.67	27.03	51.8	90.9	0.777	0.00133
	0.4613	70.00	70.27	65.6	74.3		
	0.6167	43.33	91.89	81.2	66.7		
APRI	0.1239	91.67	28.95	55.0	78.6	0.700	0.00659
	0.1601	77.78	55.26	62.2	72.4		
	0.3327	33.33	92.11	80.0	59.3		
	0.4928	5.56	97.37	66.7	52.1		

In a comparison of model performance for predicting advanced fibrosis, our model again performed best, with an AUC of 0.81 (*95% C.I.* = 0.70 – 0.89) and p-value of $6.3e^{-05}$ (Table 11). The ELF and OELF panels were the next highest performing panels, each with AUC values of 0.74 (*95% C.I.* = 0.63 – 0.84) and p-values of 0.0003 and 0.096, respectively. The APRI model (AUC = 0.68 (*95% C.I.* = 0.56 – 0.79), p-value = 0.057) and the combined ELF + NAFLD Fibrosis Score model (AUC = 0.688 (*95% C.I.* = 0.56 – 0.80), p-value = 0.027) had modest predictive power, while the NAFLD Fibrosis Score performed most poorly and did not identify patients with advanced fibrosis with any

more accuracy than would occur by chance alone (AUC = 0.52 (*95% C.I.* = 0.40 – 0.64), p-value = 0.333).

Table 11. Comparison of predictive models for the diagnosis of advanced fibrosis. "Combined" refers to the combined ELF + NAFLD Fibrosis Score model.

	Threshold	***Sensitivity***	***Specificity***	***PPV (%)***	***NPV (%)***	***AUC***	***p-value***
Our model	0.0816	93.33	35.19	28.6	95	0.807	0.00006
	0.2442	86.67	70.37	44.8	95		
	0.3640	46.67	90.74	58.3	86		
OELF	0.1034	87.5	28.81	25.0	89.5	0.739	0.09557
	0.3083	68.75	81.36	50.0	90.6		
	0.3945	31.25	93.22	55.6	83.3		
ELF	0.1561	87.5	47.46	31.1	93.3	0.742	0.00027
	0.2695	75.0	74.58	44.4	91.7		
	0.3749	31.25	89.83	45.5	82.8		
NAFLD	0.1327	84.62	36.21	22.9	91.3	0.524	0.33347
Fibrosis	0.1541	69.23	48.28	23.1	87.5		
Score	0.3103	15.38	81.03	15.4	81.0		
Combined	0.0399	92.31	25.93	23.1	93.3	0.688	0.02742
	0.2229	69.23	72.22	37.5	90.7		
	0.3429	30.77	90.74	44.4	84.5		
APRI	0.4928	7.14	96.67	33.3	81.7	0.681	0.05652
	0.1206	92.86	20.00	21.3	92.3		
	0.2256	64.29	66.67	31.0	88.9		
	0.3856	42.86	93.33	60.0	87.5		

A summary chart showing the performances (by AUC) of all models tested in this study is shown in Figure 9. This chart includes the performances of the models derived from the current data set that were based on clinical variables alone.

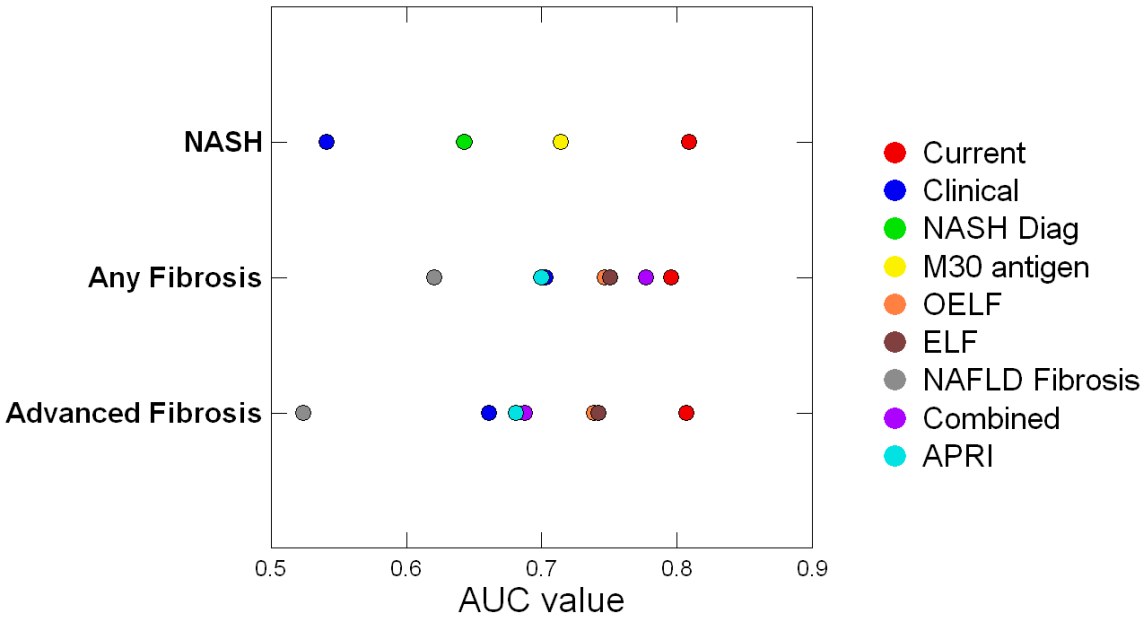

Figure 9: Comparison of model performance for the prediction of NASH, fibrosis, and advanced fibrosis.

Discussion. NAFLD is a highly prevalent liver disease which, at its more advanced stages of NASH and NASH-related fibrosis, poses a risk for chronic liver disease and liver failure (Matteoni *et al.*, 1999). NAFLD has no cure and is managed by controlling weight and metabolic abnormalities using a combination of diet and exercise regimes as well as pharmaceutical agents (Younossi, 2008); thus, early diagnosis of NAFLD is important for proactive management of the disease. There have been several reports of non-invasive, serum-based biomarkers and biomarker panels suitable for the detection of NASH or fibrosis; some markers of fibrosis have been developed with NAFLD in mind while others were tested on NAFLD patients after their discovery (see Chapter 1).

The aim of this study was to develop a non-invasive, serum-based biomarker panel for NASH and NASH-related fibrosis, incorporating various aspects of the disease process including adipose-derived signaling, apoptosis, and fibrogenesis. Multiple linear regression was used to develop three models for the prediction of NASH, fibrosis, and advanced fibrosis. Clinical, demographic, and serum-based parameters were tested as potentially significant model predictors. These parameters also were subjected to two-sample statistical comparisons to observe whether any parameters differed significantly between diagnostic groups. Finally, all models were tested for stability by 10-fold cross validation and their predictive power assessed by calculating the sensitivity, specificity, positive- and negative predictor values of the models, as well as by ROC curve analysis. The relative performance of the models was compared with that of previously published models by applying those models to our study cohort.

Our analyses showed that a vast majority (95%) of patients with NASH have some form of hepatic fibrosis and many (40%) have advanced fibrosis. Furthermore, patients with NASH were more likely to be diabetic and had significantly higher weight and serum liver enzymes (AST and ALT) compared to patients without NASH, and were higher in body mass index as well. These findings are in accordance with known clinical features of NAFLD (Hossain *et al.*, 2009). Some surprising results were that the NASH group had significantly lower total cholesterol levels compared to the non-NASH group, and in contrast to the stereotype, was significantly higher in its proportion of males. Whether these findings reflect unknown mechanisms in the pathogenesis of NAFLD or were simply by chance is uncertain. The NASH group also was significantly taller than

the non-NASH group but this finding was almost certainly confounded by the dominance of males in the NASH group. NASH patients had significantly higher serum levels of M30 antigen, TIMP-1, P3NP, and HA, suggesting elevated apoptotic and fibrogenic activities in these individuals.

Analysis of all clinical, demographic, and laboratory variables by multiple linear regression demonstrated that the presence of diabetes and male gender, together with elevated BMI, elevated serum levels of M30 antigen and increasing difference between M65 and M30 antigens and reduced serum triglycerides are highly predictive of NASH. This model had very good performance (AUC = 0.809) and outperformed other biomarker panels for NASH, specifically NASH Diagnostics (Younossi *et al.*, 2008) and the Apoptosis Biomarker (Wieckowska *et al.*, 2006); thus it appears to be robust for the prediction of NASH. Biologically, these results suggest that even when we account for demographic and clinical variables such as gender, BMI, serum triglycerides and the presence of diabetes, the levels of apoptotic and necrotic activity, as indicated by serum levels of M30 antigen and the difference between M65- and M30 antigens, respectively, are predictive of NASH and are likely to take part in the pathology of NASH, either as contributors to the disease process or as its byproducts.

NASH and hepatic fibrosis appear to be closely linked phenomena; not only did a majority of NASH patients have fibrosis, but the majority (97%) of patients with hepatic fibrosis also had NASH. Thus, ruling out other causes of liver disease, it appears that patients with NASH are likely to have fibrosis, and *vice versa*. As observed in the analysis of NASH, the fibrosis subcohort had a higher proportion of males and

significantly lower levels of total cholesterol compared to those without fibrosis. In contrast, the observations of significantly higher weight, higher BMI, and significantly higher AST- and ALT levels are in accordance with risk factors for NAFLD. Patients with fibrosis, like those with NASH, appear to have elevated apoptotic and fibrogenic activity, as evidenced by significantly higher serum levels of M30 antigen, TIMP-1, P3NP, and HA.

Incorporating all clinical, demographic and laboratory data it was noted by multiple regression that the presence of diabetes, male gender, increased BMI, decreased serum triglycerides, and elevated serum levels of M30 antigen as well as elevated difference between M65- and M30 antigens are highly predictive of NASH-related hepatic fibrosis. The model had high performance with an AUC value of 0.796 and it outperformed five other models for the prediction of fibrosis including the OELF and ELF (Rosenberg *et al.*, 2004), NAFLD Fibrosis Score (Angulo *et al.*, 2007), the combined ELF + NAFLD Fibrosis Score (Guha *et al.*, 2009), and the APRI model (Cales *et al.*, 2009; Wai *et al.*, 2003). It is important to appreciate that although the predictors in this model are the same as those in the model predicting NASH, it was tested on a different subcohort (patients grouped by fibrosis, not NASH) and new beta-values were generated during the 10-fold cross validation procedure; thus, it appears to be robust for the prediction of fibrosis. Biologically it provides a similar "message" as the model predicting NASH; namely that even when controlling for the significant clinical and demographic factors (gender, BMI, serum triglycerides and the presence of diabetes), apoptosis and necrosis are important predictors of fibrosis and presumably play a role in the pathology of NASH-related

fibrosis, either directly contributing to the disease process or resulting from other disease-related events.

Given the close association between NASH and fibrosis it was not surprising to find that 100% of patients with advanced fibrosis had NASH, compared to only 38% of the patients without advanced fibrosis. Demographic factors were not useful for distinguishing between patients with advanced fibrosis and those without it, but one clinical variable, serum AST concentration, was significantly higher in patients with advanced fibrosis. Those patients also had significantly higher levels of two secreted markers, namely M30 antigen and TIMP-1, suggesting that apoptosis and fibrogenesis are occurring in patients with advanced fibrosis, as was observed previously for patients with any hepatic fibrosis and with NASH.

Using all clinical, demographic and laboratory variables as potential predictors it was observed by multiple linear regression that the presence of diabetes along with increasing AST and TIMP-1 and decreasing triglycerides were predictive of advanced fibrosis. This model had good performance with an AUC = 0.807, and it outperformed the OELF and ELF (Rosenberg *et al.*, 2004), NAFLD Fibrosis Score (Angulo *et al.*, 2007), the combined ELF + NAFLD Fibrosis Score (Guha *et al.*, 2009), and the APRI model (Cales *et al.*, 2009; Wai *et al.*, 2003). In fact, the NAFLD Fibrosis Score was ineffective for the detection of advanced fibrosis in our study cohort. Biologically, our modeling results suggest that fibrogenesis is an important component of the disease process in cases of advanced fibrosis, since TIMP-1 is predictive even when diabetes, serum AST levels and triglycerides are statistically controlled.

Taken together, the results of this study underscored the use of markers of fibrogenesis and cell death for the prediction of NASH and NASH-related fibrosis. Biomarkers of adipose signaling, namely resistin and adiponectin, were not useful in distinguishing NAFLD disease states, even though they were useful in a previous biomarker panel developed for NASH (Younossi *et al.*, 2008).

Regarding the demographic factors evaluated in this study, it is interesting to note the significant contribution of male gender to the prediction of NASH and NASH-related fibrosis. There is growing evidence that estrogen may be protective against NAFLD, possibly explaining why females were under-represented in the NASH and fibrosis groups. Serum estradiol is significantly lower in women with NAFLD compared to those without NAFLD, even when considering only those women who are pre- or post-menopausal or have polycystic ovary syndrome (PCOS) (Gutierrez-Grob *et al.*, 2010). Similarly, breast cancer patients treated with tamoxifen, an estrogen receptor antagonist, are at increased risk for the development of steatosis and NASH (Oien *et al.*, 1999; Van *et al.*, 1996). There are a number of mechanisms by which estrogen may be protective against NAFLD. First, estrogen appears to suppress both steatosis and hepatic fibrosis, and to prevent macrophage accumulation in the liver which in turn limits the release of pro-inflammatory cytokines (*reviewed in:* Shimizu and Ito, 2007). Estrogen also has anti-diabetic functions related to insulin signaling and oxidative stress (*reviewed in:* Louet *et al.*, 2004). In terms of insulin signaling, it has been shown that estradiol-17β (E2) administered alone or as part of hormone replacement therapy improves insulin sensitivity in women lacking endogenous E2 secretion, and this improvement is thought

to take place in the liver, as experiments have shown that insulin sensitivity in skeletal muscle does not change in response to estrogen. E2 replacement in post-menopausal women is also associated with decreased visceral obesity and with increased, insulin-mediated suppression of lipolysis. Experiments with murine models underscore these observations. Both aromatase knock-out (ArKO) mice and estrogen receptor α knock-out (αERKO) mice develop insulin resistance, and in ArKO mice, hepatic insulin resistance is accompanied by excess triglyceride accumulation, increased expression of lipogenic genes and decreased expression of genes responsible for fatty acid oxidation. Regarding oxidative stress, it was discovered many years ago that several forms of estrogen, including E2, act as anti-oxidants and are protective against lipid peroxidation in the liver (Lacort *et al.*, 1995; Yoshino *et al.*, 1987). In rat hepatocytes undergoing oxidative stress, E2 up-regulates Bcl-2, a gene whose products suppress lipid peroxidation and prevent apoptosis (Shimizu and Ito, 2007; Inoue *et al.*, 2003). Taken together, these studies suggest that estrogen may to be protective against NAFLD and its actions may target various aspects of the disease process, including the development of steatosis, inflammation, fibrosis, insulin resistance, and oxidative stress.

Another result from this study that warrants further investigation is the use of BMI in predictive models of NAFLD. Body mass index was not statistically significant in any of the group comparisons at a significance level of 0.05, but it would have been significant in all of them if a significance level of 0.10 had been used. The most reasonable explanation for the lack of significance at 0.05 is that nearly the entire study cohort was morbidly obese; thus even the control groups were not representative of the general

population. However, BMI was a significant contributor to the models predicting NASH and NASH-related hepatic fibrosis, and would certainly be important to include along with markers of fibrogenesis and cell death in future efforts to refine the current biomarker panel for NAFLD.

Study Limitations. There are several limitations associated with our study, most notably the relatively small sample cohort (N = 79). As tissues and serum continue to be collected as part of the the ongoing NAFLD study conducted by the Center for Liver Diseases, it is hoped that the study will be repeated using a larger cohort of patients, providing not only greater statistical power but also the possibility of increasing the number of patients with NASH but not fibrosis. In this study, the majority of patients had both NASH and NASH-related fibrosis, such that the respective models predicting those diagnoses were the same in terms of their predictors, except in the case of advanced fibrosis. It would be helpful to know whether and how the NASH and hepatic fibrosis models would differ in composition if they were developed using a study cohort with more patients falling into distinct disease categories. Ideally, it also would be extremely helpful to have a control group consisting of individuals with no liver disease *and* presenting no features of Metabolic Syndrome, however acquiring liver specimens from such individuals would be unethical given the risks associated with liver biopsy, and the absence of liver disease would need to be inferred using a weight-of-evidence approach (*e.g.*, serum levels of liver aminotransferases within normal ranges, *and* normal weight and BMI, *and* abstinence from alcohol, etc.).

Future studies also would benefit from collections of larger volumes of serum, as this study was restricted by serum availability. As a consequence, it was not possible to conduct duplicate assays on the same patients for any of the target analytes, and intra- and inter-assay variability could not be calculated. Repeated assays along with measuring samples in triplicate would have greatly improved our ability to assess the accuracy of our results, and would have provided greater confidence that the results observed were replicable. This point cannot be understated given that the process of conducting an ELISA or EIA has some inherent variability due to subtle differences in incubation times, antibody concentrations, etc., although the use of kits certainly minimizes this variability since protocols have been optimized and reagents are standardized.

Finally, there are some improvements that could have been made to our statistical approach. Due to the small study cohort, validation of the models was conducted using a 10-fold cross-validation procedure, in which the same data set was used for both training and testing purposes. An independent validation cohort would have provided greater rigor for testing our models. Furthermore, due to the extensive computational demands of re-designing a new regression model for each of the ten training sets, only the beta values were allowed to change with each training round and these were applied to the corresponding validation set. Confidence in our models would be greatly strengthened by re-developing a new model, with potentially new predictors, during each iteration of the training procedure and applying this model to the validation set. By amassing a set of ten "best predicting" models, this approach would hopefully reveal the most robust predictors for the various diagnostic categories.

Conclusions. Our study demonstrated that secreted molecules signifying apoptosis and fibrogenesis, together with clinical and demographic parameters, can be used to distinguish patients with NASH and NASH-related fibrosis from those having only steatosis or minimal hepatic changes. The parameters that most often distinguished diagnostic groups included the presence of diabetes, gender, serum triglycerides, BMI, AST, M30 antigen, an estimate of necrosis (M65 antigen – M30 antigen), and a marker of fibrogenesis (TIMP-1). The adipokines resistin and adiponectin were not useful in predictive models of NASH or NASH-related fibrosis; however, due to the strong association between obesity and NAFLD, it would be reasonable to continue testing other adipokines for efficacy in predictive models.

4: NOVEL BIOMARKERS OF NAFLD
REFLECTING THE ROLE OF ADIPOSE TISSUE

Enrichment Analysis and Selection of Candidate Biomarkers. The enrichment analysis was initially performed using MetaCore and, based on protein names alone, it revealed that the pathways most enriched in the Younossi *et al.* (2010) data set were those relating to insulin signaling, including the IGF-1 receptor pathway (which can be triggered by insulin), insulin regulation of translation, AKT signaling (a component of the insulin, chemokine, and apoptosis signaling pathways), PIP3 signaling (a component of the insulin signaling pathway), and regulation of lipid metabolism by insulin signaling. This finding was in accordance with expected results, as the phosphoproteins chosen for study in Younossi *et al.* were those already known to play a role in insulin signaling and were selected based on the strong association between insulin resistance and NAFLD. However, using the protein array data from Younossi *et al.*, this enrichment analysis also served to illustrate which components of these pathways were most differentially phosphorylated in patients with and without NASH, thereby suggesting mechanistically how cell signaling in adipose tissue might be de-regulated. For example, in the pathway illustrating regulation of lipid metabolism by insulin, the proteins from Younossi *et al.* that were most differentially phosphorylated were insulin receptor substrate 1 (IRS-1) and its binding partner, SHC-transforming protein (SHC), and to a lesser extent 3-

phosphoinositide dependent protein kinase-1 (PDPK1), ribosomal protein S6 kinase, 70 kDa (p70-S6 kinase 2), and eIF4E-binding protein 1 (4E BP1); in all of these cases phosphorylation levels were notably lower in patients with NASH relative to those without NASH, whereas other measured proteins in this pathway (*e.g.*, mechanistic target of rapamycin (mTOR)) were not as strikingly different in their phosphorylation levels (Figure 10). Several of these phosphoproteins appeared repeatedly throughout the enriched pathways, and therefore it is not surprising why a subset of them (IRS-1, AKT) were independently predictive of NASH and NASH-related fibrosis in regression models (Younossi *et al.*, 2010). Table 12 shows in detail which of the original 27 proteins analyzed appeared in the five most prevalent pathways.

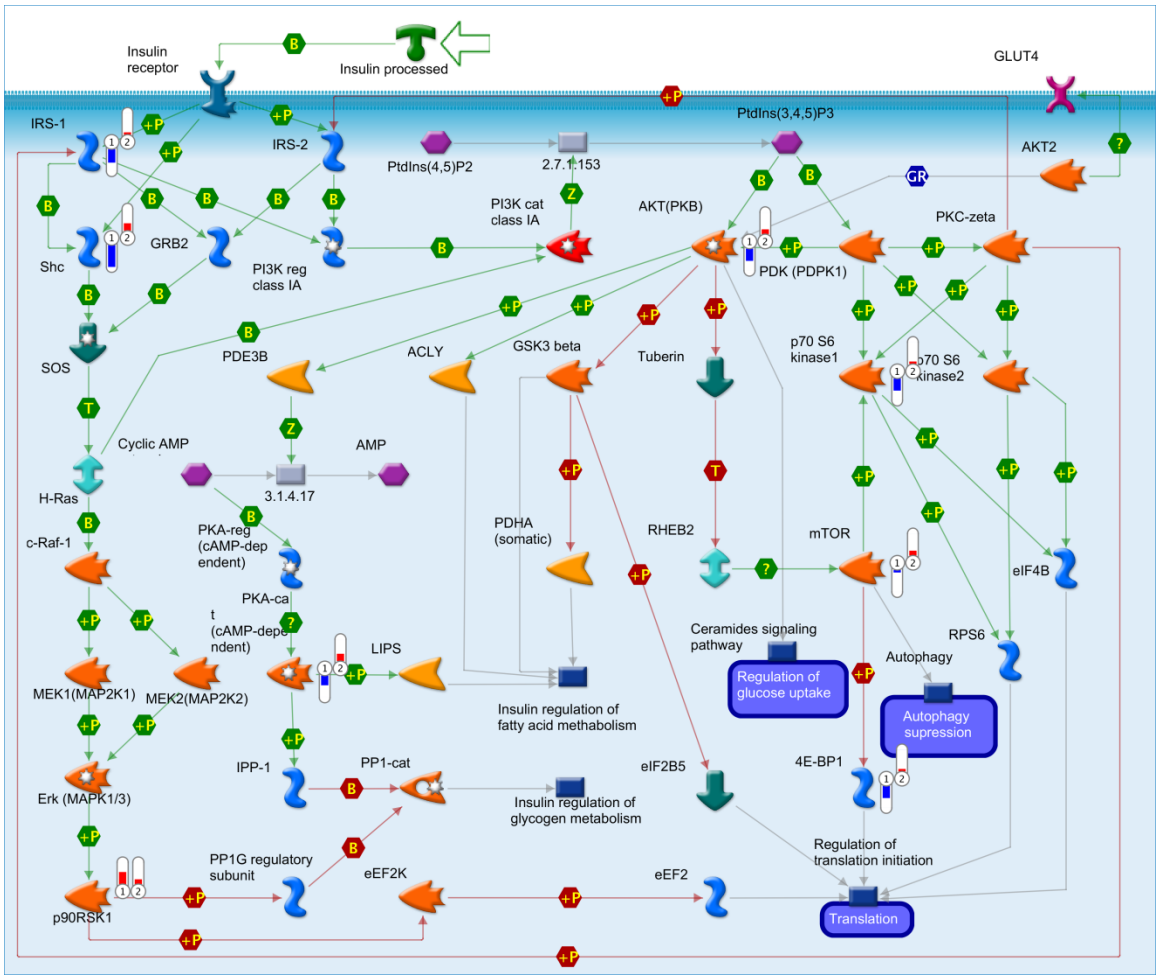

Figure 10. MetaCore output showing regulation of lipid metabolism by insulin, with relative phosphorylation levels of proteins measured in Younossi *et al.* (2010) indicated by bars (bar 1 = patients with NASH; bar 2 = patients without NASH). Bars point up (red) or down (blue) in relation to the assay normalization value; bar height indicates the degree of difference in phosphorylation from the normalization value.

Table 12. Subset of phosphoproteins analyzed in Younossi *et al.* (2010) appearing in the five most enriched pathways.

Phosphoprotein	Function	Pathway(s)
IRS-1	docking protein	regulation of lipid metabolism by insulin signal transduction by AKT signal transduction by PIP3 IGF-1 receptor signaling regulation of translation by insulin
SHC	protein domain	regulation of lipid metabolism by insulin signal transduction by PIP3 IGF-1 receptor signaling regulation of translation by insulin
AKT	kinase	regulation of lipid metabolism by insulin signal transduction by AKT signal transduction by PIP3 IGF-1 receptor signaling regulation of translation by insulin
P70-S6	kinase	regulation of lipid metabolism by insulin signal transduction by AKT signal transduction by PIP3 IGF-1 receptor signaling regulation of translation by insulin
PKA-c	kinase	regulation of lipid metabolism by insulin
mTOR	kinase	regulation of lipid metabolism by insulin signal transduction by PIP3

		IGF-1 receptor signaling regulation of translation by insulin
4E-BP1	translation	regulation of lipid metabolism by insulin signal transduction by AKT signal transduction by PIP3 IGF-1 receptor signaling regulation of translation by insulin
p90 RSK1	kinase	regulation of lipid metabolism by insulin signal transduction by PIP3 IGF-1 receptor signaling regulation of translation by insulin
GSK-3	kinase	signal transduction by AKT signal transduction by PIP3 IGF-1 receptor signaling
FOXO3A	transcription factor	signal transduction by AKT signal transduction by PIP3 IGF-1 receptor signaling
BAD	apoptosis	signal transduction by AKT signal transduction by PIP3 IGF-1 receptor signaling
CREB1	transcription factor	signal transduction by PIP3 IGF-1 receptor signaling
eIF4G	translation	regulation of translation by insulin

As the enrichment analysis was conducted, it became clear that MetaCore would not be an adequate tool for hypothesis formation regarding secreted molecules, because (1) MetaCore is optimized for the investigation of intracellular activities and is not easily manipulated for the identification of secreted molecules, and (2) it draws its information from a proprietary, "black box" database which necessarily limits the pool of potential pathways associated with a molecule of interest. Therefore, the enrichment analysis was repeated using Pathway Studio, which forms its database by scanning all open source publications for relevant information and is not limited to intracellular activities. After analyzing the full data set a series of networks were generated, and the network containing the most differentially expressed phosphoproteins, as determined by group comparison between patients with and without NASH conducted within Pathway Studio, was selected for subsequent analysis. To this network, molecules regulated by the phosphoproteins that were independently predictive of NASH and advanced fibrosis in Younossi *et al.* (2010) were added; these included targets of AKT kinase, insulin receptor substrate-1 (IRS1), glycogen synthase kinase-3 (GSK3), and protein kinase A (PKA). Diseases and cellular processes pertinent to NASH, namely liver fibrosis, insulin resistance, apoptosis, and reactive oxygen species (ROS) also were added as outcome categories. These were connected to existing entities in the network, producing a substantial collection of positive and negative relationships. Because Pathway Studio uses PubMed as its knowledge base, relationships formed during pathway queries are easily researched and networks may be edited to suit specific goals. Thus, the very large network of entities produced in this study was culled to a manageable level using the

following criteria: (1) only those relationships reflecting a direct or nearly direct connection to significant phosphoproteins from the Younossi *et al.* study or to the outcome categories were retained; (2) relationships based on scant evidence (*e.g.*, only one publication supporting a relationship) were deleted; and (3) only soluble molecules were retained, favoring small peptides over other types of secreted molecules such as steroid hormones. The final network resulting from this "culling" step revealed a set of proteins worth investigating as serum-based biomarkers of NASH and NASH-related fibrosis due to their close relationships with the significant phosphoproteins from the Younossi *et al.* study (and hence, potential release from adipose tissue) and with the disease and cellular processes relevant to NASH (Figure 11). Literature searches were then performed on individual members of the final collection of secreted proteins in order to establish (1) if they made biological sense to investigate as biomarkers of NASH and NASH-related fibrosis based on their known mechanisms of action; and (2) whether any previous associations had been made between a protein of interest and the occurrence of NASH or NASH-related fibrosis. Three cytokines, TNF-α, IL-6, and IL-8, were omitted from this analysis because these had been tested in previous studies performed in our laboratory and although they were useful in distinguishing patients with any form of NAFLD from those without it (Jarrar *et al.*, 2008), they did not perform well in a subsequent study in which a predictive model for NASH was developed (Younossi *et al.*, 2008).

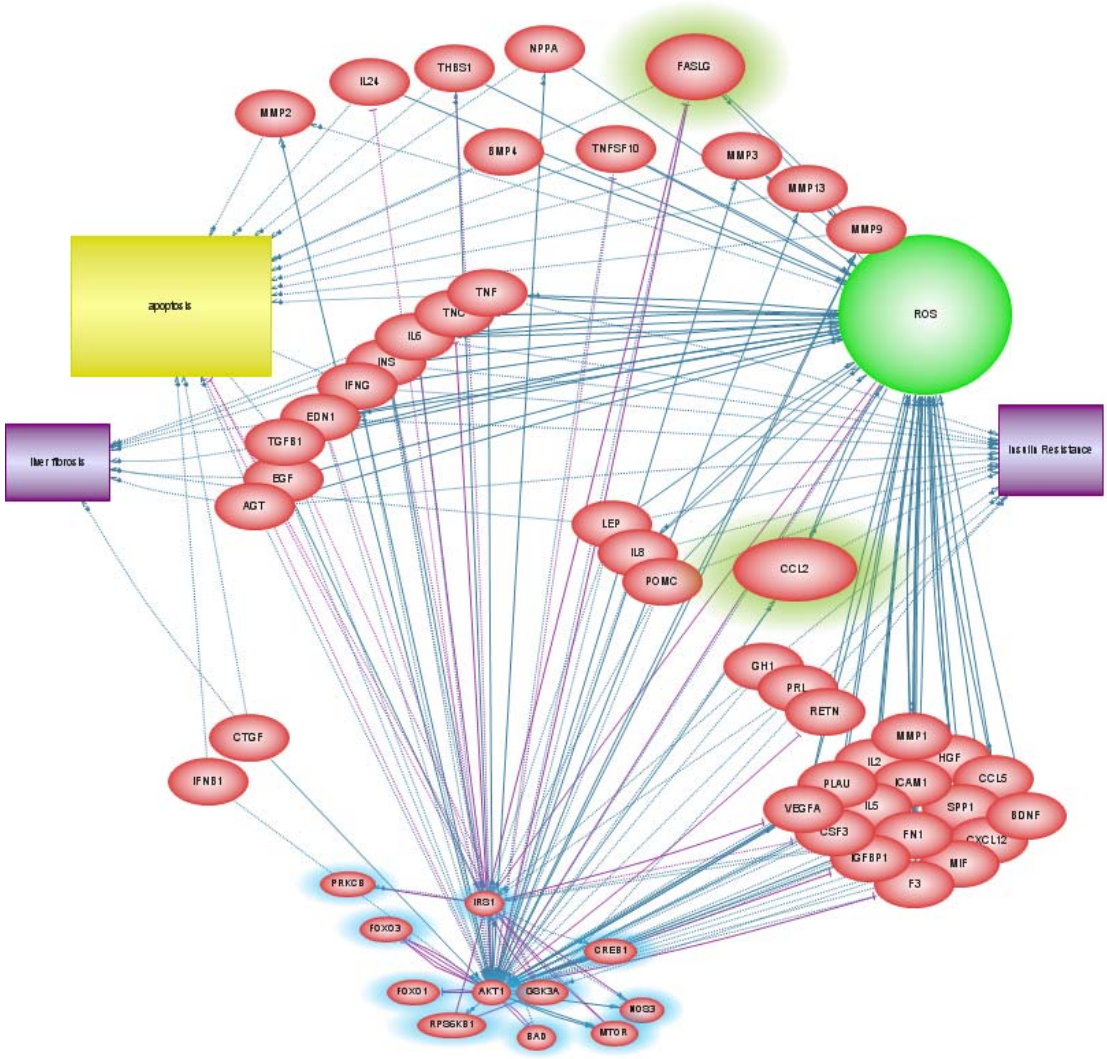

Figure 11. Final output network based on analyses performed in Pathway Studio. Proteins highlighted in blue were differentially phosphorylated in the phosphoproteomic data set. Proteins selected for testing in the NASH and NASH-related fibrosis biomarker panel are highlighted in green (Fas ligand and CCL2).

After researching the functions of the candidate proteins as well as their potential associations with NAFLD, the two proteins that appeared most suitable for testing as

biomarkers of NASH and NASH-related fibrosis were Chemokine (C-C motif) ligand 2 (CCL-2) and Fas ligand (FasL). The rationale for the use of these proteins and hypotheses regarding their roles in NAFLD is provided in the following two sections.

CCL-2. Also known as monocyte chemotactic protein-1 (MCP-1) and monocyte-chemotactic and activating factor (MCAF), CCL-2 is a small cytokine belonging to the CC chemokine family, having two adjacent cysteines near its N-terminus. The gene encoding CCL-2 is located on the q-arm of chromosome 17, within a cluster of other genes encoding CC family cytokines (Zlotnik *et al.*, 2006). At sites of inflammatory response, CCL-2 is a chemoattractant for monocytes and memory-phenotypic T-lymphocytes, and induces the migration of dendritic cells (Xu *et al.*, 1996; Carr *et al.*, 1994). CCL-2 also attracts basophils but not neutrophils or eosinophils. CCL-2 is secreted by many types of cells, including peripheral blood mononuclear cells (Seitz *et al.*, 1995), mast cells (Katsanos *et al.*, 2008), epithelial cells (Tsuboi *et al.*, 2002), endothelial cells (Sironi *et al.*, 1993), bone marrow stromal cells (Gautam *et al.*, 1995), osteoclasts (Bost *et al.*, 2001), and smooth muscle cells (Pype *et al.*, 1999). Its secretion is induced by inflammatory mediators such as interleukin-1 alpha (IL-1α), interferon-gamma (IFN-γ), and lipopolysaccharide (LPS), and potentiated by transforming growth factor beta (TGF-β) and IL-4 (Gautam *et al.*, 1995). Interleukin-10 (IL-10) may initiate or inhibit the secretion of CCL-2 depending on the cell type and extracellular conditions (Seitz *et al.*, 1995; Sironi *et al.*, 1993). CCL-2 is ligand for the seven transmembrane, G-protein coupled receptor, CCR2, and triggers signaling cascades that lead to increased cytosolic Ca^{2+} ion concentrations, stimulation of the MAPK series of kinases, and

activation of rho GTPase in monocytes (Ashida *et al.*, 2001; Yen *et al.*, 1997; Sozzani *et al.*, 1991), as well as activation of PI3K-γ in macrophages (Jones *et al.*, 2003). Other receptors binding CCL-2 include D6 and DARC, which act as decoy receptors and do not initiate a signaling cascade, and US28, a chemokine receptor that accelerates the inflammatory process (*reviewed in* Yadav *et al.*, 2010).

Functionally, CCL-2 is an inflammory chemokine that recruits leukocytes to injured or infected tissue, and therefore contributes to various healing processes ranging from the reduction of viral infection to bone remodelling (Yadav *et al.*, 2010). Elevated expression of CCL-2, however, is associated with a number of diseases including multiple sclerosis, transplant rejection, asthma, rheumatoid arthritis, atherosclerosis, inflammatory bowel disease, and cancer, although interestingly CCL-2 also has anti-tumor effects (*reviewed in* Gerard and Rollins, 2001; Yadav *et al.*, 2010). In many cases CCL-2 is functionally linked with a disease due to its ability to recruit leukocytes to tissues; for example, the ability of CCL-2 to recruit and localize monocytes in renal tissue contributes to the damage associated with inflammatory kidney diseases (*reviewed in* Yadav *et al.*, 2010). However, CCL-2 also contributes to disease processes due its effects on endothelial cells; for example it increases the permeability of the blood-brain barrier by altering cytoskeleton interactions with tight junctions in brain endothelial cells, and it contributes to angiogenesis by causing endothelial cells to migrate to, and sprout from aortal rings.

In the context of NAFLD, CCL-2 has ties to pathological functioning in both adipose tissue and the liver, and therefore has potential as a serum-based biomarker. As outlined in Chapter 2, deregulation of adipose tissue resulting from obesity contributes to the

development of insulin resistance and metabolic syndrome. Part of this pathological process involves the secretion of pro-inflammatory cytokines from adipocytes and macrophages in adipose tissue, which is elevated when adipose tissue is composed of large adipocytes (Gustafson, 2010; Sopasakis *et al.*, 2004). CCL-2 is one of the inflammatory cytokines released by adipose tissue; its expression is higher in obese *vs.* lean subjects and in visceral *vs.* subcutaneous adipose tissue (Bruun *et al.*, 2005), and it is expressed and secreted by adipocytes but the majority of its secretion from adipose tissue has been attributed to adipose-resident macrophages (Meijer *et al.*, 2011; Bruun *et al.*, 2005). Recently a study demonstrated that over-expression of CCL-2 in the adipose tissue of obese murine models resulted in increased macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis (Kanda *et al.*, 2006); conversely, knocking out *MCP-1* (CCL-2) gene function decreased macrophage infiltration, insulin resistance, and steatosis in obese, murine models; thereby suggesting a critical role for CCL-2 in the pathogenesis of NAFLD. A 2010 study supported and extended these findings by experimentally increasing circulating levels of CCL-2 in a murine model; chronically elevated CCL-2 levels resulted in insulin resistance, increased infiltration of macrophages into adipose tissue, and increased hepatic triacylglycerol content (steatosis), whereas acutely elevated CCL-2 levels resulted in insulin resistance only (Tateya *et al.*, 2010). Consequently, CCL-2 appears to induce systemic insulin resistance regardless of leukocyte activity in adipose tissue. As an inflammatory marker, CCL-2 also has been linked specifically to NAFLD in humans. Serum levels of CCL-2 were significantly elevated in patients with NAFLD compared to healthy controls, and were significantly

elevated in patients with NASH compared to those with simple steatosis (Haukeland *et al.*, 2006). Furthermore, serum levels of CCL-2 remained a predictor for the diagnosis of NAFLD after adjusting for age, sex, and BMI, and a predictor for NASH after adjusting for sex, BMI, and the presence of metabolic syndrome. Accordingly, patients with steatosis but no histological signs of inflammation have increasing gene expression of CCL-2 in proportion to liver fat content, as measured by Affymetrix gene chip and PCR (Greco et *al.*, 2008; Westerbacka *et al.*, 2007).

Taken together, the research to date on CCL-2 supports the investigation of this cytokine as a biomarker for NAFLD, and specifically for NASH and NASH-related fibrosis for the following reasons: (1) it recruits leukocytes to tissues and therefore promotes inflammation; (2) it is secreted from adipose tissue and from more than one cell type within that tissue; (3) its secretion is positively related to adiposity and linked to adipocyte size; (4) it contributes to insulin resistance and steatosis in murine models; and (5) its gene expression and serum levels correlate with the progression of NAFLD in humans. Based on these observations, I put forth the following mechanistic explanation: circulating CCL-2 is elevated in patients with NAFLD due to enlarged visceral adipose tissue; when released from adipose tissue, it causes monocyte infiltration into the liver and contributes to steatosis and insulin resistance of hepatocytes, thereby playing a role in the pathophysiology of NAFLD.

Fas Ligand. Also known as TNF superfamily member 6 (TNFSF6), CD178 antigen and CD95 ligand, Fas ligand (FasL) is a member of the tumor necrosis factor (TNF)

superfamily. FasL is a single-pass, type II transmembrane protein consisting of 281 amino acids. The gene encoding Fas ligand is located on the q arm of chromosome 1.

Binding of FasL to its receptor, FAS, a type I transmembrane protein, induces apoptosis. Both are expressed during embryonic development and in adult life (Green and Ferguson, 2001), however, not all types of cells express FAS. FAS is primarily expressed in organs (*e.g.* heart) and in the lymphoid system, while FasL is expressed in activated T cells and constitutively expressed in several tissues including the eye and testis, and in endothelial cells (Green and Ferguson, 2001; Sata and Walsh, 1998). FAS is also expressed in neutrophils, monocytes, and eosinophils, but of these, only neutrophils constitutively express FasL and as a result, easily undergo apoptosis in response to pro-inflammatory cytokines and other signaling molecules (Liles *et al.*, 1996).

The canonical pathway for FasL signaling is as follows, and is displayed in Figure 12. FasL (which is thought to exist as a homotrimer) oligomerizes FAS upon binding; when three or more FAS molecules have come together (called "cross-linking") the adaptor protein, fas-associated death domain (FADD), is recruited (Green and Ferguson, 2001). FADD binds FAS *via* its death domain, while its death-effector domain recruits and binds pro-caspase-8. The entire complex is then known as the death-inducing signal complex (DISC). Pro-caspase-8 is then cleaved and caspase-8 is released, which in turn activates other caspases and induces a cascade leading to apoptosis; however, caspase-8 at low concentration can induce apoptosis *via* the release of cytochrome c from mitochondria (Mahmood and Shukla, 2010). Other proteins can bind FADD leading to other results; for example, receptor interacting protein (RIP) has a death-effector domain and upon binding

FADD, induces caspase-independent cell death, while FADD-like IL-1β converting enzyme inhibitory protein (c-FLIP) inhibits cell death upon binding FADD (Green and Ferguson, 2001).

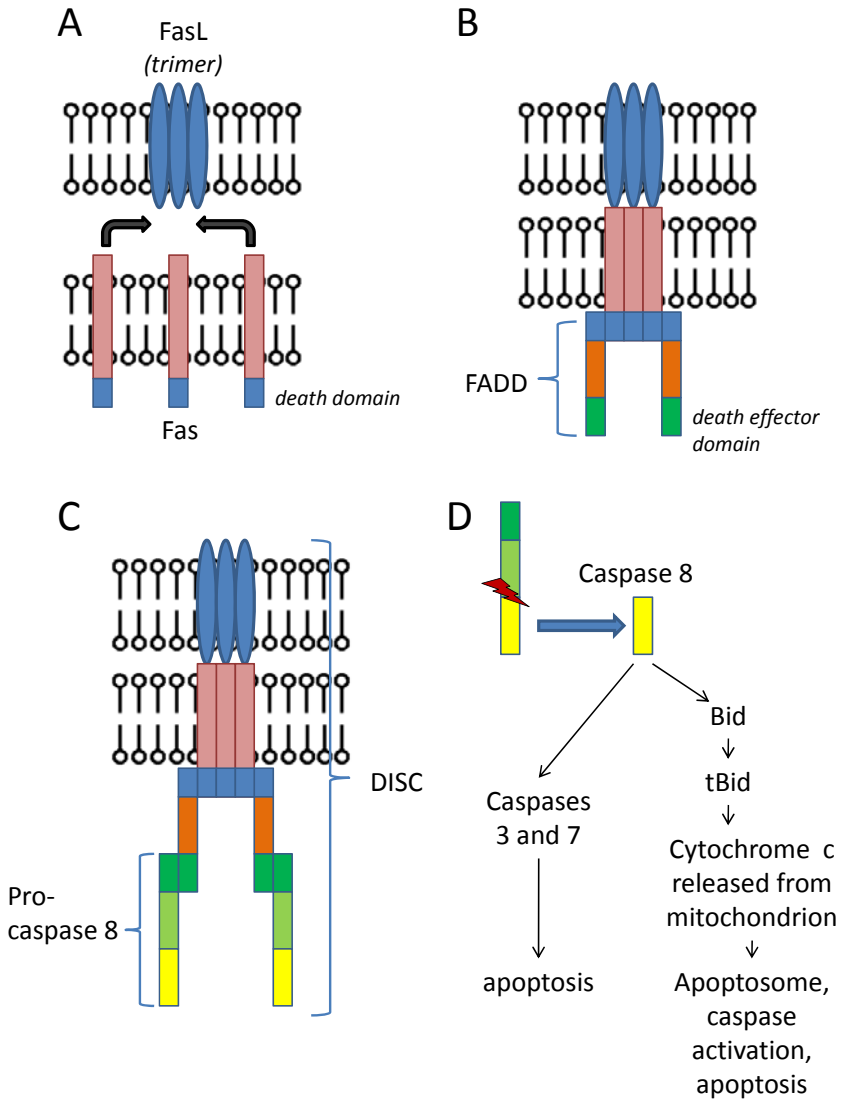

Figure 12. FasL initiation of apoptosis. Binding of FasL to its receptor, Fas, causes oligomerization of the receptor (A) and recruitment of FADD (B). FADD is an adaptor protein that in turn recruits pro-caspase 8 (C), which is then cleaved to produce caspase 8 (D). Caspase-8 in high concentration activates caspases 3 and 7, which initiate apoptosis. If caspase-8 is low in concentration, it initiates the intrinsic pathway for apoptosis by initiating a cascade that causes cytochrome c release from mitochondria.

While intact FasL is a 40 kDa protein, it can be proteolytically cleaved to produce a soluble protein (sFasL) of 26 kDa that then circulates as a trimer (Tanaka *et al.*, 1995); this cleavage event also produces TNFα (Tanaka *et al.*, 1996). Matrix metalloproteinases (MMPs), *e.g.* MMP-7 and MMP-3, cleave FasL near the transmembrane domain but at more than one site, generating distinct forms of sFasL that may be responsible for the seemingly contradictory activities attributed to sFasL (Vargo-Gogola *et al.*, 2002). For example, sFasL released by neutrophils induces cell death in lung epithelial cells (Serrao *et al.*, 2001), whereas human Jurkat cells and mouse primary hepatocytes are resistant to human sFasL-induced apoptosis, and endothelial cells resist hypoxia-induced apoptosis by releasing sFasL (Tanaka *et al.*, 1998; Mogi *et al.*, 2001). In a direct comparison of apoptotic capability between sFas and membrane-bound FasL (mFasL), sFasL was 1,000-fold less efficient than mFasL, and was not cytotoxic to hepatocytes when injected at high doses into mice (Schneider *et al.*, 1998). Mogi *et al.*, 2001 proposed two explanations for the decreased cytotoxic and even apoptotic-protective effects of sFasL: (1) upon binding to Fas, the Fas-sFasL complex is readily internalized and degraded leading to down-regulation of apoptotic signaling, whereas the Fas-mFasL complex is not easily internalized and therefore not rapidly down-regulated; and (2) Fas-sFasL binding may activate alternative pathways that promote survival factors such as nuclear factor-kappa B (NF-κB).

While the predominant role of sFasL in the regulation of apoptosis is still unknown, its function as a pro-apoptotic factor is widely accepted (Kavathia *et al.*, 2009), and recent studies of its contribution to disease suggest that at least in pathological processes,

its cytotoxic capability is most relevant. For example, in rheumatoid arthritis sFasL inhibits angiogenesis by promoting apoptosis of synovial cells, thereby reducing the production of vascular endothelial growth factor (Kim *et al.*, 2007). With regard to cancer, it has been proposed that the poor prognosis associated with elevated levels of plasminogen activator inhibitor-1 (PAI-1) can be explained by its ability to inhibit plasmin production, in that plasmin cleaves FasL to produce sFasL, promoting endothelial cell apoptosis (Bajou *et al.*, 2008). Soluble FasL also has served well as a serum-based biomarker of disease when regarded as a pro-apoptotic factor. Kavathia *et al.* (2009) first confirmed that systemic apoptosis decreases with age by noting a negative correlation between age and serum sFasL levels in both men and women, and then demonstrated that serum levels of sFasL were negatively correlated with the stage of breast and prostate cancers. These results were mirrored by serum levels of cytochrome c, which also is pro-apoptotic and is an established serum marker of apoptosis.

While the role of sFasL in the liver has not been well studied, Fas and FasL are highly expressed in the liver (Faubion *et al.*, 1999), and as a result, together with the elevated expression of other death receptors and their ligands in the liver, it is believed that hepatocyte apoptosis is predominantly mediated by extrinsic rather than intrinsic signaling (Guicciardi and Gores, 2010). Apoptosis appears to be ubiquitous among liver diseases, and Fas-mediated apoptosis is associated with NAFLD, alcoholic hepatitis, viral hepatitis, cholestatic liver disease, and others. Pertinent to NAFLD, human hepatocytes exposed to free fatty acids increase their expression of Fas and more readily undergo Fas-mediated apoposis *via* a mechanism that is still unknown (Feldstein *et al.*, 2003a).

Furthermore, expression of Fas and FasL correlate with disease severity as determined by immunohistochemistry of normal liver, steatotic liver, and NASH, and this finding supported the concordant observation of increasing apoptosis with disease progression (Feldstein *et al.*, 2003b). It was suggested that Fas-mediated apoptosis contributes to liver fibrosis *via* the following pathway: fas signaling activates caspase 8, which in turn creates mitochondrial dysfunction that triggers apoptosis and also generates the release of reactive oxygen species (ROS) in a caspase-dependent manner. ROS then promotes apoptosis (in a feedback manner) as well as tissue injury and inflammation, leading to fibrosis. This last step was based on knowledge of the close association between apoptosis and fibrosis observed in animal studies and the fact that hepatic stellate cells (HSC) are known to undergo fibrogenic activity upon engulfment of apoptotic bodies. However, the mechanisms by which apoptosis may lead to fibrosis have since been investigated in more detail and can be classified by two processes: engulfment of apoptotic bodies, and fibrosis-signaling molecules emanating from dying cells (Guicciardi and Gores, 2010). In the first type, liver injury generates excess apoptosis, such that there are too many apoptotic bodies to be engulfed by hepatic stellate cells and macrophages, and these trigger inflammation leading to fibrosis. In the second type, apoptotic cells release signaling molecules in the form of nucleotides into the extracellular space. These bind HSC and macrophages and act as chemoattractants that drive the HSCs and macrophages to the sites of injury. There, HSCs are activated to become myofibroblasts, which in turn generate the collagen that forms scar tissue. Taken

together, these studies illustrate the potential importance of the Fas/FasL system in the pathology of NAFLD.

In contrast, little work has been done to elucidate the potential role of sFasL in liver disease, and only one study investigated the use of sFasL as a serum-based biomarker of NASH (Tamimi *et al.*, 2011); published after completion of the work presented in this chapter. In that study, sFasL was not a significant predictor of NASH despite the fact that the remaining two apoptosis-related proteins forming the biomarker panel, caspase-cleaved cytokeratin-18 and the soluble fas ligand receptor, sFas, were predictive. sFasL did positively correlate with histological features of NASH including lobular inflammation and hepatocyte ballooning, but not strongly (p-values not reported). However, sFasL was not tested as a marker of NASH-related fibrosis and to date, no other studies have tested sFasL as a biomarker of NAFLD. Soluble FasL has been tested in the context of other liver diseases and has been useful as a biomarker of apoptosis for some of them. Serum levels of sFasL were useful in differentiating patients with acute liver failure from those with acute hepatitis E or with sepsis alone (Singhal *et al.*, 2009; Nakae *et al.*, 2001). However, plasma levels of sFasL were low in patients with hepatitis C (HCV) while concurrent measurements of sFas plasma levels were high, indicating an overall *decrease* in programmed cell death in the HCV disease process (Lapinsky *et al.*, 2006; Raghuraman *et al.*, 2005). Serum levels of sFasL were higher in patients with chronic but not acute hepatitis B, and highest in patients with a most severe infection (Song le *et al.*, 2004). Serum sFasL levels also were elevated in patients with alcoholic-related cirrhosis, and a concomitant, *in vitro* study of peripheral blood mononuclear cells

(PBMCs) derived from the same patient cohort showed that the PBMCs of patients with alcoholic-related cirrhosis secreted higher levels of sFasL relative to controls (Szuster-Ciesielska *et al.*, 2005).

In humans, the role of the Fas/FasL system in adipose tissue is not as well understood as that of the liver, however animal studies suggest an adipose-specific functional role for Fas/FasL in insulin resistance. *In vitro*, human preadipocytes undergo apoptosis upon exposure to FasL, and mature adipocytes derived from murine white adipose tissue express Fas (Gross *et al.*, 2009; Kim *et al.*, 2007b). Adiponectin, an adipose-derived hormone that is thought to be protective against fatty liver diseases (Wang *et al.*, 2009), suppresses interferon gamma production in natural killer (NK) cells, which in turn down-regulates their expression of Fas ligand and consequently limits their cytotoxic effects (Kim *et al.*, 2006). One study related sFasL to adiposity, finding that in comparison of patients with and without cachexia there was inverse relationship between serum levels of sFasL and measures of body composition, namely BMI and percent body fat (Takabatake *et al.*, 2005). Regarding NAFLD, Moreno (2005) postulated that Fas ligand-induced liver cell death represented a late step in a pathological process that starts with inflammation and insulin resistance in adipose tissue, releasing free fatty acids and promoting hyperinsulinemia which in turn cause steatosis and oxidative stress in hepatocytes. Studies of Fas/FasL in murine models have provided a mechanistic explanation for the contribution of adipose-expressed Fas/FasL to insulin resistance. Wueest *et al.* (2010) demonstrated that Fas expression is increased in the adipose tissue of mice with genetic- and diet-induced obesity, and then conducted a brief study in humans, finding that the

adipose tissue of obese subjects more highly expresses Fas than that of lean subjects, and that the adipose tissue of obese patients with type 2 diabetes expresses significantly higher levels of Fas than that of lean, non-diabetic subjects. Animal studies then showed that (1) Fas-knockout mice were protected from diet-induced glucose intolerance and elevated fasting insulin levels; (2) adipocyte-specific *Fas* knock-out mice (AFasKO) were significantly more insulin sensitive, both in adipocytes and systemically, relative to controls; (3) AfasKO mice expressed decreased mRNA levels of pro-inflammatory cytokines including IL-6, Cd11b, and MCP-1 (CCL-2) as well as decreased circulating levels of IL-6; (4) AfasKO mice were protected from hepatic insulin resistance and steatosis and had decreased levels of phosphorylation of IRS-1 (although increased levels were expected); and (5) murine adipocytes cultured in FasL-conditioned media developed insulin resistance. Consequently, the authors concluded that in obesity, Fas activation in adipocytes leads to increased secretion of pro-inflammatory cytokines, which in turn promote insulin resistance.

Taken together the previous studies suggest that Fas ligand is worthy of investigation as a serum-based biomarker of NASH and NASH-related fibrosis, for the following reasons: (1) it is firmly established that the Fas/FasL death receptor system initiates apoptosis, and apoptosis is an important pathological step in NAFLD; (2) the pro-apoptotic activities of sFasL has been associated with various diseases and disease processes such as rheumatoid arthritis, angiogenesis, and cancer, and its serum levels are inversely correlated with age and the progression of cancer, both of which involve decreasing levels of apoptosis; (3) the Fas/FasL system is highly expressed in the liver

and its level of expression correlates with the progression of NAFLD, up to and including NASH; (4) murine models of obesity suggest a mechanistic role for adipocyte-specific Fas/FasL in the development of systemic and hepatic insulin resistance as well as steatosis; (5) Fas-mediated apoptosis in the liver is linked to the development of hepatic fibrosis *via* the engulfment of apoptotic bodies by HSCs, which then differentiate into myofibroblasts and produce collagen; and (6) previous tests of sFasL as a serum-based biomarker for liver disease have been successful in most cases; *e.g.* sFasL was predictive of acute liver failure, hepatitis B and E, and alcoholic cirrhosis. Thus, I put forth a hypothesis that Fas ligand-mediated apoptosis is an important contributor to the development of NASH, by initiating cell death and fibrosis in response to liver injury and inflammation. Mechanistically, I hypothesize that the deregulation of cell signaling in the adipose tissue of morbidly obese patients leads to (1) increased expression of Fas/FasL which may lead to increased cytokine production and in turn hepatic insulin resistance and steatosis; and (2) increased secretion of sFasL from adipocytes, which in turn enhances apoptosis in hepatocytes by binding membrane-bound Fas; this chronic signaling and resulting tissue damage then triggers fibrogenesis as part of the liver's healing response.

Results.

Prediction of NASH. Table 13 shows all demographic, clinical, laboratory, and histological data and the outcomes of group statistical comparisons for patients with and without NASH. Although NASH can occur in absence of fibrosis, in this study cohort

100% of patients with NASH had some level of hepatic fibrosis. Thus, the prevalence of fibrosis was significantly higher ($p = 0.015$) in patients with NASH than in those without NASH but with steatosis. However, only 14% of patients with NASH had advanced fibrosis compared to 20% of patients without NASH. Surprisingly, patients with and without NASH did not differ in any clinical or demographic attributes, and were alike in terms of serum levels of the candidate biomarkers, CCL-2 and sFasL. Histologically, NASH patients were distinguished by the fact that only they presented pericellular fibrosis and ballooning degeneration; however, these features are among the criteria for diagnosing NASH and would be unlikely to occur in isolation. Likewise, portal and lobular inflammation as well as Kupffer cell hypertrophy occurred at notably higher rates (*i.e.* p-values approaching significance) in NASH patients, again consistent with diagnostic criteria.

Table 13. Demographic, clinical, and laboratory data for patients with and without NASH. Entries are counts for discrete measures (with percentage of group total given in parentheses) or mean ± S.D. for continuous measures. A p-value of ≤ 0.05 was considered significant. Significant results are shown in bold text. Chi = chi square test of homogeneity; FE = Fisher's exact test; MW = Mann-Whitney U test; 2T = two-sample t-test (2-tailed).

	NASH	***no NASH***	***P-value***	***Test***
N	22	15		
Fibrosis (any)	22 (100%)	10 (67%)	**0.015**	Chi
Advanced fibrosis	3 (14%)	3 (20%)	0.667	FE
Diabetes	8 (36%)	5 (33%)	0.872	Chi
Female	16 (73%)	9 (60%)	0.647	Chi
Caucasian	19 (86%)	12 (80%)	0.951	Chi
Age	49 ± 9	47 ± 11	0.596	2T
BMI	49 ± 11	46 ± 9	0.421	MW
Hyperlipidemia	12 (54%)	11 (73%)	0.417	Chi
Hypertension	15 (68%)	11 (73%)	0.801	Chi
AST (U/L)	23 ± 6.4	22 ± 7.4	0.760	2T
ALT (U/L)	35 ± 18	29 ± 9	0.496	MW
AST: ALT	0.74 ± 0.21	0.82 ± 0.34	0.577	MW
Albumin (g/dL)	4.1 ± 0.27	3.9 ± 0.77	0.732	MW
Bilirubin (total) (mg/dL)	0.44 ± 0.17	0.59 ± 0.38	0.263	MW
White blood cell count (10^3/uL)	7.6 ± 2.2	6.9 ± 1.6	0.246	MW
Platelet count (10^3/uL)	274 ± 78	270 ± 69	0.845	2T
Hemoglobin (g/dL)	13 ± 1.1	13 ± 1.7	0.878	2T
Glucose (mg/dL)	116 ± 43	104 ± 32	0.556	MW
Cholesterol (total) (mg/dL)	187 ± 30	190 ± 41	0.808	2T
Triglycerides (mg/dL)	179 ± 144	174 ± 83	0.458	MW
HDL (mg/dL)	47 ± 9	51 ± 11	0.182	2T
CCL-2 (pg/mL)	464 ± 118	486 ± 218	0.902	MW
sFasL (pg/mL)	89 ± 31	82 ± 34	0.516	MW
Portal fibrosis	16 (73%)	10 (67%)	0.976	Chi
Pericellular fibrosis	12 (54%)	0 (0%)		*n/a*
Ballooning degeneration	17 (77%)	0 (0%)		*n/a*
Portal inflammation	16 (73%)	6 (40%)	0.099	Chi
Lobular inflammation	22 (100%)	14 (93%)	0.076	Chi
Kupffer cell hypertrophy	17 (77%)	6 (40%)	0.051	Chi

Multiple linear regression using bidirectional stepwise selection revealed that for this patient cohort, only BMI and HDL were independent predictors for NASH (p = 0.048). Specifically, the probability of NASH = 0.7475 + (0.0197)[BMI] + (-0.0226)[HDL]. The standard deviation and p-value associated with each beta value are shown in Table 14.

Table 14. Model for the prediction of NASH.

	β-value	***± S.E.***	***p-value***
(Intercept)	0.7475	0.4333	0.0935
BMI	0.0197	0.0093	0.0416
HDL (mg/dL)	-0.0226	0.0094	0.0222

Prediction of Fibrosis. In an evaluation of patients with and without any form of hepatic fibrosis (Table 15), 69% of patients with fibrosis had NASH, and 19% of patients had advanced fibrosis. None of the demographic and clinical variables were significantly different between patients with and without fibrosis. Regarding the candidate biomarkers, serum levels of CCL-2 were similar between the diagnostic groups; however, sFasL was significantly higher (p = 0.015) in patients with fibrosis relative to those without fibrosis. Also, histological signs of liver disease including portal and pericellular fibrosis, ballooning degeneration and portal inflammation were limited entirely to the subcohort with fibrosis, and Kupffer cell hypertrophy was largely confined to that subcohort as well. Lobular inflammation was found at high levels in both subcohorts.

Table 15. Demographic, clinical, and laboratory data for patients with and without any hepatic fibrosis. Entries are counts for discrete measures (with percentage of group total given in parentheses) or mean ± S.D. for continuous measures. A p-value of ≤ 0.05 was considered significant. Significant results are shown in bold text. FE = Fisher's exact test; MW = Mann-Whitney U test; 2T = two-sample t-test (2-tailed).

	Fibrosis	***no Fibrosis***	***P-value***	***Test***
N	32	5		
NASH	22 (69%)	0 (0%)		*n/a*
Advanced fibrosis	6 (19%)	0 (0%)		*n/a*
Diabetes	10 (31%)	3 (60%)	0.321	FE
Female	23 (72%)	2 (40%)	0.304	FE
Caucasian	28 (88%)	3 (60%)	0.177	FE
Age	48 ± 10	49 ± 11	0.847	2T
BMI	48 ± 10	47 ± 13	0.564	MW
Hyperlipidemia	19 (59%)	4 (80%)	0.630	FE
Hypertension	23 (72%)	3 (60%)	0.603	FE
AST (U/L)	22 ± 6.4	22 ± 10	0.956	2T
ALT (U/L)	33 ± 16	30 ± 6.9	0.807	MW
AST: ALT	0.77 ± 0.21	0.81 ± 0.56	0.548	MW
Albumin (g/dL)	4.0 ± 0.56	4.1 ± 0.34	0.806	MW
Bilirubin (total) (mg/dL)	0.51 ± 0.29	0.44 ± 0.23	0.667	MW
White blood cell count (10^3/uL)	7.3 ± 1.9	7.4 ± 2.3	0.773	MW
Platelet count (10^3/uL)	267 ± 72	306 ± 79	0.347	2T
Hemoglobin (g/dL)	14 ± 1.3	13 ± 1.3	0.299	2T
Glucose (mg/dL)	112 ± 41	103 ± 17	0.947	MW
Cholesterol (total) (mg/dL)	191 ± 34	170 ± 35	0.267	2T
Triglycerides (mg/dL)	182 ± 126	142 ± 87	0.351	MW
HDL (mg/dL)	48 ± 8.9	51 ± 16	0.667	2T
CCL-2 (pg/mL)	457 ± 138	570 ± 279	0.374	MW
sFasL (pg/mL)	91 ± 30	54 ± 26	**0.015**	MW
Portal fibrosis	26 (81%)	0 (0%)		*n/a*
Pericellular fibrosis	12 (38%)	0 (0%)		*n/a*
Ballooning degeneration	17 (53%)	0 (0%)		*n/a*
Portal inflammation	22 (69%)	0 (0%)		*n/a*
Lobular inflammation	32 (100%)	4 (80%)	0.146	FE
Kupffer cell hypertrophy	22 (69%)	1 (20%)	0.057	FE

By multiple linear regression using stepwise bidirectional selection, both hypothesized biomarkers, CCL-2 and sFasL, as well as race were independent predictors of hepatic fibrosis ($p = 0.007$). Specifically, the probability of Any Fibrosis = 0.5464 + (0.2399)[Caucasian] + (-0.0006)[CCL-2] + (0.0047)[sFasL]. The variable "causasian" has a value of 1 if true, or 0 if false. The standard deviation and p-value associated with each beta value are shown in Table 16.

Table 16. Model for the prediction of any hepatic fibrosis.

	β-value	***± S.E.***	***p-value***
(Intercept)	0.5464	0.2268	0.0217
Caucasian	0.2399	0.1345	0.0837
CCL-2 (pg/mL)	-0.0006	0.0003	0.0591
sFasL (pg/mL)	0.0047	0.0016	0.0054

Prediction of Advanced Fibrosis. Only six patients in the study cohort had advanced fibrosis, specifically portal or pericellular fibrosis at stage 2, the highest stage of fibrosis observed in this study. No patient had both portal and pericellular fibrosis at stage 2, however. Three of the six patients with advanced fibrosis had NASH (Table 17), two of which also had diabetes; however a third patient with diabetes but not NASH also had advanced fibrosis. Thus, there was no statistical difference between patients with and without advanced fibrosis in terms of the occurrence of NASH or diabetes. The two diagnostic groups were not distinguished by demographic factors, but several clinical variables distinguished the groups including AST-, ALT-, and HDL serum levels. In fact, these variables appeared to be at "healthier" levels relative to the control group, in that

serum levels of AST and ALT were significantly lower in patients with advanced fibrosis, while HDL levels were significantly higher in that group. Serum levels of the candidate biomarkers CCL-2 and sFasL were not different between patients with and without advanced fibrosis; in fact, the mean and standard deviation of the levels of sFasL were coincidentally identical between the two groups. (For comparison the median levels of sFasL in patients with advanced or minimal to no fibrosis were 79 and 84, respectively.) None of the histological features associated with NASH and NASH-related fibrosis were significantly different between the two diagnostic groups.

Table 17. Demographic, clinical, and laboratory data for patients with and without advanced fibrosis. Entries are counts for discrete measures (with percentage of group total given in parentheses) or mean ± S.D. for continuous measures. A p-value of ≤ 0.05 was considered significant. Significant results are shown in bold text. Chi = chi square test of homogeneity; FE = Fisher's exact test; MW = Mann-Whitney U test; 2T = two-sample t-test (2-tailed).

	Adv. Fibrosis	***none to minimal Fibrosis***	***P-value***	***Test***
N	6	31		
NASH	3 (50%)	19 (61%)	0.667	FE
Diabetes	3 (50%)	10 (32%)	0.664	FE
Female	4 (67%)	21 (68%)	1.00	FE
Caucasian	5 (83%)	26 (84%)	0.561	Chi
Age	51 ± 10	48 ± 10	0.483	2T
BMI	48 ± 2.6	48 ± 11	0.564	MW
Hyperlipidemia	4 (67%)	19 (61%)	1.00	FE
Hypertension	6 (100%)	20 (65%)	0.244	Chi
AST (U/L)	20 ± 2.4	23 ± 7.2	**0.042**	2T
ALT (U/L)	23 ± 4.7	34 ± 16	**0.026**	MW
AST: ALT	0.88 ± 0.15	0.75 ± 0.29	0.122	MW
Albumin (g/dL)	4.0 ± 0.28	4.0 ± 0.57	0.804	MW
Bilirubin (total) (mg/dL)	0.52 ± 0.28	0.49 ± 0.28	0.753	MW
White blood cell count (10^3/uL)	8.0 ± 2.7	7.2 ± 1.8	0.592	MW
Platelet count (10^3/uL)	256 ± 99	276 ± 69	0.649	2T
Hemoglobin (g/dL)	12.4 ± 1.8	13.6 ± 1.2	0.156	2T
Glucose (mg/dL)	103 ± 41	113 ± 39	0.606	MW
Cholesterol (total) (mg/dL)	203 ± 15	185 ± 36	0.065	2T
Triglycerides (mg/dL)	144 ± 39	183 ± 131	0.853	MW
HDL (mg/dL)	56 ± 7.8	47 ± 9.8	**0.041**	2T
CCL-2 (pg/mL)	390 ± 103	488 ± 169	0.161	MW
sFasL (pg/mL)	86 ± 33	86 ± 33	0.805	MW
Portal fibrosis	5 (83%)	21 (68%)	0.782	Chi
Pericellular fibrosis	3 (50%)	9 (29%)	0.367	FE
Ballooning degeneration	1 (17%)	16 (52%)	0.187	FE
Portal inflammation	5 (83%)	17 (55%)	0.397	Chi
Lobular inflammation	6 (100%)	30 (97%)	0.500	Chi
Kupffer cell hypertrophy	4 (67%)	19 (61%)	1.00	FE

Analysis by multiple linear regression with stepwise bidirectional selection showed that HDL and CCL-2 were independent predictors of advanced hepatic fibrosis ($p = 0.028$). Specifically, the probability of Advanced Fibrosis = -0.2154 + (0.0142)[HDL] + (-0.0007)[CCL-2]. The standard deviation and p-value associated with each beta value are shown in Table 18.

Table 18. Model for the prediction of advanced fibrosis.

	β-value	***± S.E.***	***p-value***
(Intercept)	-0.2154	0.3122	0.4949
HDL	0.0142	0.0059	0.0214
CCL-2	-0.0007	0.0004	0.0757

Discussion. The goal of this study was to identify and test novel serum-based biomarkers of NASH and NASH-related fibrosis that could be potentially tied to deregulated cell signaling in adipose tissue. Using the phosphoproteomic data set generated by Younossi *et al.* (2010), enrichment analysis was conducted and that analysis resulted in the identification of pathways that were deregulated in the adipose tissue of patients with NASH relative to what is found in the adipose tissue of control patients. The five pathways most enriched were those relating to insulin signaling, including the IGF-1 receptor pathway, insulin regulation of translation, AKT- and PIP3 signaling, and regulation of lipid metabolism by insulin signaling. A number of molecules within those pathways were phosphorylated at lower levels in NASH patients than in patients without NASH, particularly IRS-1, SHC, PDPK1, p70-S6 kinase 2, and 4E BP1. These results

suggest that cell signaling pathways in the adipose tissue of morbidly obese patients with NASH are indeed deregulated. Further analysis revealed that a number of secreted molecules could be linked to these pathways and to disease processes associated with NAFLD (*e.g.* insulin resistance, liver fibrosis) including cytokines, chemokines and matrix metalloproteinases, among others. An extensive literature search was then undertaken to identify which members of this collection might have a functional role in liver disease and consequently be linked to the development of NAFLD, resulting in the identification of CCL-2/MCP-1 and FasL as candidate serum-based biomarkers. Based on those findings, I hypothesized that (1) circulating CCL-2, elevated in patients with NAFLD due to enlarged visceral adipose tissue, causes monocyte infiltration into the liver and contributes to steatosis and insulin resistance of hepatocytes, thereby playing a role in the pathology of NAFLD; and (2) that Fas ligand-mediated apoptosis is an important contributor to the development of NASH, initiating cell death and fibrosis in response to liver injury and inflammation, and that the deregulation of cell signaling in the adipose tissue of morbidly obese patients may lead to increased secretion of sFasL, which in turn would promote apoptosis and fibrosis in hepatocytes.

Interestingly, the results of this study suggest that both candidate biomarkers are potentially useful markers of hepatic fibrosis, yet only the second of the two hypotheses mechanistically supports this observation. It was expected that if the secretion of CCL-2 from adipose tissue contributes to the pathology of NAFLD, then its serum levels would increase with increasing progression of the disease. Instead, average serum concentrations of CCL-2 decreased with progression of the disease, such that in patients

with steatosis, NASH (with any fibrosis), fibrosis with or without NASH, and advanced fibrosis, the average concentrations were 570, 464, 457, and 445 pg/mL, respectively. Consequently, *decreasing* CCL-2 was an independent predictor of fibrosis and advanced fibrosis, although it was not a significant predictor of NASH. Differences in serum levels of CCL-2 between the diagnostic groups also were not statistically significant in any group comparisons.

Serum levels of sFasL were significantly increased in patients with any form of hepatic fibrosis relative to those without fibrosis but with steatosis. Moreover, increasing sFasL was independently predictive of hepatic fibrosis. However, serum levels of sFasL were not significantly different in group comparisons or in regression modeling relating to the prediction of NASH or advanced fibrosis. Tamimi *et al.* (2011) also did not find a statistical difference in sFasL levels between patients with and without NASH, however in both their study and mine sFasL levels were higher in NASH patients than in those without NASH. As in this study, Tamimi *et al.* found that sFasL was not a significant predictor of NASH by regression analysis.

A few additional findings in this study are worth consideration. Increasing BMI and decreasing HDL were independent predictors of NASH. Other studies of NASH have underscored the importance of these variables. BMI is positively correlated with NAFLD ($p < 0.001$) in a dose-response manner (Jiang *et al.*, 2010) and also correlates strongly ($p < 0.001$) with NAS (NAFLD activity score; a scoring system for NAFLD severity up to and including NASH), while serum HDL levels are inversely correlated ($p = 0.004$) with NAS (Puljiz *et al.*, 2010). Likewise, in group comparisons Tamimi *et al.* (2011) reported

significantly higher BMI values and somewhat lower HDL levels in NASH patients relative to those without NASH.

Whether an individual was Caucasian factored into the model predicting hepatic fibrosis. Hossain *et al.* (2009) found that NAFLD patients with moderate to severe fibrosis were more likely to be Caucasian, and a multiple regression model for the prediction of moderate to severe fibrosis included Caucasian as a significant predictor.

In the evaluation of advanced fibrosis, the some of the results are contrary to expected trends. AST and ALT were significantly lower in the group with advanced fibrosis relative to controls, but in another study they were significantly higher in patients with NAFLD and moderate to severe fibrosis and increasing levels of these aminotransferases were independent predictors for moderate to severe fibrosis by multiple regression (Hossain *et al.,* 2009). However, in this study the average AST and ALT levels were within the normal range (<40) for both diagnostic groups and therefore the finding of significant difference between the groups may not be biologically relevant. HDL was significantly higher in patients with advanced fibrosis, whereas in the study by Hossain *et al.* (2009) it was not significantly different between NAFLD patients with and without moderate to severe fibrosis nor was it a significant predictor for moderate to severe fibrosis in regression analysis. The average HDL levels measured in this study, while not at a level that is considered protective of heart disease (>60) were also not alarmingly low in either group, so again the statistical difference between the diagnostic groups may not be relevant to the progression of NAFLD. Of the three clinical variables discussed, only HDL was an independent predictor of advanced fibrosis in multiple regression analysis.

There were some limitations to this study that could be easily addressed in future testing of CCL-2 or sFasL as biomarkers of NASH and NASH-related fibrosis. One explanation for the seemingly contradictory performances of sFasL in this study, as well as the failure of CCL-2 to predict NASH, is simply lack of statistical power. My study cohort was heavily weighted toward individuals with NASH and mild fibrosis; only five subjects had steatosis alone and only six had advanced fibrosis. Furthermore, the classification of "advanced" in this study was limited to fibrosis at stage 2 (of a possible 4) and was predominantly portal in nature (4 of 6 cases), which is not as strongly associated with NASH as is pericellular fibrosis (Hall and Kirsch, 2005). Thus, it is possible that larger and better defined sub-cohorts would have led to greater statistical power to differentiate patients with various stages of NAFLD using CCL-2 and sFasL. For example, patients with stages 3 and 4 portal or pericellular fibrosis and more examples of stage 2 pericellular fibrosis, as well as greater numbers of patients with steatosis with and without accompanying inflammation would be desirable (in this study all but one subject had some degree of lobular inflammation).

Future experiments designed to test CCL-2 and sFasL as biomarkers of NAFLD should involve simultaneously testing of other, targeted molecules to better support hypotheses regarding the pathology of NAFLD. It would be particularly helpful to measure established serum biomarkers of apoptosis, such as caspase-cleaved cytokeratin 18 (M30 antigen), concurrent with screening sFasL levels so that correlations between the two could be calculated; if the two positively correlate than those data would underscore the role of sFasL as a biomarker of apoptosis. If not, perhaps it is inaccurate

to regard sFasL as a pro-apoptotic factor in the context of NASH; certainly it was not predictive of NASH in this study or that by Tamimi *et al.* (2011). In fact, Tamimi *et al.* (2011) found that sFas (receptor), which in other contexts is implicated in the suppression of apoptosis (Lapinsky *et al.*, 2006; Raghuraman *et al.*, 2005), was predictive of NASH and correlated significantly with histologic features of NASH.

Another avenue that would be interesting to explore is the relationship between CCL-2 and Fas/FasL expression in human adipocytes and hepatocyes. If the findings in Wueest *et al.* (2010) using murine models of obesity could be extended to humans, they would suggest that Fas/FasL overexpression in the adipocytes of obese individuals leads to increased secretion of pro-inflammatory cytokines into the bloodstream, which in turn promotes insulin resistance and steatosis in the liver. Schaub *et al.* (2003) showed that increased expression of the Fas/FasL system in human vascular smooth muscle cells increased secretion of CCL-2/MCP-1, as mediated by interleukin-1 alpha (IL-1α). However, in this study sFasL increased with increasing severity of fibrosis whereas CCL-2 decreased with increasing severity of fibrosis. As suggested in the second hypothesis, there may be two disparate contributions of Fas/FasL to the pathology of NAFLD; one in which adipose-specific Fas/FasL leads to hepatic insulin resistance and steatosis as mediated by inflammatory factors such as CCL-2, and another in which sFasL contributes to hepatic fibrosis. Given that all the patients in this study except five had some form of fibrosis, perhaps the second mechanism was easy to detect while the first, *i.e.* the contribution of CCL-2 to hepatic inflammation, would have been easier to detect with a larger subcohort of patients having only steatosis or NASH without fibrosis.

A few technical approaches could be improved in future experiments. Due to limited serum volumes, each patient in this study was measured only once (albeit in duplicate) for each analyte. Future experiments testing patients in triplicate would provide greater accuracy, and repeated assays would allow inter-assay variability to be assessed. Use of a larger cohort would provide more statistical power and, hopefully, more examples of the full spectrum of NAFLD, as described previously. Finally, in this study there was a mechanical failure of a plate reader, causing the CCL-2 assay to sit unmeasured for 1 hr. beyond the recommended time interval in which measurements should have been taken. Fortunately, the quality of the data did not seem compromised by this event, as the fit of the standard curve remained at an R-value of 1. Nonetheless, repeated testing of CCL-2 in patients with NAFLD would be useful to confirm the results reported here.

Conclusions. The results of this study suggest that deregulation of insulin signaling in the adipose tissue of obese patients may lead to increased secretion of the chemokine CCL-2 and the soluble, apoptosis-inducing ligand sFasL, which in turn are predictive of hepatic fibrosis. Lack of a sufficiently large control group without fibrosis may account for the failure to detect significant differences in the serum levels of these candidate biomarkers between patients with and without NASH. Nonetheless, this study is in accordance with others implicating a pathological role for CCL-2 and sFasL in the progression of NAFLD.

Results.

Two sets of analyses were conducted for this study, each investigating the relationship(s) between serum-based parameters (specifically, serum levels of selected cytokines, hormones, and metabolic factors), and fatigue. Patients were categorized as having peripheral or central fatigue as described in Chapter 2, and were compared against an appropriate control group; *i.e.*, patients with chronic liver disease who did not experience the type of fatigue in question.

Peripheral fatigue. For the analysis of peripheral fatigue, the total sample size was 23 (specifically, 20 subjects, three for whom follow-up data were available); totaling 9 in the "high MET" group and 14 in the "low MET" group. In a comparison of patients with and without peripheral fatigue, those who were fatigued (low MET) had significantly elevated serum levels of IL-6 and IL-8 ($p < 0.01$ and < 0.05, respectively) relative to those without fatigue (high MET) (Table 19). Aside from IL-6 and IL-8, no other serum-based parameters differed significantly between the high and low MET groups.

Table 19. Results of group comparisons between patients with and without peripheral fatigue. Table entries for "low MET" and "high MET" groups (except in the case of LDL) are medians (25^{th}, 75^{th} quartiles); entries for LDL are mean ± SD. Significant results are shown in bold text. MW = Mann Whitney U test; 2T = two sample, two-tailed student t-test assuming separate variances.

Parameter	***Low MET***	***High MET***	***p-value***	***Test***
Serotonin (ng/ml)	89 (27, 108)	206 (96, 246)	0.101	MW
C-peptide insulin (pg/ml)	3131 (2441, 4282)	2405 (2120, 2768)	0.131	MW
IL-8 (pg/ml)	19 (16, 25)	13 (11, 20)	**0.044**	MW
TNF-α (pg/ml)	7.8 (6.5, 11)	8.1 (4.6, 9.0)	0.705	MW
IL-6 (pg/ml)	2.9 (2.6, 3.7)	1.6 (1.2, 1.8)	**0.006**	MW
ALT (U/L)	53 (33, 63)	39 (24, 58)	0.256	MW
AST (U/L)	50 (36, 62)	40 (26, 46)	0.088	MW
AST:ALT	1.1 (0.9, 1.1)	1 (0.9, 1)	0.497	MW
TC (mg/dL)	171 (152, 227)	212 (199, 221)	0.231	MW
TRG (mg/dL)	95 (74, 156)	121 (77, 163)	0.875	MW
HDL (mg/dL)	40 (32, 50)	47 (30, 61)	0.468	MW
LDL (mg/dL)	132 ± 51	135 ± 44	0.721	2T
Non-HDL fraction (mg/dL)	126 (108, 197)	153 (132, 188)	0.450	MW
Glucose (mg/dL)	103 (96, 127)	101 (88, 106)	0.242	MW

A second analysis investigated the relationship between age and objective measures of peripheral fatigue. Patients (n = 36) were divided into 3 age categories: low (<41 years old), medium (41-50 yrs), and high (>50 yrs). The measures of fatigue included MET scores, 6-min. walk distance, and Borg Scale value. The differences among age groups for the three measures of fatigue were tested by Kruskal Wallis test. None of the differences were significant (Table 20). Thus, age does not appear to play a role in peripheral fatigue in patients with chronic liver disease.

Table 20. Results of group comparisons comparing age groups (low, medium, high) with objective measures of peripheral fatigue (MET scores, 6-min. walk distance, and 6-min. Borg Scale value).

Measure of Peripheral Fatigue	p-value (Kruskal Wallis)
6 min. walk (distance)	0.398
Borg Scale value (6 min.)	0.531
MET	0.247

Central Fatigue. For the analysis of central fatigue, repeat visits were excluded and pertinent data were lacking for one patient, bringing the total sample size to 30; of these, 13 were classified as having central fatigue and 17 did not have central fatigue. Those with and without central fatigue were compared with regard to all laboratory parameters (Table 21). Only one variable, the ratio of AST to ALT, was significantly different between the groups.

Table 21. Results of group comparisons between patients with and without central fatigue. Table entries for "yes" (central fatigue present) and "no" (central fatigue absent) groups (except in the case of LDL) are medians (25^{th}, 75^{th} quartiles); entries for LDL are mean ± SD. Significant results are shown in bold text. MW = Mann Whitney U test; 2T = two sample student t-test assuming separate variances.

Parameter	*Yes*	*No*	*p-value*	*Test*
Serotonin (ng/ml)	106 (22, 224)	118 (92, 190)	0.601	MW
C-peptide insulin (pg/ml)	2768 (2412, 3940)	2348 (2028, 2882)	0.233	MW
IL-8 (pg/ml)	20 (14, 26)	16 (13, 20)	0.217	MW
TNF-α (pg/ml)	6.3 (3.4, 8.1)	7.6 (6.5, 10)	0.082	MW
IL-6 (pg/ml)	2.7 (1.8, 3.0)	1.9 (1.6, 3.8)	0.601	MW
ALT (U/L)	58 (39, 83)	33 (24, 47)	0.082	MW
AST (U/L)	51 (37, 62)	31 (26, 58)	0.325	MW
AST:ALT	0.9 (0.7, 1)	1.1 (1, 1.1)	**0.004**	MW
TC (mg/dL)	183 (148, 221)	199 (163, 212)	0.630	MW
TRG (mg/dL)	82 (60, 158)	89 (59, 132)	0.769	MW
HDL (mg/dL)	51 (36, 58)	42 (33, 49)	0.516	MW
LDL (mg/dL)	123 ± 38	134 ± 43	0.488	2T
Non-HDL fraction (mg/dL)	147 (106, 166)	132 (117, 171)	0.676	MW
Glucose (mg/dL)	106 (101, 125)	102 (88, 112)	0.276	MW

Correlations. Using the full data set including follow-up visits (N = 36), a number of significant correlations were observed among cytokines, liver enzymes, and serum lipids (Table 22). Serum levels of IL-6 were positively correlated with levels of IL-8 and negatively correlated with MET value, and serum concentrations of IL-8 also correlated with the liver enzymes, ALT and AST. ALT and AST concentrations were positively correlated with each other. Serum levels of LDL cholesterol, not surprisingly, correlated with total cholesteral levels as well as with serum tryglyceride levels.

Table 22. Significant correlations among cytokines, liver enzymes, and serum lipids in patients with chronic liver disease.

Comparison	p-value	Type of Correlation
IL-6 vs. IL-8	0.028	positive
IL-6 vs. MET	0.013	negative
IL-8 vs. ALT	0.019	positive
IL-8 vs. AST	0.019	positive
AST vs. ALT	<0.001	positive
LDL vs. total cholesterol	<0.001	positive
LDL vs. triglycerides	0.046	positive

Discussion. The biological underpinnings of the fatigue associated with chronic disease have not been fully understood, yet fatigue can be debilitating to patients, affecting their physical, emotional, and social well-being. Chronic liver diseases in which fatigue is a symptom include NAFLD, alcoholic cirrhosis, various forms of viral hepatitis, and primary billiary cirrhosis. Fatigue associated with liver disease can be extensive, contributing to lower quality of life (Sogolow *et al.*, 2008). In NAFLD, fatigue manifests as decreased physical activity and an increase in the perception of fatigue; furthermore, it is strongly correlated with daytime sleepiness and dysfunction of the autonomic nervous system, which in turn can lead to dizziness and increase the likelihood of falling (Newton, 2010; Newton *et al.*, 2008). The extent of fatigue is not, however, related to the severity of NAFLD or to insulin resistance (Newton *et al.*, 2008). Children with NAFLD also experience fatigue, and this fatigue contributes significantly to a decline in quality of life relative to that of a healthy reference population (Kistler *et al.*, 2010). In HCV, fatigue may arise from abnormalities of the neuroendocrine pathways associated with

regulation of cortisol and the stress response (Swain, 2000; Swain and Maric, 1995). The severity of fatigue in patients with HCV is associated with elevated plasma levels of leptin and TNFα, and decreased plasma levels of L-carnitine (Anty *et al.*, 2011; Piche *et al.*, 2002).

The aim of this study was to to determine whether, in patients with chronic liver disease, there is a correlation between self-reports of physical activity-associated fatigue (peripheral fatigue) or more global lack of energy and motivation (central fatigue) with serum markers of inflammation, or with abnormalities of glucose and lipid metabolism. The results of this study suggest that serum levels of the pro-inflammatory cytokines, IL-6 and IL-8 may be important biomarkers of peripheral fatigue in patients with chronic liver disease. Both cytokines were significantly elevated in patients with low MET scores, and IL-6 also negatively correlated with MET score. Most studies relating IL-6 and IL-8 to chronic fatigue have done so in the context of chronic fatigue syndrome (CFS); in these studies and others, there appears to be concordant evidence for the role of IL-6 in fatigue, and somewhat conflicting information regarding IL-8. Circulating levels of IL-6 are significantly higher in patients with chronic fatigue syndrome (CFS) relative to controls (Nas *et al.*, 2011), possibly due to its increased secretion from peripheral blood mononuclear cells of patients with CFS (Chao *et al.*, 1991). By analysis with mutiplex technology, IL-6 was significantly elevated in the plasma of women with CFS; however, IL-8 was significantly lower relative to controls (Fletcher *et al.*, 2009). In contrast, circulating levels of both IL-6 and IL-8 were elevated in patients with CFS experiencing a flare-up of symptoms following moderate exercise. No studies to date

have compared blood levels of cytokines with measures of fatigue associated with chronic liver disease. However, a recent study, noting the association of sleep disorders with disturbances in immune response, found that individuals with HCV experiencing poor quality of sleep had significantly higher serum levels of IL-6, while HCV-infected individuals who sleep well have significantly decreased serum levels of IL-8 (de Almeida *et al.*, 2011); they hypothesized that the hypersecretion of these pro-inflammatory cytokines indicated worsening of the disease, which in turn led to disturbances in sleep.

Another finding of this study is that age does not appear to contribute to peripheral fatigue in patients with chronic liver disease. There was no significant relationship between age and measures of peripheral fatigue, including MET score, Borg scale value, and 6-min. walk distance. This finding is important given that the ages of the participants in this study spanned three decades, ranging from 33 to 63 years old; thus, peripheral fatigue in patients with chronic liver disease cannot be considered a consequence of aging.

Regarding central fatigue, the ratio of AST to ALT was significantly lower in patients with central fatigue. The median ratio value in this cohort was 0.9, compared to 1.1 in the cohort without central fatigue. The biological significance of this finding is unclear. The conventional use of this ratio is to distinguish patients with alcoholic liver disease, who tend to have a ratio ≥ 2, from NASH, having a ratio <1 (Sorbi *et al.*, 1999). However, in a direct comparison of patients with either of those two diseases, a ratio of >1 is sufficient to identify patients with alcoholic liver disease (Zamin *et al.*, 2002). The AST/ALT ratio is not diagnostic for HCV; in patients already diagnosed with HCV an AST/ALT value

>1 was formerly considered indicative of cirrhosis, however that guideline has been disputed (Bartos *et al.*, 2007; Reedy *et al.*, 1998). In this study, there were approximately equal counts of patients with NAFLD or with HCV falling into the "yes" and "no" classifications for central fatigue; therefore the significant difference in AST/ALT values between the two cohorts did not reflect disease status, as confirmed by Fisher's Exact test ($p = 0.688$). No studies have directly compared AST or ALT values to measures of fatigue in chronic liver disease; however there is scant circumstantial evidence for a relationship between altered aminotransferase levels and fatigue. For example, fatigue was reported by 50 patients with biopsy-proven NAFLD, representing the entire study cohort, and the AST/ALT was <1 for 72% of this cohort (Khurram and Ashraf, 2007). Likewise, in a cohort of 20 patients with primary biliary cirrhosis, fatigue was a major symptom while aminotransferases, although slightly elevated, were reported to be "reversed" in terms of the AST/ALT ratio (Yao *et al.*, 2002).

The remaining positive correlations observed in this study merit some discussion. IL-6 and IL-8 were positively correlated with one another, IL-8 was positively correlated with the aminotransferases, and the aminotransferases correlated positively with each other. These findings support similar results found in studies of NAFLD and HCV. Serum levels of IL-6 and IL-8 are highly correlated in patients with NAFLD (Jarrar *et al.*, 2008), and serum levels of IL-6 are significantly higher in patients with chronic hepatitis C relative to controls (Lee *et al.*, 2010; Antonelli *et al.*, 2009). Serum levels of IL-8 are significantly elevated in patients with NAFLD relative to obese and non-obese controls (Jarrar *et al.*, 2008) and increase with the progression of hepatitis C (Neuman *et al.*,

2007). In fact, IL-8 is induced by hepatitis C and its consistent elevation in the context of that disease justified its use as a marker of anti-viral drug treatment efficacy (Akbar *et al.*, 2011). AST and ALT are frequently elevated above the normal range in NAFLD and their sustained elevation in the bloodstream over time plays into the decision of whether to suspect NAFLD, and even whether to conduct a liver biopsy (Younossi, 2008). Elevated aminotransferases including AST and ALT are also associated with chronic hepatitis B and C and several other liver diseases (Liu, 2009; Maier, 2005).

Finally, in this study there was a positive correlation between LDL and total cholesterol, and between LDL and triglycerides. Lipoproteins are spherical particles released from or delivered to the liver that contain proteins (known as apolipoproteins) and lipids. The role of lipoproteins is to transport lipids through the bloodstream; among these lipids are cholesterol and triglycerides. Low density lipoproteins (LDL) deliver lipids from the liver to tissues and organs, whereas high density lipoproteins (HDL) deliver lipids from organs and tissues to the liver for processing. Consequently, the finding of positive correlations between LDL and total cholesterol and between LDL and triglycerides is not surprising and indicates a high lipid content of the blood and tissues of this patient cohort, all of whom were overweight or obese. A positive correlation between LDL and total cholesterol was identified over two decades ago (Lam *et al.*, 1990), and levels of total cholesterol and LDL were recently shown to aggregate by family to an extent that environmental factors appear negligible (de Miranda Chagas *et al.*, 2011); thus it is possible that genetics accounted for the strong association between these two variables in the current study. Previously reported correlations between LDL and

triglycerides depended on the class of LDL under consideration and whether blood levels of triglycerides exceeded a given threshold, with some correlations being highly positive and others highly negative depending on the particular combination of factors (Griffin *et al.*, 1994); however increasing serum triglycerides is associated with a concomitant increase in the triglyceride content of total LDL particles and of large but not small LDL particles (Halle *et al.*, 1999), suggesting a potential biological mechanism for the observed positive correlation between serum triglyerides and LDL levels in this study.

As a pilot study, the research presented herein represents a first step in the effort to identify objective, serum-based biomarkers of fatigue in patients with chronic liver disease. Thus, several aspects of this research could be improved in future studies continuing this area of investigation. First, larger patient cohorts would be advisable. This study may have failed to detect other relationships between fatigued and non-fatigued patients simply due to its small cohort of 31 patients. Furthermore, there were only four patients in total who had neither peripheral nor central fatigue and it would be of interest to measure serum levels of the significant parameters (IL-6, IL-8) with those levels found in a larger cohort of patients with chronic liver disease but no fatigue, as well as in patients without chronic liver disease. As mentioned in other chapters, liver biopsy would not be conducted on healthy individuals but perhaps a "healthy" control group could be identified based on a weight of evidence approach, in which the status of "no liver disease" could be surmised from lack of evidence of liver dysfunction based on clinical variables, a lack of family history of liver disease, etc. Another problem with the use of the small cohort in this study is that several parameters, including serum cytokines, were

highly skewed and therefore the significant results found for IL-6 and IL-8, for example, may have been driven by a small number of patients. Again, use of a larger cohort in future studies would help to reduce uncertainty in significant results.

Another recommendation would be to expand the number of serum markers of inflammation, since these preliminary results suggest that pro-inflammatory cytokines appear to correlate well with peripheral fatigue. In studies of cancer, fatigue is known to occur not only from pharmaceutical treatments targeting the immune system but also is a problem after treatment ceases, possibly due to the prolonged release of pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα; release of these factors by tumors may also account for fatigue in patients not receiving immunotherapy (Bower, 2007). Likewise, serum levels of C-reactive protein (CRP) were significantly higher in survivors of breast cancer suffering from chronic fatigue than in those survivors not experiencing fatigue (Reinertsen *et al.*, 2010). Regarding chronic liver disease, the recent findings of elevated leptin and TNFα and decreased L-carnitine in fatigued subjects with HCV (Anty *et al.*, 2011; Piche *et al.*, 2002) are also intriguing and would suggest that these molecules, although not all related to immune function, would be good to test in future studies of fatigue involving patients with HCV.

A final recommendation would be to investigate additional means of defining central fatigue in patients with chronic liver disease. Since the only significant finding in this study was that patients with and without central fatigue differed only in their AST:ALT ratio, usually an indicator of type of liver disease but not effective in this study, it would

be interesting to test whether other measures of central fatigue would be more helpful in differentiating patients according to blood parameters.

Conclusions. Our study demonstrated that in patients with chronic liver disease (1) the pro-inflammatory cytokines IL-6 and IL-8 are significantly higher in patients with peripheral fatigue and are therefore potentially useful biomarkers of peripheral fatigue in these patients; and (2) age does not appear to play a role in peripheral fatigue. Future studies expanding on the current findings and incorporating additional measures of central fatigue will likely be useful in better characterizing fatigue in patients with chronic liver disease.

6: CONCLUSIONS

In 2000, the National Cancer Institute (NCI) established the Early Detection Research Network (EDRN), which provided an infrastructure to coordinate the multidisciplinary efforts needed to develop tools for the early detection of cancer (Srivastava and Kramer, 2000). The NCI recognized that the preliminary work of research laboratories to identify promising biomarkers of cancer was not being used as a basis for subsequent studies further investigating the utility of these biomarkers as screening tools in clinical settings, despite a growing awareness of the importance of early detection in cancer therapy. A reason for the disparity between laboratory and clinical research was a lack of stable connections between the two types of institution and hence the lack of appropriate samples and associated diagnostic, clinical, and demographic data on which to validate and refine potential biomarkers. As a result, the EDRN established a network of laboratories designed to coordinate efforts in the identification and validation of candidate biomarkers. Members of this network proposed a 5-phase plan for the process of biomarker development (Pepe *et al.*, 2001). Phase 1 involves preclinical exploratory studies in which a wide variety of genes and proteins are analyzed for differential expression between diseased and non-diseased tissues, with the goal of discovering and prioritizing potentially useful biomarkers. Phase 2 involves the non-invasive collection of samples and measurement of the potential biomarker using a clinical assay. The clinical

assay must distinguish diseased from non-diseased individuals and be assessed for performance by measures such as sensitivity, specificity, and ROC curve analysis. Since in Phase 2 a biomarker is tested in the context of patients already with established disease, the ability of the biomarker to discern early stages of the disease cannot be assessed. Therefore, Phase 3 requires the execution of retrospective longitudinal studies, in which a biomarker is tested for its capacity to detect pre-clinical disease. These studies begin with a repository of samples collected from seemingly healthy subjects who were monitored over time for the development of cancer; samples from those who developed cancer become cases and are compared to appropriate controls, *i.e.* samples from subjects who did not develop cancer. Levels of biomarker expression are then compared between the case and control cohorts to determine whether the biomarker can distinguish the cohorts at the time of sample collection, which preceded clinical diagnosis. Phase 4 tests the biomarker's performance in a prospective manner; it is broadly applied to patients as a screening tool and those with positive results receive subsequent, definitive diagnostic testing. This phase allows further characterization of the biomarker in terms of its performance and highlights the stage(s) of disease at which it works efficiently; however, large sample sizes are needed since the tool is not applied to a targeted population, and ethical concerns arise as patients will receive diagnoses and treatments as a result of the biomarker test. In phase 5, the final step, studies are undertaken to determine whether implementation of the biomarker test reduces mortality in the population at large. Even tests that identify early stages of disease are not always helpful in reducing overall mortality, due to a lack of effective treatment for the disease, difficulty implementing the

screening program, prohibitive costs associated with executing the screening or with diagnostic testing of false positives, or over-diagnosis of disease cases that might otherwise have gone into regression.

Although the 5-phase plan for biomarker development was designed for the detection of cancer, its framework is readily applied to the development of biomarkers for other diseases (Miller *et al.*, 2011). The biomarkers described herein targeted NASH and NASH-related fibrosis from patients with NAFLD, or targeted fatigue associated with chronic liver disease. The studies identifying and validating these biomarkers largely fall within the criteria of Phase 2 development. In the first study, all candidate proteins tested as part of the biomarker panel had been separately identified in previous studies as effective in differentiating a disease state from a non-diseased state, either with regard to NAFLD or to a more generalized disease process (*e.g.* apoptosis, fibrosis). Thus, there was not a biomarker discovery *per se* but rather a "meta-discovery" that already identified biomarkers work optimally in combination to predict NASH and NASH-related fibrosis. Furthermore, these were tested and validated on non-invasively collected samples from patients that had been definitively diagnosed with established stages of NAFLD, further fitting the requirements of Phase 2. The second study, identifyng CCL-2 and FasL as potential biomarkers of NAFLD-related fibrosis, also fit the requirements for Phase 2 development in that they were measured by clinical assay in non-invasively collected samples from patients with definitive diagnoses; however, since these biomarkers were chosen by pathway analysis based on protein array data and had undergone only a limited amount of testing as biomarkers of liver disease in other

studies, they also somewhat fit the description of biomarker discovery outlined in Phase 1. The third study, identifying biomarkers of fatigue in patients with chronic liver disease, also straddled the line between Phases 1 and 2 of the 5-phase plan. Elevated levels of serum cytokines have been associated with fatigue in other diseases, including chronic fatigue syndrome, but had only rarely been tested as biomarkers of fatigue in the context of chronic liver disease, and apparently not at all in the context of NAFLD; thus, one can argue that IL-6 and IL-8 (and perhaps the AST/ALT ratio) were "discovered" biomarkers of fatigue in NAFLD and HCV. Moreover, these were tested on definitively diagosed cohorts of patients, using clinical assays and serum, and therefore also fit the requirements of phase 2.

In summary, the studies presented in this document illustrate that (1) serum markers of apoptosis and fibrosis, in combination with specific clinical and demographic data are useful in distinguishing patients with NASH and NASH-related fibrosis from those with steatosis; (2) serum levels of the chemokine CCL-2, and the apoptotic signaling molecule, sFasL, in combination with specific demographic and clinical data may be useful for distinguishing NAFLD-related fibrosis (with or without NASH) from steatosis; and (3) serum levels of the cytokines IL-6 and IL-8, and possibly the AST/ALT ratio, may be predictive of fatigue in patients with chronic liver disease. Taken together, the molecules that proved most useful in these studies suggest that the physiological processes most important to the pathology of NAFLD and perhaps chronic liver disease in general are inflammation, apoptosis, and fibrosis. While those processes have been widely implicated in numerous other studies of NAFLD, the data presented herein further

confirm that these processes are worthy of continued study in the context of chronic liver disease.

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CURRICULUM VITAE

Sandra Jean Page was born in Mount Holly, NJ and grew up in the countryside of Pennsylvania taking care of many four-legged animals. She pursued her undergraduate degree in liberal arts but elective coursework in biology convinced her to take a new direction. In 1996 she joined the Masters Program in Biology at George Mason University and earned her degree with a concentration in Environmental Science and Public Policy. She then spent a number of years working as a professional fisheries biologist for the State of Maryland, an environmental consulting firm, and the U.S. Geological Survey. In doing so she assisted toxicologists, veterinarians and pathologists in conducting a variety of animal health studies. An interest in the use of biomarkers to detect disease led her to a graduate program at North Carolina State University, where she conducted research on the molecular characterization of genes serving as biomarkers of xenoestrogens, and earned a second Masters degree. Family ties and a growing interest in human health brought her back to the northern VA region, where she first worked as a volunteer research assistant for a breast cancer researcher and then fulfilled a contractual position at Invitrogen Federal Systems. In 2008 she returned to George Mason University to pursue doctoral work in the development of biomarker panels for liver disease. When not working, she has enjoyed spending time with her loving husband, Eddie, and her joyful son, James.