COMPARATIVE PROFILING OF CIRCULATORY LEVELS OF ADIPOKINES AND CYTOKINES IN PATIENTS WITH VARIOUS TYPES OF NON ALCOHOLIC FATTY LIVER DISEASE.

by

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DEDICATION

This thesis is dedicated to the many people who have contributed in thought, work, and support to this project. Thanks also goes to all those I was privileged to work with at George Mason University, Translational Research Institute and Center for Liver Disease (Inova Fairfax Hospital). Particularly, my thanks and appreciation go to my family who has supported me these past several years in so many ways, especially my wife and kids for giving me most of the time that I have to spend with them, to my parents for their encouragement and support and to my brothers and sisters for their help. You all have been always a dedicated honest supporters and quite an inspiration in my life.

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LIST OF ABBREVIATIONS AND SYMBOLS

Term	Abbreviation/Symbol
Acyl-CoA synthetase long-chain family member 4	ACSL4/FACL4
Alcoholic Liver disease	ALD
Analysis of variance	ANOVA
Alcoholic steatoHepatitis	ASH
Aspartate aminotransferase	AST
Alanine aminotranferease	ALT
Adenosine triphosphate	ATP
Body Mass Index	BMI
Coronary Artery Risk Development in Young Adults	CARDIA
Cytokeratin 18	CK18
Connective tissue growth factor	CTGF
Cardiovascular disease	CVD
Cytochrome P450 2E1	CYP2E1
Diabetes mellitus	DM
Deoxyribonucleic acid	DNA
Extracellular matrix	ECM
European Group for study of Insulin Resistance	EGIR
Enzyme Immunoassays	EIA
Enzyme linked Immunosorbent Assay	ELISA
Epidemiology of Nonalcoholic Fatty Liver Disease	EPI-NAFLD
Endoplasmic reticulum	ER
Fatty Acid Binding Protein 4	FABP4
Free Fatty acid	FFA
Fasting plasma glucose	FPG
Glucose-6-phosphatase	G-6-P
Guanine exchange factors	GEF
Glucose transporter 4	GLUT4
Glycogen synthase kinase-3	GSK3
Hepatitis C Virus	HCV
High Density Lipoprotein	HDL
Hepatic glucose	HG
Health Insurance Portability and Accountability Act	HIPAA
Human immunodeficiency virus	HIV

3-hydroxy-3-methylglutaryl-coenzyme A synthase……………………........HMGCS2
4-hydroxynonenal…………………………………………………………HNE
Homeostatic model assessment……………………………………………...HOMA
Hypothalamic pituitary adrenal axis…………………………………….....HPA
Hepatic steatosis…………………………………………………….......HS
Hepatic stellate cells………………………………………………………HSC
Stellate cells…………………………………………………………………SC
International Diabetes Federation……………………………………......IDF
Insulin-like Growth Factor 1……………………………………………...IGF-1
I-κB kinase β…………………………………………………………………IKK- β
Interleukin…………………………………………………………….....IL
Ingenuity Pathways Knowledge Base……………………………………IPKB
Insulin Resistance………………………………………………………IR
Institution review board…………………………………………….....IRB
Insulin receptor substrate……………………………………………….IRS
Jun N-terminal kinase…………………………………………………......JNK
Mitogen-activated protein…………..MAP
Mitogen-activated protein kinase...MAPK
Methionine adenosyltransferase...MAT
Malondialdehyde...MDA
Migration inhibitory factor...MIF
Metabolic syndrome..MS
Nicotinamide adenine dinucleotide..NADH/NAD+
Non alcoholic fatty liver...NAFL
Non alcoholic fatty liver disease………………………………………......NAFLD
Non alcoholic steatoHepatitis……………………………………………NASH
National Cholesterol Education Program Adult Treatment Panel III…….NCEP ATPIII
Nuclear factor-kappa B…………………………………………………...NF-KB
Non Insulin Dependent Diabetes Milletus..NIDDM
Plasminogen activator inhibitor-1...PAI-1
Polymerase Chain Reaction...PCR
Phosphoinositide-dependent kinase...PDK
Phosphoinositide 3...PI3
Phosphatidylinositol-3'-kinase...PI3K
Phosphatidylinositol (3,4,5)-trisphosphate...PIP3
Protein Kinase A..PKA
Protein Kinase B..PKB
Protein Kinase C..PKC
Polymorphonuclear cells...PMN
Peroxisomal proliferator activated receptor α..PPARα
Protein tyrosine phospatase 1B..PTP1B
Quantitative trait loci……………………………………………………...QTL
Red blood cells……………………………………………………………RBC
Reactive Oxygen species…………………………………………………ROS

San Antonio Heart Study……………………………………………………SAHAS
SH2-containing inositol phosphatase 2…………………………………...SHIP2
Son of Sevenless……………………………………………………………..SOS
Sterol regulatory element-binding proteins………………………………SREBP
Simple steatosis……………………………………………………………...SS
Triglycerides…………………………………………………………………TG
Tumor growth factor Beta…………………………………………………...TGF-β
Tumor Necrosis Factor receptor 1..TNF-R1
Tumor Necrosis Factor Receptor 2..TNF-R2
Tumor Necrosis Factor alpha...TNF-α
TNF receptor-associated factor 2…………………………………………TRAF2
Thiazolidinediones…………………………………………………………..TZD
Very Low Density Lipoprotein……………………………………………...VLDL
World Health Organization…………………………………………………WHO

ABSTRACT

COMPARATIVE PROFILING OF CIRCULATORY LEVELS OF ADIPOKINES AND CYTOKINES IN PATIENTS WITH VARIOUS TYPES OF NON ALCOHOLIC FATTY LIVER DISEASE.

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Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome and the most common cause of liver disease. The NAFLD spectrum ranges from simple benign steatosis to NASH to fibrosis and cirrhosis. Omental adipose tissue is a biologically active organ that plays a role in pathogenesis of NAFLD. Expansion of adipose tissue mass accompanied by alteration in adipocytokines could be a prominent mechanism in the pathogenesis and progression of NAFLD. Adipocytokines play a role in insulin resistance, oxidative stress and hepatocyte apoptosis and may be there is imbalance in their serum levels in NAFLD spectrum and the degree of insulin resistance. Accordingly, we explored the differences in the level of transcription of genes in adipose tissue and liver as well as adipocytokines serum levels in different types of NAFLD. This would help to identify non-invasive diagnostic marker profiles for early distinction between NASH and steatosis.

In this study of gene expression analyses, we show that there is a prominent adipose-specific deregulation of the inflammation and immune system related genes in NASH. In addition, there are increases in the hepatic detoxification enzymes in obese patients. A number of liver and adipose-specific functional networks centered by TNFalpha, JUN/JUNB and IFNgamma were highlighted in relation to the NASH pathogenesis.

Our results also show that serum levels of TNF-α and IL-8 were significantly higher in NAFLD patients compared to both obese and non-obese controls. Univariate analyses involving all patients revealed a significant correlation between serum concentrations of TNF-α and IL-8, as well as between IL-6 and IL-8. Insulin resistance measured by homeostasis model assessment (HOMA) scores negatively correlated with serum Adiponectin levels in NAFLD patients. Serum Visfatin was significantly higher in steatosis and obese controls than the non-obese controls. Visfatin and IL-6 serum levels in NASH patients were lower in comparison to patients with steatosis implicating their possible co-regulated protective role from progression of steatosis to NASH. Multivariate analyses revealed that TNF-α serum level levels influenced mainly by serum glucose, HOMA, BMI and IL-8 in NAFLD, while IL-6 levels influenced mainly by HOMA scores and IL-8. A multivariate analysis indicated that TNF-α was the only independent predictor of histologic fibrosis in patients with NASH.

Apoptosis per se, measured by levels of Ck-18 cleaved at Asp396-neoepitope and secreated to the circulation is significantly increased in the serum of NASH patients in comparison to patients with steatosis and BMI matched morbidly obese patients. In

NAFLD and obese controls, both apoptosis and total cell death measured by serum levels of intact CK18, closely correlated with TNF-α levels and IL-8, but in NAFLD apoptosis and total cell death only correlated with TNF-α. Of special importance, NASH could be reliably predicted by a combination of apoptosis, necrosis, serum Adiponectin and serum Resistin, with sensitivity of 95.45%, specificity of 70.21%, and AUC of 0.908.

HCV infection is clearly characterized by alterations in cytokine serum levels. There are significant differences in serum levels of major pro-inflammatory cytokines (IL-6, IL-8 and TNF-α) between patients groups with HCV infection regardless of genotype or association with steatosis when compared to group of patients with steatosis only. In addition, IL-8 and TNF-α were significantly increased in HCV3 infections compared to HCV1.

Some of these cytokines play more prominent roles in development of steatosis accompanied by HCV infection. Our data shows that there is strong connection between the HCV genotype 3, steatosis and increases in IL-8 serum levels. Multiple correlation analyses showed that steatosis in HCV is mostly dependent on IL-6 and IL-8; in HCV1 on IL-6, TNF-α and IL-1B while in HCV3 is mostly dependent on IL-8. Actually HCV3 associated steatosis can be predicted by serum levels of IL-8 with 87.5% specificity, 100% sensitivity and AUC of 0.931.

Introduction

The liver is an extremely important organ of the human body that performs multiple vital functions. These functions range from blood filtration to synthesis, regulation, storage, and secretion of many important proteins and nutrients. These proteins play crucial roles in many biochemical, immunological and metabolic processes. Liver is also considered the primary detoxification organ through which many catabolic and purification reactions occur for clearing many toxins and waste products from the body. Healthy maintenance of liver structure and function is considered to be paramount in maintaining body homeostasis. Consistant with such numerous and universal functions, liver diseases are versatile, common and have detrimental consequences on a broad spectrum of the biochemical and metabolic processes in the body. A short list of common chronic diseases that affect liver include viral Hepatitis, alcoholic Hepatitis and metabolic Hepatitis. Of special emphasis is non-alcoholic fatty liver disease (NAFLD) that may develop in both obese and lean individuals.

Metabolism and Homeostasis

The metabolic processes of the human body are comprised of a complex network of enzymatic interactions performed by different tissues and organs. These reactions allow for the proper mobilization and utilization of energy sources in an efficient way.

The main objectives of metabolic process are: (1) to provide energy to all body cells according to need and priority, (2) ensure regular homeostasis and (3) maintain life under various intra- and extra-environmental circumstances. The major players in regulation of homeostasis are insulin as the master hormone, glucagon as the main counterbalancer, glucose as the major fuel currency and lipids as the main energy source. Liver is the factory that orchestrates the metabolic processes, while adipose is the main storage organ for lipids and the largest regulatory endocrine organ known to date (Hauner et al., 2005; Kershaw & Flier, 2004; Ahima & Rexford, 2006). Proper regulation and utilization of carbohydrates, lipids, and proteins in the body are the main considerations for achieving homeostasis.

Body homeostasis is influenced directly by the balance between the amount of nutrition (energy intake) and physical activities (energy expenditure) performed by the body under normal metabolic regulation. Glucose, primarily, and free fatty acids (FFA), secondarily, are the main sources of energy in the body to sustain homeostasis. After consumption, the most food is converted mainly to glucose that is used as the primary source of energy for most of the body and some of excess of energy is deposited in a form of glycogen in the liver and skeletal muscles. Because of the hydrophilic nature of the glycogen, it binds to a lot of water, that why it is not an ideal mass storage form of energy in tissues. Glycogen is a limited source of energy for only a short fasting time (Bollen et al., 1998). An excess of dietary fat is stored as triglycerides and FFA mainly within adipose tissue. Fatty acids and lipids are hydrophobic molecules which make them ideal for efficient storage in a compact form (Wolinsky, 1998; Bollen et al., 1998).

Accordingly, when the sum of the energy balance is shifted toward more energy intake and less energy expinditure, the excess of the energy is converted to fat which ultimatly stored in adipose tissue. With time, continuation of this metabolic shift would lead to increases in fat storage, expansion of adipose tissue, and ultimately obesity. The latter leads to or is accompanied by alteration in the recycling and partitioning processes of non-oxidized fatty acids between different organs and tissues. Infiltration of tissues that not designed for lipids storage leads to dramatic changes in the levels of synthesis and activity of many factors involved in molecular and biochemical processes of metabolism.

Recently, obesity and associated health complications has become a major public health problem worldwide (WHO, 1998). Indeed, obesity has become the major health concern of the 21st century. It is estimated that 1 in 3 adults in he USA are obese (Baskin et al., 2005) and 25 % of the adult population in the UK are classified as obese on the basis of body mass index (BMI) $\geq$30 kg/m^2 (Rennie & Jebb, 2005). Moreover, obesity prevalence rates increased in the United States from 13 % to 32 % between the 1960s and 2004 and by 2015, it is expected that 75 % of adults will be overweight (BMI>25) and 41 % will be obese (BMI>30) (Caballero, 2007; Wang & Beydoun, 2007). Even now, according to Surveillance Survey conducted by Center for Chronic Diseases Control and Prevention in 2002, 24% of all Virginia residents are obese, while 35% are overweight.

The main contributing factors to obesity are genetic factors, profound modifications in life style, sustained economic development and revolution in food industry. Ironically, obesity already superseded the most prevalent health issue which

dominated most of the 20th century, malnutrition. But obesity is associated with greater costs of the prevention and treatment than malnutrition. The prevalence of obesity worldwide is not restricted to developed or poor countries and doesn't mean a decrease in poverty and malnutrition. It simply reflects a shift towards fewer healthy people in a population, as they become overweight.

Various epidemiological studies have established a direct association between body fat mass and an increased risk of developing a variety of chronic disorders affecting mainly the cardiovascular system and liver. The excessive body fat, especially visceral fat, contributes to the development of a complex network of dangerous clinical conditions such as insulin resistance, glucose intolerance, dyslipidaemia, elevated blood pressure, impaired fibrinolysis and endothelial dysfunction (Bjorntorp, 1991). Such a constellation of risk factors are recognized as components of what is now called Metabolic Syndrome (MS) (Reaven, 1988; Bjorntorp, 1991; Hauner, 2002). The most common description of MS includes obesity, insulin resistance, dyslipidemia and hypertension (Moller & Kaufman, 2005). The cluster of these four parameters is also considered high risk for developing atherosclerotic cardiovascular disease and type II diabetes (Haffner et al., 1992; Isomaa et al., 2001; Moller & Kaufman 2005**).** These risk factors are also considered to be atherogenic, so individuals with MS have two- to three-fold higher probabilities of developing cardiovascular problems (Lakka et al., 2002; Sattar et al., 2003). Moreover, the same factors are considered as associated indicators and/or contributors for the development of nonalcoholic fatty liver (NAFL) (Hamaguchi et al., 2005).Currentely, Non Alcoholic Fatty Liver Disease (NAFLD) is the only liver disease

to be directly linked to disturbances in the basic metabolism processes. As a matter of fact, some consider NAFLD as the metabolic syndrome version of the liver. Moreover, the high prevalence of NAFLD in obese individuals has become public health concern.

This thesis focuses on two of the most common causes of the development of Non-Alcoholic Fatty Liver Disease: Metabolic Syndrome and infection by Hepatitis C virus.

Fatty Liver: Definition, Causes and Spectrum

Fatty liver is a general term used to describe a histopathological condition where greater than 10% of the liver is infiltrated with fat (American Liver Foundation, 2001). Accumulation of large fat droplets in hepatocytes disrupts the liver architecture and interferes with many cellular signaling and biochemical processes (American Liver Foundation, 2001). The most commonly cited contributing factors to development of the fatty liver condition are: (1) excessive alcoholic consumption, (2) exposure to environmental toxins and drugs, (3) tuberculosis, (4) obesity-related metabolic disorders, especially insulin resistance, and (5) chronic infection with Hepatitis C virus (American Liver Foundation, 2001). The common pathogenesis processes in developing NAFLD include expansion of adipose tissue, IR, adipocytokines, oxidative stress and free fatty acids (Figure 1).

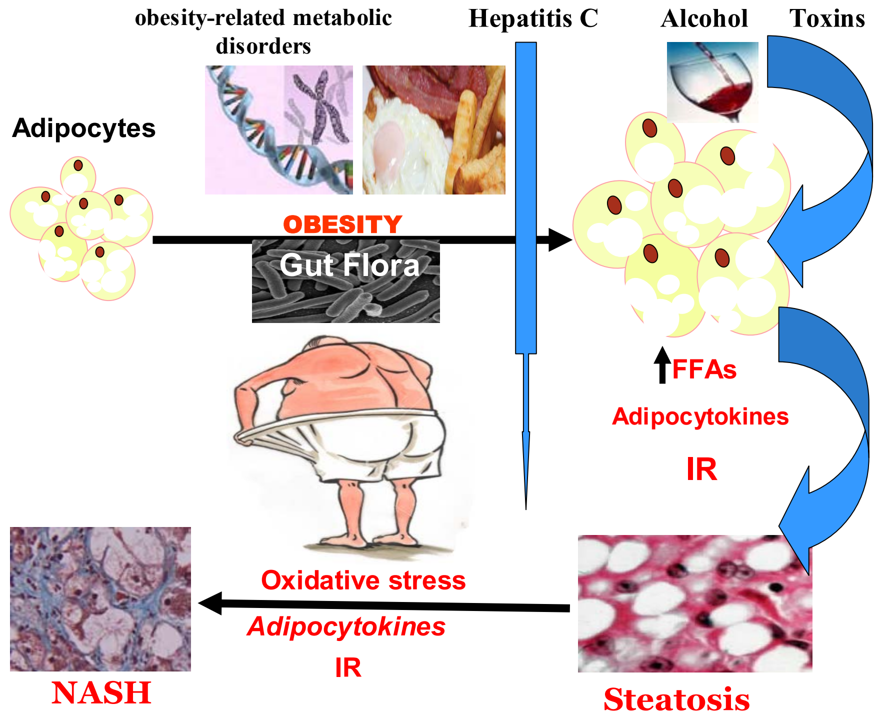

Figure 1. Major contributors to the development of fatty liver condition, with brief summary of pathogenetic process. IR-Insulin resistance, FFAs-Free fatty acids.

The severity of damage and classification of different stages of fatty liver disease depend on the relative amount of fat deposited in the liver cells, its anatomical distribution and the degree of liver inflammation. These stages range from simple non-symptomatic fatty liver called steatosis, to symptomatic steatoHepatitis, to cirrhosis and finaly to irreversible scarring of the liver (liver fibrosis) (Matteoni et al., 1999). Younossi classified the fatty liver progression stages to subtypes: type 1- hepatic steatosis; type II-steatoHepatitis; type 3- steatonecrosis; type 4- steatonecrosis with Mallory bodies or fibrosis (Younossi et al., 1998). Common diagnostics for fatty liver include

ultrasonography, computerized tomography and magnetic resonance imaging accompanied by liver function tests. However, liver biopsy with all its invasivness is the only accepted method used to ensure accurate diagnosis and to differentiate between different stages (Angulo, 2002; Brunt, 2004).

The pathogenetic process in the fatty liver that leads to its progression to inflammatory steatoHepatitis is multifactorial and still under intense debate. In the non-alcoholic version of fatty liver, a heterogeneous mixture of genetic and environmental factors heavily influences both susceptibility to the disease, its pathogenesis and its progression (Alwis & Day, 2007). Insulin resistance has emerged as the central theme for explaining and connecting the main underlying clinical features associated with NAFLD (Wallace & Matthews, 2002). Insulin resistance is multifactorial and can be attributed to a deficiency in insulin receptor signaling as a result of either genetic polymorphism or production of autoantibodies to beta cells of langerhans. In majority of cases, Insulin resistance develop as aresult of impairment in insulin signal transduction pathways distal from the insulin receptor (Wallace & Matthews, 2002) as will be discussed later in detail.

Resistance to insulin is tightly linked to obesity, which manifests as an excess in the adipocytic mass accumulated by human body. The hallmark discovery of the *Leptin* gene and its association with obesity granted more respect for the role of adipose tissue in body homeostasis (Zhang et al., 1994). This discovery served as a starting point for understanding the physiological role of adipose tissue. Circulating levels of the adipocyte-derived Leptin correlate directly with both body fat mass and fat cell size (Friedman & Halass, 1998). Leptin discovery triggered and facilitated the research that

has resulted in identification of many soluble factors synthesized and produced by adipose and other cells. Since the discovery of the Leptin, more than 100 adipokines and cytokines have been identified, many of which exert multiple effects at both local and systemic levels modulating many metabolic and inflammatory processes (Hauner et al., 2005). Such physiological processes include but are not limited to: homeostasis, inflammatory process, immunological reactions, lipid metabolism, blood pressure regulation, insulin sensitivity and angiogenesis (Hauner et al., 2005; Trayhurn & Wood, 2005; Fantuzzi, 2005; Lee &Pratley., 2005; Baranova et al., 2007).

Recently, there has been intense interest in the role of inflammation in obesity as one of the major drivers in the development of the metabolic syndrome and NAFLD (Hauner et al., 2004). There appears to be a strong association between obesity and inflammation, thereby suggesting that obesity represents a chronic inflammatory state that advances with an increase of the production of pro-inflammatory mediators by a growing adipocytic mass (Hauner et al., 2004; Baranova et al., 2007). It is highly possible that disturbance in adipokine levels may play a role in the patogenesis of the liver steatosis and its progression to steatoHepatitis and ultimately to liver fibrosis.

Cytokines disturbances and oxidative stress are common pathological mechanisms which can be attributed to both alcoholic liver disease (ALD) and NAFLD (Stewart et al., 2001; Day, 2002, 2006). In ALD most of cytokine release comes from the Kupffer cells in the liver and is trigured by portal endotoxemia resulted from ethanol and its metabolites that increases gut permeability. Kupffer cells, also called stellate cells, are specialized macrophages located in the liver that form part of the reticuloendothelial

system. The primary function of Kupffer cells is to recycle old red blood cells that no longer are functional (Hubrich, 2004).

Alternatively in NASH, the cytokine release may also come from hepatocytes in response to an increased supply of FFAs, and from adipose tissue macrophages (Day, 2006). In ALD, ethanol metabolism, Kupffer cell activation and the effect of TNF-α on hepatocyte mitochondria lead to oxidative stress. In NAFLD oxidative stress arises via the increase oxidation of FFA by mitochondria, peroxisomes, and microsomes (Day, 2006).

Current knowledge about the progression to liver fibrosis in both ALD and NAFLD is controversial. It was thought first that fibrosis in both ALD and NAFLD arise as part of the normal healing response to inflammation and injury, but new evidences indicated that IR can cause fibrosis (Day, 2006). IR leads to the stimulation of the release of connective tissue growth factor (CTGF) from hepatic stellate cells and alterations in the adipocytokine profiles. Genetic polymorphism and environmental factors affects the severity of steatosis, degree of oxidative stress, expression of adipocytokines and the severity of fibrosis (Browning et al., 2004). Many studies showed that genetic factors also define susceptibility for developing NASH and NASH-related "cryptogenic" cirrhosis (unknown cause cirrhosis) (Browning et al., 2004; Caldwell et al., 2002).

Fatty liver disease also present in almost two thirds of chronic Hepatitis C virus infection (HCV) cases (Asselah et al., 2003), ranging from 40 to 86% depending on patient population, genetic factors, accompanied metabolic risk factors and genotype of

the virus (Zekry et al., 2005, Asselah et al., 2003). In addition, insulin resistance that underlies steatosis is a common theme in both chronic Hepatitis C infection and NAFLD. However, the pathogenetic mechanisms of developing the insulin resistance and/or the liver steatosis in Hepatitis C infected patients are under intense investigation. Most researchers have focused on the independent role of Hepatitis C genotype and its interplay with the underlying metabolic factors as a direct cause of steatosis or insulin resistance development.

In Hepatitis C, understanding the pathogenic process is very complex for many reasons: First, it is not clear at this point if the steatosis seen in Hepatitis C patient's results from pro-steatotic action of the HCV core proteins, or if it develops under the indirect influence of the metabolic factors that may accompany many HCV infections. In addition, we do not yet know if HCV infection modulates the bodies' metabolism and insulin signaling. Finally, the sequence of events and relationship between steatosis, necroinflammation and fibrosis in HCV are still not well understood. Necrosis and inflammation in the liver is a histological feature characterized by uncontrolled death of hepatocytes as aresult of severe inflammation. Necro-inflammatory activity is the main predictor of liver disease outcome. Indeed, the patients with high activity are at risk of rapid fibrosis progression and cirrhosis. For example, it is not known if steatosis in HCV infection is a marker, a consequence, or a cause for necroinflammation and if it is a first step toward fibrosis in disease progression (Zekry et al., 2005). Some studies have shown that there is strong association between steatosis and necroinflammation processes in HCV infection (Zekry et al., 2005). Other studies support the hypothesis that HCV

genotype 3 with some of its core protein directly causes steatosis (Zekry et al., 2005; Polyak et al., 2001; Asselah et al., 2003). However, many studies suggest that the pathogenesis of NAFLD and chronic HCV infection share many similar biological pathways that lead to steatosis development, induction of Reactive Oxygen Species (ROS) and production of progessive inflammatory state.

It is reasonable to suggest that chronic HCV infection is responsible for a dynamic process of necroinflammation in the liver, especially as it has been shown that HCV infection increases production of TNF-alpha and Interleukin 8 (IL-8) (Neuman et al., 2001; Polyyak et al., 2001). These pro-inflammatory cytokines may hasten the progression of the benign liver steatosis to fibrosis. In addition, HCV infection may exacerbate the metabolic conditions predisposing the patients to steatosis itself.

Various types of NAFLD spectrum are accompanied by kinetic changes in expression levels of many genes, especially those encoding adipocytokines. Recently, a number of highly innovative genomics and proteomics technologies such as microarrays, mass spectrometry, reverse proteomics and multiplexed ELISA were applied to the search of the molecular basis of NAFLD (Baranova et al., 2007). In this project we used a combination of such technologies to explore the status of gene expression in adipose and hepatic tissues from Obese and NAFLD patients. In addition we profiled serum levels of adipocytokines in patients with various diseases of NAFLD spectrum and with chronic Hepatitis C or Hepatitis B viral infection. The main objectives of this study are to profile a number of human adipocytokines and derive diagnostic patterns that can be used for non-invasive differentiation between types of liver diseases in these patients and possibly

pave the way for the prevention, or for applicant of targeted therapies for fatty liver disease, both in morbidly obese patients and patients infected with HCV.

CHAPTER 1.

Metabolic Syndrome

Definition

Clinical definitions of metabolic syndrome are varying and often controversial. The World Health Organization (WHO), National Cholesterol Education Program Adult Treatment Panel III (NCEP ATPIII) and the European Group for study of Insulin Resistance (EGIR) defined different cut-off values for diagnostic measurables applicable to MS (Moller &Kaufman 2005, Alberti, 2005). According to the WHO 1999 statement, the clinical definition of metabolic syndrome involves impaired glucose tolerance or diabetes and/or insulin resistance plus any two or more of the following criteria: abdominal obesity which is defined by waist/hip ratio >0.90 in men and >0.85 in women or body mass index >30 kg/m2; hypertriglyceridemia (serum triglyceride level ≥1.7 mmol/L (150 mg/dl); low levels of HDL cholesterol <0.9 mmol/L (35 mg/dl) in men, <1.0 mmol/L (39 mg/dl) in women; high blood pressure (≥140/90 mm Hg), or microalbuminuria (albumin level in urine ≥20 µg/min or albumin: creatinine ratio ≥30 mg/g)(Alberti, 2005).

The criteria for diagnosis of the metabolic syndrome according to the NCEP ATPIII 2001 definition are any three or more of the following: Fasting plasma glucose ≥110 mg/dl; waist circumference >102 cm in men and > 88 cm in women; serum triglyceride level ≥150 mg/dl; HDL cholesterol <40 mg/dl in men and <50 mg/dl in

women; or blood pressure ≥130/85 mm Hg (Moller & Kaufman, 2005; Carnethon et al., 2004, Alberti, 2005).

A third definition was proposed by the European Group for study of Insulin Resistance (EGIR) in 2001 as a modified version of the 1999 definition by the WHO to be used mainly for non diabetic subjects. The EGIR definition for metabolic syndrome states that insulin resistance, where the hyperinsulinaemia is in the top 25% of fasting blood insulin levels among non diabetic population plus two of the following: central obesity with waist circumference ≥ 94 cm (M) and ≥ 80cm (F); increase in triglycerides ≥ 20 mmol/l and /or low HDL-cholesterol <1.0mmol/L; raised arterial pressure ≥ 140/90 mmHg (Alberti, 2005).

In 2004, the International Diabetes Federation (IDF) established a common and universal definition that hopefully can accommodate all variables and thus be more practical in predicting cardiovascular disorders and diabetes as primary complications of metabolic syndrome. According to the new IDF definition, a person has metabolic syndrome if he/she has central obesity with waist circumference greater than defined cutoffs values specific to their ethnicity group and two of the following: raised TG level ≥ 150 mg/dL (1.7 mmol/L), reduced HDL cholesterol: < 40 mg/dL (1.0 mmol/L) in males and < 50 mg/dL (1.3 mmol/L) in females, raised blood pressure with systolic ≥ 130 or diastolic ≥ 85 mm Hg or raised fasting plasma glucose (FPG) ≥ 100 mg/dL (5.6 mmol/L) (International Diabetes Federation, 2004). This universal definition emphasized the role of insulin resistance and central obesity taking into account ethnic and age discrepancies worldwide. However, this new definition doesn’t include impaired glucose

tolerance as a factor despite its high occurrence, as the oral glucose-tolerance test is not taken into account in this definition (Albertia et al., 2005).

Epidemiology

Many health statistics studies have projected obesity and a lack of physical activity as the underlying conditions that prompt the development of metabolic syndrome. Obesity and physical activity negatively correlate with each other all over the world. For example, according to the year 2000 census data, about 47 million US residents have the metabolic syndrome associated with obesity (Ford et al., 2002). However, estimations of the prevalence of metabolic syndrome lead to variable results depending on particular definition criteria, population, age and ethnic background (Alberti, 2005). This is especially true as most of epidemiological and prevalence studies about MS were done before the publication of 2004 IDF definition. Accordingly, variable prevalence results make direct comparisons between data from different epidemiological studies more difficult.

For example, in a study performed by Ford and colleagues in 2002, an analysis of the data on 8814 men and women aged 20 years or older (1988-1994 cohort) using the NCEP ATPIII 2001, definition the prevalences of metabolic syndrome in age-adjusted gender groups were 21.8% and 23.7%, respectively. The Mexican Americans had the highest age-adjusted prevalence of the metabolic syndrome (31.9%). No difference between the prevalence of metabolic syndrome between men and women in age-adjusted group were found in general, but in particular, African American women had about 57% higher prevalence of the metabolic syndrome than men (Ford et al., 2002).

On the other hand, different prevalence rates of metabolic syndrome calculated according to different definitions of the disease led to confusion in validating the credibility of publications in the case of many research studies. For example, in one study three different figures were generated for an Australian population, where the prevalence of metabolic syndrome was defined as 20.9%, 18.4% and 15.9% using the WHO, the ATP III and EGIR definitions, respectively (Figure 2) (Alberti, 2005; Dunstan et al., 2002). Only 9.2% of the Australian population fulfilled the MS criteria according to all three definitions.

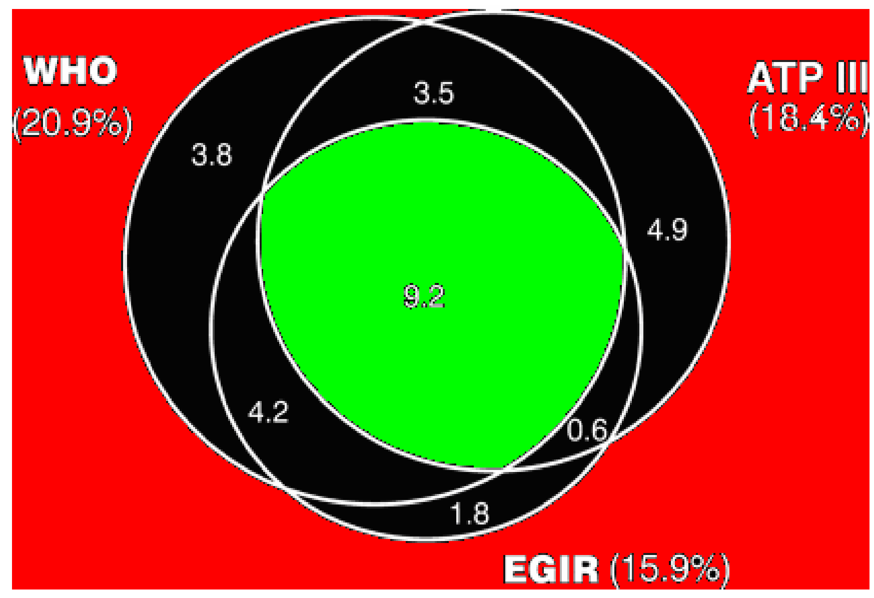

Figure 2. Data showing the percent prevalence of metabolic syndrome within the total Australian population using World Health Organization (WHO), Adult Treatment Panel III (ATPIII) and the European Group for study of Insulin Resistance (EGIR) definitions (Modified from Alberti 2005).

Moreover, the use of the clinical features associated with metabolic syndrome according to the WHO, the ATPIII or EGIR as definite predictors or high risk factors for

developing type II diabetes and cardiovascular disease (CVD) is also found to be controversial and in conclusive (Connor, 2005). For example, in a well-known research study called Coronary Artery Risk Development in Young Adults (CARDIA) that was carried out on 4,192 subjects followed for up to 15 years, the role of body mass index (BMI) and weight gain as the highest risk factors for developing MS and cardiovascular disease has been proposed (Carnethon et al., 2004). However, in another study group called the San Antonio Heart Study (SAHS), which was done on 1709 participants followed for up to 7.5 years, they concluded that using Diabetes Predicting Model (statistics multivariant predictive model for diabetes) and the Framingham Risk Score (risk assessment tool for estimating 10-year risk of developing Myocardial Infarction and Coronary Death) performs much better than clinical features of MS defined by NCEP ATPIII guidelines as a predictors for type II diabetes and CVD (Carnethon et al., 2004; Stern et al., 2004). These risk assessment tools depends on multi-clinical variables rather on one factor. For example, the risk factors included in the Framingham calculation are age, total cholesterol, HDL cholesterol, systolic blood pressure, treatment for hypertension, and cigarette smoking. Because of a larger database, Framingham estimates are more robust for total cholesterol than for LDL cholesterol. Note, however, that LDL cholesterol remains the primary target of therapy. The Framingham risk score gives estimates for "hard CHD" which includes myocardial infarction and coronary death (Stern et al., 2002; Wilson et al., 1998).

Regardless of such differences in the points of view and in line with all three MS definition criteria, metabolic syndrome is on the rise, especially in the western world. The

same is true for the most of its components, such as type II diabetes, obesity, dyslipidemia and hypertension (Xiong & Zhiping, 2006).

Pathogenesis and Complications

Molecular mechanisms of MS, as well as its main pathogenic processes at the organismal level are very complex and far from being clear. However, it is believed that the basis of this disease resides within a triangle of three factors: (1) insulin resistance, (2) hyberinsulinemia and (3) glucose intolerance that are mainly due to the defect in the insulin signal transduction pathways.

Insulin is the master hormone secreted by β-cells of pancreas that regulates a wide variety of metabolic processes in the body. It is the major player in regulating homeostasis by modulating carbohydrates, lipids and protein metabolic pathways, and as such is critical for the survival of an organism (Saltiel & Kahn, 2001). Insulin injections promote energy storage and anabolism. This is done by facilitating glucose uptake in muscles and adipose tissue, promoting glucose storage in form of glycogen in liver and muscles and encouraging protein synthesis. On the other hand, insulin prevents catabolic processes that release energy such as gluconeogenesis and glycogenolysis, ketogenesis, lipolysis and proteolysis. The counter balance regulator for insulin activities is glucagon. The balance levels and activities of these two main hormones together are necessary to keep homeostasis constant. The collective actions of insulin involve decreasing circulatory blood glucose, while glucacon actions increase blood glucose (Gaw et .al., 1995). The negative feedback mechanisms that depend on end products and nutrients levels such as circulatory glucose, free fatty acids and amino acids are controlling an

activity of both hormones. On physiological level, insulin regulates the balance between glucose output and peripheral utilization to maintain normal blood glucose levels. The major organs involved in regulating blood glucose are liver, gut, muscles, adipose, brain and red blood cells. Brain cells and red blood cells (RBCs) are entirely dependent on glucose as their only energy source and function independent of insulin. These cells are also major consumers of glucose. On the other hand, the major insulin-dependent glucose consuming tissue is muscle. Accordingly, the main determinants for circulatory glucose levels in the fasting state are hepatic glucose release and glucose consumption by muscles.

The regulatory mechanism that defines glucose availability and fate in different tissues is mainly dependent on the tissue-specific regulatory enzymes, differential expression of their mRNAs and post-translational regulation of their processing. These regulatory mechanisms are fine tuned to provide some human tissues with energy resulting from glucose catabolism and to store the excess as glycogen in others. For example, the hexokinase isozyme 4 (Glucokinase) that is found in liver hepatocytes and in kidney has high Km (lower affinity) for glucose than other isozymes, making this form of enzyme active only at very high concentration of glucose. Other tissues such as brain and muscles express other hexokinase isozymes (Hexokinases 1, 2 and 3) that have low Km (higher affinity), and, therefore, can be activated by lower concentrations of glucose. Differences in the activity of these isozymes in different tissues prioritize glucose supply for different parts of the body. When circulatory glucose levels are low, only brain and muscle hexokinases will be activated to use the scarce glucose supply. On the other hand, during long periods of glucose shortage such as in fasting, liver gluconeogenesis initiates

in. However, the production of the glucose by gluconeogenesis is relatively slow, thus, ensuring that resulting glucose levels are in line with a rate of glucose consumption. In this case glucose produced in the liver will be funneled mainly to brain and muscles through the circulation (Medical Biochemistry Pages, 2007). Another example of a tight control of the glucose levels is allosteric inhibition of hexokinases 1, 2 and 3 by an increase in their direct product Glucose 6 phospate (G6P). This regulatory mechamism does not work in the case of high Km glucokinases. This ensures that the excess of glucose immediately after a meal is transferred to the liver and converted to glycogen for storage. Another key enzyme in glycolysis pathway is pyruvate kinase that is allosterically inactivated by ATP and acetyl-CoA. However, the liver pyruvate kinase isozyme (L-type) is regulated by PKA-dependent phosphorylation, while isozymes of brain and muscles (M-types) are not. This regulatory feature helps to balance glycolysis and gluconeogenesis in liver, but not in muscles and brain cells. Moreover, Glucose-6-phosphatase that is present in hepatocytes converts G-6-P to glucose upon glucagon and epinephrine stimulation of glycogenolysis, but in muscle and brain cells lacking Glucose-6-phosphatase, G-6-P is oxidized in the process of glycolysis (Medical Biochemistry Pages, 2007).

Insulin Resistance

On molecular level, the translocation of circulating glucose into muscle and fat cells depends on the insulin ability to stimulate translocation of the vesicles that move glucose transporter 4 (GLUT4) to the cell surface in regulated manner (Shulman, 2000; Pessin & Saltiel, 2000). This insulin signaling action is mediated by activating

Phosphatidylinositol-3'-kinase (PI3K) pathway, exerting its effects on glucose and lipid metabolism and/or the mitogen-activated protein (MAP) kinase pathway which is also involved in cell growth and proliferation through MAPK-dependent modulation of gene transcription (Whiteman et .al.,2002; Reusch, 2002; Sivitz ,2004).

Loss of the sensitivity to the insulin leads to the attenuation of GLUT4 translocation to the cellular membrane and the decrease of the glucose uptake by muscle and fat cells. This is the primary molecular defect underlying insulin resistance (Baumann, 2000). Accordingly, the simplest definition of insulin resistance is “an increase in amount of insulin needed for cells to take up specific amount of glucose” (Wallace & Matthews, 2002). The continuous demand for a high level of the insulin production to compensate for this resistance leads to pancreatic B-cell exhaustion and admonishment of pancreatic function resulting in overt diabetes.

Many people develop a certain degree of insulin resistance as they age. In many cases of IR in the elderly, the developments of the overt symptoms of diabetes take along time to manifist. For example, insulin resistance could be diagnosed 10-20 years before the onset of type II Diabetes mellitus. On the other hand, it is apparent, that IR serves as the main predictor for the future development of type II diabetes in high risk patients (Warram et al., 1990; Lillioja et al., 1988). Insulin resistance is also strongly associated with dyslipedemia (Alberti, 2005).

The details of the intra-cellular signaling mechanism that mediates insulin action in human tissues and the development of the insulin resistance have been widely studied, but remain incompletely understood. Insulin binds to the two extracellular α-subunits of

insulin receptor leading to the dimerization of the receptor. Dimerization, in turn, induces conformational changes in the receptors which lead to autophosporylation of the receptors and an increase in its tyrosine kinase activity. Autophosphorylation and conformational changes in the insulin receptors make them able to bind and activate many intracellular substrates by trans-phosphorylation of their tyrosine residues. The list of the intermediate substrates include insulin receptor substrate proteins IRS-1, -2, -3 and -4, Grb2, Son of sevenless (SOS), adapter protein with a pleckstrinhomology (PH) and Src homology 2 (SH2)domain (APS), Shc and others (Avruch, 1998; Czech & Corvera 1999; Khan & Pessin 2002; Reusch, 2002; Pessin & Saltiel 2000). These substrates bind to insulin receptor and act as docking sites for recruiting, binding and phosphorylating of other signaling molecules that contain SH2 domains (White &Yenush, 1998). Depending on the state of energy balance, tissue type and levels of insulin receptor substrate proteins as well as other signaling regulators, at least one of the two types of downstream signaling pathways is activated with different consequences (Sivitz, 2004). For example, IRS proteins in some tissues will activate Phosphatidylinositol-3'-kinase (PI3K) pathway, the main pathway that is important in mediating the effects of insulin on glucose and lipid metabolism, while Grb2 will activate the mitogen-activated protein (MAP) kinase pathway which channels the action of the insulin to the processes of the cell growth and proliferation through insulin-dependent activation of gene transcription (Whiteman et al., 2002; Reusch, 2002; Sivitz, 2004). In the MAP kinase pathway, Grb2 and Shc dimerize with SOS and exchange GTP for GDP leading to activation of RAS protein which, in turn, activates RAF. This latter protein acts through the MAPK pathway to activate Mitogen-

activated protein kinase kinase (MEK), which, in turn, activates the extracellular regulated kinase (ERK) that translocates to nucleus and modulates expression of many genes involved in cell growth and metabolism (Saltiel & Kahn, 2001).

Activation of the PI3K signaling cascade by tyrosine phosphorylated IRS leads to formation of second messengers, namely phosphatidylinositol (3,4,5)-trisphosphate (PIP3,) which acts as an allosteric regulator for phosphoinositide-dependent kinase (PDK), in turn, activating phosphoinositide-dependent serine-threonine kinase super-family that include Protein Kinase C. PIP3 recruits PI3K dependent serine/threonine kinases (PDK1 and Akt (PKB) from the cytoplasm to the plasma membrane, where PDK1 phosporylates Akt (PKB) and activates it (Avruch, 1998; Czech & Corvera, 1999). Activated Akt, in turn, accelerates mobilization of glucose transporters such as GLUT4 to the plasma membrane by an incompletely understood mechanism (Pessin & Saltiel 2000). Many other proteins, for example, PKC and glycogen synthase kinase-3 (GSK3) also play important roles in this process. It is believed that PI3K is necessary, but not sufficient for a shift of the kinetic balance of a vesicular transport of GLUT4 toward exocytosis, implicating the requirement for help of other independent pathways, for example Cb1/Cap/CT10 pathway (Ribon et al., 1998, Chang et al., 2006). Negative regulators of this signaling process include lipid protein tyrosine phosphatases such as PTP1B and Phosphatidylinositol-3-phosphatases such as PTEN and SH2-containing inositol phosphatase 2 (SHIP2). These phosphotases weaken insulin signaling by dephosphorylation of the insulin receptor and its substrates, therefore causing a decrease in PIP intermediate second messengers, intervening in PKC and PKB phosphorylation

and GLUT4 translocation (Bei, 2002). All these negative regulators have been proposed as therapeutic targets for an improvement of insulin resistance and treatment of diabetes. In addition, any protein factor possessing serine/threonine phosphorylating activity and interacting with any component of PI3K pathway starting from the receptor and IRS proteins to PKC and Akt (PKB), would minimize the receptor tyrosine kinase ability for phosphorylating other intermediates and PI3 serine/threonine kinase phosphorylating role for downstream proteins, and, therefore, attenuating insulin effect on translocation of GLUT4 to membrane surface (Bei, 2002).

The differential expression and localization of all proteins involved in insulin signaling in different tissues implies that they have non-redundant, unique roles in insulin signaling pathways. For example, it has been found that IRS-1 mainly acts in skeletal muscle, while IRS-2 resides in the liver (Kido et al., 2000). On the other hand, even though both IRS-1 and IRS-2 are present in liver, glucose metabolism is mediated mainly by IRS-1 activation, while IRS-2 activation controls lipid metabolism (Taniguchi et al., 2005). It has been shown that knockout of IRS-1 in mice results in B-cell hyperplasia, resistance to an action of insulin and insulin-like growth factor 1 (IGF-1) as well as in impaired glucose tolerance (Araki et al., 1994; Tamemoto et al., 1999). Knockout of IRS-2 results in overt type II diabetes with severe insulin resistance in both skeletal muscle and liver (Withers et al., 1998). The particular roles of IRS-3 and IRS-4 are not clearly understood, but some studies have indicated that they might play a role in IGF signaling (Fantin et al., 200, Tsuruzoe et al., 2001).

Insulin resistance could lead to elevation of serum FFA and glucose resulting in a state of lipotoxicity and glucotoxicity in many peripheral tissues and organs (Sivitz, 2001). The increase in lipid deposition in peripheral tissues not designed for fat storage such as liver is pathologically harmful for cellular functions (Reich et al., 1950; Baranova et al., 2007). In addition, this abnormal metabolic situation and the resulting disturbances in the partition and the usage of energy in the body may lead to energy deprivation in some organs (Larter & Farrell, 2006). Moreover, IR hastens and impairs proper signaling interactions between liver, muscles and adipose tissue by alteration of serum adipokinome. Lately, many adipocytokines produced by the adipocytic mass have been implicated in the development of insulin resistance, thus, forming another vicious circle (Hotamisligil et al., 1993, Gurre-Millo, 2004).

Dyslipidemia and Free Fatty Acids

Another important player in the pathogenesis of metabolic syndrome is a central, or a visceral type of obesity that is characterized by intra-abdominal expansion of adipose tissue mass.

Visceral fat drains directly into the portal system and, therefore, exposes the liver to large amounts of free fatty acids, making it more vulnerable of an infiltration with fat. In addition, differences in the numbers and affinities of β adrenoreceptors in omental and subcutaneous adipose tissue as well as in different ethnic groups affects rate of lipolysis and serum FFAs. However the exact biologic significance of the differences in the β adrenergic receptors in vivo in obesity pathogenesis is still not well understood (McConnaughey et al., 2004). For example, visceral fat has a higher concentration of β

adrenoreceptors and lower sensitivity to insulin making it lipolytically more active than subcutaneous fat (wolf et al., 2005). Current data support the view that the elevation of the release of fatty acids from enlarged fat stores is the primary mechanism that plays a central role in the development of the metabolic disturbances observed in cases such as dyslipidaemia, impaired glucose metabolism and primarily insulin resistance (Boden, 1997). The other argument says that, due to low levels of insulin or insulin resistance, the effect of insulin on the lipolysis inhibition in peripheral tissues is attenuated. TGs stored in adipose undergo hydrolysis by hormone sensitive lipase (HSL) to produce FFA and glycerol (Gilham& Lehner 2004). The released FFAs bind to albumin and are transported to liver and muscles where they undergo β-oxidation and release energy stored in them. However, some FFAs will end up in the liver contributing to liver steatosis (Heijboer et al., 2005). The combined effects of the increase in peripheral lipolysis and increase in adipose mass are among of the major direct causes of liver steatosis.

It is still not clear whether insulin resistance is a cause or a consequence of dyslipidemia in Metabolic Syndrome and in NAFLD patients. One school of thought indicates that disturbances in fat metabolism and increase in circulatory FFA levels interfere with insulin sensitivity and could induce insulin resistance (Perseghin et al., 1997). For example, it has been found that the percentage of the visceral fat, systemic FFA concentrations and insulin resistance correlate positively with each other and with type II diabetes (Jansen, 2006). This theory was further substantiated when recent studies showed a strong correlation between triglyceride content in muscles and insulin resistance (Pan, 1997). Many researchers supported this suggestion, but proposed

different possible mechanisms of action for FFA to cause insulin resistance. For example, Randle and colleagues proposed that elevated FFA would exert negative feedback inhibition on glycolysis in muscles by inducing increases in muscles intramitochondrial acetyl CoA/CoA and NADH/NAD+ ratios. The latter would cause inactivation of pyruvate dehydrogenase and a subsequent increase in citrate levels, phosphofructokinase inhibition, increases in glucose-6-phosphate and, last but not least, the inhibition of hexokinase II activity. This sequence of events leads to increases in intracellular glucose concentration and decreased glucose uptake by muscles (Randle et al., 1964, 1965; Shulman, 2000).

Roden and colleagues proposed an alternative to the mechanism described by Randle by showing that increases in plasma FFA result in the decrease in intracellular glucose-6-phosphate that is further followed by the decrease in glycogen synthesis in muscles and glucose oxidation (Roden et al., 1996). In this case, the accumulation of intracellular fatty acid metabolites such as fatty acyl CoA, diacylglycerol and ceramides resulting from an increase in the delivery or a decrease in the metabolism of fatty acids by liver and muscles most probably interferes with glucose transport or insulin receptor phosphorylation capability. Specifically, these metabolites modulate the serine/threonine kinase cascade by phosphorylating serine/threonine sites on insulin receptor substrates. This would interfere with tyrosine kinase phosphorylation of IRSs by the insulin activated insulin receptor. So, phosphorylation of serine/threonine sites on insulin receptor substrates would weaken their ability to activate PI3K and downstream factors in

the pathway of the GLUT4 translocation to cell membrane (Roden et al., 1996, Shulman, 2000).

However, studies of the metabolic and morphological similarities between MS and “Cushing’s Syndrome, a disorder caused by excess synthesis and systemic secretion of glucocorticoids, pointed at the fact that central adiposity and elevated circulating FFA levels are most often the consequences of insulin resistance, but not the cause of IR (Summers &Nelson, 2005). These deductions were supported by experiments showing that regardless of level of circulating FFAs, glucocorticoids by themselves, acting within relatively short periods of time, can induce insulin resistance (Tappy et al., 1994; Pellacani et al., 1999). Additionally, in vitro cell culture experiments showed that dexamethasone can directly antagonize the effects of insulin (Weinstein et al., 1995). However, other experiments indicated that responsiveness of the peripheral tissues to insulin is markedly decreased by saturated fats, while unsaturated fats are less capable of the promotion of IR, or, in some cases, exert insulin-sensitizing effects (reviewed by Rivellese et al., 2002). Many studies of skeletal muscles have shown that the excess of the external supply of lipids lead to a decrease of the sensitivity to insulin (reviewed by Summers &Nelson, 2005) In addition, there is a strong correlation between intra-myocellular lipid concentrations and the severity of insulin resistance (McGarry, 2002).

Some scientists believe that ceramide is the primary molecular intermediate that directly contributes to metabolic syndrome and NAFLD (Schmitz-Peiffer et al., 1999; Chavez et al., 2003). Specifically, long-chain saturated FFAs is considered as the indirect cause for insulin resistance as their metabolism increases the intracellular the pool of

palmitoyl-CoA, the primary compound in ceramides synthesis (Summers &Nelson, 2005). Moreover, it has been shown that ceramides may accumulate in muscles of obese humans (Adams et al., 2004) and rodents (Turinsky et al., 1990; Gorska et al., 2004) with insulin resistance. Infusion of the lipids leads to marked elevation of the ceramide levels in the muscles of patients, while decreasing insulin sensitivity (Straczkowski et al., 2004).

The exact mechanism by which ceramides inhibit insulin signaling is not clearly determined. However, many cell culture studies showed that ceramides most often block the activation of Akt/PKB by either inhibiting its translocation to the cell membrane (Stratford et al., 2004; Powel et al., 2003) or by promoting its dephosphorylation by protein phosphatase 2A (Chavez et al., 2003; Teruel et al., 2001; Salinas et al., 2000). Other researchers suggested that unsaturated fatty acids also may induce insulin resistance through diglycerol, a lipid intermediate acting independently of a ceramide mechanism by inhibiting insulin signaling to IRS-1 and PI3K (Yu et al., 2002).

Adipokines in Metabolic Syndrome

The common perception of adipose tissue as a simple fat storage changed when Leptin was identified as an adipokine secreted by adipocytes and its concentrations were directly correlated with both body fat mass and fat cell size (Friedman & Halass, 1998). Lepton plays an important role in appetite and energy balance by its effect on hypothalamus (Zhang et al., 1994).Collectively the accumulated evidence reveals, more than 100 soluble factors have been identified to be produced and released by adipose tissue; these adipokines exert multiple effects at both the local and the systemic level modulating many different metabolic processes (Hauner et al., 2005).

Nowadays, the adipose tissue is considered to be more than a passive lipid-storing organ that only mobilizes fatty acids according to energy demands. Adipose is a very active, multifunctional endocrine organ involved in intense signaling crosstalk with other peripheral tissues (Kershaw & Flier, 2004). Accordingly, changes in the mass and the composition of adipose tissue associated with MS are accompanied by changes in the secreted adipokinome that are regulated both on the mRNA and protein levels. This indirectly indicates that adipokines could serve as major players in pathogenesis of metabolic syndrome, as they modulate complex networks of homeostatic, immunological and inflammatory processes. Specifically, alterations in the functional levels of fat-derived circulating factors (adipokines) and in cytokines produced by immune cells infiltrating adipose often have deleterious consequences on such processes. For example, Esposito *et al.* found that levels of some anti-inflammatory cytokines, such as IL-10, are decreased in metabolic syndrome patients, but remain normal in healthy obese controls (Esposito et al., 2003). In addition, there is a link between low levels of circulatory Adiponectin and high systolic blood pressure (Huang et al., 2003).

It has been already shown that regulation of insulin sensitivity in peripheral tissues is influenced by the level of many adipose derived factors such as TNF-α, Interleukin 6, Resistin, Adiponectin, and Leptin (Gurre-Millo, 2004). Other adipokines play a role in inflammatory processes such as TNF-α, IL-6 and IL-8, while others induce vasoconstriction and inhibit fibrolysis (Rajala et al., 2003; Saltiel, 2001; Gurre-Millo, 2004). Particluar effects of various adipocytokines will be discussed further in the succeeding chapters.

Some researchers have emphasized the pleiotropic role of retinoic X receptor that is also involved in the pathogenesis of metabolic syndrome (Shulman and Mangelsdorf, 2005). Metabolic syndrome can be considered as neuroendocrine disorder. This view was endorsed by the notion that in metabolic syndrome patients the function of the hypothalamic–pituitary adrenal (HPA) axis is somewhat diminished, leading to lower than normal amounts of the cortisol in the circulation due to an increase in its degradation rates. Interestingly, the production of cortisol as measured in saliva is elevated in obesity (Björntorp & Rosmond, 2000).

Links between metabolic syndrome and NAFLD are very obvious, as they share many commonly associated metabolic features, *e.g.* insulin resistance, obesity and type II diabetes. Some even consider NAFLD as an intrinsic pathological feature of metabolic syndrome (Larter & Farrell, 2006). For example, five out of six patients who have histologically proven Non Alcoholic SteatoHepatitis (NASH) also have metabolic syndrome, mainly because of the strong association of NASH with insulin resistance (Larter & Farrell, 2006). Others found that patients with metabolic syndrome tend to have more severe forms of NAFLD compared to NAFLD patients without metabolic syndrome (Tuma, 2005). Many agree that metabolic syndrome is a strong predictor for the development of the NAFLD. However, it is still argued whether any specific component of metabolic syndrome precedes liver steatosis and leads to its progression to NASH (Hemaguchi et al., 2005). Major players and possible interactions taking part in the development of NAFLD in the patients with metabolic syndrome are summarized in (Figure 3).

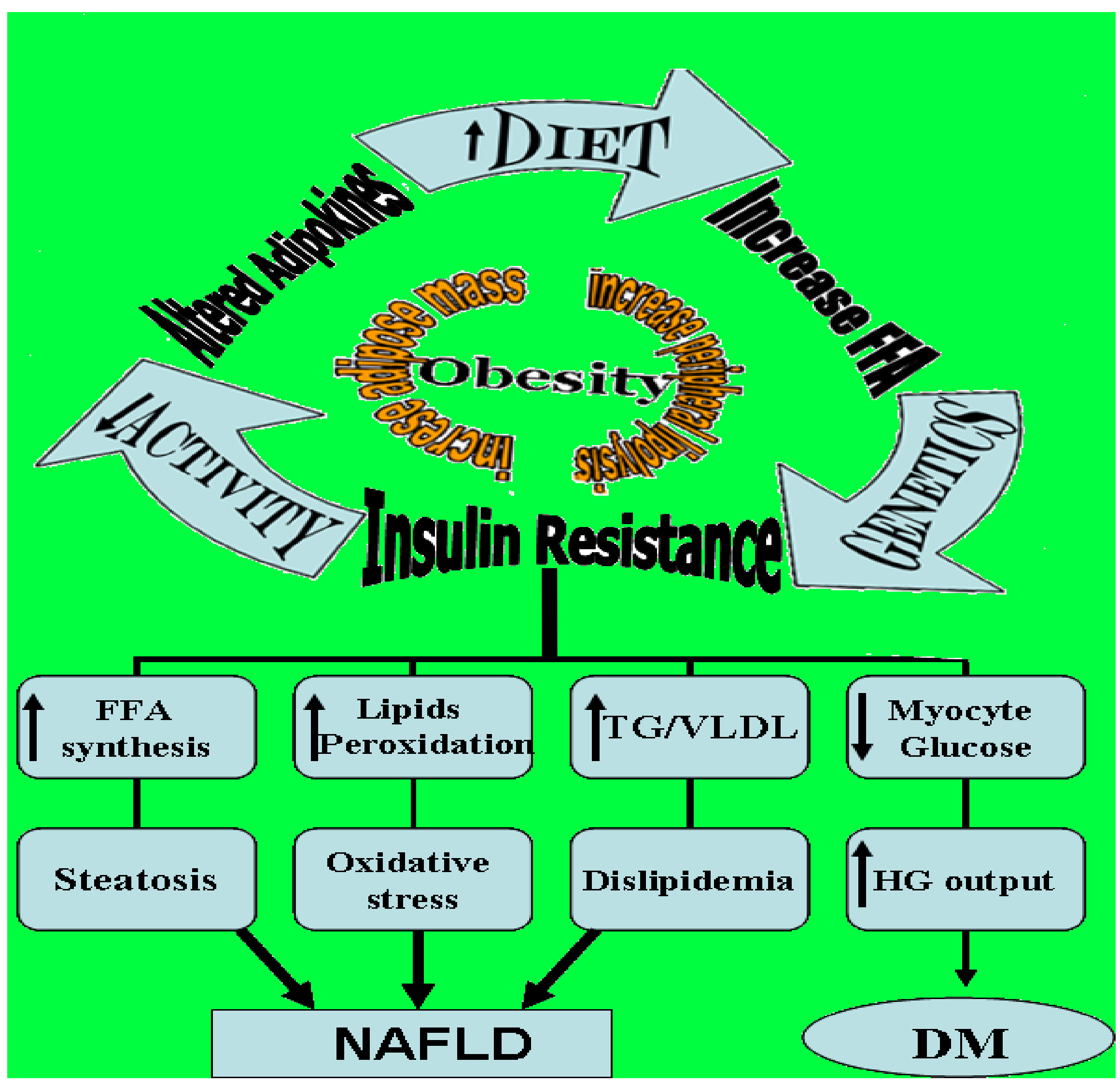

Figure 3. Schematic diagram outlining interaction of the causes and consequences in the vicious circle of obesity, insulin resistance and NAFLD in metabolic syndrome. DM – *Diabetes mellitus,* HG-Hepatic glucose, VLDL- Very low density lipoprotein, TG-Triglycerides. FFA-Free fatty acids.

CHAPTER 2.

Non Alcoholic Fatty Liver Disease

Definition and Spectrum

The non- alcoholic version of fatty liver disease (NAFLD) is a coined term for a spectrum of disorders where fat infiltrates the liver and accumulates in the hepatocyte's cytoplasm. Non-alcoholic fatty liver develops in patients that maintain daily alcohol intake at the levels less than 20g/day for women and less than 30g/day for men (Ludwig et al., 1980; Bayard et al., 2006). NAFLD is a chronic liver disease that represents one of the most dangerous complications associated with metabolic syndrome. The major common underlying risk factors for developing NAFLD are obesity, type II diabetes and insulin resistance. Estimates for prevalence of NAFLD contradict each other while being heavily dependent on the employed methodology and the studied population (Bellentani et al., 2004). However, the latest consensus figure centers around 20 to 30 % in all Western countries (Neuschwander-Tetri & Caldwell, 2003) and 16 to 23 % in USA (Collantes et al., 2004). It is also estimated that two thirds of patients with a body mass index (BMI) ≥ 30 kg/m2 and 90 % of those with a BMI >39 kg/m2 have at least asymptomatic stage of the fatty liver (Angulo, 2002). It is also believed that almost 8.6 million obese persons in the United States may progress to symptomatic stages of NAFLD at some point in a future (Angulo, 2002).

The most common physical symptom of NAFLD is hepatomegaly which is present in almost 75% of patients. Up to 25% of those also show signs of splenomegaly (Ludwig et al., 1980). Hepatic function tests in NAFLD patients demonstrate that in 65 to 95% of the patients there is a 2- to 4-fold increase in transaminase levels and that their AST/ALT ratio is less than one. AST/ALT ratio is a main diagnostic instrument allowing one to differentiate between NAFLD and alcoholic steatoHepatitis (Matteoni et al., 1999). Other liver enzymes such as alkaline phosphatase and gamma glutamyltransferase are also elevated in NAFLD, but to lesser extent than transaminases. In severe cases where cirrhosis is already present, a prothrombin time, bilirubin and an albumin also increase; however, these hepatic serological measurables also increase in many other chronic liver diseases.

The pathological spectrum of NAFLD ranges from clinically non symptomatic steatosis to non-alcoholic steatoHepatitis (NASH) and then to fibrosis and cirrhosis (Matteoni et al., 1999). Descriptive classification of NAFLD include a number of conditions: type 1- hepatic steatosis; type II- steatoHepatitis; type 3- steatonecrosis; type 4- steatonecrosis in the Mallory bodies or fibrosis (Younossi et al., 1998). Insulin resistance, obesity and type II diabetes could be considered as contributors or arguably preliminary stages in the development of the diseases of the NAFLD pathogenetic spectrum. Histopathologically, the most advanced disease of the NAFLD spectrum, NASH, is characterized by hepatic steatosis combined with hepatocyte ballooning degeneration, lobular inflammation, and, occasionally, a presence of Mallory hyaline or sinusoidal fibrosis (Matteoni et al., 1999, Teli et al., 1995). It is widely accepted that

NAFLD is a consequence of defects in lipid homeostasis leading to lipotoxicity in the non-adipose tissues. Excessive deposition of the triglycerides in the liver, particularly in the liver parenchyma, or hepatocytes, leads to steatosis (Falck-Ytter et al., 2001; Younossi et al., 2002). In simply overweight and moderately obese patients lipid accumulation most often localized in central lobular regions, but in the severely obese lipid accumulation shows diffuse distribution (Kral et al., 2004)

It is critical to conduct early distinction between NASH and the benign steatosis alone because of their differential risk for progression and management. Some patients with NAFLD develop NASH, while the majority remains in the benign steatosis stage (Matteoni et al., 1999, Pendino et al., 2005) with no symptoms for liver disease (Powell, 1990). Steatotic patients rarely develop cirrhosis, while 10-25% of NASH cases will progress to more serious stages of liver damage (Falk-Ytter et al., 2001, Matteoni et al., 2002; Ong et al., 2005). NAFLD with steatonecrosis have the worst prognosis as it most often progresses to cirrhosis that leads to death related to this hepatic condition (Younossi et al., 1998).

Pathogenesis

The most plausible theory behind NAFLD pathogenesis is the “double hit” theory where the first insult is relatively benign, but it makes liver sensitive to the second hit that causes inflammation and injury (Day & James, 1998). The first hit is indirectly triggered by insulin resistance and results from an increase in fatty acids uptake by the liver cells leading to the distortion of hepatocyte architecture and macrovesicular steatosis, where fat droplets push the hepatocyte nucleus to the periphery of the cells (Mulhall et al.,

2002). Loading of liver cells with high concentrations of free fatty acids is toxic for these cells as it alters many biochemical processes. Particularly, it increases production of VLDL-triglycerides and cholesterol and inhibits hepatic pyruvate oxidation by pyruvate kinase while stimulating gluconeogenesis and glucose output (Wolf et al., 2005). The accumulation of fatty acids in liver most often is due to imbalance in partition of energy sources, mainly fats and carbohydrates, resulting in a defect of the liver metabolic function that becomes more lipogenic than glycogenic (Jansen, 2004). The main characteristics for this metabolic imbalance are an upshift in peripheral lipolysis and hyperinsulinemia due to insulin resistance. Such an imbalance results from one or more of the following: (1) increased hepatic uptake of fatty acids due to peripheral lipolysis; (2) increase in *de novo* intrahepatic FA synthesis which is followed by their esterification into TGs; (3) glycolysis due to hyperinsulinemia; (4) decrease in removal of fatty acids from the liver due to impaired mitochondrial β-oxidation process; or (5) to a decrease of the export of fatty acids from the hepatocytes (Mulhall et al., 2002; Jansen, 2004). However, it is still under debate which is the starting point in the triangle of FFA, steatosis and insulin resistance that creates NAFLD. For example, it is not known exactly whether insulin resistance is a cause for steatosis or its consequence (Larter & Farrell, 2006).

The progression from benign steatosis to NASH relies on a lengthy and complicated process representing the second hit. Its pathogenesis remains unclear. The second "hit" in the development of NASH is multifactorial as it includes participation of mitochondrial lipid peroxidation and other types of oxidative stress, pro-inflammatory

cytokines, adipokines and endotoxemia (Mulhall et al., 2002). The sources of reactive oxygen species (ROS) are not restricted to inflammatory cells but also include hepatocytes themselves as an increased supply of FFA by their mitochondria, peroxisomes and microsomal cytochrome (CYP) P450 enzymes contribute to progression of NASH (Day, 2002).

Evidence for a role of an increase in the oxidation of the lipids, proteins and DNA has been demonstrated in animal models of NAFLD (Leclercq et al., 2000; Letteron et al., 1996), and in NASH patients as the degree of oxidative stress correlates with the severity of the disease (Albano et al., 2005; Seki et al., 2002). In addition, it has been shown that the typical diet of a NASH patient is usually low in antioxidants (Musso et al., 2003). Moreover, it has been demonstrated that in methionine adenosyltransferase 1A (MAT1A) deficient mice they develop spontaneous steatoHepatitis (Lu et al., 2001) – this enzyme serves as an antioxidant. Increased production of ROS was also revealed in Ob/Ob mice, a Leptin deficient model of NAFLD (Yang et al., 2000).

Accumulation of the lipid peroxidation products has harmful consequences on hepatic cells. Through activation of NF-κB, lipid peroxidation metabolites induce liver cells and non-parenchymal cells to produce an array of inflammatory cytokines, chemotactic factors and adhesion molecules (Moore et al., 2001; Ribeiro et al., 2004). TNF-α production is usually accompanied by activation of the chemokine IL-8 leading to recruitment of neutrophiles to infiltrate liver causing more inflammation. Additionally, induction of Fas ligand expression makes hepatocytes able to interact with Fas receptor

on other hepatocytes leading to appearance of patches of dead liver cells within the liver (Pessayre et al., 2001).

Metabolites such as 4-hydroxynonenal (HNE) and malondialdehyde (MDA) can bind to hepatic proteins causing immune reactions and the production of the proinflammatory cytokines, injuring the liver cells further (Albano et al., 2005). In addition, these products can induce the synthesis of extracellular matrix (ECM) proteins by hepatic stellate cells (HSC) leading to a crosslinking of cytokeratins and the formation of the Mallory bodies (Zamara et al., 2004). When the intrahepatic antioxidant system becomes unable to counterbalance the excess of ROS produced by β-oxidation of FFA or by action of proinflammatory molecules, the underlying steatoHepatitis get excecerbated by inducing more lipid peroxidation, cytokine production and Fas ligand induction (Pessayre et al., 2001; Benedetti, 2005; Mulhall et al., 2002). The degree of lipid peroxidation correlates with steatosis severity (Oliver & Christoph, 1998). Stress caused by ROS triggers hepatic cell death and an increase in collagen synthesis -- two main histological features seen in necrosis and fibrosis, respectively (Pessayre et al., 2001).

Some scientists believe that the increased FFA oxidation and resulting ROS production are due to steatosis-induced hepatic insulin resistance (Reddy, 2001) via mechanisms involving activation of PKC-ε, Jun N-terminal kinase (JNK), I-κB kinase β (IKK-β) and nuclear factor-kappa B (NF-κB) (Samuel et al., 2004; Cai et al., 2005). In addition, FFAs and their metabolites can serve as potent ligands for peroxisomal proliferator activated receptor α (PPARα) which controls transcription of a variety of genes encoding enzymes involved in mitochondrial, peroxisomal and microsomal fat

oxidation. For example, a defect in insulin signaling leading to a post-transcriptional upregulation of hepatocyte cytochrome P4502E1 (CYP2E1) (Woodcroft et al., 2002) could be the major contributor of the ROS and the direct cause for oxidative cell injury in NASH (Weltman et al., 1996).

Recent studies of oxidative stress as the second hit leading to the developments of NASH stressed the role of mitochondrial stress in inducing apoptosis (Feldstein et al., 2003). Mitochondrial defects by themselves could be the sole cause for oxidative stress and ROS production. Many scientists believe that NAFLD patients have mitochondrial dysfunction that leads to more ROS production (Caldwell et al., 1999; Cortez-Pinto et al., 1999; Perez-Carreras et al., 20032). Mice deficient in the gene coding for the peroxisomal fatty acid acyl-CoA oxidase develop severe NASH that is attributed to the increase in ROS that is secondary to increased FFA oxidation that proceeds via both mitochondrial and microsomal pathways (Fan et al., 1998). In addition, Ob/Ob mice showed an increase generation of hydrogen peroxide and superoxide anions from their hepatocytic mitochondria when compared to wild type mice (Yang et al., 200; Laurent et al., 2004).

Endoplasmic reticulum (ER) stress characterized by increased lipid synthesis, apoptosis and JNK-induced insulin resistance are presumed to also be implicated in NAFLD pathogenesis. Patrticularly, this can be deduced from the observation that ER stress plays an important role in alcohol induced liver disease (Ji & Kaplowitz, 2003; Luis et al., 2003) and also represents an important characteristic of obesity that links it with insulin resistance, development of diabetes and NAFLD (Ozcan et al., 2004). It is believed that lack of glucose and other nutrients, accumulation of lipids and viral

infections cause stress to the ER resulting in activation of a number of transcription factors and kinases (Ron, 2002). For example, stress-dependent activation of sterol regulatory element-binding proteins (SREBPs) leads to up-regulation of lipid synthesis. ER stress also induces transcription of pro-apoptotic genes. For example, ER stress response results in ER stress-related apoptosis by releasing pro-apoptotic caspase 12 from its inhibitor TRAF2.

Adipocytokines have been recently proposed as the central players in NAFLD pathogenesis. Hepatocyte death and liver necrosis have been shown to be modulated by many cytokines. For example, both TNFα and TGFβ produced in adipose have been directly linked to hepatocyte apoptosis, hepatic stellate cells (HSC) activation and the formation of the Mallory bodies (Pessayre et al., 2001). TNFα, IL-6 and IL-1β have all been shown to play central roles in both hepatic and systemic insulin resistance (Arkan et al., 2005). Moreover, in obese NASH patients there is increase in expression of TNF-α mRNA and the level of its receptor in liver and adipose tissue correlate with disease severity (Crespo et al., 2001). One of the core objectives of this study is a thorough quantification of the serum levels for a number of adipocytokines in various types and stages of NAFLD.

Other factors playing a role in oxidative stress and cytokine induction have also been suggested to play a role in NASH development, for example, hepatic iron accumulation and endotoxemia. It is believed that overgrowth of small intestinal bacteria especially the gram negative bacteria increases permeability of intestines for absorption of endotoxins leading to endotoxemia that leads to TNF-α activation, the master trigger

for a main inflammatory cascade (Okolo & Diehl, 1998). Some studies showed that some types of the microbial types in the gut make some people more vulnerable for developing NAFLD. For example, it has been shown that the relative abundance of certain bacteria, *e.g. Bacteroides ssp*. and *Firmicutes* in the gut increases intestinal capacity to harvest energy from food and, therefore, contributes to obesity (Turnbaugh et al., 2006). However, whether the alteration in the relative abundance of normal flora or other types of secondary opportunistic microbes in the gut of an obese individual is a secondary phenomenon to medications, food consumption habits and lack of physical activities or a direct contributor to obesity needs further investigation.

The sequential order of the molecular events and relative effects of each of the pathogenesis factors involved in progression of NAFLD are not clearly known. However, we believe that the progression of steatosis to NASH to necrosis and fibrosis depends on the cumulative effect of all these factors.

Genetics

The genetic factors predisposing individuals to obesity and metabolic syndrome are poorly understood. Some researchers believe that clinical disorders associated with metabolic syndrome have a genetic basis in certain DNA polymorphisms (Bouchard & Perusse, 1996). This implies the heterogeneity of the pathological and clinical manifestations of metabolic syndrome in different people. Unfortunately, identifying unique and specific abnormalities on the genomic level is difficult, and large cohorts of patients need to be profiled to obtain solid data (Bouchard & Perusse, 1996). Types of the studies aimed at revealing the genetic influence of common diseases include family

linkage studies, classical case control studies, candidate gene and allele association studies. However, these studies are difficult to perform, take a long time, have limited genetic outcomes and are associated with many pitfalls generating both false positive and false negative results (Daly&Day, 2001). Both obesity and metabolic syndrome related features are influenced by multiple interacting genes. Identification of the genetic factors influencing these disorders will help to develop prevention strategies and novel non-invasive biomarkers and will lead to identification of novel treatment targets. There is a hope that hypothesis free approaches, such as by using high throughput microarray technologies, proteomic studies and quantitative trait loci (QTL) analysis will significantly increase the chances of identifying novel genetic factors involved in susceptibility to NAFLD (Rangnekar et al., 2006).

Early evidence of the genetic causes of NAFLD came from a study on seven members of the same family having either NASH or cryptogenic cirrhosis, or both (Struben et al., 2000). Later, another study showed that 18% of 90 surveyed patients with NASH had an affected first degree relative or family member (Willner et al., 2001). Browning *et al*. showed that the prevalence of cryptogenic cirrhosis in the Hispanic population is threefold higher than in European American patients, while in African Americans fourfold lower despite a similar prevalence of type II diabetes (Browning et al., 2004). Some studies identified genetic loci influencing major clinical phenotypes associated with metabolic syndrome. For example, pedigree-based analysis pointed at a quantitative trait locus (QTL) on chromosome 3q27 strongly linked to weight, waist circumference, Leptin, insulin, insulin/glucose ratio, and hip circumference with possible

epistatic interaction with a second QTL on chromosome 17p12 that is strongly linked to plasma Leptin levels (Kissebah et al., 2000). Recently, Frayling *et al.* identified a common variant of the gene *FTO* (fat mass and obesity associated) on chromosome 16q12.2 that is consistently strongly associated with early-onset and severe obesity in both adults and children of European ancestry. This common variant of *FTO* increases the risk of obesity by almost 67 % and was implicated in the development of obesity in 13 studies involving over 38,000 subjects (Frayling et al., 2007). Recently, Xu *et al*. identified a circulating protein called Adipocyte Fatty Acid Binding Protein (FABP4) and showed that its high level in serum predicts the development of metabolic syndrome (Xu et al., 2007). Molecular genetic analysis of the common SNP polymorphisms have also been used to study the association of certain genes with some clinicopathological factors of metabolic syndrome and their interactions with different ethnic and gender backgrounds (McCarthy et al., 2003; Robitaille et al., 2004). For example, in the US, the highest rates of metabolic syndrome have been registered in Mexican American women (Park et al., 2003).

Recently, microarray technology has become a routine research tool for the investigation of the molecular underpinnings of disease pathology (Deaciuc et al., 2004). Candidate genes identified from such microarray analyses could be used later in association studies aimed at the identification of genetic risk markers. As a matter of fact, microarrays have been applied to study several chronic diseases including liver disease, diabetes, and obesity (Baranova et al., 2005) primary biliary cirrhosis (Shakel et al., 2001) Hepatitis C virus infection (Shakel et al., 2002) ALD, and NAFLD (Seth et al.,

2006; Sreekumar et al., 2003; Younossi et al., 2005). For example Younossi and colleagues identified thirty-four genes differentially expressed in NASH livers compared to the livers of non-obese controls. Of specific interest, a mitochondrial enzyme for increasing ketogenesis, 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGCS2) and a regulator of lipid metabolism acyl-CoA synthetase long-chain family member 4 (ACSL4/FACL4) were among the upregulated genes (Younossi et al., 2005).

Adipokines Role in the Pathogenesis of NAFLD

Adipokines, also called adipocytokines, are a group of pharmacologically active proteins characterized by a wide range of molecular weights. Adipokines produced by adipose tissue and other cells, such as leukocytes, actively participate in the regulation of many physiological and pathological processes in the human body, including inflammation, lipid metabolism, blood pressure regulation, glucose uptake and angiogenesis (Hauner et al., 2005; Trayhurn & Wood, 2005). Examples of adipokines produce by the adipose are Leptin, Adiponectin, Resistin, Apelin, Visfatin, Vaspin And Zinc-Alpha2-Glycoprotein, as well as the "traditional" cytokines and chemokines, such as TNF-alpha, plasminogen activator inhibitor-1 (PAI-1), IL-6, IL-8, IL-1B, monocyte chemoattractant protein 1, and others (Fantuzzi, 2005; Lee &Pratley., 2005).

In NAFLD, expansion of adipose tissue and infiltration of adipose by lymphocytes disturbs the balance of adipokines in these tissues and in the whole body. High levels of proinflammatory adipokines secreted by adipose tissue lead to a systemic inflammation that has direct and indirect consequences on many physiological processes and contribute to many disorders such as vasculopathy, cardiovascular diseases and

NAFLD (Anders et al., 2005). The imbalance in the synthesis and secretion levels of such adipokines has been directly linked to many metabolic syndrome associated factors such as insulin resistance and obesity (Hong & Heber, 2006). However, the molecular links between adipocytokines, insulin resistance and obesity in NAFLD are not well characterized. More specifically, the primary cause or sequence of events in steatosis development has not been determined. Insulin resistances, together with increases in FFAs and adipocytokines have been prioritized as candidates for causing steatosis by various researchers (Reviewed by Rodent, 2006). In addition, the influence of genetics, diet and physical activity on NAFLD pathogenesis has been established making it more complicated (Figure 4).

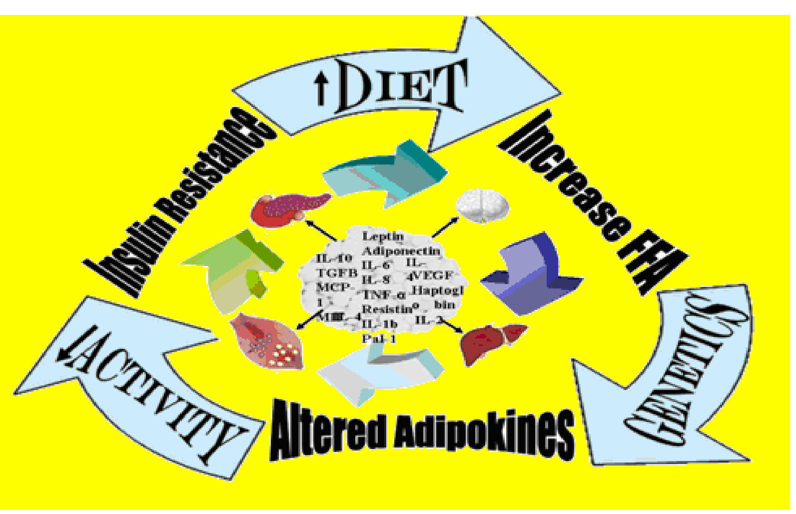

Figure 4. The triangle of the main driving forces of NAFLD pathogenesis with an emphasis on the role of adipokines.

Changes in adipocytokine levels in serum may be benificial or detrimental depending on the particular adipokine. For example, up-regulation of some of these

factors could have a detrimental effect as seen with the case of TNF-α or have a beneficial effect in the case of Apelin and Visfatin as these are thought to protect against obesity-related pathologies (Bełtowski, 2006). Similarly, down-regulation of some adipokines could also have different effects; for example low Adiponectin levels could contribute to pathologies associated with obesity while low Resistin levels are beneficial. Regardless, the imbalance in circulating levels of various pro- and anti-inflammatory cytokines, including adipokines, has a direct role in the pathogenesis of NASH in both the first and second hits (Jansen, 2004; Tilg &Diehl, 2000; Kugelmas et al., 2003; Fantuzzi, 2005; Lee & Pratley, 2005).

However, the pathological mechanisms by which adipokines exert their biological influences in NAFLD pathogenesis are very complex and affected by many factors.

First, the cellular and tissue sources of adipokines at different stages of NAFLD are expected to be different. It has been proposed that adipocytes may serve as significant contributors in the pathological process of NAFLD by increasing systemic inflammation in obese patients (Lee & Pratley, 2005). Recent studies suggest that obesity is also associated with an increased infiltration of adipose tissue by macrophages attracted by migration inhibitory factor (MIF) (Skurk et al., 2005) which also participates in or exacerbates the inflammatory process through the elaboration of more cytokines (Wellen et al., 2003). These findings were substantiated when a reduction in the macrophage infiltration of adipose tissue was found to accompany weight loss that lead to a decrease in the expression of the pro-inflammatory genes. Accordingly, it is expected that obesity associated pathological conditions such as insulin resistance and consequently peripheral

tissue damage are influenced by adipokines produced by both adipocytes and macrophages infiltrating adipose tissue (Bastard et al., 2006).

Moreover, adipose tissue contains many types of cells other than adipocytes and macrophages such as stromal cells, endothelial cells, sympathetic nerve fibres and monocytes (Hauner et al., 2005). So, the modulatory effect of adipokines in NAFLD pathogenesis is not limited to adipocytes and macrophages or adipose. Liver cells such as hepatocytes, Kupffer cells and stellate cells also synthesize many pro-inflammatory and anti-inflammatory cytokines that contribute to the shift in balance towards the pro-inflammatory side, influencing steatoHepatitis progression and necrosis (Tilg & Deihl, 2000; Wellen & Hotamisligil, 2003). The exact contribution of each of these cellular components in the "pro-inflammatory" state of obesity is not clear especially at different stages of NAFLD. For example, it is believed that the majority of the adipokines released from adipose tissue, except for Adiponectin and Leptin, originated from nonfat cells embedded in the extracellular matrix (Fain et al., 2004). Important adipokines such as Resistin and Adiponectin can be produced elsewhere in the body and at any stage of NAFLD. Adipokine synthesis outside the adipose tissue has been confirmed by protein and mRNA quantification as well as by gene expression analyses of corresponding UniGene clusters (Minn et al., 2003; Savage et al., 2001). These data suggest that serum adipokine measurements in patients most likely reflect secretions by various cells from various tissues, including adipocytes.

Second, adipokines modulate their function on different targets in different ways. For example, adipokines secreted by any cell could have autocrine effects by altering its

self function, or paracrine effects by altering neighboring cells or endocrine effects by affecting distant cells and organs such as hypothalamic neurons and skeletal muscles (McDermott, 2001). Moreover, cytokine network interactions are very complex. Cytokines could have multiple biological activities, different cytokines could have the same activity and some cytokines, such as TNF-α, can modulate the synthesis, secretion and activity of many others (McDermott, 2001). For example, TNF-α modulates levels of IL-8 and IL-6, and Visfatin induces the production of IL-6 in human CD14 (+) monocytes (El-Assal et al., 2004) whereas IL-6 negatively regulates Visfatin gene expression in 3T3-L1 adipocytes (Teoh et al., 2006). To add more complexity, many adipokine related proteins and receptors are expressed and localized differentially in different tissues. It is possible that these adipokines have different functions in different tissues in response to different local and systematic stimuli involved in homeostasis and metabolism. So the imbalance in synthesis and secretion of adipokines and their related proteins have differential pleiotropic consequences in NAFLD pathogenesis.

Third, the exact molecular mechanism underlying the role of any cytokines in the pathogenesis of NAFLD is not clearly understood. For example, the role(s) of some pro-inflammatory cytokines have been linked directly to the insulin signaling pathway. It has been shown that TNF-alpha increases serine phosphorylation of insulin receptor substrate (IRS)-1 therefore impeding insulin receptor tyrosine phosphorylation, leading to insulin resistance (Hotamisligil et al., 1993). Other studies stress that the complexity of TNF-α modulation in the insulin signaling pathway is multiplied by the fact that TNF-α may bind to different receptors. Thus, the outcome of the signaling process is affected

depending on whether TNF-α s present in the soluble or membrane bound form (Hube et al., 1999). It has been demonstrated that the membrane bound TNF-α can bind to both TNF-R1 and R2 (Hube et al., 1999; Sethi et al., 2000). However, its binding to TNF-R2 is important for an induction of insulin resistance and lipolysis, while by its binding to TNF-R1 plays a more important role in stimulating lipolysis and apoptosis (Hube et al., 1999; Sethi et al., 2000; Tuncer et al., 2003). On the other hand, the soluble form of TNF-alpha has greater affinity for TNF-R1. This complexity increases when TNF receptors are expressed differentially in different parts of cells and tissues. For example, it has been found that TNF-α, TNF-R1 and TNF-R2 are expressed more in subcutaneous adipose tissue than in omental tissue (Hube et al., 1999a).

Like TNF-α, other adipocytokine mediated signaling effects are influenced by a variety of factors such as particular isoform, tissue source, function, receptor expression levels and localization which makes understanding their exact mechanism of action and role in the pathogenesis of NAFLD open for debate (reviewed in Baranova et al., 2007). Source, targets and effects of a panel of main adipocytokines evaluated in this thesis are summarized in Appendix Table.1.

CHAPTER 3.

NAFLD and Hepatitis C Infection

Hepatitis C virus (HCV) is one of the most common blood borne pathogens that causes devastating chronic liver diseases.

HCV infection can lead to fibrosis, cirrhosis, liver cancer and even death (Hoofnagle, 2002). More than two thirds of acute case infections become chronic (Alter et al., 2000; Dubois F et al., 1994). More than 170 million people are infected world wide (Negro, 2006; Alter et al., 2000), and almost 2.3 % of adults 20 years of age or older in United States are positive for anti-HCV-antibody (Alter et al., 1999). The methods of transmission of HCV infection are numerous ranging from percutaneously, through blood and related products, to haemodialysis, organ transplantation, injections and sexual intercourse making it a highly epidemic problem (Alter, 1996, 1997, 1999, 2002) . The opportunistic nature of HCV makes its prevalence and rate of infection much higher and more lethal in HIV patients and drug users than others (Sulkowski et al., 2000).

Hepatitis C is very infectious and has serious health consequences. It replicates rapidly inside the body generating almost 1 trillion new virions each day and has developed many subtypes and quasi-species by continuously mutating itself to avoid the immune system defense mechanisms (Franciscus, 2006). Consequently, many infected people develop chronic infection and no treatment or vaccine is yet available (Franciscus,

2006). In addition, in every infected person there are millions of different HCV quasi-species with unique characteristics in response to a patient's unique immune system. Currently there are only 6 confirmed genotypes, but more than 50 subtypes of HCV are known worldwide, that differ in infectivity, viral load, disease progression, epidemiology and response to current medications (reviewed in Choi & Ou, 2006; Franciscus, 2006). For example, genotypes 4-6 are most common in certain areas of the world, while genotypes 1-3 are found worldwide. Genotype 2 and 3 respond better to current medications than others, while genotype 1 is the least responsive (reviewed in Franciscus, 2006).

Hepatic steatosis (HS) is the most common histopathological finding in both NAFLD and HCV infection. Moreover, NAFLD and HCV infection can both coexist in the same individual further exacerbating their liver disease (Zekry et al., 2005). Actually, steatosis has been found to be present in around 50% of patients suffering from chronic Hepatitis C (Lo Iacono et al., 2007; Petit et al., 2005). Others estimated HS in HCV infections to range from 40-86% depending on the patient population, genetic factors, accompanied metabolic risk factors (obesity, diabetes and dyslipedemia) and genotype of the virus, which is most commonly Geno type 3a (Zekry et al., 2005, Asselah et al., 2003).

It is not clear whether the steatosis seen in Hepatitis C patients results from pro-steatotic action of the HCV core proteins or if it develops under the indirect influence of the metabolic factors that may accompany HCV infection. While in many cases, HCV seems to coexist independently with NAFLD, some forms of the virus, specifically HCV genotype 3a, appear sufficient to cause virally-induced steatosis (Lo Iacono et al., 2007,

Wang et al., 2005, Petit et al., 2005). This is evidenced by correlations between the severity of steatosis and replication levels of the HCV as well as with successful antiviral therapy and the amelioration of steatosis (Negro, 2006). On the other hand, HCV infection could modulate the metabolic processes in the body that lead to steatosis. Some evidence shows that steatosis can in turn influence the progression of HCV, possibly affecting replication rates and its ability to spread through hepatic cells (Negro, 2006). In fact, it has been shown that the nucleocapsid protein of the Hepatitis C virus is by itself capable of inducing an accumulation of lipids within liver cells (Negro, 2006). Moreover, the sequence of events and relationship between steatosis, necroinflammation and fibrosis in HCV are still arguable. It is not known if steatosis in HCV infection is a marker, a consequence, or a cause of the necroinflammation, or if it is a first step toward fibrosis in disease progression (Zekry et al., 2005). Some studies have shown that there is an association between the steatosis and necroinflammation processes, while others support the hypothesis that genotype 3 HCV directly causes steatosis which could lead to necroinflammation (Zekry et al., 2005; Polyak et al., 2001; Asselah et al., 2003).

Reactive oxygen species play important roles in both HCV-associated NAFLD progression, especially in NASH manifestation, and HCV pathogenesis. Many researchers have shown that HCV infection directly causes an increase in ROS production via different mechanisms (reviewed by Choi and Ou, 2006). These mechanisms include: (1) activation of NADPH oxidase in Kupffer cells and PMN cells by NS3 protein during inflammation, (2) increase iron overload and lipid peroxidation or (3) increased production of mitochondrial ROS by the electron transport chain due to

some core proteins of HCV such as NS5A. Others propose that HCV decreases the gene expression of antioxidants, and increases the gene expression of cytokines that increase ROS as well as inflammation related enzymes such as COX-2 and CYP2E1 (reviewed in Choi & Ou, 2006).

Both insulin resistance and steatosis are frequent and common features in chronic Hepatitis C and NAFLD. However, the pathogenic mechanism of insulin resistance development and/or steatosis in Hepatitis C infection, and which one is the cause and the consequence in this triad is under intense investigation. Researchers have focused most of their investigation on the independent or combinational role of Hepatitis C genotype and the underlying metabolic factors in developing steatosis or insulin resistance. Some researchers have indicated that insulin resistance is indeed the cause of steatosis and fibrosis in Hepatitis C genotype 1 patients, rather than a consequence (Fartoux et al., 2005). Others showed that HCV genotype 3 infection by itself through its HCV core protein can lead to both steatosis by inhibition of microsomal triglyceride transfer protein and insulin resistance by inhibiting insulin-signaling pathways through elevating TNF-α and other factors (reviewed in Decock et al., 2007). The most common themes that explain the causes of developing steatosis in HCV infection are that, steatosis in Hepatitis C genotype 3 is directly caused by the virus, while steatosis in Hepatitis C of other genotypes is caused indirectly by a host of metabolic risk factors such as insulin resistance, obesity and hyperlipidemia (Castera, 2006).

Many researchers have suggested that alterations in adipocytokines produced by expanded adipose (adipokines) play an important role in the pathogenesis and

progression of NAFLD. This implies that changes in adipokine levels may serve as the second hit in the development of both metabolic NAFLD and steatosis seen in HCV infection (Tuncer et al., 2003, Neuman et al., 2001; Polyyak et al., 2001; Vechiet et al., 2005; Opal & DePalo, 2000). For example, it has been shown that there is an association between plasma interleukin-18 levels and liver injury in chronic Hepatitis C virus infection and NAFLD since enhanced expression of IL-18 in patients with HCV and NAFLD has been found (Vecchiet et al., 2005). In addition, it is also reasonable to suggest that chronic HCV infection induces a dynamic process of necroinflammation in the liver, especially as it has been shown that HCV infection increases production of TNF-alpha and IL-8 (Neuman et al., 2001; Polyyak et al., 2001). These processes may hasten the progression of the chronic liver disease to fibrosis. In addition, some HCV infections may exacerbate the metabolic conditions predisposing the patients to overt steatosis. This may explain why steatosis is not seen in every HCV infection. Accordingly, it is of great value to study the effects of HCV on the balance of adipocytokines and their relationship to steatosis. In this research, we intend to examine the relationship between steatosis and Hepatitis C, as well as possible serological markers for identifying the extent of both metabolically-induced and virally-induced steatosis. Adiponectin, Visfatin, Resistin, Leptin, TNF-α, IL-8, IL-6, IL-1B, IL-1raand sIL-6r were quantified in serum of patients infected by HCV genotypes 1 and 3. Genotype 1 was chosen as it is the most common strain and the most difficult to treat, while genotype 3 was chosen for its apparent ability to induce steatosis in the absence of NAFLD causative factors.

CHAPTER 4.

Hypothesis and Research Objectives

The incidence of NAFLD, the most common cause of liver abnormalities among adults, is on the rise in parallel with global epidemic of obesity. NAFLD is expected to be the most grave health issue of the twenty first century (Pendino et al., 2005; Suzuki et al., 2005). Accordingly, the most urgent and pressing issues are, elucidating the mysterious pathogenesis mechanisms of NAFLD, finding new diagnostic profiles for early detection, and finding distinction between various manifestations of the disease and exploring new therapies.

The impact of NAFLD relates to its prevalence and potential for progression. So it is critical to differentiate between different stages of NAFLD due to their differential risk for progression and management. Recent data suggest that up to 10 to 24 % of the general population and 50 to 90 % of obese individuals are affected by NAFLD (Younossi et al., 2002; Benedetti, 2005). The prevalence of NASH, which requires histological confirmation, is estimated as 1.2 - 4 %. However, in patients with risk factors such as those observed in morbidly obese, prevalence of NASH is higher, and is estimated as 20 - 47 % (Ong et al., 2005). The steatosis stage is benign and comprises the majority of NAFLD. Steatosis rarely develops to cirrhosis; however 10-25% of NASH will progress

to the more serious stages of liver damage such as cirrhosis and hepatic carcinoma (Falk-Ytter et al., 2001; Matteoni et al., 2002; Ong et al., 2005).

Central obesity and insulin resistance, the most common features and risk factors associated with NAFLD, can be used as indicators for having NAFLD but not for diagnosis. Also current known clinical symptoms and serological findings in NAFLD patients do not correlate with the histological findings, and are therefore unreliable for diagnosis or early detection for any stage of NAFLD. Unfortunately, the only definitive diagnostic tool for confirmation of NASH, fibrosis and cirrhosis is liver biopsy with all its disadvantages (Sebastiani & Albert, 2006). Moreover, the histological similarities between NASH and alcoholic steatoHepatitis (ASH) are numerous, making pathological distinction between them very difficult. The most common histological features between NASH and ASH are presence of macrovesicular steatosis, lobular mixed inflammatory infiltration, hepatocellular ballooning, and necrosis (Bacon et al., 1994)

However, obesity associated with NAFLD is characterized by two main pathophysiological changes: first, an expanded adipose tissue mass and second, a chronic mild inflammation characterized by a disturbed balance in the expression and secretion of many adipokines specifically from the biologically active white adipose tissue (Hauner et al., 2004). Many researchers have found that some adipokines are upregulated in NAFLD such as Visfatin, apelin TNF-alpha, IL-8 and IL-6; and some are downregulated such as Adiponectin. However, the relative increase or decrease of these adipokines in different stages of NAFLD progression and with different degrees of insulin resistance has not been investigated. Although the role of proinflammatory cytokines (TNF-α, IL-6) in the

pathogenesis of NASH is also well documented (Tuncer et al., 2003), the role of such cytokines in the pathogenesis of NAFLD progression is a recent observation and needs to be explored especially with regards to the degree of insulin resistance. Also it is not well known whether the imbalance in adipokine synthesis and secretion is directly due to expanded adipose tissue mass or indirectly, and consequently to an alteration in signaling pathways of these adipokines as a result of internal or external signals (Hauner et al., 2004). Moreover, determining the exact role of each adipokine in the pathogenesis of NAFLD is complicated by the interaction of adipokines with each other and with insulin resistance. Most studies have not controlled for these important factors when they evaluated the status of adipokines in NAFLD.

At the beginning of this research we believed that adipokine production levels could be correlated with the progression of the disease stages of the NAFLD spectrum and with the degree of insulin resistance. These progressive changes in adipokine levels could be influenced by: (1) continuous expansion of adipose tissue, (2) infiltration of adipose with other cells and especially macrophages exacerbating production of adipokines, (3) due to the complex interplay between various adipokines in different stages of NAFLD spectrums.

The exact source and effect of adipokines in patients with NAFLD remains unclear. This is due to many unpredicted reasons such as: the cellular composition of adipose tissue which depends on many variables such as anatomical location, body weight and other factors that vary substantially (Hauner, 2005). Second, whether the adipokine has local or systematic effects and the relative amount of each adipokine

produced by each type of cell in adipose. However, most of the adipokines that have direct systemic effects, regardless of its cellular adipose source, would have a relative level in the circulatory system as the best route to reach all targets. Additionally, most adipokines that exert local paracrine action may also induce a secondary systemic action. Therefore it is reasonable to say that many adipose secreted products could be evaluated in the circulation, and by using sensitive measurement techniques we would be able to evaluate these products at any stage of NAFLD**.** Accordingly, we preferred to quantify adipocytokines in serum samples as their levels would probably reflect a “net” biological effect of the secretion by various cells in the whole body.

Moreover, hepatic and omental tissues are the major sources of molecular influences in NAFLD pathogenesis. Changes in omental adipose tissue may result in comparable changes in adipocytokines that affect hepatic tissues. For example, an increase in the “pro-inflammatory” or “pro-oxidative” factors can alter the hepatic environment, favoring development of NASH or its progression (Choi & Diehl, 2005). All such underlying biological changes in omental adipose tissues and liver are accompanied by changes in gene expression. Differences in transcriptional regulation of these two organs may give important clues into the pathogenesis of NAFLD and its progression. Driven by such objectives; we performed a high-throughput gene expression study of liver and visceral adipose specimens of morbidly obese patients undergoing bariatric surgery, as compared to those of lean subjects.

Many studies showed that NAFLD and HCV infection share common biological phenomena such as steatosis development, reactive species induction and a progessive

inflammatory state. For such connections, it is reasonable to compare the status of adipocytokines between NAFLD and HCV infection. More importantly, the status of serum adipocytokines in relation to HCV genotype, NAFL and underlying metabolic disorders has not been fully characterized. Finding adipokine profile panels characteristic for a certain HCV genotype or NAFL or both is important for elucidating the links between HCV, NAFLD and adipocytokines.

The main objectives of this research are:

Driven by the current debates about the main contributors in pathogenesis of NAFLD and the ability to distinguish progressive stages, we intended to do this descriptive analytical study. Mainly we concentrated on answering of the following questions: (1) what are the differences in adipokine serum levels in various types of NAFLD? (2) Why some patients with very high BMI (>45) stay free of liver disease? (3) Are there some protective or predisposing factors involved? (4) Are these factors present in the liver or in the fat? More specifically, we sought to:

1. Evaluate the status of adipokinome in different stages of NAFLD progression by measuring serum levels of some major adipokines in different stages of NAFLD spectrum with different degrees of insulin resistance.

This will give a clear idea of the kinetic status of adipokines and their correlation with each other in different spectrums of NAFLD as well as with the degree of insulin resistance. Such comparative analysis may provide a meaningful correlation between various adipokines in the pathogenesis and progression of NAFLD. Finding diagnostic profiles as well as associations of adipokines with specific spectra of NAFLD or insulin

resistance would be of great value in early detection, distinction and perhaps in prognostic evaluations. Clear evaluation for the adipokinome status in different stages of progression of NAFLD and characterization of possible profiles that may explain specific signaling interactions between specific adipokines would likewise be helpful for targeted drug therapy and design.

2. We also intend to explore gene expression profile differences between omental adipose and hepatic tissues in obese and NAFLD patients. Characterizing expression changes on different levels of NAFLD could explain or identify the genomic basis for underlying pathological causes of NAFLD and obesity. In addition this would allow us to clarify signaling links and molecular interactions between different proposed molecular events involved in NAFLD pathogenesis.

3. Compare the profile of major adipocytokine levels in serum of patients diagnosed with NAFLD, HCV infection of different genotypes or both. Specifically, HCV genotypes 1 and 3 were compared to determine if the level of certain adipocytokines could be used as a diagnostic and biomarker tool or to explain the role of some adipocytokines in the pathogenic process of both NAFLD and HCV.

The specific aims of this research:

1. To measure the fasting serum levels of glucose, insulin, Adiponectin, TNF-α, Visfatin, Resistin, TNF-α, IL-8, IL-6, and CK18-Asp396 neo-epitope (M30) as a marker for apoptosis and total soluble CK18 (M65) as a marker for both apoptosis and necrosis, respectively in four groups of patients. 1) NASH (N = 26); 2) Simple Steatosis (SS, N=19); 3) Morbidly Obese Controls without NAFLD (Control I, N=38); and 4) Non-

Obese Controls who were healthy blood donors (Control II, N =12). In addition, groups of NASH, SS, and Obese controls will be divided in to subgroups according to insulin resistance as measured by homeostatic model assessment (HOMA) scores. These subgroups are subdivided as High HOMA (>3.0); Low HOMA (<1.8); and Mid-range HOMA (1.8-3.0) scores.

2. To perform and compare gene expression profiling using two sets of microarrays with a total of 5,297 genes on 6 groups of omental adipose tissues and liver biopsies. These groups are: adipose of obese NASH patients (N=10), adipose of non-Obese Controls (N=9) and adipose of Obese Controls (N=17). Liver biopsies of obese patients with NASH (N=27), liver biopsies of non-Obese Controls (N=6) and liver biopsies of Obese Controls (N=7).

3- To quantify serum level of Adiponectin, Resistin, Visfatin, Leptin, TNF-α, IL-1B, IL-6, sIL-6r, IL-1ra and IL-8 level in the sera of 7 groups of patients with chronic HCV infection. These groups are classified as follow: HCV genotype 3 (N=17), HCV genotype 3 and steatosis (N=9), HCV genotype 3 and no steatosis (N=8), HCV genotype 1 (N=80) HCV genotype 1 and steatosis (N=37), HCV genotype 1 and no steatosis (N=43) and Patients diagnosed with HBV only as controls (N=20).

CHAPTER 5.

Research Design and Protocol

Patient Cohorts and Samples

To perform this study we used existing clinical data, serum samples and tissues from the Epidemiology of Nonalcoholic Fatty Liver Disease Database (EPI-NAFLD) study. This study has been created by enrolling consecutive patients undergoing bariatric surgery at INOVA Fairfax Hospital from October 2001 to the present. EPI-NAFLD contains the extensive clinical and laboratory data routinely collected for each bariatric surgery patient after obtaining informed consent. Patients with evidence of excessive alcohol use (≥10 g/d), patients under PPARgamma agonists' therapy or patients with other known causes of liver disease were excluded from this study. Additionally, patients who have any other apparent or known diseases that could influence adipokine serum levels were also excluded. Insulin resistance was defined as a homeostatic model assessment index (HOMA) >2.2. Serum samples from each patient were collected at the time of biopsy and stored at -81°C. These frozen serum samples were aliquoted as 200 µl samples which were subsequently used for biochemical analyses such as glucose and insulin measurements, liver function tests and for adipocytokine profiling.

The cytokine profiling study was performed on four groups of patients: NASH (N = 26); Simple Steatosis (SS, N=19); Morbidly Obese with Healthy Livers designated as control I (MOHL, N=38) and Healthy Blood Donors (HBD, N =12) designated as control

II. NASH, SS and Obese control patient's groups were BMI and HOMA-matched (see Table 1). Furthermore, each of these three groups was subdivided according to HOMA scores as High HOMA (>3.0); Low HOMA (<1.8) and Mid-range HOMA (1.8-3.0) subgroups for the subsequent detailed analysis. Frozen fasting serum samples were used for biochemical analyses of glucose and insulin measurements as well as adipocytokines profiling. Clinical and demographic data describing these patients are summarized in Table 1. There are no clinical data available for healthy blood donors except HOMA scores that were normal.

Table 1. Clinical and laboratory data for patients used for profiling adipocytokines in NAFLD spectrum (mean ± SD or %).*P-values: Fasting serum glucose ($P < 0.015$); AST ($P < 0.0001$); ALT ($P < 0.0001$); Fasting serum triglyceride ($P < 0.05$). NS: Non-significant difference between groups.

Clinical Data	Simple Steatosis (N = 19)	NASH (N = 26)	Obese Controls (N=38)
Age, years (NS)	37 ± 9.2	43.9 ± 11.4	40 ± 9.5
Female, %	89.47% (17)	57.69% (15)	86.8% (33)
Caucasian, %	73.68% (14)	76.9% (20)	78.95%(30)
Hip-to waist ratio (NS)	1.06 ± 0.11	1.01 ± 0.1	1.07 ± 0.13
Body mass index (NS)	47.2 ± 7.5	47.5 ± 8.3	47.5 ± 9.4
AST level, IM/L (*)	20.6 ± 8.1	35.1 ± 25.3	18.7 ± 3.9
ALT level, IM/L (*)	24.4 ± 14.6	45.96 ± 30.4	21.7 ± 7.5
AST/ALT (NS)	1.02 ± 0.58	0.83 ± 0.32	0.94 ± 0.3
Fasting serum triglyceride, mg/dL (*)	147 ± 82.2	191 ± 99.4	136 ± 66.3
Fasting serum cholesterol, mg/dL (NS)	196.1 ± 39.7	191.3 ± 30.4	183.1 ±31.1
Fasting serum glucose, mg/dL (*)	109.8 ± 23.4	131.5 ± 49.9	105.1 ±26.4
Fasting serum insulin (NS)	10.8 ± 7.5	10.6 ± 6.3	11.1 ± 14.3
HOMA score	3.5 ± 6.6	2.4 ± 2.2	3.5 ± 2.3

Microarray comparisons were performed for the group of patients also represented in the Epidemiology of Nonalcoholic Fatty Liver Disease (EPI-NAFL) database. This is a database containing clinical and health data about patients having liver diseases in center for liver diseases at INOVA Fairfax hospital. Liver biopsy and snap-frozen omental adipose tissue samples were obtained at the time of surgery. In addition, adipose samples from the non-obese kidney donors and liver specimens from patients undergoing liver resections (liver mass without liver disease) were used as "controls".

Liver biopsies were used for histopathological assessments and classification of NAFL spectrum into groups. These same liver and visceral adipose tissue specimens were also used for gene expression profiling. Three liver and three adipose tissue groups were used in this study. Adipose of obese NASH patients (N=10), adipose of non-Obese Controls (N=9) and adipose of Obese Controls (N=17). Liver biopsies of obese patients with NASH (N=27), liver biopsies of non-Obese Controls (N=6) and liver biopsies of Obese Controls (N=7). The mean and standard deviation of main clinical and demographic data for patients of hepatic tissues and omental adipose tissues are summarized in Tables 2 and 3 respectivelly.

Table 2. Clinical and demographic data of the patients with hepatic tissue available for gene expression profiling showing the Mean ± STD. Clinical parameters that have statistically significant differences between Obese NASH and Obese Controls = none. The only clinical parameter that have statistically significant differences between Obese Controls and Non-Obese Controls: BMI (*) (adjusted p-value = 0.0004).

Descriptive Parameter	Obese NASH (N=27)	Obese Controls (N=7)	Non-Obese Controls (N=6)
Age	39.5±9.3	40.5±14.7	45.2±17.8
Waist (cm)	138.04±25.14	127.14±28.96	NA
Body mass index (BMI)	50.3±9.0	42.3±4.1 (*)	24.4±4.2 (*)
ALT, μ/L	37.4±30.2	26.0±16.3	27.0±15.1
AST, μ/L	32.5±26.9	23.9±7.6	27.4±11.1
ALT/AST	1.2±0.4	1.0±0.4	1.1±0.4
Fasting Serum Glucose, mg/dl	107.7±24.28	92.4±33.4	107.0±20.6
insulin, units	12.3±7.6	7.3±2.2	NA
HOMA	3.7±2.7	1.8±0.7	NA
Fasting Serum Cholesterol, mg/dL	200.6±35.9	191.3±42.9	NA
Fasting Serum Triglyceride, mg/dL	179.4±70.8	125.2±79.1	NA
Female (%)	74%	100%	50%
Caucasian (%)	70%	71%	100%
Type II Diabetes (%)	15%	0	17%
Hyperlipidemia (%)	29%	43%	NA

Table 3. Clinical and demographic data of the patients that underwent adipose tissue sampling. Statistically significant differences between Obese NASH and Obese Controls: ALT (*), (adjusted p-value = 0.003), AST (**) (adjusted p-value =.006), glucose (#) (adjusted p-value = 0.043) and triglycerides (##) (adjusted p-value = 0.007). Statistically significant differences between Obese Controls and Non-Obese Controls: HOMA scores (@) (adjusted p-value = 0.001), GLU (&) (adjusted p-value = 0.004), INS (&&) (adjusted p-value = 0.001), waist (^) (adjusted p-value = 0.021), BMI (^^) (adjusted p-value = 0.001).

Descriptive Parameter	Obese NASH (N=10)	Obese Controls (N=17)	Non-Obese Controls (N=9)
Age	46.6±12.8	41.6±11.00	33.2±6.7
Waist (cm)	139.6±13.8 (^)	137.3±19.1	89.1±4.2 (^)
Body mass index (BMI)	47.1±4.1 (^^)	46.8±8.9	24.0±4.0 (^^)
ALT, µ/L	65.22±36.3 (*)	19.7±6.1(*)	45.1±27.4
AST, µ/L	53.7±40.1(**)	19.5±4.5(**)	28.4±10.9
ALT/AST	1.4±0.3	1.0±0.3	1.5±0.4
Fasting Serum Glucose, mg/dl	130.11±37.5 (#) (&)	98.2±22.9 (#)	83.2±7.0 (&)
Insulin, units	14.35±7.13 (&&)	15.52±20.12	5.36 ± 3.23 (&&)
HOMA	4.31±2.0 (@)	5.42±9.6	1.4±0.8 (@)
Fasting Serum Cholesterol, mg/dL	192.7±34.4	182.6±32.4	NA
Fasting Serum Triglyceride, mg/dL	230.8±115.1 (##)	115.1±52.4 (##)	NA
Female (%)	20%	65%	33%
Caucasian (%)	90%	71%	56%
Type II Diabetes (%)	30%	18%	0
Hyperlipidemia (%)	30%	18%	0

In addition to the two patients cohorts described above we quantified adipocytokines in serum samples of patients infected with Hepatitis C and assessed for a presence of liver steatosis. For this purpose, serum samples were collected from patients diagnosed with HCV infection of different genotypes, at the Center for Liver Diseases of Inova Fairfax Hospital. The groups of patients selected for this study were screened to exclude those with chronic inflammatory disorders. Blood samples were collected using red top (no anticoagulant) vacutainers, centrifuged at 3000 RPM/min for 5 min and serum

aliquoted and stored at -80 °C. Serum samples were collected from five groups of patients: Patients infected with Hepatitis C genotype 3 and steatosis (N=9), HCV genotype 3 and no steatosis (N=8), HCV genotype 1 and steatosis (N=37), HCV genotype 1 and no steatosis (N=43) and Patients diagnosed with HBV only as controls (N=20). In addition to these five groups, the same group of patients that have only steatosis in part A of this research was used for comparative analysis of serum adipocytokines between groups. Clinical data about patients in each of these groups were assessed at Inova Fairfax Hospital (Table 4).

Table 4.Clinical and demographic data for the HCV patients groups showing the Mean ±STD. TRIG: Triglycerides, TCHOL: Total cholesterol, GLU: Glucose in serum.

Group	**AGE**	**BMI**	**ALT (U/L)**	**AST (U/L)**	**TRIG (mg/dl)**	**TCHOL (mg/dl)**	**Viral load (thousands of copies)**	**GLU (mg/dl)**
HBV	60±5	26±5	74±133	49±83	NA	NA	349±853	100±23
HCV3	43±9	26±5	81±62	77±55	90±77	221±50	70±2708	124±60
HCV1	45±6	29±6	66±44	63±53	135±85	183±32	2036±2566	111±60
HCV3 (SS)	39±9	25±3	88±72	76±54	NA	NA	1327±428	129±60
HCV3 (No SS)	46±8	27±6	75±51	73±57	66±36	185±71	2026±3086	114±61
HCV1 (SS)	45±7	30±7	63±35	66±63	131±81	187±30	2006±2592	99±33
HCV1 (No SS)	44±6	29±5	67±50	61±44	135±69	179±23	2059±7037	120±75

Pathological Assessments

This research study was done as a collaborative project with the Centers for Liver Diseases and Translational Research Institute at INOVA Faifax Hospital.

Histopathological studies for assessment and classification of various spectrums of NAFLD were carried out by INOVA Faifax hospital, department of Pathology as part of previous study approved by INOVA Faifax hospital and George Maso University. Samples and all patient information were de-identified in compliance with Health Insurance Portability and Accountability Act (HIPAA) regulations and INOVA Fairfax Hospital institution review board (IRB) guidelines. The general procedure for the pathological assessment is summarized as follow: Each liver biopsy was fixed in formalin, routinely processed for histology, sectioned, and stained with hematoxylin-eosin and Masson trichrome. All biopsies were evaluated by a single hepatopathologist (ZG) at INOVA Faifax Hospital. The degree of steatosis was assessed in the hematoxylin-eosin-stained sections and 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 hematoxylin-eosin sections included portal inflammation, lymphoplasmacytic lobular inflammation, polymorphonuclear lobular inflammation, Kupffer cell hypertrophy, apoptotic bodies, focal parenchymal necrosis, glycogen nuclei, hepatocellular ballooning, and Mallory bodies.

These histological features were graded as follows: 0 - none; 1 - mild or few; 2 - moderate; and 3 - marked or many. Fibrosis was assessed with the Masson trichrome stain. This stain used to differentiate collagen fibers from other type of tissues by staining it intense green. Portal fibrosis and interlobular pericellular fibrosis were graded as follows: 0 - none; 1 - mild; 2 - moderate; and 3 - marked. When present, bridging fibrosis was noted as few or many bridges, and cirrhosis was identified when parenchymal

nodules surrounded by fibrous tissue were noted. Cirrhosis was further categorized as incomplete or established, depending on the degree of loss of acinar architecture. Each liver biopsy was assigned to one of four diagnostic categories: 1) no fatty liver disease present, 2) steatosis alone, 3) steatosis with nonspecific inflammation, or 4) NASH. NAFLD was defined as a biopsy showing changes consistent with diagnostic categories 2 to 4. Patients were defined as having steatosis alone if they had any degree of hepatocellular fat accumulation as their sole pathology. Patients with steatosis and nonspecific inflammation had, in addition to fat, spotty hepatocellular dropout with focal inflammation or Kupffer cell hypertrophy. NASH was identified when in addition to fat, lobular and hepatocellular inflammation was identified on the hematoxylin-eosin stain. The presence of least one unequivocal Mallory body and some degree of zone three pericellular fibrosis or bridging fibrosis on the Mason trichrome stain was also consistent with the diagnosis of NASH (Ong et. al., 2005). For the purpose of analysis, fibrosis was categorized as follows: 1 = no evidence of fibrosis; 2 = minimal fibrosis (grade 1) either pericellular or portal; 3 = moderate fibrosis (grade 2); and 4 = marked fibrosis (stage 3 or 4).

Research Compliance and Cooperation

The serum and tissue samples used in this research and protocol have been previously used for another study that also have been approved by INOVA Fairfax Hospital's IRB and were stored in -80 °C freezer. These same serum samples, tissues and corresponding clinical data have been de-identified in compliance with HIPAA regulations and INOVA Fairfax Hospital's IRB guidlines. This research project on these

samples has been done in cooperation and support of both Center for Liver Diseases at INOVA Fairfax Hospital/Translational Research Institute and Center for the Study of Genomics in Liver Diseases at George Mason University. This research and protocol using these samples has been granted a certificate of exemptions with the name of investigators from both INOVA Fairfax Hospital IRB (IRB number: 06.065) under the requirement of 45-CFTR-46 101 and from Human Subjects Research Review at George Mason University (Protocol NO.: 4925) under the same requirement of 45-CFTR-46 101. This research study was reviewed and approved by the institutional review board of INOVA Fairfax Hospital and of George Mason University.

Additionally, the resulting data were coded and de-identified during the samples processing and testing, and the access to these data is limited only to the investigators at INOVA Fairfax Hospital and/or George Mason University-INOVA Health System Translational Research Centers.

Methods and Procedures

1. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a powerful tool for measurement of many molecules in serum and any body fluids or solutions and so it has been applied in many research protocols, diagnostic and clinical settings in medicine. Its main principle is using an antigen- antibody reaction which generally involves linking a specific antibody or antigen to a 96-well microtiter plate. The main components of an ELISA assay are an antibody specific to the antigen or any molecule under study, a second labeled antibody and a substrate solution.

The main characteristics of this technique are high specificity for using antibody and sensitivity for using enzyme substrate reaction. Other advantages of using enzyme immunoassays are the cost effective and highly efficient diagnostic technology uses small volume samples and it could be used for quantitative or qualitative purposes. Moreover, ELISA technology is safer and more practical compared to Radioimmunoassay (Porstmann & Kiessig, 1992).

Adipocytokines Measurements

Serum levels of adipokines and cytokines were measured with commercially available Enzyme Immunoassays (EIA) according to the manufacturer's instructions. Each measurement was performed in duplicate with controls and standards. Serum levels of TNF-α, IL-6, IL-1B, sIL-6r, and IL-8 were quantified by using Compact ELISA kits from RDI Division of Fitzgerald Industries Intl (Concord, MA, USA). Pelikine tool set reagent and buffer kits were used for each of these ELISA kits. The serum levels of Resistin, Adiponectin and Leptin were quantified by using kits provided by BioVendor Laboratory Medicine, Inc. (Candler, NC, USA). The serum Visfatin was measured with competitive ELISA assays from Phoenix Pharmaceuticals, Inc (Belmont, CA, USA). Finally, serum levels of IL-1ra were measured by kits provided by Biosource (Nivelles, Belgium).

To identify and quantify cell death mode in each group of samples representative for NAFLD spectrum we measured apoptosis and necrosis by measuring cytokeratin CK18 (M65 antigen, a measurement of overall cell death due to both apoptosis and necrosis) and caspase-cleaved CK18 (M30 antigen, a specific measurement of apoptosis)

(Leers MP et al., 1999; Ueno T. et al., 2005). For this purpose, we used M30-Apoptosense ELISA kit and the M65 ELISA kit from AXXORA (San Diego, CA) to measure levels of CK18-Asp396 neo-epitope as a marker for apoptosis and total soluble CK18 as a marker for both apoptosis and necrosis, respectively. All procedures and measurements were used according to manufacturers' instruction manuals.

Insulin serum levels were measured in serum samples by using human insulin ELISA Kit from LINCO Research (Missouri, USA) according to the instruction manual. This is a Sandwich ELISA based immunoassay that is very sensitive, reproducible and most importantly very specific to human insulin with no cross reactivity to either human proinsulin or its intermediates.

Homeostasis model assessment (HOMA) scores, a measure of insulin resistance, were calculated for all subjects from both serum fasting glucose and insulin levels of patients using HOMA calculator software, version 2.2 (http://www.dtu.ox.ac.uk/).

The calibration curves were constructed by plotting the net average absorbances of the standards on the Y-axis and the concentrations on the X-axis using the recommended data reduction method by the manufacturers' instruction manuals to linearize and draw the best fitting curves. The absorbances of each sample for the adipocytokines were measured using a BioTeck ELx800 plate reader and concentrations were calculated from the calibration curves using Gen5 software (Winooski, Vermont, USA). The correlation coefficients were linear in a concentration range between 1 and 700 pg/mL for TNF-α (r=0.973); 2 and 300 pg/mL for IL-8 (r=0.989); 1.5-400 pg/ml for IL-6 (r=0.968); 1.82-49 ng/ml for Visfatin (r=0.977); 1.5-50 ng/ml for Resistin (r=0.969);

0.3-100 μg/ml for Adiponectin (r=0.975); 0-50 ng/ml for Leptin (r=0.9775 and 2-200 μu/ml for insulin (r=0.982). In addition, linearity was 0.4-300 pg/ml for IL-1B (r=0.965), 10-200 pg/ml for sIL-6r (r=0.989), 4-2000 pg/ml for IL-1ra (r=0.998), 25-1000 μ/L for CK18 Asp396 (r=0.995) and 184-5000 μ/L for total CK 18 (r=0.995)), 0.4-300 pg/ml for IL-1B (r=0.965), 4-2000 pg/ml for IL-1ra (r=0.998), 25-1000 μ/L for CK18 Asp396 (r=0.995) and 184-5000 μ/L for total CK 18 (r=0.995). Samples with higher concentrations of analytes were quantified after dilutions.

A-ELISA Principle and Procedure for Cytokines: TNF-α, IL-8, IL-1B, sIL-6r and IL-6

The Pelikine human TNF-α, IL-8, IL-1B, sIL-6r and IL-6 ELISA kits have the same principle. It is a "sandwich-type" of enzyme immunoassay in which a monoclonal anti-human TNF-α, IL-8, IL-1B, sIL-6r or IL-6 antibody is bound onto polystyrene microplate wells. Human TNF-α, IL-8, IL-1B, sIL-6r or IL-6 present in a measured volume of sample or standard is captured by the antibody on the microplate plate, and non-bound material is removed by washing. Subsequently, a biotinylated polyclonal antibody to human TNF-α, IL-8, IL-1B, sIL-6r or IL-6 is added.This antibody binds to the TNF-α, IL-8, IL-1B, sIL-6r or IL-6 antibody complex present in the microplate well. Excess biotinylated antibody is removed by washing, followed by addition of horseradish peroxidase (HRP) conjugated streptavidin, which binds onto the biotinylated side of the TNF-α, IL-8, IL-1B, sIL-6r or IL-6 sandwich. After removal of non-bound HRP conjugate by washing, a substrate solution is added to the wells. A colored product is formed in proportion to the amount of TNF-α, IL-8, IL-1B, sIL-6r or IL-6 present in the

sample or standard. After the reaction has been terminated by the addition of a stop solution, absorbance is measured in a microplate plate reader. From the absorbance of samples and those of a standard curve, the concentration of TNF-α, IL-8, IL-1B, sIL-6r or IL-6 can be determined by interpolation with the standard curve.

All TNF-α, IL-8, IL-1B, sIL-6r and IL-6 ELISA kit contents were stored at 2-8'C except streptavidine-HRP conjugate separately below -18'C according to instructions.

Protocol

At Day 0: the coating antibody was brought to room temperature (18-25 °C). Coating antibodies for TNF-α, IL-8 and IL-6 were diluted 1:100 in coating buffer, then 100 ml were added to all wells, plate(s) were covered and incubated overnight at room temperature.

At Day 1: all reagents were brought to room temperature, with the exception of streptavidin-HRP, the plate(s) were washed five times with PBS, and then all wells blocked by 200 ml blocking buffer provided by the kit and incubated for one hour at room temperature. Standard, controls and samples were diluted according to instruction with dilution buffer provided by the kit.

100 ml of diluted standard, controls and sample were added to the appropriate wells except the blank and incubated for one hour at room temperature with shaking at 300 RPM.

After plate(s) were washed five times with washing buffer, dilute biotinylated antibody 1:100 was added to all wells except the blank, and incubated again for one hour at room temperature.

Again, plate(s) were washed five times with washing buffer and 100 ml of the diluted (1:10,000) streptavidin-HRP conjugate were added to each well except blank and incubated for 30 minutes at room temperature.

Plates washed again five times with washing buffer, 100 ml substrate solution added to all wells including the blank wells, and incubated for 30 minutes at room temperature in the dark. Finally, 100 ml stop solution was added to all wells and plates were read at 450 nm.

IL-1ra Protocol

Human IL-1ra in serum, standards, and quality controls reacts with captured monoclonal antibody coated on microtiter plate and with second biotinylated monoclonal antibody forming a sandwich. After incubation and washing, streptavidin-peroxidase is added which will bind to the biotinylated antibody. After incubation, the unbound enzyme is removed by washing and substrate solution is added. The reaction stopped with the addition of acid, and then the plate absorbance was read at 450.

In summary, 100 μL of standards, specimens and controls were added into appropriate. 50 μL antigen-specific Biotin conjugate were added to each well. Plates were incubated at room temperature for 2 hours, washed 5 times with with Working Wash Buffer provided by the kit and 100 μL streptavidin-HRP working conjugate were added to each well. After Incubation for 60 minutes at room temperature, plates were washed again 4 times with working wash buffer. 100 μL of substrate added and incubated at room temperature for 30 minutes in the dark. Finally 100 μL of stop solution were added to each well and plates read at 450 absorbance.

B-ELISA Principle and Procedure for Major Adipokines.

Resistin

Human Resistin ELISA, calibrators or samples are incubated with a goat polyclonal anti-human Resistin antibody coated in microtiter wells. After one-hour incubation and a washing, biotin-labelled polyclonal anti-human Resistin antibody is added and incubated with captured Resistin. After a thorough wash, streptavidin-horseradish peroxidase conjugate is added. After one hour incubation and the last washing step, the remaining conjugate is allowed to react with the substrate H2O2-tetramethylbenzidine (TMB). The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance is proportional to the concentration of Resistin. A standard curve is constructed by plotting absorbance values versus Resistin concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

Adiponectin

Human Adiponectin ELISA, standards, quality control and samples of sera (or plasma) are incubated in microtitration wells coated with recombinant human Adiponectin together with horse radish peroxidase-labelled anti-Adiponectin antibody (conjugate). After a thorough wash, the conjugate bound to the Adiponectin coated wells is allowed to react with the hydrogen peroxide-TMB substrate solution. The reaction is stopped by addition of sulfuric acid solution and absorbance of the resulting yellow color product is measured spectrophotometrically at 450 nm. The absorbance is inversely proportional to the Adiponectin concentration. A standard curve is constructed by

plotting absorbance values versus concentrations of serum Adiponectin standards, and the concentrations of unknown samples are determined (μg/ml) using this standard curve.

Leptin

In the BioVendor's Human Leptin ELISA, standards, quality controls and samples of sera are incubated in microtitration wells coated with anti-human Leptin antibody. After a thorough wash, anti-human Leptin antibody labelled with horseradish peroxidase (HRP) is added to the wells and incubated with the immobilized antibody-Leptin complex. Following another washing step, the remaining HRP-conjugated antibody is allowed to react with the substrate tetramethylbenzidine. The reaction is stopped by addition of acidic solution, and absorbance of the resulting yellow product is measured spectrophotometrically at 450 nm. The absorbance is proportional to the concentration of Leptin. A standard curve is constructed by plotting absorbance values versus Leptin concentrations of standards, and concentrations of unknown samples are determined using this standard curve.

2. Biochemical Analysis

Glucose levels were measured by Glucose Oxidase-based kits (product code GAGO-20) from (Sigma-Aldrich, Missouri, USA) according to manufacturer's protocol. This enzymatic method is very sensitive, specific, reproducible and so ideal for analytical and quantitative purposes of glucose in any fluid.

3. Gene Expression Analysis

Gene expression experiments were completed in the course of previous studies performed ar the lab (Younossi et al., 2005, Baranova et al., 2005). The experimental

details of these customized cDNA microarray profiling experiments, microarray data processing and Quantitative Real Time PCR verification have previously been reported (Younossi et al., 2005). The two sets of microarrays had a total of 5,297 genes in common, which became the basis of this study. Resulting datasets were used for this analysis that was a superposition and comparison of two independently performed gene expression studies focusing on 5297 genes which were present on both expression microchips (Younossi et al., 2005, Baranova et al., 2005). Using both sets of gene expression data, we employed indirect comparisons of the expression levels as previously described (Baranova et al., 2005, 2006). In this approach, each group of patients was compared to its own group of non-obese controls, and the results of these comparisons were cross-compared. Four comparisons of this kind were executed, including two adipose-specific and two liver-specific comparisons: adipose of obese NASH patients (N=10) vs. adipose of non-Obese Controls (N=9) (Appendix Table 2), adipose of Obese Controls (N=17) vs. adipose of non-Obese Controls (N=9) (Appendix Table 3), liver biopsies of obese patients with NASH (N=27) vs. liver biopsies of non-Obese Controls (N=6) (Appendix Table 4), liver biopsies of Obese Controls N=7) vs. liver biopsies of non-Obese Controls (N=6) (Appendix Table 5).

Data Analysis

We performed pairwise comparisons between various groups of patients for the serum concentrations of insulin, glucose, Visfatin, TNF-α, Resistin, Adiponectin, IL-8, and IL-6 and other adipocytokines using non-parametric Mann-Whitney test. One-way ANOVA test or the Kruskal-Wallis test has been used to compare three or more groups.

We also performed linear regression analysis using S-Plus v. 7.0. from Insightful Corporation (Seattle, Washington). The sensitivity, specificity, positive predictive values, negative predictive values and confidence intervals (CIs) were assessed using receiver operating characteristic (ROC) curve analysis using MedCalc demo (http://www.medcalc.be/). The associations between the concentration levels for pairs of adipokines and cytokines of interest were tested with use of Pearson correlation coefficients after appropriate log-normalizations of concentration values. Additionally, multivariate linear regressions with stepwise variable selection were used to test for significant relations in continuous data with adjustment for possible confounders (Hastie et al., 2003). Categorical data were subjected to univariate and multivariate ordered probit regression analysis. Unless otherwise noted, we used 2-tailed hypothesis tests and $P<0.05$ was considered significant. A complete list of the descriptive parameters and P values associated with each comparison can be found in Appendix table 6.

For Adipocytokines in HCV groups, data analysis was done in a similar way to adipocytokines in NAFLD spectrum. Mainly, this included descriptive statistics and groups comparisons using Mann-Whitney ranksum test. P-values ≤ 0.01 were considered significant except other wise noted.

Gene expression levels across groups were assessed by computing the ratios of average group expression levels. Genes exhibiting a difference of at least 2-fold across groups were identified, and Mann-Whitney ranksum tests were performed on them to determine statistical significance. A multiple testing correction was performed to control for the false discovery rate, which is the expected proportion of false positives in the

rejected hypotheses (Benjamini & Hochberg, 1995). Genes with significant differences between groups were identified and examined for their role in specific molecular pathways using IPA 4.0 software. This software uses the Ingenuity Pathways Knowledge Base (IPKB), a curated database of biological networks consisting of millions of individually modeled peer-reviewed pathway relationships. A more detailed description of this database and IPA 4.0 software can be found at the website (http://www.ingenuity.com) (Mayburd et al., 2003; Sharma et al., 2006).

CHAPTER 6.

Results and Discussion

NAFLD, one of complications of metabolic syndrome, is on the rise in parallel with a global epidemic of obesity. The pathological spectrum of NAFLD ranges from simple steatosis to NASH to fibrosis and cirrhosis. Obesity and insulin resistance could be preliminary stages of NAFLD. In addition, HCV infection has been coincidently observed in many cases by steatosis, a hallmark feature seen in NAFLD. Factors defined by HCV genotype and host metabolic factors could both be the direct causes for statosis. Scientists believe that adipocytokines have role in the pathogenesis of steatosis, as it characterized by imbalance in production of many adipocytokines. In addition, adipose enlargement and hepatic infiltration with fat causes gene expression changes on different levels affecting the underlying pathological symptoms of NAFLD and obesity.

The impact of NAFLD relates with its prevalence and potential for progression. It is critical to differentiate between different stages of NAFLD because of its differential risk for progression and management. The status of adipocytokines in different stages of NAFLD progression and its interaction with insulin resistance has not been investigated in detail. In addition, the difference in gene expression between omental adipose and hepatic tissues has not been investigated.

We hypothesized that adipocytokines' production levels could be correlated with the progression of NAFLD, with the degree of insulin resistance and, therefore, could be used to non-invasively diagnose these diseases and as well to diagnose steatosis in HCV infection subjects. In addition we hypothesized that changes in gene expression could shed more light on the role of the adipokinome in NAFLD pathogenesis.

To that end, we performed comparative profiling of the serum levels of a number of adipocytokines in the matched groups of subjects representative of various types of NAFLD progression and of different degrees of insulin resistance measured by HOMA scores.

Serum Adipocytokines in NAFLD Spectrum

In this study we measured the fasting serum levels of insulin, glucose, Visfatin, Resistin, Adiponectin, TNF-α, IL-8 and IL-6 on four groups of patients: 1) NASH (N = 26); 2) Simple Steatosis (SS, N=19); 3) Morbidly Obese Controls without NAFLD (Control I, N=38); and 4) Non-Obese Controls who were healthy blood donors (Control II, N =12). The NASH and obese-control groups were further subdivided into high HOMA (HOMA > 3.0), low HOMA (HOMA < 1.8) and mid HOMA (3 > HOMA > 1.8). The NASH group contained 12 high HOMA samples, 4 mid HOMA samples and 7 low HOMA samples; the obese-control group contained 8 high HOMA samples, 11 mid HOMA samples and 17 low HOMA samples.

In addition, total CK18 (M65, a measurement of overall cell death due to both apoptosis and necrosis) and caspase-cleaved CK18 (M30, a specific measurement of

apoptosis) were measured on a cohort of 69 patients of three groups (22 patients with NASH, 15 patients with simple steatosis and 32 morbidly obese controls). All measurments were done from 200 µl aliquots of serum samples that have been stored at -81°C after thawing to room temperature for analysis. Clinical and demographic data are summarized in Table 1.

Adiponectin

As previous researchers reported, our results also confirmed that by groupwise comparisons, NASH patients have a significantly ($P < 0.001$) lower Adiponectin level than those with SS and the obese controls (NASH 6.7± 6.5 µg/ml, SS 12.2±7.56 µg/ml, obese control (Control I) (10.2± 7.4 µg/ml). In addition, we also showed that HOMA scores negatively correlated with serum Adiponectin levels in all NAFLD patients ($R=-0.44$, $p < 0.0032$) (Figure 5A), but this correlation is weaker in NASH (Figure 5B) and in steatosis (Figure 5C) subgroups separately and not in the Control I cohort. However, stratification of patients based on HOMA scores (High-HOMA vs. Low-HOMA groups) failed to show a significant difference in Adiponectin levels among different groups.

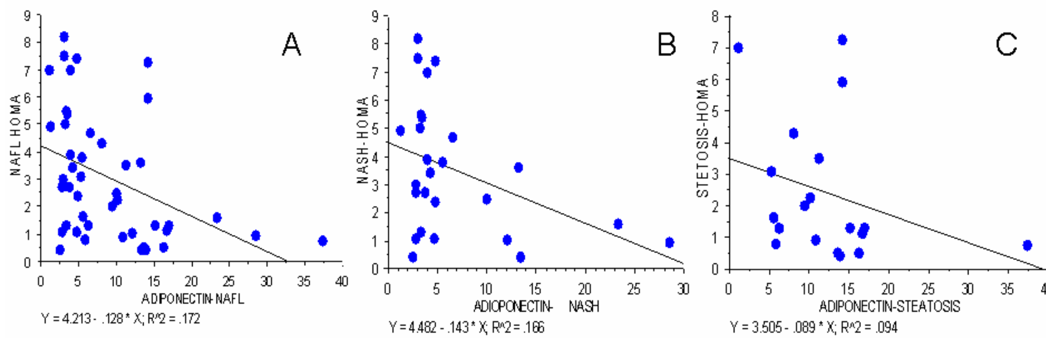

Figure 5. Correlation between HOMA scores and Adiponectin in NAFL, NASH and Steatosis.

Resistin

There were no statistically significant differences in serum Resistin in comparisons of NASH, SS and the obese control (Control I) (Appendix table 6). Additionally, HOMA scores, serum insulin, and serum glucose levels did not correlate with serum Resistin levels in the obese patients regardless of their liver histology. Furthermore, a comparison of High-HOMA vs. Low-HOMA groups did not reveal any significant differences in serum Resistin levels.

Visfatin

Serum Visfatin levels were significantly higher in obese controls compared to the healthy, nonobese controls: Control I: 26.8±15.2 ng/ml vs. Control II: 17.11±6.24 ng/ml, $p<0.05$). Interestingly, Visfatin levels in NASH patients were lower in comparison to patients with SS and the obese controls (NASH: 17.11±6.24 ng/ml, SS: 45.14±60.86 ng/ml, and Control I: 26.8±15.2 ng/ml, $p< 0.05$) which could potentially predispose them to this form of progressive liver disease (Figure 6A).

Correlation analysis of pro-inflammatory cytokine and adipokine levels:

As shown in Figure 6, serum level of TNF-α and IL-8 were significantly higher in NAFLD patients compared to both obese and nonobese controls. Additionally, obese controls showed lower serum levels of TNF-α than non-obese controls (Control I: 1.91±0.25 pg/ml vs. Control II: 2.3±0.4, $P< 0.0001$) (Figure 6B and 6C).

IL-6 profiling revealed statistically significant differences between NAFLD and the obese controls, between NASH and obese controls, and between SS patients and the non-obese controls (Figure 6D). When patients with SS were compared to the obese

controls and to patients with NASH, the differences failed to reach statistical significance (Figure 6D).

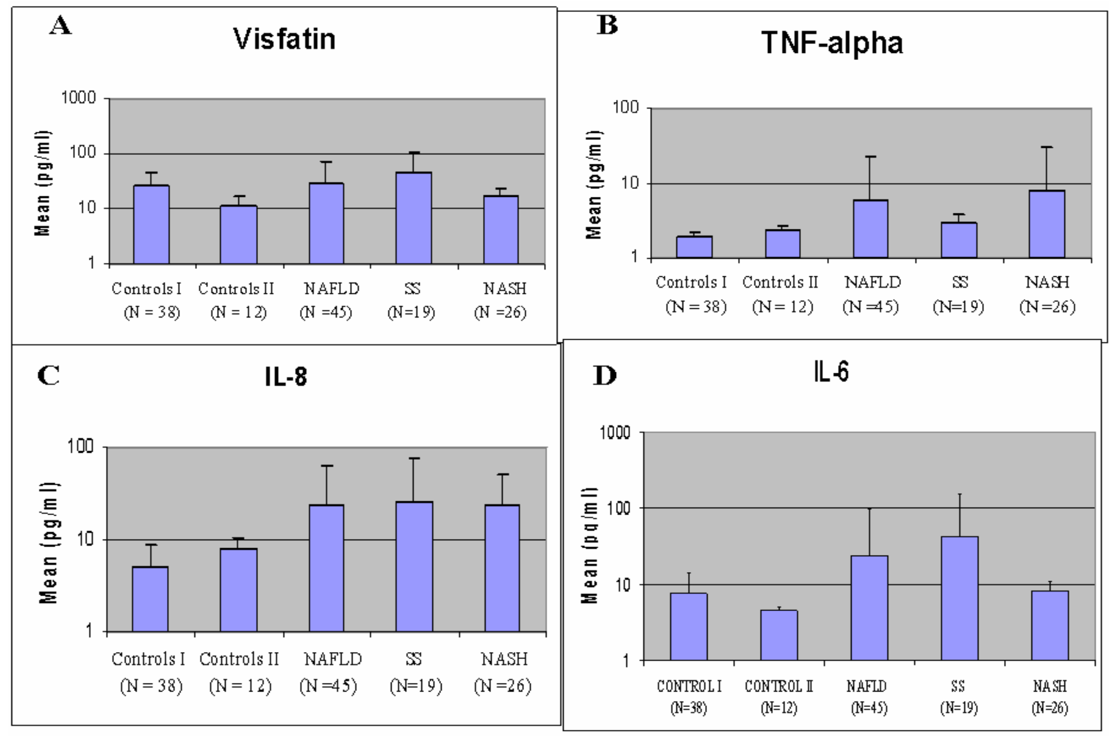

Figure 6. Visfatin and Pro-inflammatory cytokines in the serum of morbidly obese patients with and without NAFLD: A) Visfatin. B) TNF-α. C) IL-8. D) IL-6. Descriptive parameters and P values for every comparison can be seen in Appendix Table 1.

When all the patients profiled in this study were stratified according to their HOMA scores (high-HOMA group vs. low-HOMA group), the differences in TNF-α, IL-8 and IL-6 serum levels between these groups remained significant ($P < 0.01$).

Univariate analysis involving all patients revealed significant correlations between serum concentrations of TNF-α and IL-8 ($R = 0.5276$, $P < 6.319e^{-08}$), as well as between IL-6 and IL-8 ($R = 0.7079$, $P \leq 5.271e^{-15}$). On the other hand, levels of TNF-α

and IL-6 showed no correlation. Interestingly, the association of TNF-α and IL-6 in patients with SS reached statistical significance (R = 0.4583, P < 0.05). Similarly, this correlation was significant when obese controls and SS patients were profiled together (R = 0.4689, P < 0.0003). The multivariate analysis indicated that in NAFLD patients, IL-6 levels mostly depend on HOMA scores (P< 0.0027) and IL-8 levels (P< 0.0006) (Table 5).

Table 5. Best fitting multiple linear regression models showing relationship between IL-6 and other clinical parameters. Regression coefficient β represents Slope estimate ± Standard Error of the estimate (SE).

GROUP	**Independent variable**	**Regression coefficient β and SE**	**P-values of independent variables**	**P-value of the whole model**
	(Intercept)	1.676 ± 0.355	$<10^{-04}$	
Obese Controls	Insulin	-0.981 ±0.527	<0.0724	P < 0.001
	HOMA	1.095 ± 0.451	<0.0213	
	TNF-α, pg/ml	-1.221 ± 0.663	<0.0752	
	(Intercept)	1.859 ± 0.993	<0.0689	
	Glucose	-0.868 ± 0.425	<0.0798	
	HOMA	0.587 ± 0.183	<0.0027	
NAFLD	TNF-α, pg/ml	-0.455 ± 0.241	<0.0665	P < 0.00083
	IL-8, pg/ml	0.630 ± 0.167	<0.0006	
	Adiponectin, mkg/ml	0.267 ± 0.161	<0.1053	
	(Intercept)	0.133 ± 0.144	<0.388	
ALL OBESE PATIENTS PROFILED	HOMA	0.271 ± 0.079	<0.001	$P < 2.325e^{-07}$
	IL-8, pg/ml	0.432 ± 0.100	$<10^{-04}$	
	Resistin, ng/ml	0.283 ± 0.144	<0.053	

Furthermore, in NAFLD patients, TNF-α level depend on serum glucose concentration, HOMA, BMI and IL-8 (P-values < 0.01).

Of special note, when all obese patients were subjected to multiple regression analysis, the concentrations of TNF-α were significantly dependent on IL-8 ($P< 0.0001$) and Resistin levels ($P < 0.01$) (Table 6). On the other hand, in obese controls, and in NAFLD patients, correlations of TNF-α and Resistin serum levels were insignificant but followed opposite trends.

Table 6. Best fitting multiple linear regression models showing relationship between TNF-α and other clinical parameters. Regression coefficient β represents Slope estimate ± Standard Error of the estimate (SE).

GROUP	**Independent variable**	**Regression coefficient β and SE**	**P-values of independent variables**	**P-value of the whole model**
Obese Control	(Intercept)	-3.0775 ± 1.5765	<0.0606	P < 0.01656
	Glucose	1.2510 ± 0.6087	<0.0490	
	Insulin	1.3436 ± 0.6010	<0.0332	
	HOMA	-1.3200 ± 0.6019	<0.0365	
	IL-8, pg/ml	0.1027 ± 0.0446	<0.0286	
	IL-6, pg/ml	-0.0690 ± 0.0391	<0.0877	
NAFLD	(Intercept)	0.4776 ± 0.9365	<0.6131	$P < 7.701e^{-06}$
	Glucose	-1.0073 ± 0.3303	<0.0042	
	Insulin	-0.6064 ± 0.2575	<0.0239	
	HOMA	0.7190 ± 0.2315	<0.0036	
	BMI	1.2745 ± 0.4593	<0.0086	
	IL-6, pg/ml	0.1531 ± 0.0885	$<10^{-4}$	
	IL-8, pg/ml	0.4399 ± 0.0869	<0.0921	
ALL OBESE PATIENTS PROFILED	(Intercept)	0.2119 ± 0.0660	<0.0020	$P < 2.304e^{-011}$
	IL-8, pg/ml	0.3343 ± 0.0421	$<10^{-4}$	
	Resistin, ng/ml	-0.1629 ± 0.0619	<0.0103	

Adipokines and cytokines levels to differentiate NAFLD from Obese Controls:

The serum levels of all the profiled cytokines (TNF-α, IL-6 and IL-8) were significantly higher in NAFLD patients compared to the Obese Controls. On the other hand, Visfatin, Resistin and Adiponectin levels were not different (Table 7). Observed differences remained significant after multiple test adjustment. After taking into account all additional clinical parameters listed in Table 1, no single non-cytokine parameter seemed to be independently associated with NAFLD. According to the multivariate analysis, TNF-α positively correlated with NAFLD ($P < 0.02$), but was interdependent with the levels of the Visfatin.

Table 7. Comparisons of serum adipokines and pro-inflammatory cytokines for patients with NAFLD and Obese Controls. P-value ≤ 0.05 is considered significant.

Adipocytokine	NAFLD (N=45)	Obese Controls (N=38)	p-value	Adjusted p-value
TNF-α pg/ml	6.0±16.6	1.9±0.3	$4.32096e^{-13}$	$2.592576e^{-12}$
IL-8 pg/ml	24.1±38.5	7.8±3.6	$5.55826e^{-06}$	$1.667478e^{-05}$
IL-6 pg/ml	23.1±72.9	7.6±6.3	0.005	0.01
Visfatin pg/ml	28.9±41.6	26.8±19.0	0.40	0.40
Adiponectin µg/ml	9.3±7.5	10.2±7.5	0.18	0.28
Resistin ng/ml	6.8±3.7	7.6±3.8	0.39	0.40

Adipokines and cytokines levels differentiate NASH from Simple Steatosis:

Serum levels of adipocytokines in patients with NASH and Simple Steatosis revealed some interesting differences. Levels of TNF-α were higher in NASH, whereas levels of IL-8, Visfatin, and Adiponectin were lower in NASH patients. However, after

multiple test adjustment of p-values, only the differences in TNF-α and Adiponectin remained significant (both with P-values < 0.003) (Table 8).

Table 8. Comparisons of serum adipokines and pro-inflammatory cytokines for patients with simple steatosis and NASH. P-value ≤ 0.05 is considered significant.

Adipocytokine	**NASH (N=26)**	**Simple Steatosis (N=19)**	**p-value**	**Adjusted p-value**
TNF-α pg/ml	8.2±21.7	2.9±0.9	0.0007	0.002
IL-8 pg/ml	23.1±27.7	25.5±50.2	0.04	0.06
IL-6 pg/ml	8.1±2.9	42.5±109.4	0.71	0.71
Visfatin pg/ml	17.1±6.2	45.1±60.9	0.03	0.06
Adiponectin μg/ml	6.7±6.5	12.2±7.6	0.0005	0.002
Resistin ng/ml	6.0±2.9	7.8±4.3	0.09	0.2

When additional clinical parameters were taken into account (Table 1), a multivariate analysis revealed that age and ALT level positively correlated with histologic NASH (P values < 0.01 and < 0.02, respectively), whereas IL-8 and Adiponectin negatively correlated with histologic NASH (P values < 0.05 and < 0.03, respectively). On the other hand, both TNF-α and HOMA-IR failed to be independently associated with NASH.

Adipokine and cytokine levels differentiate different stages of hepatic fibrosis:

We also assessed the relationships between hepatic fibrosis and serum adipokines and cytokines. A multivariate regression analysis was aimed at probing whether any of the adipokine or cytokine levels predicted the stage of fibrosis. This analysis indicated that only serum TNF-α was an independent predictor of histologic fibrosis in patients

with NASH (P < 0.0004). No other variable tested independently or in combination behaved as reliable predictor of the stage of fibrosis.

Measurements of apoptosis and necrosis:

To assess relative levels of apoptosis and necrosis in NAFLD patients and in morbidly obese controls, three interdependent surrogate end-point measures were employed: a total level of CK18 neoepitope (M30 antigen released in the process of the caspase cleavage and reflecting an apoptosis), the total released amount of CK18 (M65 antigen released from all dying cells and reflecting total cell death including both apoptosis and necrosis) and necrosis-reflecting parameter calculated as (M65)-(M30).

Groupwise comparisons showed that NASH patients have significantly (P-value< 0.02) higher levels of M30 antigen in the serum than those with SS and the obese Controls (NASH 307.0± 278.2 IM/L, SS 127.3±62.2 IM/L, and Obese Controls 137.4± 36.8 IM/L) respectively (Figure 7).

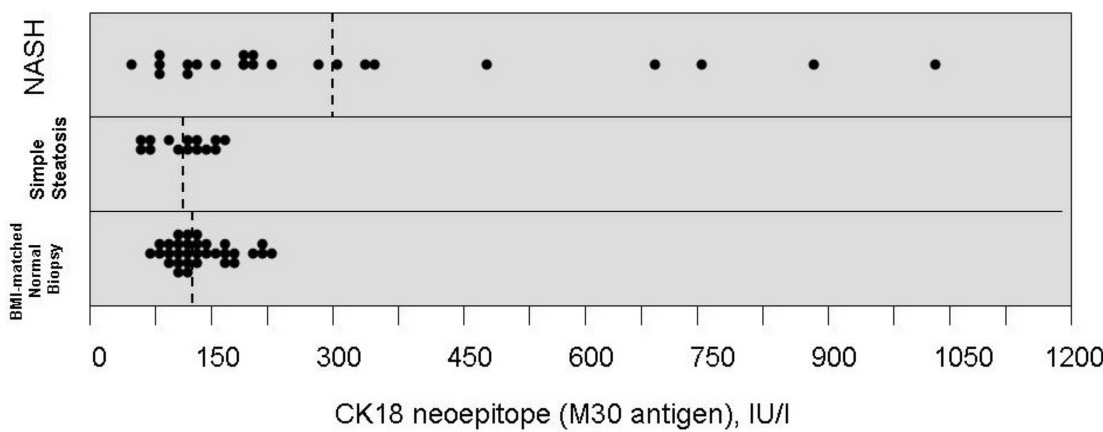

Figure 7. CK-1 neoepitope (M30 antigen) levels are significantly increased in the serum of NASH patients in comparison to patients with Simple Steatosis and BMI-Matched Morbidly Obese Patients with Normal Biopsy. On this scatter plot, each dot represents one subject, and dashed line represents the mean value for each group. IU/l: International Unit/Liter.

Differences in concentrations of M30 antigen between patients with Simple Steatosis and Obese Controls were not significant. In NAFLD patients, but not in Obese Controls, levels of apoptosis closely correlated with HOMA scores ($R = 0.5106$, $p \leq 0.0013$). However, when NAFLD patients were subdivided according to their HOMA scores (High HOMA scores (N=15) vs. Mid- and Low- HOMA scores (N =22)), the differences in the levels of apoptosis were highly significant ($P < 0.0011$).

Quantification of M65 followed the same trend as M30 measures, with significantly higher levels in NASH vs. SS ($P < 0.0032$), but not in SS vs. Obese Controls. In NAFLD patients, correlation of overall cell death (M65) and HOMA scores was significant ($R = 0.51$, $p \leq 0.002$). When NAFLD patients were subdivided according to their HOMA scores as described above, the differences in the levels of total cell death remained significant ($P < 0.00021$).

The parameter most closely reflecting necrotic cell death is calculated as M65-M30. Simple Steatosis patients differ from NASH patients according to this parameter ($P < 0.05$), but not from Obese Controls. Correlation of the levels of the necrosis and HOMA scores was not significant, although remained positive.

Relationship between levels of CK18 antigens and adipocytokines in NAFLD patients:

Systemic apoptosis values as measured by levels of M30 antigen in the serum were closely correlated with TNF-α levels and IL-8 levels in all morbidly obese patients profiled ($R = 0.4986$, $p \leq 1.395e-05$ and $R = 0.3052$, $p \leq 0.0108$, respectively), while in the NAFLD subgroup M30 antigen levels correlated with TNF-α only ($R = 0.4816$, $p \leq$

0.002626). Similarly, measurements of a total cell death revealed positive correlations both with TNF-alpha and IL-8 cytokines in all morbidly obese patients ($R = 0.3277$, $p \leq 0.006007$, and $R = 0.3095$, $p \leq 0.009697$, respectively), but in NAFLD patients correlation remained significant only for TNF-alpha ($R = 0.3049$, $p \leq 0.06669$). There were no significant correlations of levels of CK-18 derived epitopes with any other adipocytokine profiled.

Models Predicting Histologic NASH:

Additional analysis revealed that levels of M30 antigen (cleaved CK18) predicted histologic NASH with 70% sensitivity and 83.7% specificity and AUC = 0.711, $p < 10^{-4}$), while predictive value of the levels of intact CK-18 (M65) was somewhat higher (63.6% sensitivity and 89.4% specificity and AUC = 0.814, $p < < 10^{-4}$). On the other hand, the multivariate analysis revealed that histologic NASH could be reliably predicted by a combination of cleaved CK-18 (apoptosis), a product of the subtraction of cleaved CK-18 level from intact CK-18 level (necrosis), serum Adiponectin and serum Resistin (95.45% sensitivity, 70.21% specificity, AUC = 0.908, $p < 10^{-4}$) (Table 9).

Table 9. Best fitting multiple linear regression models allow distinguishing NASH patients within the cohort of NAFLD and non-NAFLD controls; Regression coefficient β represents slope estimate ± Standard Error of the estimate (SE). P-value ≤ 0.05 is considered significant.

Model	Independent variable	Regression coefficient β and SE	P-values of independent variables	P-value of the whole model
Prediction of histologic NASH	(Intercept)	0.4909 ± 0.1351	<0.0006	$p < 1.232e^{-6}$
	M30, IU/L	0.0011 ± 0.0003	<0.0001	
	M65-M30, IU/L	0.0003 ± 0.0001	<0.0548	
	Adiponectin, μg/ml	-0.0153 ± 0.0069	<0.0316	
	Resistin, ng/ml	-0.0418 ± 0.0125	<0.0014	

Despite its remarkable AUC, the overall significance of the model based on the serum concentrations of TNF-α was much lower than that based on CK-18 measurements. Performance-related characteristics of the models described above are summarized in the Table 10.

Table 10. Performance of the multivariate and univariate models predicting NASH in the cohort the morbidly obese patients. Positive predictive value: +PV, Negative predictive value: -PV, 95% confidence interval: (CI 95% confidence interval. Cut-off values corresponds to the highest accuracy value (minimal false positive and false negative results). (*) Significance of the model based on the serum concentrations of TNF-α is much lower than that based on CK-18 measurements. P-Value ≤ 0.05 is significant.

Model	Cut-off	Sensitivity (95% CI), %	Specificity (95% CI), %	+PV, %	–PV, %	Negative likelihood ratio	AUC (95% CI)	Model p-value
Full model	0.2772	95.45 (77.1-99.2)	70.21 (55.1-82.6)	60.0	97.1	0.065	0.908 (0.814-0.964)	$<10^{-5}$
	0.3499	77.27 (54.6-92.1)	87.23 (74.2-95.1)	73.9	89.1	0.26		
	0.2075	100.00 (84.4-100.0)	46.81 (32.1-61.9)	46.8	100.0	0.00		
TNF-α (univariate) pg/ml (*)	2.915	100.00 (84.4-100.0)	89.36 (76.9-96.4)	81.5	100.0	0.00	0.954 (0.875-0.989)	< 0.05
	3.25	86.36 (65.1-96.9)	91.49 (79.6-97.6)	82.6	93.5	0.15		
	4.245	40.91 (20.7-63.6)	97.87 (88.7-99.6)	90.0	78.0	0.60		
M30 (univariate) IU/L	174.1	63.64 (40.7-82.8)	87.23 (74.2-95.1)	70.0	83.7	0.42	0.711 (0.589-0.814)	$<10^{-4}$
	111.6	81.82 (59.7-94.7)	29.79 (17.4-44.9)	35.3	77.8	0.61		
	261.35	36.36 (17.2-59.3)	97.87 (88.7-99.6)	88.9	76.7	0.65		
M65 (univariate) IU/L	384.3	63.64 (40.7-82.8)	89.36 (76.9-96.4)	73.7	84.0	0.41	0.814 (0.702-0.897)	$<10^{-4}$
	242.9	86.36 (65.1-96.9)	65.96 (50.7-79.1)	54.3	91.2	0.21		
	545	36.36 (17.2-59.3)	95.74 (85.4-99.4)	80.0	76.3	0.66		

Validation of the Combinational Models Predicting Histologic NASH:

An attempt to validate the performance of the combinational NASH diagnostic model, additionally validation cohort of the patients with biopsy-proven NASH or biopsy proven Simple Steatosis (N=32) was randomly selected from the same database. BMI-matched controls were excluded from validation cohort, as serum samples of all patients

enrolled in EPI-NAFLD study and matching description of the cohort 3 were profiled during initial set of measurements. Therefore, model was tested under more rigorous conditions. Histology and the clinical characteristics of the validation cohort were blinded for all investigators. Institutional Review Boards of Inova Fairfax Hospital and George Mason University approved this protocol. Histology and the biochemical measurements were blinded until completion of the analysis.

Cleaved CK-18 (apoptosis), a product of the subtraction of Cleaved CK-18 level from Intact CK-18 level (necrosis), serum Adiponectin and serum Resistin were profiled on this blind cohort of patients. The performance of the combinational panel in the validation was characterized by an AUC of 0.732 and 95% confidence interval (CI) of 0.55 to 0.87. A threshold of 0.3825 was associated with a sensitivity of 71.4%, a specificity of 72.7%, a positive predictive value of 83.3% % and a negative predictive value of 57.1 %. Full list of thresholds for the validation set is given in Table 11.

Table 11. Results of the blinded validation of combinational model predicting NASH. Positive predictive value: PPV, Negative predictive value: NPV, True Positives: TP, True Negatives: TN False Positives: FP, False Negatives: FN. AUC (Area under the Curve) of this model is 0.732 with 95% confidence interval of (0.546 – 0.872). Model was tested on blinded cohort (N=32) of 21 NASH patients and 11 patients with Simple Steatosis.

Threshold	**TP**	**FP**	**TN**	**FN**	**Sensitivity**	**Specificity**	**PPV**	**NPV**
0.2085	19	8	3	2	90.5%	27.3%	70.4%	60.0%
0.2793	19	8	3	2	90.5%	27.3%	70.4%	60.0%
0.2805	19	8	3	2	90.5%	27.3%	70.4%	60.0%
0.3199	17	6	5	4	81.0%	45.5%	73.9%	55.6%
0.3377	16	5	6	5	76.2%	54.5%	76.2%	54.5%
0.3692	15	4	7	5	75.0%	63.6%	78.9%	58.3%
0.3825	15	3	8	6	71.4%	72.7%	83.3%	57.1%
0.5500	11	2	9	10	52.4%	81.8%	84.6%	47.4%

Adjustement of the Combinational Model according to Extended Dataset:

In order to improve performance of the combinational model the training dataset was extended to N = 101 by inclusion of the subjects whose clinical data were uncovered after completion of the validation phase. This extension resulted in a better model ($p < 2.1e-7$) with AUC of 0.854 (CI 95% 0.770 to 0.917) and an increase of the optimal threshold to 0.4320 cutting off NASH cases with sensitivity of 72.1% and specificity of 91.4%.

Discussion

This study provides an in-depth analysis of a large number of cytokines and adipokines in well characterized cohorts of patients with NAFLD. The rationale for this study was to cover the gap in the existing literature describing the relationship between serum adipokine and cytokine levels potentially involved in the development of NAFLD and its progressive form (NASH). The three most important adipokines (Adiponectin, Resistin and Visfatin), and three pro-inflammatory cytokines (TNF-α, IL-6, IL-8) were profiled in well matched cohorts of NAFLD patients and controls with extensive clinical and pathologic data. This study confirms some of previously reported findings as showing that hypoadiponectemia is negatively associated with HOMA (Musso et al., 2005; Vuppalanchi et al.,2005), lower levels of Adiponectin are associated with histologic NASH and the absence of a significant association between NAFLD subtypes and serum Resistin levels (Baranova et al., 2006).

New results reported here show that TNF-α serum levels in SS patients are much higher than in non-obese controls and TNF-α levels increased significantly from SS to

NASH. Interestingly, TNF-α serum levels were lower in obese patients compared to lean controls. These results emphasize the potential role of TNF-α in obese patients with NAFLD. Earlier researchers hypothesized that TNF-α plays a role in the pathogenesis of the diseases related to IR, including NAFLD and NASH (Diehl, 2004). Indeed, elevated TNF-α production has been described in the cultures of peripheral blood cells collected from obese patients with NAFLD (Poniachik et al., 2006). Nevertheless, direct evidence of TNF-α involvement in the earlier stages of NAFLD has not been described previously. The pathogenic role of TNF-α may be to attract inflammatory leukocytes to the liver, or increase SREBP-1c (sterol regulatory element binding protein-1c) dependent on intrahepatic fat deposition (Endo et al., 2007). Our observations corroborate findings by Satapathy and coworkers who treated patients with histologically proven NASH with a TNF-α inhibitor; pentoxifylline (Satapathy et al., 2004). Pentoxifylline treatment resulted in a significant reduction of serum TNF-α levels accompanied by normalization of both ALT and AST, and improvements in the insulin resistance index. Together, these studies support for the potential role of anti-TNF-α in the treatment of NASH.

The role of IL-6 in the pathogenesis of NASH is less clear. A previous study showed that both IL-6 and sIL-6r levels increased significantly in NASH patients compared to patients with simple steatosis and healthy volunteers (Abiru et al., 2006). In our study, the serum levels of IL-6 were higher in obese controls and in patients with SS. In contrast, IL-6 levels were significantly lower in NASH patients in comparison to SS patients (Figure 6). This pattern can be explained by the failure of IL-6 and/or Visfatin-dependent hepatoprotection associated with onset of overt NASH. The protective

function of IL-6 in steatotic livers relies on suppression of ethanol-induced oxidative stress and mitochondrial dysfunction (Sun et al., 2003, El-Assal et al.,2004) and preventing the release of reactive oxygen species (ROS), mitochondrial permeability transition (MPT), and ethanol-mediated depletion of adenosine triphosphate (ATP) (El-Assal et al.,2004). IL-6 also provides hepatoprotection in ischaemic preconditioning models (Teoh et al., 2006) and prevents obesity- and alcohol-associated fatty liver transplant failure in rats (Sun et al., 2003, Gao, 2004).

To check whether IL-6 does indeed not depend on other factors participating in the development of NASH, we performed stepwise multiple regression analysis and found best subsets of variables to fit IL-6 in each of NASH, SS and MOHL (control I) groups separately with an attempt to build best fitting models (Table. 5). In our cohort of NASH patients, IL-6 levels indeed were almost independent of other soluble molecules potentially participating in the development of NASH. On the other hand, in the entire NAFLD cohort, IL-6 also depended on HOMA scores ($P < 0.0027$) and IL-8 ($P < 0.0006$) levels, but not on TNF-α levels.

Visfatin levels in various patient groups resemble IL-6 levels, suggesting that they may be co-regulated. Levels of IL-6 and Visfatin might be correlated because of the presence of both negative and positive feedback loops. For example, Visfatin induces the production of IL-6 in human CD14 (+) monocytes (El-Assal et al., 2004), whereas IL-6 negatively regulates Visfatin gene expression in 3T3-L1 adipocytes (Teoh et al., 2006). Visfatin serum levels were significantly higher in each of the subgroup of obese patients when these groups were compared to non-obese controls (Control II). On the other hand,

Visfatin levels were lower in NASH patients than in Obese Controls (Control I) and in patients with SS. To our knowledge, this is the first clinical observation pointing to the potential involvement of Visfatin in the pathogenesis of NAFLD.

Our analysis also focused on the use of adipocytokines to differentiate NAFLD from appropriately matched controls. Our data suggests that only TNF-α was independently associated with NAFLD. On the other hand, in comparison to SS, NASH was independently associated with age, ALT, Adiponectin, and IL-8. These important observations suggest a role for TNF-α in promoting NAFLD. Furthermore, these low levels of Adiponectin and IL-8 in NASH may contribute to the development of NASH.

In addition, we also analyzed the association between hepatic fibrosis and serum adipokine-cytokine levels. This analysis revealed that TNF-α is the only factor involved in the stage of hepatic fibrosis in NASH ($P < 0.0004$). These findings are in agreement with observations collected on the mouse model of NASH (TNFRDKO), which is deficient in TNF receptors 1 (TNFR1) and 2 (TNFR2). TNFRDKO and wild-type mice were fed a methionine and choline deficient (MCD) diet (Tomita et al., 2006). Despite the MCD diet, TNFRDKO mice showed impairments in both the activation of hepatic stellate cells and mRNA expression of tissue inhibitor of metalloproteinase 1 (TIMP-1), leading to a less fibrogenic liver phenotype (Tomita et al., 2006). Further analysis of the TNF-α related fibrogenesis in the human NASH livers is needed.

The role of cell death in the development of NASH has been previously suggested (Ramalho RM et al., 2006; Younossi ZM et al., 2005; Ribeiro PS. et al, 2004). Hepatic gene expression data (Younossi ZM et al., 2005), assessment of apoptosis-related

molecules in the liver biopsies (Ramalho RM et al., 2006) and data from animal models (Diehl AM et al., 2005) have all provided evidence to support this role. Recently, two cytokeratin 18–derived antigens, M30 and M65, released in the patient's serum in the process of the cellular death, were highlighted as possible non-invasive biomarkers of NASH (Yilmaz U. et al., 2007; Wieckowska A. et al., 2006). However, due to complexity of the pathogenesis of NASH, it is likely that other pathways including cytokines and adipokines play critical roles in the development of NASH. Therefore, a set of biomarkers that include both markers of apoptosis and adipocytokines may provide a better mean to discriminate NASH from other, less progressive, types of NAFLD. Such panel of biomarkers can reduce the need for liver biopsy in the management of patients with NAFLD, reducing both biopsy-related costs and its associated risks.

For such purposes, we compared the predictive values of cytokeratin 18-based serum markers as well as a number of important cytokines and adipokines (Garcia Glaiano D et al., 2007; Yalniz M. et al., 2006; Baranova et al., 2006) in patients with different spectrum of NAFLD. M30 was able to predict histologic NASH with a sensitivity of 63.6% and specificity of 87.2% (AUC = 0.710), confirming previous observations (Yilmaz U. et al., 2007; Wieckowska A. et al., 2006). Additionally, analysis of correlations revealed that the levels of this apoptosis-related marker closely correlated with levels of pro-inflammatory cytokines (TNF-α and IL-8), but not with levels of any of the adipokines.

Predictive model based on concentrations of M30 antigen out performed the model based only on the level of TNF-α. Interestingly, multivariate logistic regression

analyses of the complete set of non-invasive tests demonstrated relative importance of adipokine levels and significantly improved the NASH predictive power of the model improving both its sensitivity and AUC, while adding overall confidence in the model performance up to $P < 1.232e^{-6}$. It is important to note that having cleaved CK18 fragment M30 as a major component of the best performing model allowed us to eliminate the need in quantification of TNF-α and IL-8 in patient's serum, and, thus, reduce the number of measurements. Levels of apoptosis were also found to be closely correlated with HOMA scores, thus, confirming an importance of insulin resistance in the progression of NAFLD (Chitturi S. et al., 2002).

Data reflecting the total amount of cell death (M65) followed the same trend as the results of the quantification of apoptosis (M30). This is not surprising, as M30 antigen represents a fragment that is located within the larger molecule of cytokeratin 18 that can be released both in apoptotic and in the necrotic processes. According to our observations, the levels of necrosis (M65-M30) itself in the cohort of morbidly obese patients did not significantly correlate with histologic NASH, but serves as a valuable component in the full multiparametric model.

There are some limitations influencing the outcome of this study. One possible limitation of the use of M30 neoantigen for the prediction of NASH is its intrinsic inability to discriminate between NASH and other chronic diseases that involve apoptosis, for example, cholangitis and cholestasis (Yagmur E. et al., 2007), chronic infection with Hepatitis C (Bantel H. et al., 2004) and various malignancies (De Bruin EC et al., 2006; Olofsson et al., 2007). In addition, these levels may be influenced by trauma (Roth GA. et

al., 2004). Our study indicates that this limitation may be removed by use of the relatively simple mini-panel that will include both M30 antigen and measurements of serum adipokines. Such a panel of biomarkers could become very useful in the clinical management of patients with NAFLD. Moreover, cytokine measurements were done on clinically pre-separated populations of patients with no longitudinal observations which made it difficult to standardize and control for unknown specific external health factors. Possible drug therapy taken by patients can have an effect on the outcome of cytokine levels in each population. However, utmost caution was taken not to include any subjects with any apparent other clinical disorders that might influence adipokines level or under any treatments.

We used NASH diagnostic models on blinded set of 32 patients with NAFLD and confirmed its diagnostic value, despite the fact that the model was tested in rigorous conditions. Specifically, the validation set contained only NASH and Simple Steatosis cases, while morbidly obese patients whose liver biopsy did not show NAFLD and who are expected to have very low NASH scores were excluded. If this model would be utilized for minimization of the use of liver biopsies at the threshold of 0.3825 even in this, relatively skewed, cohort of morbidly obese patients, 15 out of 21 patients with NASH (71.4%) would avoid biopsy as being diagnosed with NASH non-invasively. As at this threshold the negative predictive value of the model is somewhat lower, patients who are non-invasively diagnosed as non-NASH would still have to be biopsied. Nevertheless, depending on the particular cohort use of non-invasive test for a presence of NASH can lead to the significant decrease in the biopsies performed.

One way to improve performance of the model is to adjust its threshold after testing of additional subjects. When training dataset was extended to include all morbidly obese patients profiled (N=101), the optimal NASH threshold became reset to 0.4320 and AUC of the model was improved to 0.854 with sensitivity of 72.1% and specificity of 91.4%. Further extension of the training dataset and subsequent independent validation experiments are recommended.

In this study we attempted to predict a presence of NASH in a cohort of morbidly obese patients by application of the combinational panel consisting of two adipokines and two biomarkers of cellular death. This panel of biomarkers, the so-called NASH Diagnostics©, can reliably predict NASH, thus, decreasing the number of biopsies required. NASH Diagnostics™ needs to be extensively tested in various cohorts of the patients with NAFLD, e.g. in non-obese and/or HCV-positive subjects. After further validation, it may become extremely useful in the clinical management of these patients.

In conclusion, this study provides an in-depth assessment of several important adipokines and cytokines in a well described and well controlled group of patients with NAFLD. Our findings confirm that hypoAdiponectinemia and IL-8 are consistently associated with NASH. TNF-α is implicated in the development of NAFLD, and Visfatin and IL-6 may play a protective role in NAFLD. Collectively, these data indicate the complexity of the interactions between various adipokines, IR, and the pathogenesis of NAFLD. More importantly, M30 apoptosis measurement may serve as an important biomarker of NASH. A combination of ELISA-based measurements of cleaved and intact

CK-18, serum Adiponectin, and serum Resistin can be used as relatively simple serum marker panel for non-invasive NASH diagnosis in a cohort of morbidly obese patients. Such a panel of biomarkers can reduce the need for liver biopsy in the management of patients with NAFLD, reducing both biopsy-related costs and its associated risks.

Gene Expression in NAFLD

In this part of research we explored the gene expression profile differences between omental adipose and hepatic tissues in obese and NAFLD group's of patients. This was done on adipose of obese NASH patients (N=10), adipose of non-Obese Controls (N=9) and adipose of Obese Controls (N=17). Liver biopsies of obese patients with NASH (N=27), liver biopsies of non-Obese Controls (N=6) and liver biopsies of Obese Controls N=7).

Gene Expression Comparison of Visceral Adipose Tissue in Patients with and without NASH:

A total of 97 genes expressed in human adipose tissue were independently identified as statistically significant in the comparisons of obese NASH patients (N=10) vs. non-Obese Controls (N=9), and comparisons of Obese Controls (N=17) vs. non-Obese Controls (N=9) (Appendix Table 7). Differential expression of these genes is probably related to excessive accumulation of adipose tissue itself, but not to the accumulation of the lipids in the liver. The majority of these genes (78 of 97) have been previously reported as differentially expressed in various adipose-profiling experiments (Baranova et al., 2005). This finding lends confidence to the chosen approach.

A total of 133 genes were revealed in only one of the two comparisons described above. A total of 72 genes that are potentially important in the development of NASH have been identified (Appendix Table 8). These are genes with an increased levels of expression in visceral adipose of obese patients with NASH, but not in the visceral adipose of obese patients with no liver complications (Ratios > 2.0), and genes

suppressed in visceral adipose of obese patients without liver complications, but not in the visceral adipose of obese patients with NASH (Ratios < 0.5). Many of these genes were overexpressed in adipose of obese with NASH were encoding mostly secreted pro-inflammatory cytokines. The results in general demonstrated prominent deregulation of immune defense related genes and genes encoding various plasma proteins, supporting the hypothesis that adipocyte secretion is important in the development of non-alcoholic liver disease.

Additionally, another 61 genes have significant changes in two of the three comparisons with a potential to exert a protective effect against the development of NASH (Appendix Table 9). These genes are suppressed in visceral adipose of obese patients with NASH, but not in the visceral adipose of obese patients with no liver complications (Ratios < 0.5), and genes with an increased levels of expression in visceral adipose of obese patients without liver complications, but not in the visceral adipose of obese patients with NASH (Ratios > 2.0).

Gene Expression in the Liver Tissue of Patients with and without NASH:

A total of 14 genes expressed in hepatic tissue were independently identified as statistically significant in the comparisons of obese NASH patients (N=27) vs. non-Obese Controls (N=6), and obese (N=7) vs. non-Obese Controls (N=6) (Appendix Table 10). Changes in the expression levels of these genes are probably related to the excessive adipose tissue load, but not to secondary inflammatory processes in the liver itself. Most of these genes (13/14) have been previously reported (Younossi et al., 2005). The only novel gene reported here encodes attractin, which is capable of interacting with

melanocortin receptors involved in the regulation of the energy homeostasis (Yeo et al., 2003).

A set of 67 genes showed significant changes in expression levels in one of the two liver-specific comparisons described above. Among these, 11 genes have potential functional importance in the development of NAFLD and NASH (Appendix Table 11). These are genes with increased expression levels in the hepatic biopsies of obese patients with NASH, but not in the livers of obese patients with no liver complications (Ratios > 2.0). It also shows the genes suppressed in the livers of obese patients without liver complications, but not in the livers of obese patients with NASH (Ratios < 0.5).

Another 56 genes identified probably exert a potentially protective effect against development of NASH (Appendix Table 12). These are genes that are suppressed in the hepatic biopsies of obese patients with NASH, but not in the livers of obese patients with no liver complications (Ratios < 0.5). It also displays genes with increased expression levels in the livers of obese patients without liver complications, but not in the livers of obese patients with NASH (Ratios > 2.0).

In general, a compensatory increase in the expression of many detoxification related genes has been identified in obese patients without liver complications. This might be beneficial for the maintenance of the liver function under morbidly obese conditions.

Exploration of the Molecular Pathways Associated With the Development of NASH:

We used the Ingenuity Pathways Knowledge Base (IPKB) and IPA 4.0 software for functional analyses of the gene sets with a potentially hepatoprotective role, and for gene sets potentially involved in the pathogenesis of NASH. Targets of the proinflammatory action of TNFα and IL-6 include multiple adipose-expressed genes that may be functionally engaged in the development of NASH (Figure 8 A).

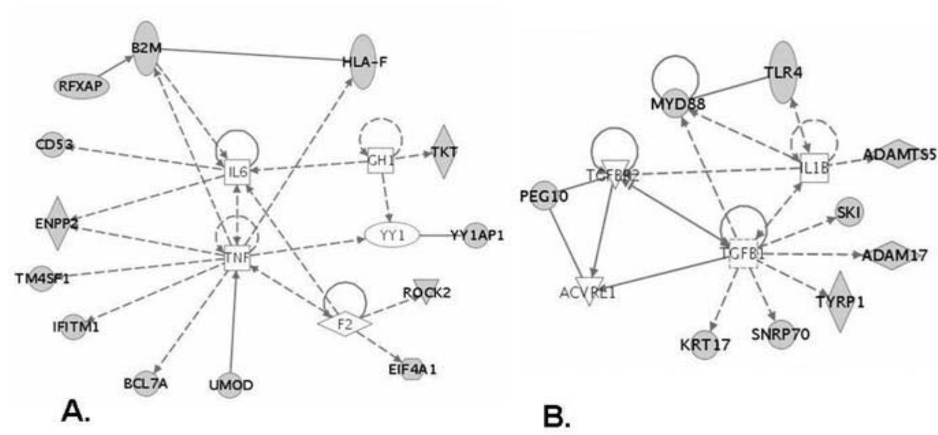

Figure 8. A. TNFalpha and IL-6 regulated genes expressed in adipose play a prominent role in the development of NASH. For the complete list of genes and their functions, see Appendix Table 8. B. Genes that are regulated by TGF-beta signal in adipose are important for the development of the primary phenotype of the morbid obesity. For the complete list of genes and their functions, see Appendix Table 7.

When the same type of the analysis was performed on a list of adipose-expressed genes (Appendix Table 7), the largest network centered at the differentiation related TGFβ. Analysis of the hepatoprotective genes expressed in the liver revealed three

additional networks: IFNγ (Figure.9A), JUN/JUNB (Figure.9B), and Leptin/TNFα (Figure.9C) (Fig. 2). Furthermore, analyses of a set of hepatoprotective genes expressed in the adipose tissue, and a set of NASH promoting genes expressed in the liver indicated the importance of interferon/STAT3 and TNFα coordinated gene networks (not shown). The latter networks involve a great proportion of secondary and tertiary gene mediators.

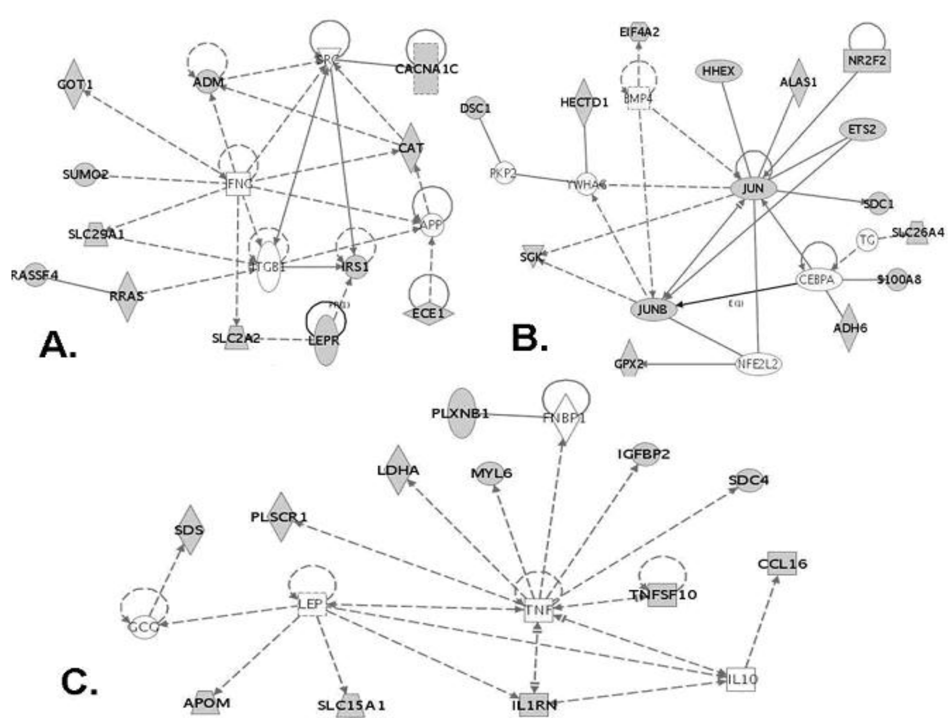

Figure 9. IFN-gamma (A), JUN/JUNB (B), and Leptin/TNFalpha (C) regulated genes protect the livers of a subgroup of morbidly obese patients against the development of steatosis. For the complete list of genes and their functions, see Appendix Table 12.

Gene expression results were corroborated with ELISA measurements of TNFα and IL-6 levels in the serum samples available for 12 NASH patients and 13 obese controls. As expected, concentrations of serum TNFα were significantly higher in the

obese NASH patients compared to the obese controls (3.79 ± 0.75 pg/mL vs. 1.95 ± 0.35 pg/mL, p-value < 0.001), but the comparison of IL-6 levels showed no statistically significant differences.

Discussion

The era of high-throughput gene expression profiling has revolutionized our approach to studying chronic diseases such as NASH. Several recent reports recognize the importance of white adipose tissue in the pathogenesis of NASH. This study attempts to connect hepatic gene expression to gene expression in the adipose tissue of patients with NASH. We selected a matched group of patients with biopsy-proven NASH and a control group without NASH. Note that these groups were well–matched with respect to both BMI and HOMA scores. Therefore, insulin resistance or differences in the extent of obesity alone do not explain the development of NASH.

Both liver and adipose-specific comparisons were performed against similar control samples collected from lean patients. Genes identified by comparing both NASH vs. lean control, and obese control (without NAFLD) vs. lean control, were classified according to the morbidly obese state itself, but not to the development of the liver disease. Note that the majority of genes had previously been identified as obesity related genes (Younossi et al., 2005; Baranova et al., 2005). Interestingly, functional analysis of the 97 adipose specific genes linked to morbid obesity itself revealed a network centered on TGF-β (Figure 8B), a cytokine known to orchestrate many differentiation programs and, particularly, to suppress adipogenesis (Roelen & Dijke, 2003). Because the release of TGF-β by human adipose tissue is enhanced in obesity (Fain et al., 2005), we

hypothesize that TGF-β may play a role in the maintenance of the adipocyte tissue mass in morbid obesity.

The remaining significant genes were segregated into adipose- and liver-specific genes exerting either NASH-promoting or liver protecting effects. Functional analysis of the adipose-specific genes potentially associated with NASH revealed the relative importance of the molecules represented in the TNFα network (Figure 8A). According to our analysis, the functioning of 13 out of 72 (18%) genes that were differentially expressed in the visceral fat of NASH patients is influenced by TNFα and/or IL-6. These findings were confirmed by the measurements of the corresponding serum levels of TNFα in the morbidly obese patients with NASH.

A prominent NASH-associated deregulation of inflammation- and immune system-related genes, and genes encoding various plasma proteins were also detected in the adipose tissue. Most of them (13 out of 16) were over-expressed in visceral adipose tissue of obese patients with NASH, but their levels were unchanged in the visceral adipose tissue of obese patients with no liver complications. Nevertheless, some immunity–related genes were highlighted as liver protective, particularly gene IL1RAP. This gene encodes a secreted decoy receptor that antagonizes the action of the pro-inflammatory cytokine IL1 (Smith et al., 2003). An increased secretion of the IL1RAP protein may balance the elevated production of the IL1 previously demonstrated in NAFLD obese patients (Poniachik et al., 2006).

Most of the NASH promoting genes expressed in adipose tissue encode soluble serum-circulating proteins, namely: CCL26, CCL20, IL18, cathepsin B, β-2

microglobulin, uromodulin, protein C, clusterin, ficolin, and thromboplastin. In addition, the adipose tissue of the obese NASH patients revealed prominent suppression of the PGDS gene. This gene encodes the prostaglandin D (2)-synthesizing enzyme that produces anti-inflammatory metabolites that binds to the nuclear peroxisome proliferator-activated receptor-γ (PPARγ) (Herlong & Scott, 2006). Prostaglandin D2 also antagonizes VLDL secretion in hepatocytes that are maintained in pro-inflammatory conditions (Perez et al., 2006). Suppressed PGDS expression in the adipose tissue may lead to decreased serum levels of prostaglandin D2 and to the absence of its beneficial effects on liver homeostasis. All of these findings strongly support the importance of adipocyte secretion in the development of NASH.

Eleven genes expressed in the livers of the morbidly obese patients were related to the development of NASH. Three of them (27.3%): ACSL4, PRSS3, and SH3BGRL2, were previously identified in a comparative study of NAFLD (Youonossi et al., 2005). Among the novel genes revealed here, we noted CCL7/MCP-3, which attracts macrophages during inflammation. CCL7 production is suppressed at the transcription level in the liver biopsies of morbidly obese patients without liver complications, thus limiting its pro-inflammatory effects.

A total of 66 genes might play a role in hepatoprotection. Most of these genes encode different detoxification enzymes. The liver tissue of the NASH patients expresses less of the adrenomedullin encoding gene ADM, which may exert a negative influence on hepatic stellate cells (Wang et al., 2005). Therefore, the decreased synthesis of adrenomedullin in the NASH hepatocytes may make them more vulnerable to hepatic

injury. Another interesting gene with a potentially hepatoprotective role encodes transcription factor NR2F2/COUP-TFII, which controls a number of other genes (LPAL2, APOM, APOL3, glycogenin 2, CYP7A1, IL1RN and others).

Functional analysis of the genes that appear to protect the livers of the morbidly obese patients from the developments of NAFLD revealed JUN/JUNB, IFN-γ, and Leptin/TNFα centered networks. TNFα centered networks generated in this analysis and in the analysis of the pro-NASH genes expressed in adipose differ in their appearance; in the livers protected from NAFLD, TNFα remains under tight anti-inflammatory control exerted by Leptin, IL1RN, and IL10. Involvement of JUN/JUNB in the development of the steatosis has been reported earlier (Schattenberg et al., 2006). JUN and JUNB represent central pieces of the corresponding network suppressed in the hepatic biopsies of obese patients with NASH, but not in the liver tissue of obese patients with no hepatic complications (Appendix table 12). The importance of the INFγ-centered network in the development of NASH is less clear, because most of the studies aimed at measurements of IFN-gamma in the patients' sera were performed in the cohort infected with hepatitic B and C (Falasca et al., 2006; Takegoshi et al., 2004).

In conclusion, our gene expression analysis of the liver and visceral adipose tissues of morbidly obese patients with NAFLD reveals prominent adipose-specific deregulation of inflammation- and immune system-related genes, and the genes encoding various plasma proteins. The findings support the hypothesis that adipocyte secretion is important for the development of non-alcoholic fatty liver disease. A number of liver and adipose-specific functional networks, including these centered at TNFα, JUN/JUNB, and

IFNγ, were highlighted in relation to NASH pathogenesis. Among other interesting findings are compensatory increases in the hepatic expression of the genes encoding detoxification enzymes, and decreases in the mRNA levels for the glucose and cholesterol metabolism-related genes that are controlled by transcription factor NR2F2/COUP-TFII.

Profiling of Serum Adipocytokines in Patients infected with HCV

Metabolic syndrome, ALD, and HCV infections are the most common causes of fatty liver. Steatosis coexists with HCV infection in 50-70% of cases and further exacerbates the inflammatory process in the liver (Asselah et al., 2003). The severity of steatosis accompanied HCV infections depends on many factors including patient population, genetic factors, accompanied metabolic risk factors and genotype of the virus (Zekry et al., 2005, Asselah et al., 2003). For example, it has been found that mild to moderate steatosis is more frequent with HCV genotype 1 infections and, together with BMI and age, seems to influence therapeutic response negatively. In contrast, severe steatosis is more frequent in patients infected with HCV genotype 3a and these patients are more responsive to successful therapy (Soresi et al., 2006).

Many studies have shown that pro-inflammatory cytokines play role in pathogenesis of NAFLD, ALD and HCV infection. For example, it has been found that serum levels of TNF-α, IL-1 and IL-6 are increased in serum of patients with autoimmune diseases (Tilg et al., 1992), with NAFLD (McClain et al., 1999; Fan et al., 2003), and with alcoholic fatty liver (Thurman et al., 1999). However, the difference in serum adipocytokine profile between NAFLD-type of simple steatosis and steatosis associated with chronic infection by HCV is not well known. The coexistence of steatosis and HCV infections in many patients could produce different signature profiles of adipocytokines than in steatosis alone or HCV infection alone, and this possibility is explored in this thesis.

The role of the agonists and antagonists of pro-inflammatory cytokines in HCV and NAFLD pathogenesis also has been under investigation. For example, inflammatory IL-1 receptor antagonist (IL-1ra), one of the negative regulators to IL-1 signaling, is considered to be hepatoprotective and anti-inflammatory as it competitively binds and blocks the functional receptor for IL1B (IL-1 receptor type-I) without triggering its activation (Arend et al., 1998; Dinarello et al., 1998). IL-1rais produced by hepatocytes, macrophages, and monocytes (Gabay et al., 2001). It is probable that the imbalance between the inflammatory cytokines, their receptors, and IL-1raantagonist is critical for HCV pathogenesis and should be evaluated. HCV and HCV-associated steatosis effects on serum levels of pro-inflammatory cytokines and their agonists or antagonists is also of importance as it can provide better understanding of the role of the viral and host factors in the process of liver deterioration in chronically infected patients.

We profiled some of most common adipocytokines in different groups of HCV patients infected with HCV genotypes 1 and 3 and who either with or without liver steatosis. Adiponectin, Resistin, Visfatin, and Leptin, TNF-α, IL-6 and its agonist sIL-6r, IL-8, IL-1B, and IL-1rawere measured in serum samples of 6 patients groups. These groups are classified as follow: Steatosis only (N =19), HCV genotype 3 with steatosis (N=9), HCV genotype 3 with no steatosis (N=8), HCV genotype 1 with steatosis (N=36), HCV genotype 1 with no steatosis (N=46) and patients diagnosed with HBV only as side controls (N=20).

Differences in Adipocytokine serum levels between HCV and HBV groups:

There were statistically significant differences in serum levels of most of cytokines between the HBV and the HCV patient groups (Table 12, Appendix Table 13).

Table 12. Comparison of serum levels for different adipocytokines between patients infected HCV and HBV. Mean values are in black color on top and P-values are in italic bellow. P-values are calculated with Mann-Whitney test. P-value ≤ 0.01 is considered significant.

Adipocytokine	HCV vs HBV	HCV-G3 vs HBV	HCV-G1 vs HBV
IL-6	**18.56/13.91** ***(0.04)***	**20.98/13.91** ***(0.0018)***	**18.06/7.48** ***(0.10)***
IL-8	**36.67/36.6** ***(0.00002)***	**55.59/36.67** ***(0.00004)***	**32.75/36.67** ***(0.000001)***
TNF-α	**36.67/16.62** ***(0.00002)***	**32.78/16.62** ***(0.0061)***	**34.25/16.62** ***(0.000004)***
Adiponectin	**18.50/20.02** ***(0.90)***	**15.92/20.02** ***(0.25)***	**19.08/20.02** ***(0.85)***
Resistin	**4.51/3.80** ***(0.06)***	**6.08/3.80** ***(0.0003)***	**4.19/3.80** ***(0.26)***
Visfatin	**55.18/93.69** ***(0.012)***	**47.30/93.69** ***(0.004)***	**57.18/93.69** ***(0.03)***
IL-1B	**10.77 /4.41** ***(0.034)***	**5.26/4.41** ***(0.52)***	**11.92/4.41** ***(0.02)***
sIL-6r	**38.10/14.91** ***(0.00002)***	**57.88/14.91** ***(0.00003)***	**33.95/14.91** ***(0.000016)***
Leptin	**20.79 /7.48** ***(0.00004)***	**13.85/7.48** ***(0.23)***	**22.25/7.48** ***(0.000006)***
IL-1ra	**272.65/124.59** ***(0.00002)***	**188.36/7.48** ***(0.0017)***	**290.33/7.48** ***(0.00002)***

The serum levels of the pro-inflammatory cytokines IL-8 and TNF-α were significantly higher in the HCV patient groups than in the HBV patient group regardless of HCV genotype (Table 12, Appendix Table 13). Moreover, levels of IL-6 agonist, sIL-6r, were also significantly higher in HCV group than in HBV group regardless of HCV

genotype, but levels of IL-6 were only significantly higher in HCV-G3 group when compared to HBV group (Table 12, Table 13).

There were no differences between serum levels of IL-1B between the HBV and HCV groups regardless of genotype, but the serum levels of its antagonist- IL-1rawere significantly higher in the HCV groups than in the HBV group. Interestingly, Leptin serum levels were significantly higher in the HCV-G1 group only when compared to the HBV group. Descriptive parameters and results of the group's comparison can be found in Table 12 and Table 13.

Our results also show that there are significant differences in serum levels of Resistin and Visfatin between the HBV and HCV-G3 groups only. Resistin was higher in the HCV-G3 group compared to the HBV group while Visfatin was lower.

Differences in serum level of Adipocytokines between patients infected with HCV genotypes 1 or 3 with or without steatosis and NAFLD type of steatosis:

There were significant differences in serum levels of major pro-inflammatory cytokines (IL-6, IL-8 and TNF-α) between patients with HCV infection regardless of genotype or association with steatosis when compared to the group of patients with only the NAFLD-type of steatosis. However, serum levels of Adiponectin and Resistin showed significant differences only when HCV genotype 1 infected patients were compared to patient with NAFLD-type of steatosis (Table 13).

Table 13. Serum levels of adipocytokines in patients infected with HCV genotypes 1 or 3 with or without steatosis and patients with the NAFLD-type of simple steatosis (SS). Mean and Standard Deviations are showen for each group of patients. Levels of significance for each comparison are discussed below.

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Adipocytokine	NAFLD-type SS	HCV-G3 (SS)	HCV-G3 (No SS)	HCV-G1 (SS)	HCV-G1 (No SS)
TNF-α (pg/ml)	2.9±0.9	45.45±44	37.7±40.7	29.5+/16.3	18.5±3.7
IL-8 (pg/ml)	25.5±50.2	74.01±40.3	32.9±19.3	32.6±26.3	34.9±11.3
IL-6 (pg/ml)	42.5±109.4	22.6±5.1	19.95±10.4	14.99±7.7	19.1±8.0
Visfatin (ng/ml)	45.1±60.9	50.43±63.4	64.9±84.3	47.88±31.8	40.1±39.3
Adiponectin (µg/ml)	12.2±7.6	13.05±6.8	18.0±7.5	20.5±11.5	18.5±3.7
Resistin (ng/ml)	7.8±4.3	6.11±2.0	4.3±2.3	4.0±1.6	6.0±1.67
sIL-6r(ng/ml)	N/A	56.64±14.6	59.27±18.8	32.08± 16.5	35.27± 23.0
IL-1B (pg/ml)	N/A	6.66±6.9	3.69±1.2	11.05± 13.2	12.42±30.3
IL-1ra (pg/ml)	N/A	203.56±68.2	171.3± 69	292.56±179	285.6±125.6
Leptin (ng/ml)	N/A	14.64± 16.0	13± 15.6	22.02 ±18.9	22.32±18.9

TNF-α:

As can be seen from Table 13 and Figure 10, serum levels of TNF-α were significantly increased in the following comparisons: HCV-G3 with SS, HCV-G3 without SS, HCV-G1 with SS and HCV-G1 without SS when compared to NAFLD patient group with SS. In addition, levels of TNF-α were higher in the HCV-G3 groups compared to HCV-G1 groups, especially when patients when different HCV genotypes without steatosis were compared to each other (Figure 10). These findings emphasize the pro-inflammatory role of TNF-α in both HCV infection and NAFLD pathogenesis. As we have seen earlier, TNF-α was also implicated in insulin resistance and steatosis development, and an increase in its level due to HCV infections could exacerbate NAFLD pathogenesis.

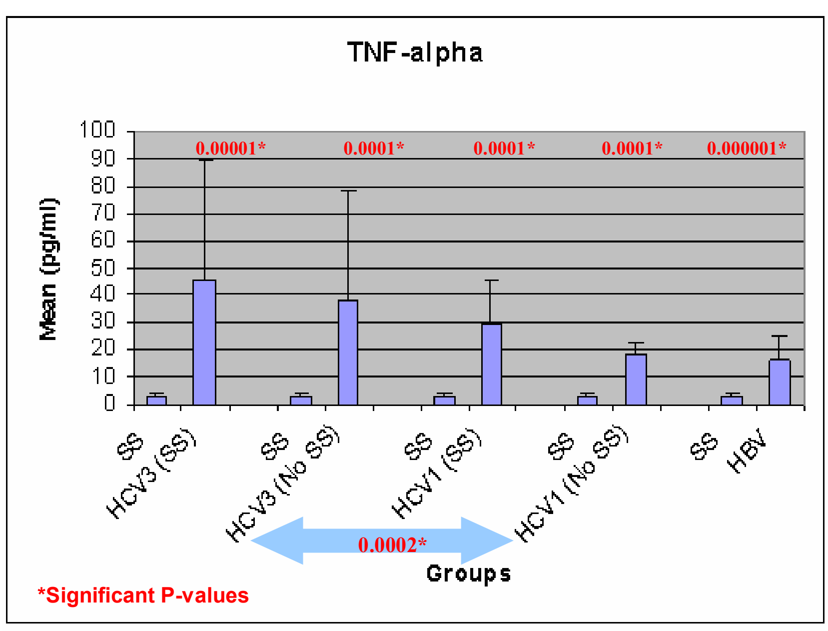

Figure 10. The significant differences in averages of serum levels for TNF-α between patient groups with different HCV genotypes with or without Steatosis compared to NAFLD-type steatosis group (SS). *P-value ≤ 0.01 are considered significant and highlighted in red. SS: Simple Steatosis. Significnt differences between other groups shown by down arrows.

IL-8:

IL-8 followed the same pattern as TNF-α. Specifically, its serum levels were increased in HCV-G3 with SS, HCV-G3 without SS, HCV-G1 with SS, and HCV-G1 without SS when compared to the NAFLD-type of SS (Figure 11). As previously demonstrated in the first part of this study, both TNF-α and IL-8 were increased in NAFLD-type of SS compared to obese and normal controls, thus, emphasize the co-regulatory relationship between these two cytokines and their functional role both in NAFLD pathogenesis and in inflammatory processes accompanying HCV infection.

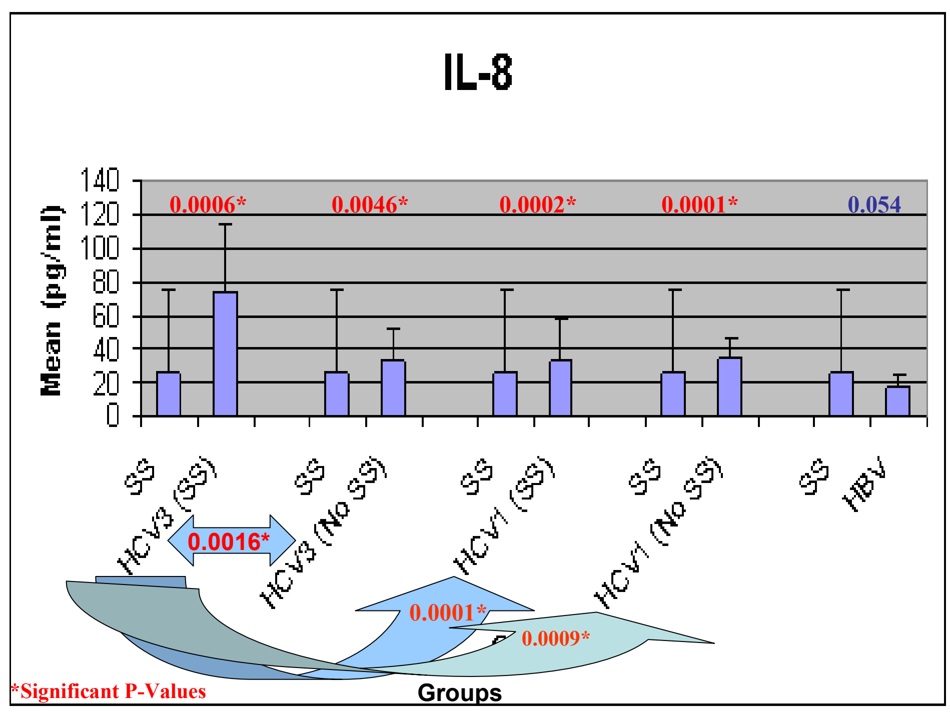

Figure 11. The significant differences in averages of serum levels for IL-8 between patient groups with different HCV genotypes with or without Steatosis compared to NAFLD-type steatosis only group. *P-value ≤ 0.01 are considered significant and highlighted in red. SS: Simple Steatosis. Significnt differences between other groups shown by down arrows.

One of the important findings is that serum levels of IL-8 are significantly higher in HCV-G3 (SS) when compared to HCV-G1 (SS) groups (Figure 11, Table 14). At the same time serum levels of IL-8 are significantly higher in HCV-G3 with SS compared to HCV-G3 without SS, and when compared to HCV-G1 groups with or without steatosis (Figure 11). This data shows that there is strong connection between HCV-G3 genotype, steatosis and increase in IL-8 serum levels.

Table 14. Comparison of serum levels of different adipocytokines in patients infected with HCV genotypes 1 or 3 with or without steatosis and patients with NAFLD-type of SS. Mean values are in black color on top and P-values are in italic bellow. P-values are calculated by of Mann-Whitney test. P-value ≤ 0.01 are considered significant.

Adipocytokine	HCV-G3 vs HCV-G1	HCV-G3 vs HCV-G1 (SS)	HCV-G3 vs HCV-G1 (no SS)
IL-6	**20.98 /18.06** ***(0.08)***	**22.63/14.99** ***(0.005)***	**19.11/19.95** ***(0.97)***
IL-8	**55.59/32.75** ***(0.00016)***	**74.01/32.56** ***(0.0001)***	**34.87/32.94** ***(0.30)***
TNF-α	**32.78/34.25** ***(0.07)***	**45.45/29.50** ***(0.57)***	**18.54/37.77** ***(0.0002)***
Adiponectin	**15.92/19.08** ***(0.1)***	**13.05/20.71** ***(0.03)***	**18.71/17.96** ***(0.91)***
Resistin	**6.08 /4.19** ***(0.00011)***	**6.11/20.71** ***(0.0020)***	**6.04/4.33** ***(0.01)***
Visfatin	**47.30/57.18** ***(0.09)***	**50.43/47.88** ***(0.33)***	**40.06/64.88** ***(0.16)***
IL-1B	**5.26/11.92** ***(0.06)***	**6.66/11.06** ***(0.35)***	**3.69/12.42** ***(0.09)***
sIL-6r	**57.88/33.95** ***(0.00002)***	**56.64/32.08** ***(0.0006)***	**59.27/35.27** ***(0.0016)***
Leptin	**13.85 /22.25** ***(0.01)***	**14.64/22.02** ***(0.12)***	**12.96/22.32** ***(0.046)***
IL-1ra	**188.36 /290.33** ***(0.0009)***	**203.56/292.56** ***(0.06)***	**171.27/285.59** ***(0.008)***

IL-6:

Surprisingly, IL-6 serum levels were significantly lower in patient groups HCV–G3 with SS, HCV-G3 without SS, HCV-G1 with SS, and HCV-G1 without SS when compared to patient groups with NAFLD SS only. In addition IL-6 serum levels were higher in HCV-G1 (SS) group compared to HCV-G3 (SS) (Table 13, Figure 12). However, IL-6 in these groups were close to normal ranges in healthy subjects (<20 pg/ml), further complicating understanding its role in NAFLD and HCV infection

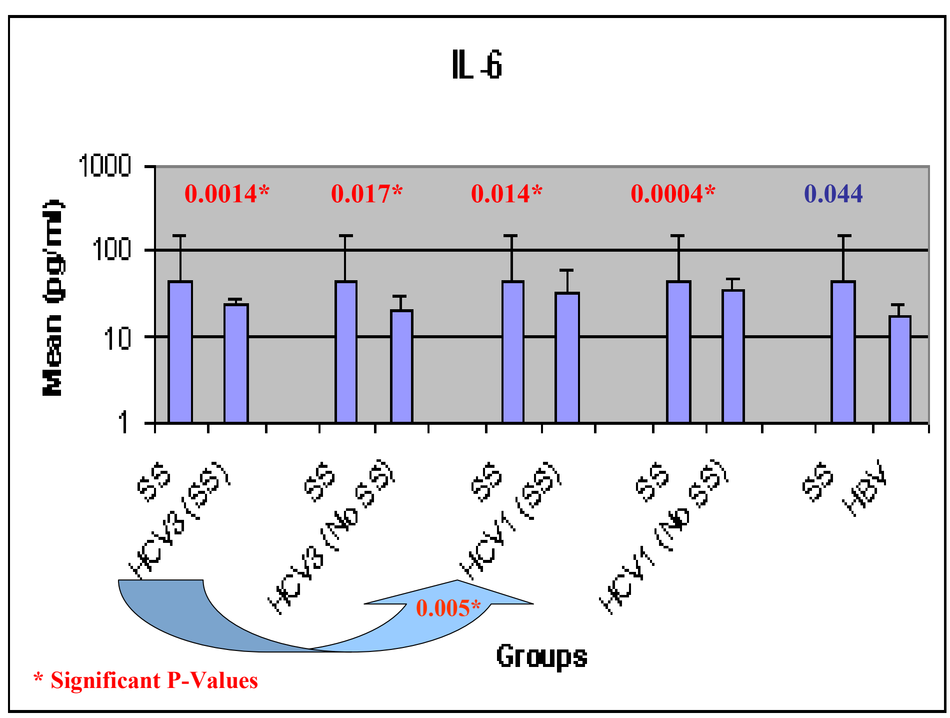

Figure 12. The significant differences in averages of serum levels for IL-6 between patient groups with different HCV genotypes with or without Steatosis compared to NAFLD-type steatosis only group. *P-value ≤ 0.01 are considered significant and highlighted in red. SS: Simple Steatosis. Significnt differences between other groups shown by down arrows.

Adiponectin:

Interestingly, Adiponectin serum levels were significantly higher in HCV-G1 groups with or without steatosis compared to SS only groups (Figure 13). No significant differences in Adiponectin serum levels were revealed when HCV groups were compared.

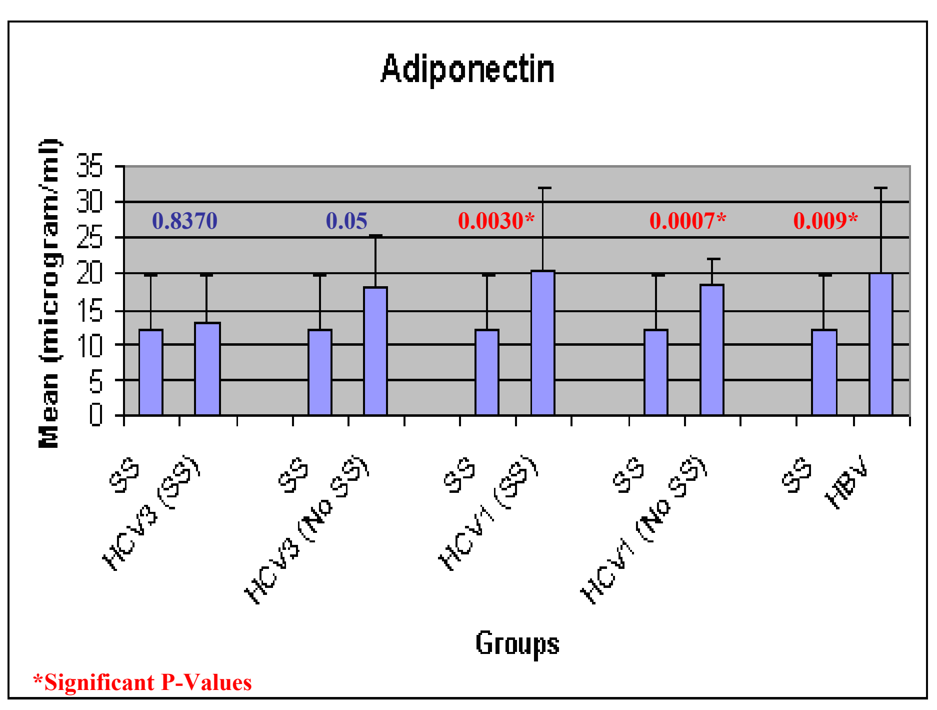

Figure 13. The significant differences in averages of serum levels for Adiponectin between patient groups with different HCV genotypes with or without Steatosis compared to NAFLD-type steatosis only group. *P-value ≤ 0.01 are considered significant and highlighted in red. SS: Simple Steatosis.

Resistin:

In contrast, serum levels of Resistin were significantly lower in HCV-G1 groups with and without steatosis compared to NAFLD-type SS only group (Figure 14). Comparisons between HCV groups show that HCV-G3 with SS had higher Resistin serum levels than HCV-G1 with SS. Also these levels were higher in HCV-G1 without SS when compared to HCV-G3 without SS (Figure 14, Table 14). However, no significant differences in serum levels of Resistin were revelead between HCV groups with steatosis and without, regardless of HCV genotypes. If influences of HCV genotype-

specific factor are excluded, these results are confirming our previous finding that serum Resistin levels are not significantly different between obese controls, steatosis and NASH groups (Appendix Table 6). These combined findings minimize the possible direct role of Resistin in developing steatosis, but pointing out that HCV genotype 3 virus itself may play a role in modulating Resistin serum levels.

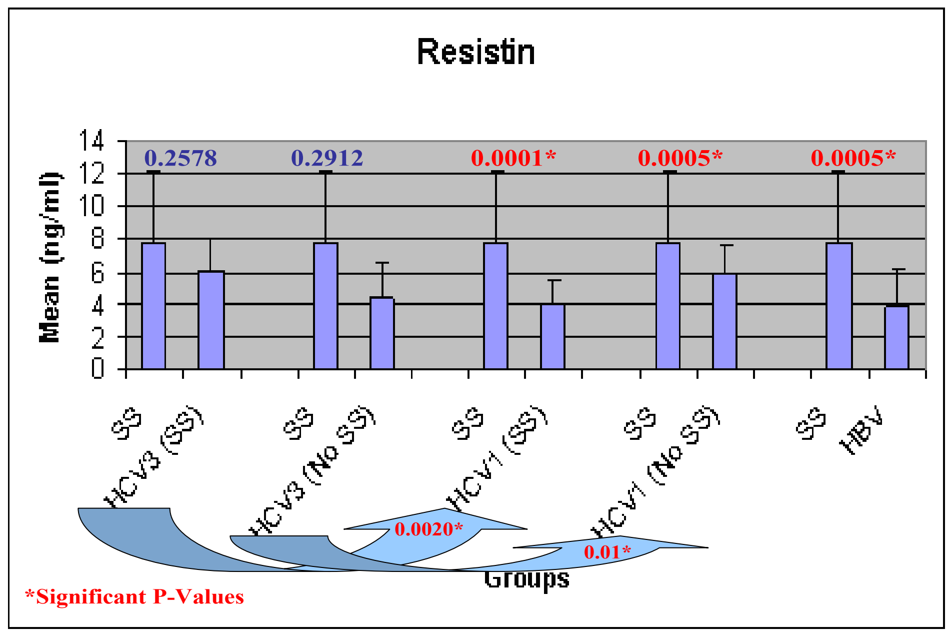

Figure 14. The significant differences in averages of serum levels for Resistin between patient groups with different HCV genotypes with or without Steatosis compared to NAFLD-type steatosis only group. *P-value ≤ 0.01 are considered significant and highlighted in red. SS: Simple Steatosis. Significnt differences between other groups shown by down arrows.

IL1B and Visfatin:

There were no significant differences in serum levels of IL-1B and Visfatin between any groups of patients infected with any HCV genotypes with or without steatosis (Table 13, Table 14).

IL-1ra and Leptin:

Serum levels of IL-1ra and Leptin were significantly higher in HCV-G1 group of patients compared to HCV-G3 groups in general (Table 14 and Figure 15). This highly significant difference in IL-1ra serum levels was mainly seen when HCV-G1 groups with or without steatosis compared to HCV-G3 groups with no steatosis (Figure 15). However, there were no significant differences in IL-1ra between HCV-G1 groups of patients with and without steatosis or between HCV-G3 groups with and without steatosis.

These results might indicate that HCV-G1 has direct influence on serum levels of IL-1ra. On the other hand, the significant differences in serum levels of IL-1ra between HCV-G3 groups when compared to HBV could not directly relate to HCV genotype as most of their values were close to the average of IL-1ra in healthy individuals (119 pg/ml), but rather could be explained by phenotypic heterogeneity of the patients. As IL-1B serum levels did not show any significant changes between any of the groups compared, this also may indicate that HCV infection has no direct influence on IL-1B levels.

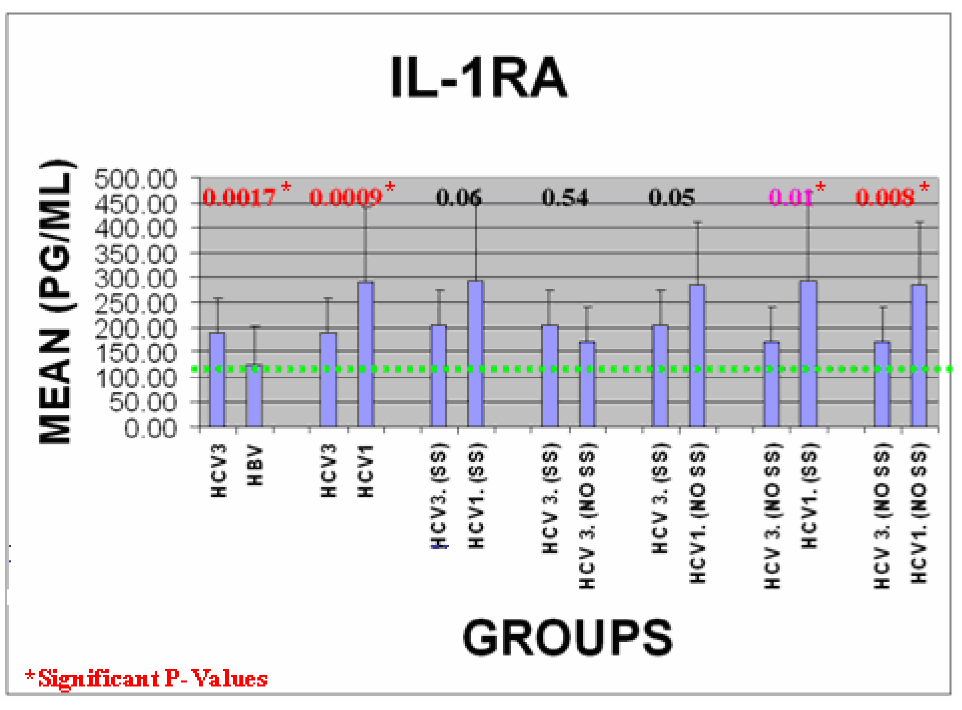

Figure 15. The significant differences in averages of serum levels for IL-1ra between patient groups with different HCV genotypes with or without Steatosis compared to NAFLD-type steatosis only group. *P-value ≤ 0.01 are considered significant and highlighted in red. SS: Simple Steatosis. The green dotted line is the average of IL-1ra in healthy individuals.

SIL-6r:

According to our measurements, serum levels of sIL-6r were higher in HCV-G3 patients group compared to HCV-G1 groups of patients in general. Interestingly, this was true regardless of presence or absence of steatosis with either genotype (Table 14, Figure 16). There were no significant differences in sIL-6r serum levels between between HCV-G1 groups of patients with or without steatosis or between HCV-G3 group with or without steatosis. However, most of the serum levels of sIL-6r in HCV-G3 group were

within the normal range for normal healthy subjects (54 ng/ml), but in HCV-G1 group, serum levels of sIL-6r were much lower of the average.

IL-6 serum levels were only significantly higher in HCV-G3 with steatosis compared to HCV-G1 with steatosis (Figure 16).

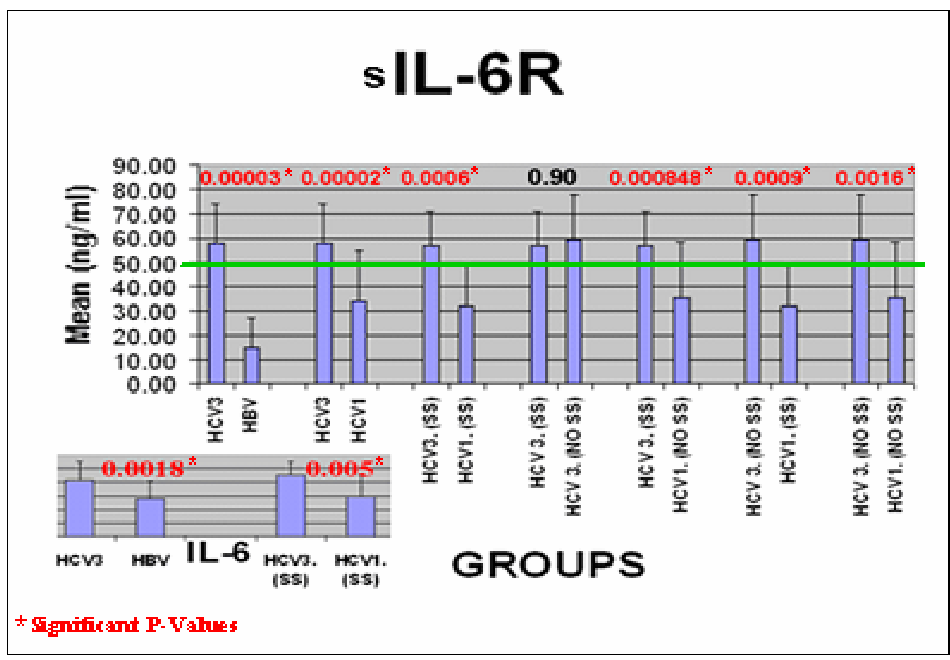

Figure 16. The significant differences in averages of serum levels for sIL-6r and IL-6 between patient groups with different HCV genotypes with or without Steatosis compared to NAFLD-type steatosis group. *P-value ≤ 0.01 are considered significant and highlighted in red. SS: Simple Steatosis. The green line is the average of sIL-6r in healthy individuals.

Discussion

The coexistence of both steatosis and HCV infection is very common and more detrimental for the liver. On the other hand, simultaneous presence of both steatosis and HCV infection in one subject makes it an ideal system to study the role of both inflammation and metabolic factors in NAFLD pathogenesis. In this part of this study we profiled some of the common adipocytokines in different groups of HCV patients infected with viruses of genotypes 1 and 3, with or without underlying steatosis. Adiponectin, Resistin, Visfatin, and Leptin, TNF-α, IL-6, sIL-6r, IL-8, IL-1B and its antagonist IL-1ra were measured across the serum samples that belong to 6 patients groups. These groups are classified as follow: Steatosis only (N=19), HCV genotype 3 with steatosis (N=9), HCV genotype 3 with no steatosis (N=8), HCV genotype 1 with steatosis (N=36), HCV genotype 1 with no steatosis (N=46) and patients diagnosed with HBV only as controls (N=20).

Our results show that most of adipocytokines serum levels are generally increased in HCV infection compared to that of HBV. All adipocytokines in HCV-G3 infected groups, except Adiponectin, IL-1B, and Leptin were significantly higher than in HBV group. The difference in HCV-G3 groups and HCV-G1 groups when compared to HBV is that Resistin significantly increased while Visfatin decreased in HCV-G3 groups, but Leptin was significantly increased in HCV-G1 group.

The physiological role of Resistin in NAFLD and metabolic syndrome pathogenesis has been controversial, especially in humans. In rodents, Resistin has been implicated in insulin resistance as it has been found to be increased during diet-induced

obesity, genetic obesity, and diabetes type 2 (Steppan et al., 2001). Manipulating plasma Resistin concentrations in mice demonstrated that its levels correlate with glucose levels and the degree of insulin resistance (Steppan et al., 2001; Muse et al., 2004).

It has been shown recently that Resistin is an intrahepatic proinflammatory cytokine affects hepatic stellate cells (HSCs) (Bertolani et al., 2006) by stimulating an expression of chemokines particularly monocyte chemoattractant protein-1 and interleukin-8. It is believed that Resistin expression correlates with degrees of inflammation and fibrosis.

Our results showed that there is no difference beween serum level of Resistin between HCV groups with and without steatosis regardless of HCV genotypes. These findings agree with previous studies that have shown that Resistin does not contribute to steatosis in HCV-infected patients (Petit et al., 2006). However, surprisingly, our results show that HCV-G1 groups with or without steatosis have lower Resistin serum levels than the NAFLD-related steatosis group and lie within the normal range for healthy individuals (4-12 ng/ml). This further underscores the role of Resistin in the development of the steatosis in HCV.

Our results also show that there is no significant difference in serum Adiponectin levels between HCV-G3 groups with steatosis or without when compared to NAFLD-related SS group. This result contradicts the previous finding that in patients with chronic HCV-G3 there is hypoadiponectinemia, acondition which is significantly associated with the development of liver steatosis (Petit et al., 2005). It is even more puzzling as in HCV-G1 group there was a significant increase in Adiponectin serum levels when compared to

NAFLD-related SS groups. These contradictory results may be explained by the fact that patients in Petit and colleagues (2005) study were infected with HCV-G3 genotype only while the ones in our study where both HCV-G3 and HCV-G1. On the other hand, all serum Adiponectin levels in our groups were still within normal ranges (men: 5.6-13.4, women: 7.1-19.3), so the significant differences seen in serum Adiponectin levels, especially between HCV-G1 groups could be possibly explained by patients population factors, e.g. their BMI, gender and age differences.

The most prominent feature accompanying HCV infection is clearly seen in the alterations in cytokine serum levels. In addition to the fact that these pro-inflammatory cytokine changes are compatible with the inflammatory nature of HCV infection, their levels are also interdependent with steatosis development. Multiple linear regression analyses showed that steatosis in HCV mostly depends on IL-6 and IL-8; in HCV-G1 on IL-6, TNF-α and IL-1B while in HCV-G3 it depends mostly on IL-8 (Table 15). Multilinear regression was performed by M. Stepanova (Statistician) according to the data provided by our lab.

Table 15. Best fitting multiple linear regression models showing relationship between steatosis in HCV and adipocytokines. Regression coefficient β represents slope estimate ± Standard Error of the estimate (SE). P-value ≤ 0.01 is considered significant.

GROUP	Independent variable	Regression coefficient β and SE	P-values of independent variables	P-value of the whole model
Steatosis in HCV-G1	(Intercept)	0.8163+/-0.1348	0.00004	0.02467
	IL-6 (pg/ml).	-0.0133+/-0.0056	0.0186	
	TNF (pg/ml).	-0.0069+/-0.0034	0.0442	
	IL-1B(pg/ml).	0.0076+/-0.0045	0.0990	
Steatosis in HCV-G3	(Intercept)	0.0776+/-0.2012	0.7054	0.01835
	IL-8 (pg.ml).	0.0081+/-0.0031	0.0183	
Steatosis in all HCV	(Intercept)	0.5068+/-0.1250	0.0001	0.095
	IL-6 (pg/ml).	-0.0089+/-0.0053	0.1006	
	IL-8 (pg/ml).	0.0031+/-0.0019	0.1100	

IL-8 belongs to the CXC subfamily of pro-inflammatory chemokines and is known to induce chemotaxis and cell adhesion and to modulate cell growth (Reviewed in Baggiolini, 2001). IL-8 is synthesized by hepatocytes, Kupffer cells and macrophages and functions by activating neutrophils, leading to more pronounced induction of TNF-alpha and IL–6. Both of these cytokines are involved in promoting insulin resistance and steatosis. Our results clearly show that IL-8 and TNF-α levels increase significantly in HCV infection more than in HBV. These increases are more significant in HCV-G3 groups compared to HCV-G1 groups. For TNF-α, there was a significant increase between HCV–G3 groups without steatosis when compared to HCV-G1 groups without steatosis. TNF-α was incresed in non-steatosis groups indicating it is affected mainly by HCV, not by liver steatosis. More importantly, serum levels of IL-8 were significantly

higher in the HCV-G3 group with steatosis compared to same genotype without steatosis, and when compared to HCV-G1 groups with or without steatosis (Figure 17).

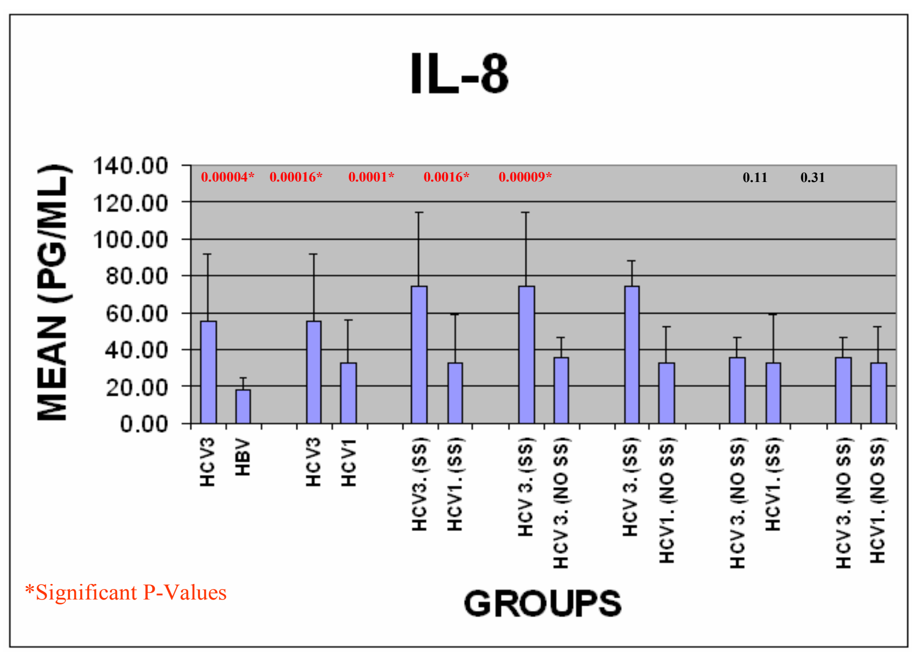

Figure 17. The significant differences in averages of serum levels for IL-8 between patient groups with different HCV genotypes with or without Steatosis compared to NAFLD-type steatosis group. *P-value ≤ 0.01 are considered significant and highlighted in red. SS: Simple Steatosis.

These findings clearly link the increase in IL-8 to steatosis, specifically, caused by HCV-G3 infection. IL-8 may directly promote steatosis in HCV-G3 patients. It has been shown previously that serum levels of IL-8 in HCV patient's correlate with liver fibrosis (Kaplanski et al., 1997), and that the expression level of intrahepatic IL-8 is associated with hepatic inflammation and fibrosis (Fukuda et al., 1996). From our results we can link serum levels of IL-8 in HCV-G3 patients to steatosis. Actually, HCV-G3 associated

steatosis can be predicted by serum levels of IL-8 with 87.5% specificity, 100% sensitivity and AUC of 0.931 (Figure 18, Table 16). Multilinear regression was performed by M. Stepanova.

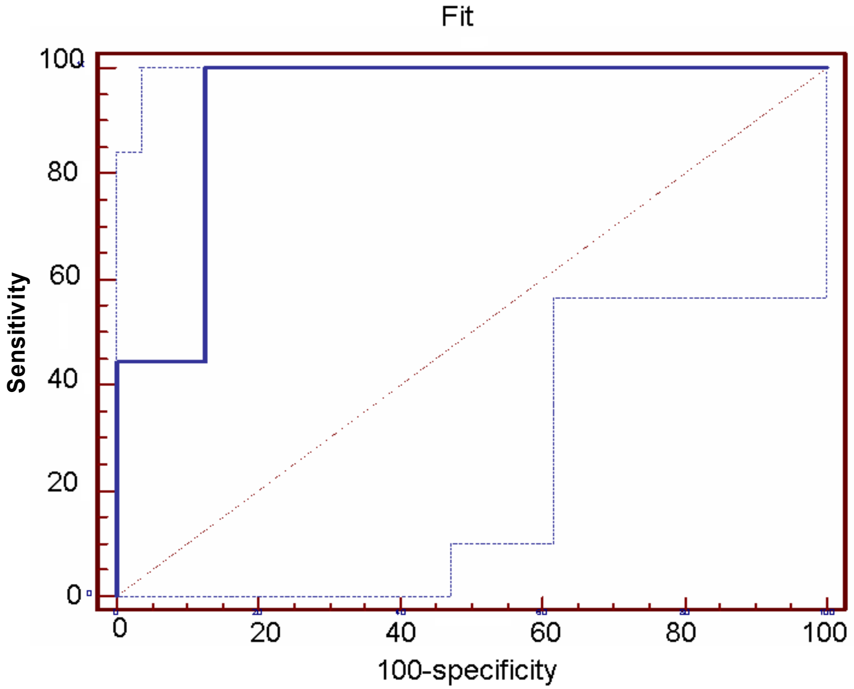

Figure 18. AUC: area under the curve reflecting a trade off between sensitivity and specificity for IL-8 based model predicting steatosis in HCV-G3 patients.

AUC is a popular method of assessing the predictive discrimination of a test is to use a receiver-operating characteristic (ROC) curve that plots the sensitivity of the test against the corresponding false-positive rate (Hanley, 1983). It is a measure of how well a continuous variable can predict the outcome of interest: If the sensitivity increases steeply as the threshold for diagnosis is relaxed, with only a relatively slow accumulation of

false-positive results, the area under the ROC curve will be large; conversely, if the sensitivity increases slowly as the threshold for diagnosis is relaxed, with a rapid accumulation of false-positive results, the area under the ROC curve will be correspondingly smaller. The differences in the areas under two curves may be tested to see whether the apparent superiority of one continuous variable over another is statistically significant.

In the present context, sensitivity here means the percentage of HCV-G3 cases where IL-8 initial value increases, above a given cut point, among all cases that later found to have steatosis is 100%. The false-positive rate (-PV), which refers to the percentage of cases in which IL-8 initial values increases and were above the given cut point among all cases who remained free of steatosis, is very low.

Table 16. Comparative performance of models predicting steatosis in HCV groups. AUC: area under the curve +LR: Positive likelihood ratio; -LR: Negative likelihood ratio; +PV: Positive predictive value; -PV: Negative predictive value. 95% confidence interval: (CI 95%). P-value ≤ 0.01 is considered significant.

Steatosis in HCV-G3	**IL-8, pg/ml**	*p-value*	0.01835	95% CI
		AUC	0.931	0.514 - 0.732
		sensitivity	100	43.5 - 76.8
		specificity	87.5	52.0 - 80.5
		+LR	8	
		-LR	0	
		+PV	90	
		-PV	100	
		threshold	0.3978	
Steatosis in HCV-G1	**IL-6, pg/ml TNF-alpha, pg/ml IL-1B, pg.ml**	*p-value*	0.04788	95% CI
		AUC	0.628	0.698 - 0.990
		sens	61.1	66.2 - 100.0
		spec	67.4	47.4 - 97.9
		+LR	1.87	
		-LR	0.58	
		+PV	59.5	
		-PV	68.9	
		threshold	0.4708	
Steatosis in all HCV patients	**IL-6, pg/ml. IL-8, pg/ml.**	*p-value*	0.09487	95% CI
		AUC	0.618	0.515 - 0.714
		sens	62.2	46.5 - 76.2
		spec	66.7	52.5 - 78.9
		+LR	1.87	
		-LR	0.57	
		+PV	60.9	
		-PV	67.9	
		threshold	0.4656	

The molecular details of the functional connection between the increases in IL-8 in HCV-G3 and the developing steatosis are not clearly understood. HCV proteins, such as core protein HCV NS5A can trigger the release of inflammatory chemokine IL-8 that can cause endothelial apoptosis (Balasubramanian et al., 2005). Through a different mechanism, IL-8 can also act as anti-apoptotic signal leading to an increase in the HCV-

linked necrosis and necro-inflammation. It has been shown that IL-8 protects liver cells from galactosamine and endotoxin-mediated apoptosis by signaling through phosphatidylinositol-3 kinase (PI-3K) (Hanson et al., 2006). HCV-induced increases in serum levels of IL-8 correlates with level of resistance to antiviral therapy by interferon (IFN), virus persistence, and disease progression (Polyak et al., 2001). Now we may add to this list the possibility that this chemokine may plays a role in the pathogenesis of fatty liver disease in humans.

It has been previously reported that chronic Hepatitis C induces synthesis of IL–1Ra as reflected by an increase in its serum levels when compared to normal controls. The increase in IL-1Ra serum levels have been shown to be positively correlated with viral load and response for IFN therapy (Lee et al., 2002). Our results add that IL-1Ra and Leptin serum levels are increased in parallel only in HCV-G1 infection, and are significantly higher in HCV-G1 group of patients when compared to HCV-G3 groups and when compared to the HBV group (Figure 19).

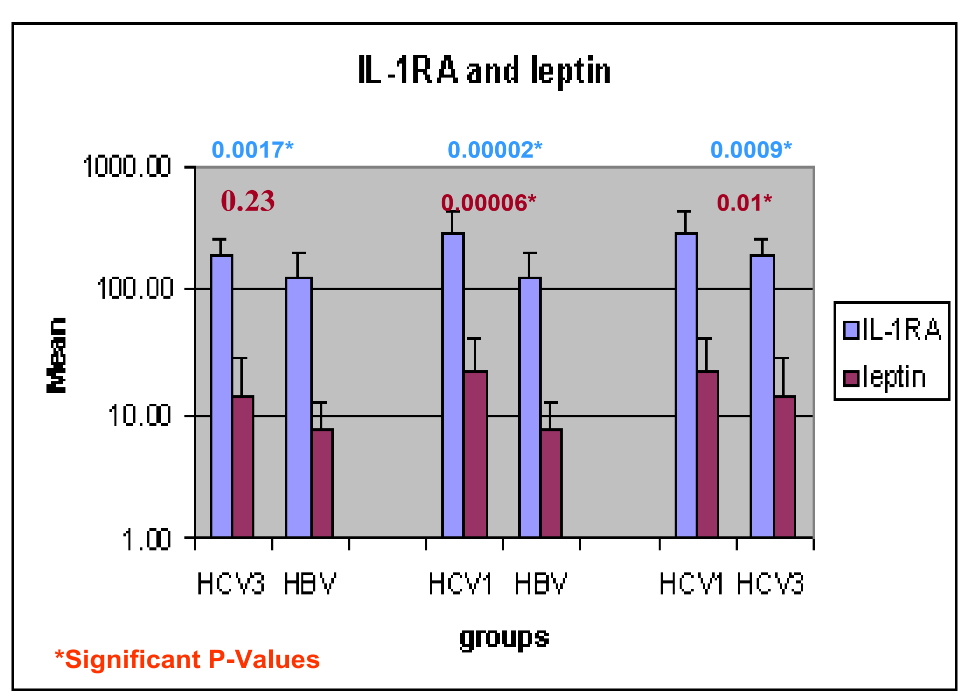

Figure 19. The significant differences in averages of serum levels for IL-1ra and Leptin between patient groups with different HCV genotypes with or without Steatosis compared to NAFLD-type steatosis group. *P-value ≤ 0.01 are considered significant and highlighted in red. SS: Simple Steatosis.

An increase in IL-1Ra production is both positive and negative for the health. IL-1Ra is a negative regulator to IL-1 signaling and is considered hepatoprotective and anti-inflammatory as it competitively binds to and blocks the functional receptor (IL-1 receptor type-I) without triggering its activation (Arend et al., 1998; Dinarello et al., 1998). So IL-1Ra could affects IL-1 activity, but not its expression level or serum level. That might explain why IL-1B serum levels were not different in any of the groups compared. Other probability is that IL-1Ra agonistic effect is more specific to IL-1A, other form of IL-1 cytokine.

Luheshi and colleagues (1999) have found that IL-1Ra is increased in the serum of obese patients and is correlated with BMI and insulin resistance. They proposed that it induces Leptin resistance by antagonizing the action of Leptin at the hypothalamic level (Luheshi et al., 1999). The role of IL-1Ra as a regulator of adipogenesis, food intake, and energy expenditure was further substantiated by showing that IL-1Ra (-/-) mice have a lean phenotype (Somm et al., 2005), and on the other hand that when IL-1Ra deficient mice IL-1Ra (-/-) were fed with atherogenic diet for 20 weeks their livers showed severe steatosis and pericellular fibrosis and contained many inflammatory foci (Sawada et al., 2006).

However, in our results, the highly significant differences in IL-1RAa serum levels were mainly seen when HCV-G1 groups with either steatosis or without were compared to HCV-G3 group with no steatosis (Figure 16). There were no significant differences in IL-1Ra levels between HCV-G1 groups of patients with or without steatosis, or between HCV-G3 groups with steatosis and without. From these results, we may deduce that IL-1Ra in conjunction with Leptin is more directly influenced by HCV-G1 virus, and that they could contribute to steatosis development in patients infected by the virus with this genotype.

IL-6 is a pleiotropic cytokine involved in the regulation of many inflammatory, immunologic and metabolic processes (Reviewed in Taga & Kishimoto, 1997). IL-6 exerts its biological activities by binding to the alpha subunit (gp 80) of sIL-6r receptor, then homodimerized with the larger subunit gp130 that is mainly involved in down-stream signaling processes by binding tyrosine kinases and activating STAT1 and STAT3

transcription factors. As a result of either proteolytic cleavage or by alternative splicing of the membrane moiety of the alpha subunit (gp 80) of the receptor (IL-6r), a soluble form called sIL-6r can be found in biological fluids such as serum and urine (Thomson, 1991; Aggerwal & Gutterman, 1992). Apoptosis is a natural stimulus of sIL-6r shedding (Chalaris et al., 2007). Even though some data indicate that sIL-6r is upregulated in female ethanol consuming animals (Randle M et al., 2004), it is not clearly known if there is a relation between sIL-6r activation and liver steatosis.

In our measurement, we show that there are significant differences in serum levels of sIL-6r between HCV-G3 patient groups and HBV patient groups as well as HCV–G1 regardless of the presence or absence of steatosis (Figure 17). These differences are especially prominent in HCV-G1 and HBV patient groups compared to HCV-G3 groups. On the other hand, serum levels of IL-6 were higher in HCV-G3 patient groups with steatosis compared to HCV-G1 with steatosis. The limited agonistic effect of sIL-6r on IL-6 in the presence of steatosis might indicate that steatosis is influenced by factors other than IL-6, or that the steatosis itself might directly influence IL-6 serum levels. This data also sheds light on the possibility that the HCV-G1 virus itself plays a direct role in modulating serum levels of sIL-6r by lowering it. This conclusion was emphasized by the fact that there were no significant differences in serum levels of sIL-6r between HCV-G3 groups with steatosis compared to HCV-G3 without steatosis, nor between HCV-G1 group with steatosis compared to HCV-G1 without steatosis. It is prossible that the sIL-6r agonist effect is more influential in modulating the pleiotrpic functions of IL-6 rather than its serum levels or that this agonist effect is also influenced by presence of steatosis.

SIL-6r can bind its ligand and induce cellular responses by association with gp130 even in cells that express only the membrane gp130, thus, acting as an IL-6 agonist. The association of IL-6 with the soluble form of sIL-6r-α (gp 80) is capable of cellular activation, called "trans-signalling". In trans-signalling, cells that are capable of responding to IL-6/sIL-6r-alpha complexes add a whole new spectrum of IL-6 activities (John & Neurath, 2004). For example, IL-6 can function as a pro-inflammatory cytokine by enhancing leukocyte recruitment by up-regulating chemokine production and adhesion molecule expression (Modur et al., 1997; Biswas et al., 1998; Romano et al., 1997) and has been implicated in insulin resistance. In contrast, IL-6 has been shown to inhibit TNF-α expression (Benveniste et al., 1995) or induce the expression of soluble TNF-α receptors and the IL-1R antagonist thus acting as anti-inflammatory cytokine (Tilg et al., 1994). IL-6 with its agonist sIL-6r has been shown to have role in liver regeneration and reversing severe hepatocellular injury (Galun et al., 200). The protective function of IL-6 in steatotic livers relies on suppression of ethanol-induced oxidative stress and mitochondrial dysfunction (Sun et al., 2003, El-Assal et al.,2004) and preventing the release of reactive oxygen species (ROS), mitochondrial permeability transition (MPT), and ethanol-mediated depletion of adenosine triphosphate (ATP) (El-Assal et al.,2004). IL-6 also provides hepatoprotection in ischaemic preconditioning models (Teoh et al., 2006) and prevents obesity- and alcohol-associated fatty liver transplant failure in rats (Sun et al., 2003, Gao, 2004).

In conclusion, the biochemical interactions between metabolic and inflammatory processes influence NAFLD development and pathogenesis. The dual roles of many

adipocytokines in both processes complicate profiling them as pro-NAFLD and anti-NAFLD molecular factors. However, the changes in the serum level of certain adipocytokines are a more prominent feature of certain types of NAFLD, emphasizing their distinguished role in establishing the NAFLD phenotype and so the hope to use these profiles for diagnostic purposes.

Future studies should focus on identifying molecular links between inflammatory process and changes in the metabolism that promote the development of NAFLD.

Appendices

Table 1: Breif summary of site of production, targets, functions and regulators of major adipocytokines described in this thesis.

Adipokine	Sites of Production	Sites of action	Level in MS/ NAFL	Functional role in NAFLD	Positive regulators	Negative regulators
Adiponectin	Mainly in adipose, but also from skeletal muscle cells, cardiac myocytes and endothelial cells.	heart, muscles, liver	↓	Anti-oxidant Ani-inflamatory, anti-diabetic, Anti-fibrotic, anti-atherosclerotic. Suppresses IR by activation of AMPK	PPAR γ agonists TZDs	PGD2 Insulin TNF-alpha IL-6 Testosterone
Leptin	Mainly in Adipose, but also in placenta, bone marrow, stomach, gastric epithelium, muscle placenta and CNS	Mainly hypothalamus, CNS, pancreas beta and delta cells, probably stomach, intestine, liver,	variable	Pro-oxidant, pro-inflammatory. Neuroendocrine hormone effects the reproductive system,immune system, hemopoietic system, autonomic nervous system, bone development and hypothalamo-pituitary-adrenal and thyroidal axes. Pro-Fibrogenic. IR suppression by activation of AMPK. Also activate JAK/STAT signaling pathway, which leads to stimulation of phagocytosis, production of oxygen and nitrogen reactive species, and also to increase in secretion of pro-inflammatory cytokines.	Insulin, glucocorticoids, TNF-alpha, estrogens and Catecholamines	PGD2 Alpha-MSH. TZDs Insulin, sex steroids, Glucocorticoids FFAs, Growth hormone

Resistin	Mainly in adipose, but also in muscle, pancreatic cells and mononuclear cells	muscle, liver, vascular system	?	Possibly pro-oxidant, pro-inflamatory as it ↑ TNF,↑ IL-1β ↑ IL-6, ↑ IL-12 ↑ NF-κB, ↑ Endothelial adhesion molecules (VCAM1). Possibly fibrogenic and involved in insulin resistance; difficult to study in humans	pro-inflammatory cytokines IL-1, IL-6 TNF LPS	TZDs Pituitary, Steroid & thyroid hormones adrenaline β3-Adrenoreceptor activation, NF-κB inhibitor
Visfatin	adipose, skeletal muscles, liver, osteoblasts	pancreas, vascular system, lungs	variable	Unclear of oxidative effect, Probably pro-inflamatory as it ↑ IL-6 ↑ IL-8, ↓ Apoptosis of Neutrophils, Unknown Fibrogenic effect. Insulin mimetic, it binds to and activates the insulin receptor on different sites from the insulin	Macrostemonoside A. Hypoxia Dexamethasone	IL-6 TNF alpha Growth hormone
TNF-α	adipose, macrophages, monocytes, T & B cells, NK cells	liver, muscles ,adipose, leukocytes	↑	Pro-oxidant, pro-Fibrogenic. Impairs insulin signaling: Induce production of IKKB Or JNK1 or by phosphorylating IRS1 directly or through ceramides	Resistin, Leptin stress factors LPS ROS 15-d-PGJ_2	Thalidomide Adalimumab Etanercept PGE2 15-d-PGJ_2
IL-6	adipose tissue, astrocytes fibroblasts, macrophages, endothelial cells, T cells immune cells (T-cells), skeletal muscle	spleen CNS brain T & B cells, thymocytes, hepatocytes, neurons macrophages	↑	Pro-inflammatory, defense mechanisms, regulate thermogenesis and estrogen metabolism Neurotrophi, adipocyte differentiation marker Differentiation of myeloid cells, induction of acute phase proteins, tropic for neurons. Probably pro-fibrogenic. Play role in insulin resistance (exerts long term inhibitory effects on the gene transcription	PGD2 TNF-α Leptin LPS Catecholamines Insulin IL-1B sIL-6r	PI3K inhibitors Akt inhibitors Glucocorticoids statins

				of IRS-1, GLUT-4, and peroxisome proliferator-activated receptor γ) mostly trough SOCS-3		
IL-1Beta	macrophages, endothelial cells, keratinocytes, lymphocytes, fibroblasts, osteoblasts	CNS monocytes macrophages B cells	**?**	Unknown role in insulin resistance. Proinflammatory, stimulates fibroblasts and bone catabolism, neuroendocrine effects (fever, sleep, anorexia, corticotropin release). Unknown Fibrogenic effect	TNF-α 15-d-PGJ_2	15-d-PGJ_2
IL-8	monocytes, neutrophils, fibroblasts, endothelial cells, keratinocytes, T cells	Neutrophils, basophils, T cells, endothelial cells	↑	Possibly pro-insulin resistance, Proinflammatory, activates neutrophils, enhances keratinocyte growth, stimulate endothelial cells to attract monocytes, promote vascular smooth muscle cell migration, Pro-atherogenesis, Probably pro-fibrogenic.	IL-8 15-d-PGJ2 NF-κß ROS	NF-κß Inhibitors statins

Table 2. Genes identified from comparison of adipose of obese NASH patients (N=10) vs. adipose of non-Obese Controls (N=9). MnOBNASH: Mean of gene expression in Obese NASH. MnCTRLS=Mean of gene expression in normal controls.

Accession #	GENE NAME	GENE FUNCTION /ADJACENT GENE FUNCTION	MnOBNASH/ MnCTLS
AI371874	EST, 3' area of TLR4	Toll-like receptor 4	0.19
AA975301	CALCRL	calcitonin receptor-like	0.27
AA995128	FIGF	c-fos induced growth factor (vascular endothelial growth factor D)	0.31
AI123732	EBI2	Epstein-Barr virus induced gene 2 (lymphocyte-specific G protein-coupled receptor)	0.34
W72103	SPTBN1	spectrin beta, non-erythrocytic 1	0.36
AI092288	PAX9	paired box gene 9	0.36
AA425649	DMD	dystrophin (muscular dystrophy, Duchenne and Becker types)	0.37
AA425545	DCUN1D1	DCN1, defective in cullin neddylation 1, domain containing 1	0.37
R59192	HLF	hepatic leukemia factor	0.4
AI375048	ADAMTS5	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 5 (aggrecanase-2)	0.41
H69878	EST, 3' area of RARB	retinoic acid receptor, beta	0.43
H99427	PTPRM	protein tyrosine phosphatase, receptor type, M	0.43
AA181307	AHR	aryl hydrocarbon receptor	0.44
N93476	EST, 3' area of EDG1	endothelial differentiation, sphingolipid G-protein-coupled receptor1	0.45
AA928584	AASS	aminoadipate-semialdehyde synthase	0.46
AI002403	COX7A2	cytochrome c oxidase subunit VIIa polypeptide 2 (liver)	0.46
AI262129	EST, 3' area of POSTN	periostin, osteoblast specific factor	0.46
AI350508	FAM107A	Family with sequence similarity 107, member A	0.46
T53302	MAN2A2	mannosidase, alpha, class 2A, member 2	0.46
AA086038	PLA2R1	phospholipase A2 receptor 1, 180kDa	0.47
AA620426	AMPH	amphiphysin (Stiff-Man syndrome with breast cancer 128kDa autoantigen)	0.47
AI268937	EST, 3' area of CCL8	chemokine (C-C motif) ligand 8	0.47
AI356028	GPRC5B	G protein-coupled receptor, family C, group 5, member B	0.48
AA179391	OXCT	3-oxoacid CoA transferase	0.49
AA282092	GTF2F1	general transcription factor IIF, polypeptide 1, 74kDa	2
R56251	EMG1	EMG1 nucleolar protein homolog	2
AA497029	LDHA	lactate dehydrogenase A	2.01

AA598470	DDX23	DEAD (Asp-Glu-Ala-Asp) box polypeptide 23	2.01
AA669452	EIF2S1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	2.01
AI284347	UMOD	uromodulin (uromucoid, Tamm-Horsfall glycoprotein)	2.01
AI358848	MARS	methionine-tRNA synthetase	2.01
AA016290	RBBP6	retinoblastoma binding protein 6	2.02
W92769	CAPZA1	capping protein (actin filament) muscle Z-line, alpha 1	2.02
AA436374	TMED4	Transmembrane emp24 protein transport domain containing 4	2.04
AA876147	EST, 3' area of IRAK2	interleukin-1 receptor-associated kinase 2	2.04
AA987261	ECE2	endothelin converting enzyme 2	2.04
AI279788	EST, 3' area and intron of ARIH2	ariadne homolog 2	2.04
AA058323	IFITM1	interferon induced transmembrane protein 1 (9-27)	2.06
R02346	SNRP70	small nuclear ribonucleoprotein 70kDa polypeptide (RNP antigen)	2.06
AA280677	ZMYM6/ZNF258	Zinc finger, MYM-type 6	2.07
H01164	STK17A	serine/threonine kinase 17a (apoptosis-inducing)	2.07
H63077	ANXA1	annexin A1, 1565	2.07
AI339502	PRAMEF8	PRAME family member 8 and genes similar to it (LOC653622, LOC390999)	2.07
AA070358	TKT	transketolase (Wernicke-Korsakoff syndrome)	2.08
AA465723	PPM1G	protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform	2.08
AA285043	EIF2S3	eukaryotic translation initiation factor 2, subunit 3 gamma, 52kDa	2.08
AA479977	KLHL21	Kelch-like 21	2.08
AA775241	ALDOA	aldolase A, fructose-bisphosphate	2.08
AI018066	CYLC2	cylicin, basic protein of sperm head cytoskeleton	2.08
H65334	YLPM1	YLP motif containing 1	2.08
H91169	BRD8	bromodomain containing 8	2.08
AA434391	NAB2	NGFI-A binding protein 2 (EGR1 binding protein 2)	2.09
N55461	EPB49	erythrocyte membrane protein band 4.9 (dematin)	2.09
H96775	PRKAA1	protein kinase, AMP-activated, alpha 1 catalytic subunit	2.09
R26732	PMP22	peripheral myelin protein 22	2.1
AI077350	C19orf37	Chromosome 19 open reading frame	2.1

		37	
H90147	BCL7A	B-cell CLL/lymphoma 7A	2.1
AA775803	PRDX1	peroxiredoxin 1	2.11
AI160964	DSC1	desmocollin 1	2.11
H56918	EIF4A1	eukaryotic translation initiation factor 4A, isoform 1	2.12
AA411202	AHSA1	AHA1, activator of heat shock 90kDa protein ATPase homolog 1	2.12
AI021885	PROC	protein C (inactivator of coagulation factors Va and VIIIa)	2.12
AA885311	BCHE	butyrylcholinesterase	2.13
H62527	YWHAB	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide	2.13
W88942	EST, not supported by gene evidences	not applicable	2.13
AA406326	EST, 3' area of DKFZp686K16132	similar to BMP2 inducible kinase	2.13
AI129421	IL18	interleukin 18 (interferon-gamma-inducing factor)	2.14
AI365246	WSB1	WD repeat and SOCS box-containing 1	2.14
R32739	EST, not supported by gene evidences	not applicable	2.14
R69522 | AI732288	RAB27B	RAB27B, member RAS oncogene family	2.14
H78609	EST, 3' area of LOC220729	similar to Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial precursor (Fp) (Flavoprotein subunit of complex II)	2.15
T60068	MSN	moesin	2.15
AA598955	TNC	tenascin C (hexabrachion)	2.15
R20625	EVL	Enah/Vasp-like	2.16
R80790	UBE2C	ubiquitin-conjugating enzyme E2C	2.16
H23255	SERINC3	Serine incorporator 3	2.17
W90520	ZNF652	Zinc finger protein 652	2.18
AA488173	YY1AP1	YY1 associated protein 1	2.18
AI127671	ENDOGL1	endonuclease G-like 1	2.18
AA055504	TRIM32	tripartite motif-containing 32	2.23
AA130017	CLU	clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)	2.23
N58130	EST in the intron of CG016 transcript	not applicable	2.23
T61078	CPS1	carbamoyl-phosphate synthetase 1, mitochondrial	2.24
AA633818	EST in the 3' area of ACSL4 and three other	acyl-CoA synthetase long-chain family member 4	2.24

	intergenic and intronic hits		
R20646	EHD1	EH-domain containing 1	2.24
AI680557	COL4A6	collagen, type IV, alpha 6	2.25
AI583623	SFRS10	splicing factor, arginine/serine-rich 10 (transformer 2 homolog,Drosophila)	2.26
N53169	APOC3	apolipoprotein C-III	2.27
AA521416	LOC390284	similar to signal recognition particle 14kDa (homologous to Alu RNA binding protein)	2.27
T47483	PPID	peptidylprolyl isomerase D (cyclophilin D)	2.27
AA398233	LAPTM4A	lysosomal-associated protein transmembrane 4 alpha	2.28
H56428	LOC644582 and 3 other intronic hits	similar to Ribosome biogenesis protein BMS1 homolog	2.28
AA598950	CTSB	cathepsin B	2.29
AA099408	No hits in the human genome	not applicable	2.29
AA598990	No hits in the human genome	not applicable	2.29
AA928058	COX11	cytochrome c oxidase assembly protein COX11	2.29
AI376113	TINP1	TGF beta-inducible nuclear protein 1	2.29
T56880	AFP	alpha-fetoprotein	2.29
H50677	RBM6	RNA binding motif protein 6	2.3
AA878951	ADAM17	a disintegrin and metalloproteinase domain 17 (tumor necrosis factor,alpha, converting enzyme)	2.3
H81046	ASNA1	arsA arsenite transporter, ATP-binding, homolog 1 (bacterial)	2.31
H91167	HELLS	helicase, lymphoid-specific	2.33
N95243	MEIS1	Meis1, myeloid ecotropic viral integration site 1 homolog (mouse)	2.33
H62527	YWHAB	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein,beta polypeptide	2.34
AA031770	NUDT21	Nudix (nucleoside diphosphate linked moiety X)-type motif 21	2.36
AA877213	CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	2.36
AI474891	SFRS9	Splicing factor, arginine/serine-rich 9	2.36
AI261401	PLA2G4B	phospholipase A2, group IVB (cytosolic)	2.37
AA099554	ADAM12	a disintegrin and metalloproteinase domain 12 (meltrin alpha)	2.38
AA490466	GJB2	gap junction protein, beta 2, 26kDa (connexin 26)	2.38
AA056465	NONO	non-POU domain containing, octamer-binding,	2.38

AA668457	TYRP1	tyrosinase-related protein 1	2.38
W56356	PEG10	paternally expressed 10	2.38
AA424956	EIF2S1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	2.39
AA402907	ROCK2	Rho-associated, coiled-coil containing protein kinase 2	2.4
AI380324	PLK4	polo-like kinase 4	2.4
AA676484	CAPNS1	calpain, small subunit 1	2.41
AA451895	ANXA5	annexin A5	2.43
AI365571	RFXAP	regulatory factor X-associated protein	2.43
AI218585	MRPL4	mitochondrial ribosomal protein L4	2.44
AI240559	TCF2	transcription factor 2, hepatic, LF-B3, variant hepatic nuclear factor	2.44
H18068	PKN1	Protein kinase N1	2.44
AA984728	KIF5A	kinesin family member 5A	2.45
R99354	LEREPO4	likely ortholog of mouse immediate early response, erythropoietin 4	2.46
AI094611	FADS2	Fatty acid desaturase 2	2.47
AI383794	PRMT3	protein arginine N-methyltransferase 3	2.48
AA132090	CD53	CD53 antigen	2.49
AA464528	PLP2	proteolipid protein 2 (colonic epithelium-enriched), 612	2.49
AA424938	PRKCA	protein kinase C, alpha, 206	2.49
T98491	EST, intronic hit in ACTN4	actinin, alpha 4	2.49
H15246	HECW1	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 1	2.5
AI360761	ARPC4	Actin related protein 2/3 complex, subunit 4, 20kDa	2.51
AI139968	SLC26A4	Solute carrier family 26, member 4	2.53
AI285199	CCL20	chemokine (C-C motif) ligand 20	2.53
AI361349	SLC43A3	solute carrier family 43, member 3	2.53
AI263095	RDX	radixin	2.54
AI219155	CACNA1C	Calcium channel, voltage-dependent, L type, alpha 1C subunit	2.58
AI360108	DLL1	delta-like 1	2.58
H15095	CXorf40B	Chromosome X open reading frame 40B	2.61
AI050071	MEGF8	Multiple EGF-like-domains 8	2.61
R26462	Integenic hits	not applicable	2.61
AA916906	TRADD	TNFRSF1A-associated via death domain	2.62
AA031770	NUDT21	Nudix (nucleoside diphosphate linked moiety X)-type motif 21	2.64
AA775378	MGAT1	mannosyl (alpha-1,3-)-glycoproteinbeta-1,2-N-acetylglucosaminyltransferase	2.65

AI478107	TMEM123/PORIMIN	Transmembrane protein 123/ pro-oncosis receptor inducing membrane injury gene	2.65
AA988615	HLA-F	major histocompatibility complex, class I, F, 266	2.66
AA031770	NUDT21	Nudix (nucleoside diphosphate linked moiety X)-type motif 21	2.67
AI218994	HBXIP	Hepatitis B virus x interacting protein, 401	2.67
AI263109	SFRS5	Splicing factor, arginine/serine-rich 5	2.68
H40681	MYD88	myeloid differentiation primary response gene (88)	2.7
AA995464	NIPSNAP1	nipsnap homolog 1 (C. elegans)	2.7
AI270906	KLF12	Kruppel-like factor 12	2.74
AA598517	KRT8	keratin 8	2.76
AA487893	TM4SF1	Transmembrane 4 L six family member 1	2.78
AI359519	CCL26	chemokine (C-C motif) ligand 26	2.78
AI129113	BIRC4BP	XIAP associated factor	2.82
AI261561	INPP5B	Inositol polyphosphate-5-phosphatase, 75kDa	2.82
AI174255	KCNH3	potassium voltage-gated channel, subfamily H (eag-related), member 3	2.83
AA962407	YWHAB	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein,beta polypeptide	2.86
AI345617	EST, two intergenic hits	not applicable	2.88
AA670408	B2M	beta-2-microglobulin	2.94
AI457695	CGI-115	CGI-115 protein	2.98
AA668520	NARS	asparaginyl-tRNA synthetase	2.99
AI049675	GFRA1	GDNF family receptor alpha 1	3.03
AA995045	MAGEA6	melanoma antigen, family A, 6	3.07
AI201344	SUZ12P	Suppressor of zeste 12 homolog pseudogene	3.08
AA464250	KRT17	keratin 17	3.12
AA913480	KLRC1	Killer cell lectin-like receptor subfamily C, member 1	3.25
AI313387	F3	coagulation factor III (thromboplastin, tissue factor)	3.26
R64359	LIN28B	Lin-28 homolog B	3.3
AI219333	MAN1C1	mannosidase, alpha, class 1C, member 1	3.32
AI393043	GNAT2	guanine nucleotide binding protein (G protein), alpha transducingactivity polypeptide 2	3.32
AI076396	EST, intronic hit in CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	3.33
AA911236	MYBL1	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	3.38

AI129891	EST, intronic hit in RCC1	regulator of chromosome condensation 1	3.4
AI018607	SULT1A2	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 2	3.44
AI200443	MAGEA10	Melanoma antigen family A, 10	3.49
N39101	EST, intergenic hit	not applicable	3.66
AI340883	TACSTD1	tumor-associated calcium signal transducer 1	3.89
AI369218	NAPSA	Napsin A aspartic peptidase	3.93
H92070	SKI	V-ski sarcoma viral oncogene homolog (avian)	4.16
H73261	Intronic hit in WDR25	WD repeat domain 25	4.68

Table 3. Genes identified from comparison of adipose of obese controls (N=17) vs. adipose of non-obese controls (N=9). MnOBnonNASH: Mean of gene expression in obese-non NASH. MnCTRLS: mean of gene expression in normal controls.

Accession #	GENE NAME	GENE FUNCTION	MnOBnon NASH /MnCTRLS
AI371874	EST in the 3' area of TLR4	toll-like receptor 4	0.2
AA995128	FIGF	c-fos induced growth factor (vascular endothelial growth factor D)	0.26
AI123732	EBI2	Epstein-Barr virus induced gene 2 (lymphocyte-specific G protein-coupled receptor)	0.26
AA991578	VMD2	vitelliform macular dystrophy (Best disease, bestrophin)	0.28
AA975301	CALCRL	calcitonin receptor-like	0.35
AI375048	ADAMTS5	a disintegrin-like and metalloprotease (reprolysin type) withthrombospondin type 1 motif, 5 (aggrecanase-2)	0.36
R53548	FLJ23703	hypoth prot FLJ23703	0.36
AA425545	DCUN1D1	DCN1, defective in cullin neddylation 1, domain containing 1	0.36
AI206447	PGDS	prostaglandin D2 synthase, hematopoietic	0.39
W96155	JUN	v-jun sarcoma virus 17 oncogene homolog	0.4
AI268937	CCL8	chemokine (C-C motif) ligand 8	0.4
AA009609	FGF7	fibroblast growth factor 7 (keratinocyte growth factor)	0.41
AA476508	ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	0.41
AI349250	FCN1	ficolin (collagen/fibrinogen domain containing) 1	0.43
R10285	HMMR	hyaluronan-mediated motility receptor (RHAMM)	0.44
AI022531	PTPRB	protein tyrosine phosphatase, receptor type, B	0.44
AA425649	DMD	dystrophin (muscular dystrophy, Duchenne and Becker types)	0.47
AI362933	DAPK2	death-associated protein kinase 2	0.48
AA181307	AHR	aryl hydrocarbon receptor	0.49
H69878	RARB	retinoic acid receptor, beta	0.5
T53302	MAN2A2	mannosidase, alpha, class 2A, member 2	0.5
AA458981	PTMS	parathymosin	2
AA987261	ECE2	endothelin converting enzyme 2	2
AI085840	Multiple hits in the human genome	not applicable	2.01
R42785	LHFP	Lipoma HMGIC fusion partner	2.01
T56880	AFP	alpha-fetoprotein	2.01

T63779	KLF9	Kruppel-like factor 9	2.01
H17334	CBX6	chromobox homolog 6	2.03
AA451895	ANXA5	annexin A5	2.04
AA916906	TRADD	TNFRSF1A-associated via death domain	2.05
AA995464	NIPSNAP1	nipsnap homolog 1 (C. elegans)	2.05
AI218585	MRPL4	mitochondrial ribosomal protein L4	2.06
AI348451	FLJ33790	hypoth prot FLJ33790, 66	2.06
AA031770	NUDT21	Nudix (nucleoside diphosphate linked moiety X)-type motif 21	2.08
AA046701	ATP5G1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c(subunit 9), isoform 1	2.08
AA056465	NONO	non-POU domain containing, octamer-binding	2.08
AA424938	PRKCA	protein kinase C, alpha	2.08
H50677	RBM6	RNA binding motif protein 6	2.08
H18068	PKN1	Protein kinase N1	2.09
T64192	PRSS1	Protease, serine, 1 (trypsin 1)	2.1
AA282219	ECE1	Endothelin converting enzyme 1	2.1
AA664135	RPP38	Ribonuclease P/MRP 38kDa subunit	2.1
N95243	MEIS1	Meis1, myeloid ecotropic viral integration site 1 homolog	2.1
R53966	CHN1	chimerin (chimaerin) 1	2.1
AA279662	SNRPB	small nuclear ribonucleoprotein polypeptides B and B1	2.11
AI376113	TINP1	TGF beta-inducible nuclear protein 1	2.11
H81304	FAM63A	Family with sequence similarity 63, member A	2.11
H40681	MYD88	myeloid differentiation primary response gene (88)	2.12
R56251	EMG1	EMG1 nucleolar protein homolog	2.13
AA872379	SUMO3	SMT3 suppressor of mif two 3 homolog 3	2.14
N53169	APOC3	apolipoprotein C-III	2.14
W80701	No hits in the human genome	not applicable	2.16
AA877213	CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	2.16
AA029997	COL4A5	collagen, type IV, alpha 5 (Alport syndrome)	2.17
AA459947	SNRP70	small nuclear ribonucleoprotein 70kDa polypeptide (RNP antigen)	2.17
AA406326	DKFZp686K1 6132	similar to BMP2 inducible kinase	2.19
AA504461	LDLR	low density lipoprotein receptor (familial hypercholesterolemia)	2.2
N26562	MLANA	Melan-A	2.2
N64014	PIP5K1A	phosphatidylinositol-4-phosphate 5-kinase, type I, alpha	2.2
AI338791	SH2D1A	SH2 domain protein 1A, Duncan's disease (lymphoproliferative syndrome)	2.21

AA885311	BCHE	butyrylcholinesterase	2.22
AA434391	NAB2	NGFI-A binding protein 2 (EGR1 binding protein 2)	2.25
H90906	EST, 5’ non-coding area of CHDH	choline dehydrogenase	2.25
AA461467	ODC1	ornithine decarboxylase 1	2.26
AA977307	NME6	non-metastatic cells 6, protein expressed in (nucleoside-diphosphatekinase)	2.26
AI583623	SFRS10	splicing factor, arginine/serine-rich 10 (transformer 2 homolog,Drosophila)	2.27
AA031770	NUDT21	Nudix (nucleoside diphosphate linked moiety X)-type motif 21	2.28
H19109	KLHL7	Kelch-like 7	2.28
T47483	PPID and other hits	peptidylprolyl isomerase D (cyclophilin D)	2.28
AA112979	VRK1	vaccinia related kinase 1	2.29
AI023541	CA9	carbonic anhydrase IX	2.29
AI479347	MAGEB2	melanoma antigen, family B, 2	2.29
AA031770	NUDT21	Nudix (nucleoside diphosphate linked moiety X)-type motif 21	2.3
AA426352	No hits in the human genome	not applicable	2.31
AI383794	PRMT3	protein arginine N-methyltransferase 3	2.32
W87751	TIMELESS	timeless homolog (Drosophila)	2.33
AI493835	Est, intronic hit in HFM1 and two other hits	HFM1, ATP-dependent DNA helicase homolog	2.35
R20646	EHD1	EH-domain containing 1	2.35
AA775241	ALDOA	aldolase A, fructose-bisphosphate	2.36
AA521388	RUVBL1	RuvB-like 1 (E. coli)	2.36
AA280677	ZMYM6/ZNF258	Zinc finger, MYM-type 6	2.37
AA099408	No hits in the human genome	not applicable	2.37
AA404284	IL1RAP	interleukin 1 receptor accessory protein	2.37
AA436374	TMED4	Transmembrane emp24 protein transport domain containing 4	2.37
T62635	SRP72	signal recognition particle 72kDa	2.39
AA465723	PPM1G	protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform	2.42
H81046	ASNA1	arsA arsenite transporter, ATP-binding, homolog 1 (bacterial)	2.43
H77332	TRIM58	Tripartite motif-containing 58	2.44
H91167	HELLS	helicase, lymphoid-specific	2.45
AI263095	RDX	radixin	2.48
H15246	HECW1	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 1	2.5
R99354	LEREPO4	likely ortholog of mouse immediate early	2.5

		response, erythropoietin 4	
AI077350	C19orf37	Chromosome 19 open reading frame 37	2.51
AI160964	DSC1	desmocollin 1	2.51
R64640	EST, intronic hit to SPTLC2 protein	SPTLC2 protein	2.51
W90506	U2AF1	U2(RNU2) small nuclear RNA auxiliary factor 1	2.52
AI680557	COL4A6	collagen, type IV, alpha 6	2.53
AA775378	MGAT1	mannosyl (alpha-1,3-)-glycoproteinbeta-1,2-N-acetylglucosaminyltransferase	2.54
N27147	DCT	dopachrome tautomerase (tyrosine-related protein 2)	2.54
R26462	Intergenic hits	not applicable	2.56
T60068	MSN	moesin	2.57
H27912	KDELR1	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 1	2.61
R20625	EVL	Enah/Vasp-like	2.62
AI474891	SERS9	Splicing factor, arginine/serine-rich 9	2.65
AA913480	KLRC1	Killer cell lectin-like receptor subfamily C, member 1	2.66
AI361349	SLC43A3	solute carrier family 43, member 3	2.68
AI380324	PLK4	polo-like kinase 4 (Drosophila)	2.68
T61078	CPS1	carbamoyl-phosphate synthetase 1, mitochondrial	2.69
AA056465	NONO	non-POU domain containing, octamer-binding	2.71
N59790	NFX1	nuclear transcription factor, X-box binding 1	2.73
T98491	EST, intronic hit in the ACTN4	actinin, alpha 4	2.74
AI203233	WDHD1	WD repeat and HMG-box DNA binding protein 1	2.75
AI270906	KLF12	Kruppel-like factor 12	2.76
AA031770	NUDT21	Nudix (nucleoside diphosphate linked moiety X)-type motif 21	2.78
AA878951	ADAM17	a disintegrin and metalloproteinase domain 17 (tumor necrosis factor,alpha, converting enzyme)	2.8
AA633818	EST in the 3' area of ACSL4 and three other intergenic and intronic hits	acyl-CoA synthetase long-chain family member 4	2.84
AA775241	ALDOA	aldolase A, fructose-bisphosphate	2.86
AI345617	Two hits in hypothetic transcripts	not applicable	2.87
AA285043	EIF2S3	eukaryotic translation initiation factor 2,	2.89

		subunit 3 gamma, 52kDa	
AI050071	MEGF8	Multiple EGF-like-domains 8	2.9
AI240559	TCF2	transcription factor 2, hepatic, LF-B3, variant hepatic nuclear factor	2.9
AI478107	TMEM123/PORIMIN	Transmembrane protein 123 (pro-oncosis receptor inducing membrane injury gene)	2.95
H56428	LOC644582 and 3 other intronic hits	similar to Ribosome biogenesis protein BMS1 homolog	2.96
H73261	Introinic and intergenic hits in WDR25	WD repeat domain 25	2.97
AI018607	SULT1A2	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 2	2.99
AI200443	MAGEA10	Melanoma antigen family A, 10	2.99
R69603	OSTM1	Osteopetrosis associated transmembrane protein 1	3
AA962407	YWHAB	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide	3.08
AA775241	ALDOA	aldolase A, fructose-bisphosphate	3.09
AA464250	KRT17	keratin 17	3.13
AA668520	NARS	asparaginyl-tRNA synthetase	3.15
H92070	SKI	V-ski sarcoma viral oncogene homolog (avian)	3.18
W56356	PEG10	paternally expressed 10	3.18
AA668457	TYRP1	tyrosinase-related protein 1	3.19
W92769	CAPZA1	capping protein (actin filament) muscle Z-line, alpha 1	3.22
AA598955	TNC	tenascin C (hexabrachion)	3.25
AI201344	SUZ12P	Suppressor of zeste 12 homolog pseudogene	3.25
AI263109	SFRS5	Splicing factor, arginine/serine-rich 5	3.26
AI393043	GNAT2	guanine nucleotide binding protein (G protein), alpha transducingactivity polypeptide 2	3.27
AI362866	PTTG1	pituitary tumor-transforming 1, 329	3.31
R34327	No hits in human genome	NA	3.36
AI174255	KCNH3	potassium voltage-gated channel, subfamily H (eag-related), member 3	3.43
AA911236	MYBL1	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	3.62
AI457695	CGI-115	CGI-115 protein	3.63
AI000677	ST3GAL1	ST3 beta-galactoside alpha-2,3-sialyltransferase 1	3.68
AI139968	SLC26A4	Solute carrier family 26, member 4	3.69
H65334	YLPM1	YLP motif containing 1	3.71
AA424956	EIF2S1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	3.74

AI049675	GFRA1	GDNF family receptor alpha 1	3.79
AI340883	TACSTD1	tumor-associated calcium signal transducer 1	3.79
AI219333	MAN1C1	mannosidase, alpha, class 1C, member 1	3.84
AI261561	INPP5B	Inositol polyphosphate-5-phosphatase, 75kDa	3.87
AA521416	LOC390284	similar to signal recognition particle 14kDa (homologous Alu RNA binding protein)	3.95
AI219155	CACNA1C	Calcium channel, voltage-dependent, L type, alpha 1C subunit	4.14
AI129891	RCC1	regulator of chromosome condensation 1	4.26
AI369218	NAPSA	Napsin A aspartic peptidase	4.35
AI076396	EST, intronic hit in CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	4.36
AA598990	No hits in the human genome	not applicable	4.76
R64359	LIN28B	Lin-28 homolog B (C. elegans)	5.42
AA995045	MAGEA6	melanoma antigen, family A, 6	6.73

Table 4. Genes identified from comparison of liver biopsies of obese patients with NASH (N=27) vs. liver biopsies of non-obese controls (N=6). AveNASH: average of gene expression in NASH, AveCTRLS: average of gene expression non-obese controls.

Accession #	GENE NAME	GENE FUNCTION	AveNASH/ AveCTRLS
H21042	ATF3	activating transcription factor 3	0.125
AA233185	IGFBP1	insulin-like growth factor binding protein 1	0.138
W96155	JUN	v-jun avian sarcoma virus 17 oncogene homolog	0.208
T72089	NNMT	nicotinamide N-methyltransferase	0.323
T71363	SDS	serine dehydratase	0.341
AA086471	S100A8	S100 calcium-binding protein A8 (calgranulin A)	0.343
R38383	NA	R38383	0.356
H22856	GOT1	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	0.376
AA669637	B4-2	proline-rich protein with nuclear targeting signal	0.381
N99003	ABR	active BCR-related gene	0.394
H79047	IGFBP2	insulin-like growth factor binding protein 2 (36kD)	0.396
AA461467	ODC1	ornithine decarboxylase 1	0.398
AI375353	SGK	serum/glucocorticoid regulated kinase	0.405
H96235	ETS2	v-ets avian erythroblastosis virus E26 oncogene homolog 2	0.406
AA461467	ODC1	ornithine decarboxylase 1	0.435
T72877	IL1RN	interleukin 1 receptor antagonist	0.449
AA446120	ADM	adrenomedullin	0.45
AA677287	FGL1	fibrinogen-like 1	0.451
N25945	PLSCR1	phospholipid scramblase 1	0.451
AA054754	IL15RA	interleukin 15 receptor, alpha	0.453
T99236	JUNB	jun B proto-oncogene	0.458
R36467	TGFB1	transforming growth factor, beta 1	0.461
AA633811	NFIL3	nuclear factor, interleukin 3 regulated	0.467
AA447761	ALAS1	aminolevulinate, delta-, synthase 1	0.482
AA148737	SDC4	syndecan 4 (amphiglycan, ryudocan)	0.484
AA453467	LDHC	lactate dehydrogenase C	0.488
T68274	-	T68274	0.496
AA434115	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	0.497
AI355949	CALM3	calmodulin 3 (phosphorylase kinase, delta)	2.01
H89512	TF	transferrin	2.04
H19327	-	Homo sapiens clone 25085 mRNA sequence	2.106
AA677706	LTF	lactotransferrin	2.11
AA496149	HMGCS2	3-OH-3-methylglutaryl-Coenzyme A synthase 2 ,MT	2.14
AI308916	PRSS4	protease, serine, 4 (trypsin 4, brain)	2.144
N47484	GSTA4	glutathione S-transferase A4	2.234
AA683500	ATRN	attractin	2.256
AA633818	FACL4	fatty-acid-Coenzyme A ligase, long-chain 4	2.517
T59043	AFP	alpha-fetoprotein	2.769
R28686	FACL4	fatty-acid-Coenzyme A ligase, long-chain 4	2.828

Table 5. Genes identified from comparison of liver biopsies of obese controls N=7) vs. liver biopsies of non-obese controls (N=6). AveOBESECTRLS: average of gene expression in obese controls. AveCTRLS: average of gene expression in normal controls.

Accession #	Name	Gene Function	AveOBESECTRLS/ AveCTRLS
H21042	ATF3	activating transcription factor 3	0.129
AA233185	IGFBP1	insulin-like growth factor binding protein 1	0.254
N99003	ABR	active BCR-related gene	0.338
AA434115	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	0.349
H45668	KLF4	Kruppel-like factor 4 (gut)	0.428
AA464600	MYC	v-myc avian myelocytomatosis viral oncogene homolog	0.44
AA972853	CBL	Cas-Br-M (murine) ecotropic retroviral transforming sequence	0.444
T72089	NNMT	nicotinamide N-methyltransferase	0.446
AA054754	IL15RA	interleukin 15 receptor, alpha	0.452
AA669637	B4-2	proline-rich protein with nuclear targeting signal	0.456
R36467	TGFB1	transforming growth factor, beta 1	0.46
R02789	-	ESTs	0.462
AA461467	ODC1	ornithine decarboxylase 1	0.464
AI263095	-	Homo sapiens mRNA; cDNA DKFZp434I0812 (from clone DKFZp434I0812); partial cds	0.466
R38383	-	R38383	0.469
AA040170	SCYA7	small inducible cytokine A7 (monocyte chemotactic protein 3)	0.485
AA489616	-	AA489616	0.487
AA633811	NFIL3	nuclear factor, interleukin 3 regulated	0.492
H72375	BTN3A1	butyrophilin, subfamily 3, member A1	2.003
N66061	-	Homo sapiens cDNA: FLJ22119 fis, clone HEP18852	2.01
R64640	-	R64640	2.013
N58558	SERPINA4	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4	2.017
T58775	SCYA16	small inducible cytokine subfamily A (Cys-Cys), member 16	2.042
AA677706	LTF	lactotransferrin	2.05
AI356174	KIAA1131	KIAA1131 protein	2.05
AA460841	IRS1	insulin receptor substrate 1	2.052
AI208285	LEPR	Leptin receptor	2.059
AA953459	-	Homo sapiens mRNA; cDNA DKFZp586N1720 (from clone DKFZp586N1720)	2.084
AA404264	OS4	conserved gene amplified in osteosarcoma	2.089
AA129135	SLC29A1	solute carrier family 29 (nucleoside transporters), member 1	2.096
AA488346	MYL6	myosin, light polypeptide 6, alkali, smooth muscle	2.107

		and non-muscle	
AA598791	HP1-BP74	HP1-BP74	2.108
AI050071	EGFL4	EGF-like-domain, multiple 4	2.113
H89512	TF	transferrin	2.115
AA971543	APOL3	apolipoprotein L, 3	2.123
AI241088	SLC6A13	solute carrier family 6 (neurotransmitter transporter, GABA), member 13	2.132
AA135152	GPX2	glutathione peroxidase 2 (gastrointestinal)	2.139
AA496565	PLXNB1	plexin B1	2.141
AA700808	CPO	coproporphyrinogen oxidase (coproporphyria, harderoporphyria)	2.145
AA055076	NR2F2	nuclear receptor subfamily 2, group F, member 2	2.151
AI160964	DSC1	desmocollin 1	2.152
H61283	SMT3H2	SMT3 (suppressor of mif two 3, yeast) homolog 2	2.152
N47484	GSTA4	glutathione S-transferase A4	2.178
AI219155	-	AI219155	2.186
H04789	GYG2	glycogenin 2	2.189
AA683500	ATRN	attractin	2.2
AA441895	GSTTLp28	glutathione-S-transferase like; glutathione transferase omega	2.206
AA999990	EIF4A2	eukaryotic translation initiation factor 4A, isoform 2	2.217
AI285592	HHEX	hematopoietically expressed homeobox	2.222
H59614	IGF2	insulin-like growth factor 2 (somatomedin A)	2.238
R00833	SLC2A2	solute carrier family 2 (facilitated glucose transporter), member 2	2.241
AI368184	RRAS	related RAS viral (r-ras) oncogene homolog	2.244
AA621535	-	Homo sapiens AD037 mRNA, complete cds	2.264
AA598990	-	AA598990	2.265
AI015641	SDC1	syndecan 1	2.297
AI139968	SLC26A4	solute carrier family, member 4	2.318
AI262933	EIF1A	eukaryotic translation initiation factor 1A	2.338
N47484	GSTA4	glutathione S-transferase A4	2.356
AA418910	SFPQ	splicing factor proline/glutamine rich (polypyrimidine tract-binding protein-associated)	2.413
W96163	TOB1	transducer of ERBB2, 1	2.422
AI167784	-	AI167784	2.43
R63065	GSTM3	glutathione S-transferase M3 (brain)	2.449
N74679	G3A	apolipoprotein M	2.453
AI018607	SULT1A2	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 2	2.494
AA705395	ARSE	arylsulfatase E (chondrodysplasia punctata 1)	2.579
AA002162	-	ESTs, Highly similar to 1313352A apolipoprotein a [H.sapiens]	2.58

AI244615	ADH6	alcohol dehydrogenase 6 (class V)	2.591
AA454856	GPX4	glutathione peroxidase 4 (phospholipid hydroperoxidase)	2.624
H54629	TNFSF1-	tumor necrosis factor (ligand) superfamily, member 10	2.655
AI000677	SIAT4A	sialyltransferase 4A (beta-galactosidase alpha-2,3-sialytransferase)	2.668
AA282219	-	AA282219	2.67
R44730	-	EST	2.698
N54994	CAT	catalase	2.883
AA406180	SLC22A1L	solute carrier family 22 (organic cation transporter), member 1-like	2.884

Table 6. Adipokines and pro-inflammatory cytokines in serum of morbidly obese patients with and without NAFLD: Descriptive parameters and results of the group comparison by non-parametric Mann Whitney tests. CONTROL I: serum from morbidly obese patients with healthy livers. CONTROL II: serum from healthy blood donors. SS: Simple Steatosis. NAFLD: Non-alcoholic Fatty Liver Disease. NASH: Non-Alcoholic SteatoHepatitis.P-Value ≤ 0.5 is considerd statistically significant.

Group 1	**Mean and SD of cytokine concentration in Group 1**	**Group 2**	**Mean and SD of cytokine concentration in Group 2**	**P-VALUE**
TNF-α, pg/ml				
NAFLD (N = 45)	6.0±16.6	CONTROL II (N = 12)	2.3±0.39	< 0.0001
NAFLD (N = 45)	6.0±16.6	CONTROL I (N = 38)	1.9±0.25	4.472e-13
CONTROL I (N = 38)	1.91±0.25	CONTROL II (N = 12)	2.3±0.39	< 0.0001
NASH (N =26)	8.2±21.7	CONTROL II (N = 12)	2.3±0.39	< 0.000002
NASH (N =26)	8.2±21.7	SS (N=19)	2.95±0.9	0.0006
NASH	8.2±21.7	CONTROL I (N=38)	1.91±0.25	1.878e-11
SS (N=19)	2.95±0.9	CONTROL II (N = 12)	2.3±0.39	< 0.05
SS (N=19)	2.95±0.9	CONTROL I (N = 38)	1.91±0.25	3.018e-07
IL-8, pg/ml				
NAFLD (N =45)	24.1±38.5	CONTROL II (N = 12)	5.1±2.4	< 0.00002
NAFLD (N =45)	24.1±38.5	CONTROL I (N = 38)	7.83±3.6	< 0.000006
CONTROL I (N = 38)	7.83±3.6	CONTROL II (N = 12)	5.1±2.4	< 0.005
NASH (N =26)	23.1±27.7	CONTROL II (N = 12)	5.1±2.4	< 0.000004
NASH (N =26)	23.1±27.7	SS (N=19)	25.45±50.2	Not significant
NASH (N =26)	23.1±27.7	CONTROL I (N = 38)	7.83±3.6	< 0.0002
SS (N=19)	25.45±50.2	CONTROL II (N = 12)	5.1±2.4	< 0.002
SS (N=19)	25.45±50.2	CONTROL I (N = 38)	7.83±3.6	< 0.04
IL-6, pg/ml				
NAFLD (N=45)	23.1±72.9	CONTROL II (N = 12)	4.5±0.49	< 0.0005
NAFLD (N=45)	23.1±72.9	CONTROL I (N = 38)	7.57±6.3	< 0.015
CONTROL I	7.57±6.3	CONTROL II	4.5±0.49	Not

(N = 38)		(N = 12)		significant
NASH (N =26)	8.1±2.9	CONTROL II (N = 12)	4.5±0.49	< 0.0001
NASH (N =26)	8.1±2.9	SS (N=19)	42.5±109.4	Not significant
NASH (N =26)	8.1±2.9	CONTROL I (N = 38)	7.57±6.3	< 0.03
SS (N=19)	42.5±109.4	CONTROL II (N = 12)	4.5±0.49	< 0.004
SS (N=19)	42.5±109.4	CONTROL I (N = 38)	7.57±6.3	Not significant
Visfatin, pg/ml				
NAFLD (N=45)	28.95±41.6	CONTROL II (N = 12)	11.44±5.7	< 0.005
NAFLD (N=45)	28.95±41.6	CONTROL I (N = 38)	26.79±19.0	Not significant
CONTROL I (N = 38)	26.79±19.0	CONTROL II (N = 12)	11.44±5.7	< 0.009
NASH (N =26)	17.11±6.2	CONTROL II (N = 12)	11.44±5.7	< 0.03
NASH (N =26)	17.11±6.2	SS (N=19)	45.14±60.9	Not significant
NASH (N =26)	17.11±6.2	CONTROL I (N = 38)	26.79±19.0	< 0.05
SS (N=19)	45.14±60.9	CONTROL II (N = 12)	11.44±5.7	< 0.003
SS (N=19)	45.14±60.9	CONTROL I (N = 38)	26.79±19.0	Not significant
Adiponectin, µg/ml				
NAFLD (N=45)	9.03±7.46	CONTROL I (N = 38)	10.20±7.47	Not significant
NASH (N =26)	6.70±6.5	SS (N=19)	12.21±7.56	p < 0.0008
NASH (N =26)	6.70±6.5	CONTROL I (N = 38)	10.20±7.47	P < 0.001
SS (N=19)	12.21±7.56	CONTROL I (N = 38)	10.20±7.47	Not significant
Resistin, ng/ml				
NAFLD (N=45)	6.80±3.66	CONTROL I (N = 38)	7.61±3.84	Not significant
NASH (N =26)	6.00±2.90	SS (N=19)	7.83±4.31	Not significant
NASH (N =26)	6.00±2.90	CONTROL I (N = 38)	7.61±3.84	Not significant
SS (N=19)	7.83±4.31	CONTROL I (N = 38)	7.61±3.84	Not significant

Table 7. A total of 97 genes expressed in human adipose tissue were independently identified as statistically significant in the comparisons of obese NASH patients (N=10) vs. non-obese controls (N=9), and comparisons of obese controls (N= 7) vs. non-obese controls (LEAN) (N=9). MO=morbid obese. GE=gene expression.NGO=non glycemic obese. DO=diabetes obese. Numbers in first column correspond to ratios of gene expression in adipose obese NASH compared to adipose of non-obese controls (LEAN). Numbers in second column correspond to ratios of gene expression in adipose of obese non-NAFLD compared to adipose of non-obese controls (LEAN).

Accession #	GENE NAME	GENE FUNCTION	GE in adipose obese NASH/ GE in adipose LEAN	GE in Adipose obese non-NAFLD/ GE in adipose LEAN	Confirming previous study (Baranova et al., 2005)
METABOLISM					
N53169	APOC3	apolipoprotein C-III	2.27	2.14	YES (MO)
AA885311	BCHE	butyrylcholinesterase	2.13	2.22	YES (MO)
T61078	CPS1	carbamoyl-phosphate synthetase 1, mitochondrial (hepatic urea cycle)	2.24	2.69	YES (MO)
AA775241	ALDOA	aldolase A, fructose-bisphosphate	2.08	Three independent hits 2.36. 2.86. 3.09	YES (MO)
AA633818	EST in the 3' area of ACSL4 and three other intergenic and intronic hits	acyl-CoA synthetase long-chain family member 4	2.24	2.84	YES (MO)
DETOXIFICATION					
AI018607	SULT1A2	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 2	3.44	2.99	YES (MO)
AA181307	AHR	aryl hydrocarbon receptor	0.44	0.49	YES (MO)
AA877213	CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	2.36	2.16	NOVEL
H81046	ASNA1	arsA arsenite transporter, ATP-binding, homolog 1 (bacterial)	2.31	2.43	YES (MO)
AA031770	NUDT21	Nudix (nucleoside	Three	Four	YES (MO)

		diphosphate linked moiety X)-type motif 21	independ ent hits 2.36. 2.64. 2.67	independent hits 2.08. 2.28. 2.30. 2.78	
IMMUNE RESPONSE AND INFLAMMATION RELATED					
AI371874	TLR4	toll-like receptor 4	0.19	0.2	YES (MO, NGO, DO)
AI123732	EBI2	Epstein-Barr virus induced gene 2 (lymphocyte-specific G protein-coupled receptor)	0.34	0.26	YES (MO, NGO, DO)
AI268937	CCL8	chemokine (C-C motif) ligand 8	0.47	0.4	YES (MO, NGO, DO)
H69878	RARB	retinoic acid receptor, beta	0.43	0.5	YES (MO)
H40681	MYD88	myeloid differentiation primary response gene (88) MyD88 is a Key Mediator of Anorexia, But Not Weight Loss TLR4 signaling	2.70	2.12	YES (MO)
AA913480	KLRC1	Killer cell lectin-like receptor subfamily C, member 1	3.25	2.66	YES (MO)
CYTOSKELETON					
AA425649	DMD	dystrophin (muscular dystrophy, Duchenne and Becker types)	0.37	0.47	YES (MO, NGO, DO)
AI263095	RDX	radixin	2.54	2.48	YES (MO)
T60068	MSN	moesin	2.15	2.57	NOVEL
AI680557	COL4A6	collagen, type IV, alpha 6	2.25	2.53	YES (MO)
R20625	EVL	Enah/Vasp-like	2.16	2.62	NOVEL
T98491	EST, intronic hit in the ACTN4	actinin, alpha 4	2.49	2.74	YES (MO)
AA464250	KRT17	keratin 17	3.12	3.13	YES (MO)
W92769	CAPZA1	capping protein (actin filament) muscle Z-line, alpha 1	2.02	3.22	YES (MO)
AI076396	EST, intronic hit in CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	3.33	4.36	YES (MO)

EXTRACELLULAR MATRIX AND ECM PROCESSING					
AI160964	DSC1	desmocollin 1	2.11	2.51	NOVEL
AI375048	ADAMTS 5	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 5 (aggrecanase-2)	0.41	0.36	YES (MO, NGO, DO)
AA878951	ADAM17	a disintegrin and metalloproteinase domain 17 (TNFalpha converting enzyme)	2.3	2.8	YES (MO)
AI369218	NAPSA	Napsin A aspartic peptidase	3.93	4.35	YES (MO)
AA987261	ECE2	endothelin converting enzyme 2	2.04	2	NOVEL
AI340883	TACSTD 1	tumor-associated calcium signal transducer 1 (Ep-CAM)	3.89	3.79	YES (MO)
AA598955	TNC	tenascin C (hexabrachion)	2.15	3.25	YES (MO)
SOLUTE TRANSPORTERS AND OTHER NON-RECEPTOR TRANSMEMBRANE PROTEINS					
AI361349	SLC43A3	solute carrier family 43, member 3 (EEG1, found in rapidly growing tissues)	2.53	2.68	YES (MO)
AI174255	KCNH3	potassium voltage-gated channel, subfamily H (eag-related), member 3	2.83	3.43	YES (MO)
AI139968	SLC26A4	Solute carrier family 26, member 4 (chloride/anion exchanger)	2.53	3.69	YES (MO)
AI219155	CACNA1 C	Calcium channel, voltage-dependent, L type, alpha 1C subunit	2.58	4.14	YES (MO)
AA436374	TMED4	Transmembrane emp24 protein transport domain containing 4 (putative NFkB activating protein HNLF)	2.04	2.37	NOVEL
AI478107	TMEM12 3/PORIMI N	Transmembrane protein 123 (pro-oncosis receptor inducing membrane injury gene)	2.65	2.95	YES (MO)
LIGANDS AND RECEPTORS					
AA975301	CALCRL	calcitonin receptor-like (adrenomedullin receptor)	0.272	0.35	YES (MO, NGO, DO)
AA995128	FIGF	c-fos induced growth factor (vascular endothelial	0.31	0.26	YES (MO, NGO,

		growth factor D)			DO)
AI049675	GFRA1	GDNF family receptor alpha 1	3.03	3.79	YES (MO)
KINASES AND OTHER INTRACELLULAR SIGNAL TRANSDUCERS					
AA424938	PRKCA	protein kinase C, alpha	2.49	2.08	YES (MO)
H18068	PKN1	Protein kinase N1	2.44	2.09	YES (MO)
AI380324	PLK4	polo-like kinase 4 (Drosophila)	2.40	2.68	YES (MO)
AA465723	PPM1G	protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform	2.08	2.42	YES (MO)
AI393043	GNAT2	guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2	3.32	3.27	YES (MO)
AI261561	INPP5B	Inositol polyphosphate-5-phosphatase, 75kDa (Calcium signaling)	2.82	3.87	YES (MO)
AA406326	DKFZp686K16132	similar to BMP2 inducible kinase	2.13	2.19	NOVEL
TRANSCRIPTION FACTORS					
AA056465	NONO	non-POU domain containing, octamer-binding	2.38	Two independent hits 2.08, 2.71	YES (MO)
N95243	MEIS1	Meis1, myeloid ecotropic viral integration site 1 homolog tr fac	2.33	2.1	NOVEL
AI270906	KLF12	Kruppel-like factor 12	2.74	2.76	YES (MO)
AI240559	TCF2/HNF1B	transcription factor 2, hepatic, LF-B3, variant hepatic nuclear factor	2.44	2.9	YES (MO)
AA911236	MYBL1	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	3.38	3.62	YES (MO)
H92070	SKI	V-ski sarcoma viral oncogene homolog (avian)	4.16	3.18	Yes (MO)
CHROMATIC REMODELING					
H91167	HELLS	helicase, lymphoid-specific (LSH homologue)	2.33	2.45	YES (MO)
AA434391	NAB2	NGFI-A binding protein 2 (EGR1 binding protein 2)	2.09	2.25	YES (MO)
AI129891	RCC1	regulator of chromosome condensation 1	3.4	4.26	YES (MO)
AI201344	SUZ12P	Suppressor of zeste 12 homolog pseudogene	3.08	3.25	NOVEL

POST-TRANSLATION MODIFICATION OF THE PROTEINS, THEIR TRANSPORT AND DEGRADATION					
AA425545	DCUN1D 1	DCN1, defective in cullin neddylation 1, domain containing 1	0.37	0.36	YES (MO, NGO, DO)
AI383794	PRMT3	protein arginine N-methyltransferase 3	2.48	2.32	YES (MO)
R20646	EHD1	EH-domain containing 1 recycling of membrane proteins	2.24	2.35	YES (MO)
H15246	HECW1	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 1	2.5	2.5	NOVEL
AI219333	MAN1C1	mannosidase, alpha, class 1C, member 1	3.32	3.84	YES (MO)
T53302	MAN2A2	mannosidase, alpha, class 2A, member 2	0.46	0.5	YES (MO)
AA775378	MGAT1	mannosyl (alpha-1,3-)-glycoproteinbeta-1,2-N-acetylglucosaminyltransferase	2.65	2.54	YES (MO)
RNA PRODUCTION AND SPLICING					
H50677	RBM6	RNA binding motif protein 6	2.3	2.08	YES (MO)
AA459947	SNRP70	small nuclear ribonucleoprotein 70kDa polypeptide (RNP antigen)	2.06	2.17	NOVEL
AI583623	SFRS10	splicing factor, arginine/serine-rich 10 (transformer 2 homolog,Drosophila)	2.26	2.27	YES (MO, NGO, DO)
AI263109	SFRS5	Splicing factor, arginine/serine-rich 5	2.68	3.26	YES (MO)
AA521416	LOC3902 84	similar to signal recognition particle 14kDa (homologous Alu RNA binding protein)	2.27	3.95	YES (MO)
R64359	LIN28B	Lin-28 homolog B (C. elegans)	3.3	5.42	YES (MO)
RIBOSOME BIOGENESIS AND PROTEIN BIOSYNTHESIS					
AI218585	MRPL4	mitochondrial ribosomal protein L4	2.44	2.06	YES (MO)
R56251	EMG1	EMG1 nucleolar protein homolog	2.00	2.13	NOVEL
H56428	LOC6445 82 and 3 other intronic hits	similar to Ribosome biogenesis protein BMS1 homolog	2.28	2.96	NOVEL

AA285043	EIF2S3	eukaryotic translation initiation factor 2, subunit 3 gamma, 52kDa	2.08	2.89	YES (MO)
AA424956	EIF2S1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	2.39	3.74	YES (MO)
AA668520	NARS	asparaginyl-tRNA synthetase	2.99	3.15	YES (MO)
RELATED TO MELANOGENESIS					
AA995045	MAGEA6	Melanoma antigen, family A, 6	3.07	6.73	YES (MO)
AI200443	MAGEA10	Melanoma antigen family A, 10	3.49	2.99	YES (MO)
AA962407	YWHAB	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide	Three independent hits 2.13, 2.34, 2.86	3.08	YES (MO)
AA668457	TYRP1	tyrosinase-related protein 1	2.38	3.19	YES (MO)
APOPTOSIS					
T47483	PPID and other hits	peptidylprolyl isomerase D (cyclophilin D)	2.27	2.28	NOVEL
W56356	PEG10	paternally expressed 10	2.38	3.18	YES (MO)
MISCELLANEOUS					
H65334	YLPM1	YLP motif containing 1 (ZAP3)	2.08	3.71	YES (MO)
R99354	LEREPO4	likely ortholog of mouse immediate early response, erythropoietin 4	2.46	2.5	YES (MO)
T56880	AFP	alpha-fetoprotein	2.29	2.01	YES (MO)
AA995464	NIPSNAP1	nipsnap homolog 1 (C. elegans)	2.7	2.05	YES (MO)
AA280677	ZMYM6/ ZNF258	Zinc finger, MYM-type 6	2.07	2.37	YES (MO)
AI376113	TINP1	TGF beta-inducible nuclear protein 1	2.29	2.11	NOVEL
AI050071	MEGF8	Multiple EGF-like-domains 8	2.61	2.9	YES (MO)
AI077350	C19orf37	Chromosome 19 open reading frame 37	2.1	2.51	YES (MO)
NO FUNCTIONAL INFORMATION AVAILABLE					
AI457695	CGI-115	CGI-115 protein	2.98	3.63	YES (MO)
H73261	Introinic and	WD repeat domain 25	4.68	2.97	NOVEL

	intergenic hits in WDR25				
R26462	Intergenic hits	NA	2.61	2.56	NOVEL
AI345617	Two hits in hypothetic transcripts	NA	2.88	2.87	YES (MO)

Table 8. Potential NASH-related genes expressed in adipose tissue. Table is comprised of genes with an increased levels of expression in visceral adipose of obese patients with NASH, but not in the visceral adipose of obese patients with no liver complications (Ratios > 2.0), and genes suppressed in visceral adipose of obese patients without liver complications, but not in the visceral adipose of obese patients with NASH (Ratios < 0.5).

Accession #	Gene name	Gene function	Gene expression ratios
METABOLISM			
AA497029	LDHA	lactate dehydrogenase A	2.01
AA070358	TKT	transketolase (Wernicke-Korsakoff syndrome)	2.08
AI094611	FADS2	Fatty acid desaturase 2	2.47
H78609	LOC220729	similar to Flavoprotein subunit of complex II	2.15
AA928058	COX11	cytochrome c oxidase assembly protein COX11	2.29
DETOXIFICATION			
AA775803	PRDX1	peroxiredoxin 1	2.11
IMMUNE RESPONSE AND INFLAMMATION RELATED			
AI206447	PGDS	prostaglandin D2 synthase, hematopoietic	0.39
AA476508	ENPP2	ectonucleotide pyrophosphatase/ phosphodiesterase 2 (autotaxin)	0.41
AI129421	IL18	interleukin 18 (interferon-γ inducing factor)	2.14
AI359519	CCL26	chemokine (C-C motif) ligand 26	2.78
AI285199	CCL20	chemokine (C-C motif) ligand 20	2.53
AA876147	EST, 3' area of IRAK2	interleukin-1 receptor-associated kinase 2	2.04
AA464528	PLP2	proteolipid protein 2 (colonic epithelium-enriched)	2.49
AA598950	CTSB	cathepsin B	2.29
AA132090	CD53	CD53 antigen	2.49
AA670408	B2M	beta-2-microglobulin	2.94
AA988615	HLA-F	major histocompatibility complex, class I, F	2.66
AI284347	UMOD	uromodulin (Tamm-Horsfall glycoprotein)	2.01
CYTOSKELETON			
AA984728	KIF5A	kinesin family member 5A	2.45
N55461	EPB49	dematin	2.09
AI018066	CYLC2	cylicin, basic protein of sperm head cytoskeleton	2.08
AI360761	ARPC4	Actin related protein 2/3 complex, subunit 4, 20kDa	2.51
AA598517	KRT8	keratin 8	2.76
CELL CONTACTS, EXTRACELLULAR MATRIX AND ECM PROCESSING			
H63077	ANXA1	annexin A1	2.07
R26732	PMP22	peripheral myelin protein 22	2.1
AA490466	GJB2	gap junction protein, beta 2, 26kDa (connexin 26)	2.38
R10285	HMMR	hyaluronan-mediated motility receptor (RHAMM)	0.44

AA099554	ADAM12	Disintegrin&metalloproteinase domain12 meltrinα	2.38
SOLUTE TRANSPORTERS AND OTHER NON-RECEPTOR TRANSMEMBRANE PROTEINS			
AA058323	IFITM1	interferon induced transmembrane protein 1	2.06
AA991578	VMD2	vitelliform macular dystrophy (bestrophin)	0.28
AA487893	TM4SF1	Transmembrane 4 L six family member 1	2.78
AA398233	LAPTM4A	lysosomal-associated protein transmembrane 4α	2.28
PLASMA PROTEINS			
AI021885	PROC	protein C (inactivator of Va and VIIIa)	2.12
AI349250	FCN1	ficolin (collagen/fibrinogen domain containing) 1	0.43
AA130017	CLU	clusterin (apolipoprotein J)	2.23
AI313387	F3	coagulation factor III (thromboplastin)	3.26
LIGANDS AND RECEPTORS			
AA009609	FGF7	fibroblast growth factor 7	0.41
AI022531	PTPRB	protein tyrosine phosphatase, receptor type, B	0.44
AI360108	DLL1	delta-like 1	2.58
KINASES AND OTHER INTRACELLULAR SIGNAL TRANSDUCERS			
H01164	STK17A	serine/threonine kinase 17a (apoptosis-inducing)	2.07
H96775	PRKAA1	protein kinase, AMP-activated, α1 catalytic subunit	2.09
AI261401	PLA2G4B	phospholipase A2, group IVB (cytosolic)	2.37
AA402907	ROCK2	Rho-associated, coiled-coil containing protein kinase 2	2.4
R69522	RAB27B	RAB27B, member RAS oncogene family	2.14
TRANSCRIPTION FACTORS			
AA282092	GTF2F1	general transcription factor IIF, polypeptide 1, 74kDa	2
H91169	BRD8	bromodomain containing 8 (thyroid receptor coactivating protein)	2.08
AA488173	YY1AP1	YY1 associated protein 1	2.18
W96155	JUN	v-jun sarcoma virus 17 oncogene homolog	0.4
AI365571	RFXAP	regulatory factor X-associated protein	2.43
POST-TRANSLATION MODIFICATION OF THE PROTEINS, THEIR TRANSPORT AND DEGRADATION			
AA055504	TRIM32	tripartite motif-containing 32	2.23
R80790	UBE2C	ubiquitin-conjugating enzyme E2C	2.16
AI365246	WSB1	WSB1 ubiquitin ligase	2.14
AA411202	AHSA1	AHA1 activator of heat shock 90kDa protein	2.12
AI279788	ARIH2	ariadne homolog 2	2.04
RNA PRODUCTION AND SPLICING			
AI474891	SFRS9	Splicing factor, arginine/serine-rich 9	2.36
AA598470	DDX23	DEAD (Asp-Glu-Ala-Asp) box polypeptide 23	2.01
AA016290	RBBP6	retinoblastoma binding protein 6	2.02
RIBOSOME BIOGENESIS AND PROTEIN BIOSYNTHESIS			

AA669452	EIF2S1	eukaryotic translation initiation factor 2 subunit 1α	2.01
H56918	EIF4A1	eukaryotic translation initiation factor 4A isoform 1	2.12
AI358848	MARS	methionine-tRNA synthetase	2.01
APOPTOSIS			
AI127671	ENDOGL1	endonuclease G-like 1	2.18
AI218994	HBXIP	Hepatitis B virus x interacting protein	2.67
AI129113	BIRC4BP	XIAP associated factor A	2.82
MISCELLANEOUS			
H23255	SERINC3	Serine incorporator 3	2.17
W90520	ZNF652	Zinc finger protein 652	2.18
H90147	BCL7A	B-cell CLL/lymphoma 7A	2.1
AA676484	CAPNS1	calpain, small subunit 1	2.41
H15095	CXorf40B	Chromosome X open reading frame 40B	2.61
R53548	FLJ23703	Hypothetical protein FLJ23703	0.36

Table 9. Potentially hepatoprotective genes expressed in adipose. Table comprises of genes that are suppressed in visceral adipose of obese patients with NASH, but not in the visceral adipose of obese patients with no liver complications (Ratios < 0.5), and genes with an increased levels of expression in visceral adipose of obese patients without liver complications, but not in the visceral adipose of obese patients with NASH (Ratios > 2.0).

Accession #	Gene name	Gene function	Gene expression ratios
METABOLISM			
AA928584	AASS	aminoadipate-semialdehyde synthase	0.46
AA179391	OXCT/ SCOT	3-oxoacid CoA transferase	0.49
H90906	CHDH	choline dehydrogenase	2.25
AA461467	ODC1	ornithine decarboxylase 1	2.26
AA504461	LDLR	low density lipoprotein receptor	2.2
R64640	SPTLC2	Serine palmitoyl-CoA transferase long chain 2	2.51
AI002403	COX7A2	cytochrome c oxidase subunit VIIa polypeptide 2	0.46
AA046701	ATP5G1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c(subunit 9), isoform 1	2.08
IMMUNE RESPONSE AND INFLAMMATION RELATED			
AA404284	IL1RAP	interleukin 1 receptor accessory protein	2.37
AI338791	SH2D1A	SH2 domain protein 1A, Duncan's disease	2.21
CYTOSKELETON			
W72103	SPTBN1	spectrin beta, non-erythrocytic 1	0.36
AA029997	COL4A5	collagen, type IV, alpha 5	2.17
AA620426	AMPH	amphiphysin	0.47
CELL CONTACTS EXTRACELLULAR MATRIX AND ECM PROCESSING			
AI262129	POSTN	periostin, osteoblast specific factor	0.46
T64192	PRSS1	Protease, serine, 1 (trypsin 1)	2.1
AA282219	ECE1	Endothelin converting enzyme 1	2.1
AI000677	ST3GAL1	ST3 β-galactoside α-2,3-sialyltransferase 1	3.68
AI023541	CA9	carbonic anhydrase IX	2.29
NON-RECEPTOR TRANSMEMBRANE PROTEINS			
R69603	OSTM1	Osteopetrosis associated transmembrane protein	3.0
LIGANDS AND RECEPTORS			
AI356028	GPRC5B	G protein-coupled receptor, family C, group 5, member B	0.48
H99427	PTPRM	protein tyrosine phosphatase, receptor type, M	0.43
N93476	EDG1	endothelial differentiation, sphingolipid G-protein-coupled receptor 1	0.45
AA086038	PLA2R1	phospholipase A2 receptor 1	0.47
KINASES AND OTHER INTRACELLULAR SIGNAL TRANSDUCERS			

N64014	PIP5K1A	phosphatidylinositol-4-phosphate 5-kinase type I α	2.2
AA451895	ANXA5	a phospholipase A2 and protein kinase C inhibitory protein with calcium channel activity	2.04
R53966	CHN1/ RHOGAP2	chimerin 1	2.1
AA112979	VRK1	vaccinia related kinase 1	2.29
TRANSCRIPTION FACTORS			
AI092288	PAX9	paired box gene 9	0.36
R59192	HLF	hepatic leukemia factor TF	0.4
T63779	KLF9	Kruppel-like factor 9	2.01
N59790	NFX1	nuclear transcription factor, X-box binding 1	2.73
W87751	TIMELESS	timeless homolog	2.33
CHROMATIC REMODELING			
AA458981	PTMS	Parathymosin	2
H17334	CBX6	chromobox homolog 6	2.03
AI493835	HFM1	HFM1, ATP-dependent DNA helicase homolog	2.35
AA521388	RUVBL1	RuvB-like 1 (E. coli)	2.36
AI203233	WDHD1	WD repeat and HMG-box DNA binding protein 1	2.75
POST-TRANSLATION MODIFICATION OF THE PROTEINS, THEIR TRANSPORT AND DEGRADATION			
AA916906	TRADD	TNFRSF1A-associated via death domain	2.05
AA872379	SUMO3	SMT3 suppressor of mif two 3 homolog 3	2.14
H27912	KDELR1	KDEL endoplasmic reticulum protein retention receptor 1	2.61
RNA PRODUCTION AND SPLICING			
W90506	U2AF1	U2(RNU2) small nuclear RNA auxiliary factor 1	2.52
AA664135	RPP38	Ribonuclease P/MRP 38kDa subunit	2.1
AA279662	SNRPB	small nuclear ribonucleoprotein polypeptides B and B1	2.11
AI474891	SERS9	Splicing factor, arginine/serine-rich 9	2.65
T62635	SRP72	signal recognition particle 72kDa	2.39
RELATED TO MELANOGENESIS			
N27147	DCT	dopachrome tautomerase (TYRP2)	2.54
N26562	MLANA	Melan-A	2.2
AI479347	MAGEB2	melanoma antigen, family B, 2	2.29
APOPTOSIS			
AA916906	TRADD	TNFRSF1A-associated via death domain	2.05
AA977307	NME6	nucleoside-diphosphatekinase	2.26
MISCELLANEOUS			
AI350508	FAM107A	Family with sequence similarity 107, member A	0.46
H81304	FAM63A	Family with sequence similarity 63,	2.11

		member A	
R42785	LHFP	Lipoma HMGIC fusion partner	2.01
H19109	KLHL7	Kelch-like 7	2.28
AI362866	PTTG1	pituitary tumor-transforming 1	3.31
AI348451	FLJ33790	Hypothetical protein FLJ33790	2.06
H77332	TRIM58	Tripartite motif-containing 58	2.44

Table 10. A total of 14 genes expressed in hepatic tissue were independently identified as statistically significant in the comparisons of obese NASH patients (N=27) vs. non-obese controls (N=6), and obese controls (OB ctrls) (N=7) vs. non-obese controls (ctrls) (N=6).Ave: average of gene expression. Ave Nash: average of gene expression in Nash. Ave ctrls: average of gene expression in non-obese contrls. Numbers correspond to ratios of gene expression between the specified groups.

Accession #	**Gene Name**	**Gene Function / Adjacent Gene Function**	**Ave Nash/ Ave ctrls**	**Ave OB ctrls/ Ave ctrls**	**Shown In Previous Papers Youonossi et al., 2005; Baranova et al., 2005**
Metabolism					
T72089	NNMT	nicotinamide N-methyltransferase	0.323	0.446	yes
AA461467	ODC1	ornithine decarboxylase 1	Two independent hits : 0.398 and 0.435	0.464	yes
Detoxification and oxidative stress					
H89512	TF	transferrin	2.04	2.115	yes
AA677706	LTF	lactotransferrin	2.11	2.05	yes
N47484	GSTA4	glutathione S-transferase A4	2.234	2.178	yes
Transcription Factors					
H21042	ATF3	activating transcription factor 3	0.125	0.129	yes
Immune response and inflammation related					
R38383	ZFP36	zinc finger protein 36, C3H type (tristetraprolin)	0.356	0.469	yes
AA054754	IL15RA	interleukin 15 receptor, alpha	0.453	0.452	yes
R36467	TGFB1	transforming growth factor, beta 1	0.461	0.46	yes
AA633811	NFIL3	nuclear factor, interleukin 3 regulated	0.467	0.492	yes
AA683500	ATRN	attractin	2.256	2.2	NO
Kinases and other intracellular signal transducers					
AA669637	PNRC1	Proline-rich nuclear receptor coactivator 1	0.381	0.456	yes
AA233185	IGFBP1	insulin-like growth factor binding protein 1	0.138	0.254	yes
N99003	ABR	active BCR-related gene	0.394	0.338	yes
Cell contacts, extracellular matrix and ECM processing					
AA434115	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	0.497	0.349	yes

Table 11. Potentially NASH related genes expressed in the liver. This table displays the genes with increased expression levels in the hepatic biopsies of obese patients with NASH, but not in the livers of obese patients with no liver complications (Ratios > 2.0). It also shows the genes suppressed in the livers of obese patients without liver complications, but not in the livers of obese patients with NASH (Ratios < 0.5).

Accession #	**Gene name**	**Gene function**	**Gene expression ratios**
METABOLISM			
AA496149	HMGCS2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)	2.14
AA633818	ACSL4	Acyl-CoA synthetase long-chain family member 4	2.517 and 2.828
IMMUNE RESPONSE AND INFLAMMATION RELATED			
AA040170	CCL7	Chemokine (C-C motif) ligand 7	0.485
TRANCRIPTION FACTORS			
H45668	KLF4	Kruppel-like factor 4 (gut)	0.428
AA464600	MYC	v-myc oncogene homolog	0.44
CELL CONTACT, EXTRACELLULAR MATRIX AND ECM PROCESSING			
AI263095	RDX	radixin	0.466
AI308916	PRSS3	Protease, serine, 3 (mesotrypsin)	2.144
R02789	NUCB2	Nucleobindin 2	0.462
POST-TRANSLATION MODIFICATION OF THE PROTEINS, THEIR TRANSPORT AND DEGRADATION			
AA972853	CBL	Cas-Br-M ecotropic retroviral transforming sequence	0.444
MISCELLANEOUS			
AA489616	OLFML2B	olfactomedin-like 2B	0.487
H19327	SH3BGRL 2	SH3 domain binding glutamic acid-rich protein like 2 (thioredoxin-like)	2.106

Table 12. Potentially hepatoprotective genes expressed in the liver. This table displays genes that are suppressed in the hepatic biopsies of obese patients with NASH, but not in the livers of obese patients with no liver complications (Ratios < 0.5). It also displays genes with increased expression levels in the livers of obese patients without liver complications, but not in the livers of obese patients with NASH (Ratios > 2.0).

Accession #	Gene name	Gene function	Gene expression ratios
METABOLISM			
T71363	SDS	serine dehydratase	0.341
H04789	GYG2	glycogenin 2	2.189
H22856	GOT1	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	0.376
AA453467	LDHA	lactate dehydrogenase A	0.488
N25945	PLSCR1	phospholipid scramblase 1	0.451
AA447761	ALAS1	δ- aminolevulinate synthase 1	0.482
R64640	SPTLC2	Serine palmitoyltransferase, long chain base subunit 2	2.013
AA700808	CPOX	coproporphyrinogen oxidase (heme pathway)	2.145
AA002162	LPAL2	Lipoprotein, Lp(a)-like 2	2.58
N74679	APOM	apolipoprotein M	2.453
AA971543	APOL3	apolipoprotein L3	2.123
DETOXIFICATION			
AA441895	GSTO1	Glutathione S-transferase omega 1	2.206
N54994	CAT	catalase	2.883
AA953459	CBR4	Carbonyl reductase 4	2.084
AA446120	ADM	Adrenomedullin	0.45
AA705395	ARSE	arylsulfatase E (chondrodysplasia punctata 1)	2.579
AI018607	SULT1A1	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	2.494
AI244615	ADH6	alcohol dehydrogenase 6 (class V)	2.591
AA454856	GPX4	glutathione peroxidase 4 (phospholipid hydroperoxidase)	2.624
AA135152	GPX2	glutathione peroxidase 2 (gastrointestinal)	2.139
R63065	GSTM3	glutathione S-transferase M3 (brain)	2.449
IMMUNE RESPONSE AND INFLAMMATION RELATED			
T72877	IL1RN	interleukin 1 receptor antagonist	0.449
T58775	CCL16	Chemokine (C-C motif) ligand 16	2.042
H54629	TNFSF10	TNF (ligand) superfamily, member 10	2.655
AA086471	S100A8	S100 calcium-binding protein A8 (calgranulin A)	0.343
T68274	C8B	complement component 8, beta polypeptide	0.496
CYTOSKELETON			
AA488346	MYL6	myosin, light polypeptide 6, alkali, smooth muscle and non-muscle	2.107
EXTRACELLULAR MATRIX AND ECM PROCESSING			
AA677287	FGL1	fibrinogen-like 1	0.451
AI160964	DSC1	desmocollin 1	2.152
AA148737	SDC4	syndecan 4 (amphiglycan, ryudocan)	0.484
AI015641	SDC1	syndecan 1	2.297

AI000677	ST3GAL1	ST3 β-galactoside α-2,3-sialyltransferase 1	2.668
AA282219	ECE1	Endothelin converting enzyme 1	2.67
SOLUTE TRANSPORTERS AND OTHER NON-RECEPTOR TRANSMEMBRANE PROTEINS			
AA129135	SLC29A1	solute carrier family 29 (nucleoside transporters), member 1	2.096
R00833	SLC2A2	solute carrier family 2 (facilitated glucose transporter), member 2	2.241
AA406180	SLC22A18	solute carrier family 22 (organic cation transporter), member 18	2.884
AI241088	SLC6A13	solute carrier family 6 (neurotransmitter transporter, GABA), member 13	2.132
AI167784	SLC15A1	solute carrier family 15 (oligopeptide transporter), member 1	2.43
AI139968	SLC26A4	solute carrier family 26, member 4	2.318
AI219155	CACNA1C	calcium channel, voltage-dependent, L type, alpha 1C subunit	2.186
R44730	KCNH7	potassium voltage-gated channel, subfamily H (eag-related), member 7	2.698
TRANSCRIPTION FACTORS			
H96235	ETS2	v-ets avian erythroblastosis virus E26 oncogene homolog 2	0.406
W96155	JUN	v-jun avian sarcoma virus 17 oncogene homolog	0.208
T99236	JUNB	jun B proto-oncogene	0.458
AA055076	NR2F2	nuclear receptor subfamily 2, group F, member 2 (COUP-TF II)	2.151
AI285592	HHEX	Homeobox, hematopoietically expressed	2.222
AA404264	CTDSP2	CTD small phosphatase 2 (attenuates AR and GR transcriptional activity)	2.089
LIGANDS AND RECEPTORS			
AI208285	LEPR	Leptin receptor	2.059
KINASES AND OTHER INTRACELLULAR SIGNAL TRANSDUCERS			
H79047	IGFBP2	insulin-like growth factor binding protein 2 (36kD)	0.396
AA460841	IRS1	insulin receptor substrate 1	2.052
H59614	C11ORF43	Chromosome 11 open reading frame 43 (Insulin-like growth factor II-associated protein)	2.238
W96163	TOB1	transducer of ERBB2, 1	2.422
AA496565	PLXNB1	plexin B1	2.141
AI375353	SGK	serum/glucocorticoid regulated kinase	0.405
N66061	RABL3	RAB, member of RAS oncogene family-like 3	2.01
AI368184	RRAS	related RAS viral (r-ras) oncogene homolog	2.244
AA621535	RASSF4	Ras association (RalGDS/AF-6) domain family 4	2.264
CHROMATIC REMODELING			
AA598791	HP1BP3	Heterochromatin protein 1 binding protein 3	2.108
RNA PRODUCTION AND SPLICING			
AA418910	SFPQ	splicing factor proline/glutamine rich	2.413

		(polypyrimidine tract-binding protein-associated)	
POST-TRANSLATION MODIFICATION OF THE PROTEINS, THEIR TRANSPORT AND DEGRADATION			
H61283	SUMO2	SMT3 suppressor of mif two 3 homolog 2	2.152
RIBOSOME BIOGENESIS AND PROTEIN BIOSYNTHESIS			
AA999990	EIF4A2	eukaryotic translation initiation factor 4A var 2	2.217
AI262933	EIF1AP1	Eukaryotic translation initiation factor 1A pseudogene 1	2.338
MISCELLANEOUS			
AI050071	MEGF8	Multiple EGF-like-domains 8	2.113
AI356174	HECTD1	HECT domain containing 1	2.05
H72375	BTN3A1	butyrophilin, subfamily 3, member A1 (BTF5)	2.003
N58558	SERPINA4	Kallistatin	2.017

Table 13A1-Adipokines and pro-inflammatory cytokines in serum of HCV and HBV patients: Descriptive parameters and results of the group's comparison by non-parametric Mann Whitney tests. P-values ≤ 0.01 was considered significant.

Groups	**Statistics**	**IL-6** ***pg/ml***	**IL-8** ***pg/ml***	**TNF-α** ***pg/ml***	**ADIPONECTI N*µg/ml***
HCV	Mean	18.56	36.67	34.00	18.50
	STD	9.39	26.46	32.61	9.41
	P-val	0.04	0.00002	0.00002	0.90
HBV	Mean	13.91	17.61	16.62	20.02
	STD	6.31	6.88	7.95	12.13
HCV 3	Mean	20.98	55.59	32.78	15.92
	STD	6.67	35.70	34.17	8.39
	P-Val	0.002	0.00004	0.006	0.24
HBV	Mean	13.91	17.61	16.62	20.02
	STD	6.31	6.88	7.95	12.13
HCV 1	Mean	18.06	32.75	34.25	19.08
	STD	9.82	22.47	32.48	9.55
	P-Val	0.10	0.000001	0.000004	0.85
HBV	Mean	13.91	17.61	16.62	20.02
	STD	6.31	6.88	7.95	12.13
HCV 3	Mean	20.98	55.59	32.78	15.92
	STD	6.67	35.70	34.17	8.39
	P-val	0.08	0.0002	0.07	0.10
HCV 1	Mean	18.06	32.75	34.25	19.08
	STD	9.82	22.47	32.48	9.55

Table 13A2-Adipokines and pro-inflammatory cytokines in serum of HCV and HBV patients: Descriptive parameters and results of the group's comparison by non-parametric Mann Whitney tests. P-values ≤ 0.01 was considered significant.

Groups	**Statistics**	**RESISTIN** *ng/ml*	**VISFATIN** *ng/ml*	**IL-1B** *pg/ml*	**sIL-6r** *ng/ml*	**Leptin** *ng/ml*	**IL-1ra** *pg/ml*
HCV	Mean	4.51	55.18	10.77	38.10	20.79	272.65
	STD	2.11	64.47	22.35	21.72	18.50	144.94
	P-val	0.06	0.01	0.03	0.00002	0.00004	0.00002
HBV	Mean	3.80	93.69	4.41	14.91	7.48	124.59
	STD	2.36	130.85	2.59	12.06	4.82	77.18
HCV 3	Mean	6.08	47.30	5.26	57.88	13.85	188.36
	STD	1.82	52.12	5.15	16.22	15.33	68.39
	P-Val	0.0003	0.004	0.52	0.00003	0.23	0.002
HBV	Mean	3.80	93.69	4.41	14.91	7.48	124.59
	STD	2.36	130.85	2.59	12.06	4.82	77.18
HCV 1	Mean	4.19	57.18	11.92	33.95	22.25	290.33
	STD	2.03	66.85	24.34	20.46	18.85	150.70
	P-Val	0.26	0.03	0.02	0.00002	0.00001	0.00002
HBV	Mean	3.80	93.69	4.41	14.91	7.48	124.59
	STD	2.36	130.85	2.59	12.06	4.82	77.18
HCV 3	Mean	6.08	47.30	5.26	57.88	13.85	188.36
	STD	1.82	52.12	5.15	16.22	15.33	68.39
	P-val	0.0001	0.09	0.06	0.00002	0.01	0.0009
HCV 1	Mean	4.19	57.18	11.92	33.95	22.25	290.33
	STD	2.03	66.85	24.34	20.46	18.85	150.70

Table 13B1-Adipokines and pro-inflammatory cytokines in serum of HCV patients with and without NAFLD: Descriptive parameters and results of the group's comparison by non-parametric Mann Whitney tests. Y=Yes, N=NO. SS=Simple Steatosis.

Groups	Statistics	IL-6	IL-8	TNF-α	ADIPONECTIN	RESISTIN
HCV 3 (SS)	Mean	22.63	74.01	45.45	13.05	6.11
	STD	5.07	40.32	44.05	6.80	2.04
	P-value	0.005	0.0001	0.568	0.028	0.002
HCV 1 (SS)	Mean	14.99	32.56	29.50	20.71	4.00
	STD	7.72	26.26	16.33	11.54	1.60
HCV 1	Mean	19.95	32.94	37.77	17.96	4.33
	STD	10.39	19.29	40.77	7.48	2.31
	P-value	0.97	0.31	0.0002	0.91	0.01
HCV 3	Mean	19.11	34.87	18.54	18.71	6.04
	STD	8.04	11.33	3.69	9.43	1.67
HCV 3 (SS)	Mean	22.63	74.01	45.45	13.05	6.11
	STD	5.07	40.32	44.05	6.80	2.04
	P-value	0.42	0.002	0.07	0.20	0.96
HCV 3	Mean	19.11	34.87	18.54	18.71	6.04
	STD	8.04	11.33	3.69	9.43	1.67
HCV 1 (SS)	Mean	14.99	32.56	29.50	20.71	4.00
	STD	7.72	26.26	16.33	11.54	1.60
	P-value	0.02	1.00	0.32	0.64	0.99
HCV 1	Mean	19.95	32.94	37.77	17.96	4.33
	STD	10.39	19.29	40.77	7.48	2.31
HCV 3 (SS)	Mean	22.63	74.01	45.45	13.05	6.11
	STD	5.07	40.32	44.05	6.80	2.04
	P-value	0.15	0.00009	0.90	0.02	0.01
HCV 1	Mean	19.95	32.94	37.77	17.96	4.33
	STD	10.39	19.29	40.77	7.48	2.31
HCV 3	Mean	19.11	34.87	18.54	18.71	6.04
	STD	8.04	11.33	3.69	9.43	1.67
	P-value	0.22	0.11	0.01	0.82	0.002
HCV 1 (SS)	Mean	14.99	32.56	29.50	20.71	4.00
	STD	7.72	26.26	16.33	11.54	1.60

Table 13B2-Adipokines and pro-inflammatory cytokines in serum of HCV patients with and without NAFLD: Descriptive parameters and results of the group's comparison by non-parametric Mann Whitney tests. Y=Yes, N=NO. SS=Simple Steatosis.

Groups	Statistics	VISFATIN	IL-1B	sIL-6r	Leptin	IL-1ra
HCV 3 (SS)	Mean STD	50.43 63.43	6.66 6.87	56.64 14.60	14.64 15.95	203.56 68.23
	P-value	0.33	0.35	0.0006	0.12	0.06
HCV 1 (SS)	Mean	47.88	11.06	32.08	22.02	292.56
	STD	31.81	13.24	16.50	18.89	179.27
HCV 1	Mean	64.88	12.42	35.27	22.32	285.59
	STD	84.30	30.29	23.00	18.84	125.60
	P-value	0.16	0.09	0.002	0.05	0.008
HCV 3	Mean	40.06	3.69	59.27	12.96	171.27
	STD	39.31	1.18	18.81	15.63	68.85
HCV 3 (SS)	Mean	50.43	6.66	56.64	14.64	203.56
	STD	63.43	6.87	14.60	15.95	68.23
	P-value	0.61	0.74	0.89	1.00	0.54
HCV 3	Mean	40.06	3.69	59.27	12.96	171.27
	STD	39.31	1.18	18.81	15.63	68.85
HCV 1 (SS)	Mean	47.88	11.06	32.08	22.02	292.56
	STD	31.81	13.24	16.50	18.89	179.27
	P-value	0.51	0.40	0.77	0.94	0.72
HCV 1	Mean	64.88	12.42	35.27	22.32	285.59
	STD	84.30	30.29	23.00	18.84	125.60
HCV 3 (SS)	Mean	50.43	6.66	56.64	14.64	203.56
	STD	63.43	6.87	14.60	15.95	68.23
	P-value	0.14	0.60	0.001	0.08	0.05
HCV 1	Mean	64.88	12.42	35.27	22.32	285.59
	STD	84.30	30.29	23.00	18.84	125.60
HCV 3	Mean	40.06	3.69	59.27	12.96	171.27
	STD	39.31	1.18	18.81	15.63	68.85
	P-value	0.29	0.04	0.001	0.09	0.01
HCV 1 (SS)	Mean	47.88	11.06	32.08	22.02	292.56
	STD	31.81	13.24	16.50	18.89	179.27

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CURRICULUM VITAE

Mohammed Hussein Jarrar was born and raised in Palestine in a small town called al-Jadeideh. He is the oldest of 8 brothers and 4 sisters from the same parents. He graduated from Seelat El-Thaher high school in Jenin County, Palestine in 1984, and then attended Al-Quds University in Jerusalem where he received B.S degree in Medical Technology in 1988. After working as a Medical Technologist at Patients Friends Society in Jenin, Palestine, he was awarded an internship through AMIDEAST (A program that is funded by the United States Agency for International Development) to the Johns Hopkins Medical Institutions for training on the latest technologies about diagnosis of hemoglobinopathies and various immunoassays applied in clinical laboratory in 1990. From 1994 till 1997 he established and worked in his own Jarrar Medical laboratory in Jenin, Palestine. In 1998, he then returned to Johns Hopkins Hospital, Baltimore, USA and worked as Clinical Laboratory Scientist in Pathology department for 8 years. He attended Johns Hopkins University where he received a M.S in Biotechnology in 2001. He then worked as a senior research Assistant at Kennedy Krieger Institute in Neurogenetics for 2 years, and during that period he attended George Washington University where he got a graduate certificate in Clinical research Administration in 2003.