

HORMONAL IMBALANCE IN MORBIDLY OBESE BARIATRIC PATIENTS WITH
LIVER STEATOSIS AND NON-ALCOHOLIC STEATOHEPATITIS (NASH)

by

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ABSTRACT

HORMONAL IMBALANCE IN MORBIDLY OBESE BARIATRIC PATIENTS WITH LIVER STEATOSIS AND NON-ALCOHOLIC STEATOHEPATITIS (NASH)

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George Mason University, 2015

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Non-alcoholic fatty liver disease (NAFLD) is an increasingly common condition affecting about 70% of obese individuals and is now considered a major cause of liver-related morbidity and mortality. NAFLD encompasses a spectrum of hepatic pathologies with excessive accumulation of fat in the liver in patients consuming less than 20 grams of alcohol per day. NAFLD related liver injury ranges from simple hepatic steatosis to more severe forms of the disease including NASH, which can progress to fibrosis, cirrhosis, and ultimately organ failure. There is evidence that causally links NAFLD with imbalance of various hormones, especially these also involved in the development of insulin resistance and obesity.

The research presented herein involves quantitation of various soluble hormones in serum of morbidly obese bariatric patients with different confirmed

stages of NAFLD. The first study explores the role of vitamin D deficiency in NAFLD/NASH patients. Serum samples of 210 morbidly obese bariatric patients were profiled for the following markers: 25(OH)D₃; Vitamin D Binding protein (VDBP); Intact Parathyroid Hormone (1-84) and Cytokeratin 18 (M30) levels. The second study herein profiles three small gastric peptides, acylated ghrelin (AG), non-acylated ghrelin (DAG), and Obestatin, and a number of proinflammatory cytokines in circulation of patients with obesity related NAFLD. In both studies, correlation analysis was conducted to associate these biomarkers with clinical parameters routinely measured in NAFLD patients, including histological scores of inflammation and fibrosis of their liver biopsies, and predictive regression models were built. A third study reported herein investigates the interplay of circulatory melanogenesis-related hormones, α -Melanocyte Stimulating Hormone (α -MSH) and Melanin Concentrating Hormone (MCH), with NAFLD and obesity-induced low grade chronic.

CHAPTER 1

Non-Alcoholic Fatty liver Disease (NAFLD) and Non-Alcoholic Steatohepatitis (NASH)

NAFLD is a multifactorial disorder, which results from the fat accumulation in the liver in the absence of excessive alcohol consumption (less than 20g/day for women and less than 30g/day for men). A seminal paper by Day and James (1998) suggests a “multi-hit” hypothesis that describes the pathogenesis of NAFLD as a result of sequential “hits” (Day *et al.*, 1998). The first “hit” is considered to be the development of insulin resistance. This resistance results in increased production of free fatty acids (FFA) by adipose and muscle cells that oxidize lipids which are later absorbed by the liver resulting in the hallmark phenotype steatosis, or hepatic fat accumulation (Day *et al.*, 1998) (Figure 1).

It is important to mention that patients with steatosis alone can be asymptomatic and have normal liver tests. However, some patients progress past a steatosis stage to non-alcoholic steatohepatitis (NASH) that is accompanied by varying degrees of fibrosis. What seems to precipitate simple steatosis progressing to NASH in some patients are “secondary hits” such as various biochemical insults, including an exposure to oxidative stress and increased levels of inflammatory cytokines.

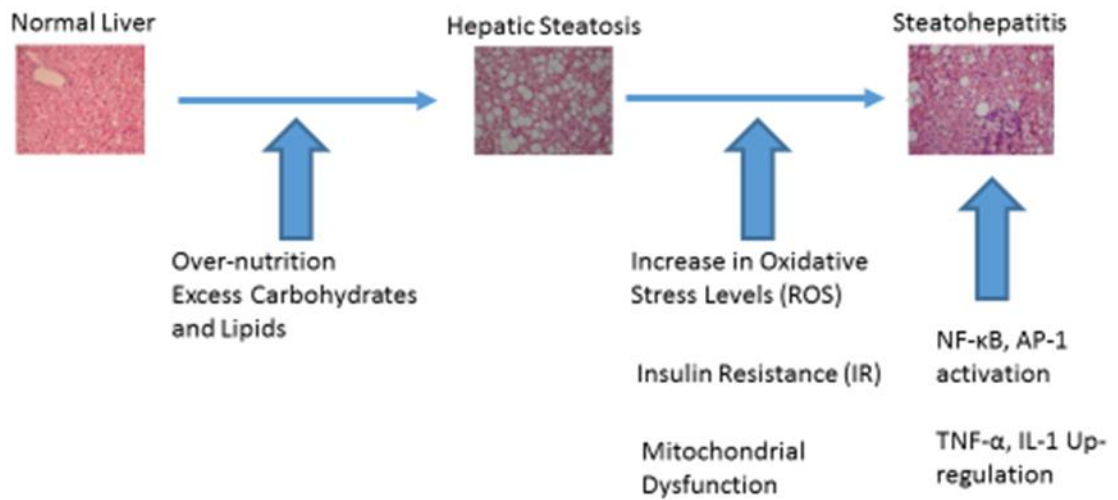


Figure 1. Schematic of obesity associated progression of NAFLD. (*Adapted from Valenzuela et al., 2011*).

No internationally accepted, evidence-based consensus clinical guidelines are developed for NAFLD management. As a result, clinical uncertainties persist in the area of diagnosis, risk stratification, treatment and long-term management. Considering the fact that much of the population is at risk for NAFLD due to high prevalence of the risk factors, namely, obesity and/or insulin resistance (IR), early diagnosis of NAFLD is a necessity. Stratifying patient risk can greatly improve identification of those with the severe form of the disease, NASH, who are at greatest risk for other liver related

morbidities, including cirrhosis and hepatocellular carcinoma (HCC). As can be expected, prognosis of patients with NAFLD depends on disease state. While simple steatosis can be symptomless or benign, a small percentage of patients progress to the more advanced form of the disease, NASH, with varying degrees of inflammation, fibrosis, cirrhosis and ultimately, HCC.

Current approaches to the treatment of NAFLD are aimed at gradual weight reduction through lifestyle alterations (diet and exercise) and/or bariatric surgery, amelioration of the metabolic syndrome i.e. insulin resistance, through use of insulin sensitizers (Metformin and Thiazolidinediones) and lipid lowering agents such as fibrates, fish oils and antioxidants (vitamin E, N-acetylcysteine and Betaine).

Epidemiology of NAFLD: Prevalence and Risk Factors

The prevalence of NAFLD is increasing on par with the prevalence of obesity, which is considered a major risk factor for the development of NAFLD. According to Centers for Disease Control and Prevention (CDC) more than one-third (34.9% or 78.6 million) of U.S. adults are obese (<http://www.cdc.gov/obesity/data/adult.html>) and the prevalence of obesity is linked to the prevalence of NAFLD worldwide (Loomba *et al.*, 2013). Most

studies in the United States report a 10-35% prevalence rate of NAFLD with variation due to study population and platforms, including liver biopsy, radiological tests such as ultrasonography, and serological tests used to diagnosis the disease (Vernon *et al.*, 2011). However, considering the fact that approximately one-third of the US population is obese, the prevalence of NAFLD in the US population is likely to be about 30% (Vernon *et al.*, 2011).

In fact, NAFLD is the most common cause of chronic liver disease in American adults (Weston *et al.*, 2005). The risks of having NAFLD are up to 95% in patients with obesity and 70% for patients with type 2 diabetes (T2D) (Cornier *et al.*, 2008). Furthermore, of these high-risk groups, 20-30% develops the advanced form of the disease, NASH, with subsequent increase in rates of cirrhosis, hepatocellular carcinoma and ultimately liver failure (McCullough, 2004).

Non-alcoholic fatty liver disease (NAFLD) is a multifactorial disease involving the interplay between various risk factors. The major risk factors for NAFLD and NASH are various components of metabolic conditions (i.e. type II diabetes, hypertension, hyperlipidemia and polycystic ovary syndrome), obesity, and chronic infections (i.e. hepatitis C virus, HCV) (Vernon *et al.*, 2011). In addition, there are a number of demographical and clinical factors that have shown strong associations with NAFLD and NASH, including age (Tiniakos *et*

al., 2010; Vernon *et al.*, 2011). However, a possible explanation of the relationship between age and the degree of the fibrosis in NAFLD may be a function of the chronicity of disease (Vernon *et al.*, 2011). Another factor is gender. Originally, it was well accepted that NAFLD is more common in women than in men. However, Chen and colleagues have reported that in a study of 26,527 Asian subjects receiving medical checkups, the prevalence of NAFLD was 31% in men and 16% in women (Chen *et al.*, 2008). Systematic reviews of the role of gender in NAFLD suggest the need for further studies using larger population based cohorts (Vernon *et al.*, 2011).

Importantly, in a majority of NAFLD cohorts, African Americans are significantly underrepresented and Hispanic and Asians are overrepresented when compared to Europeans (Guerrero *et al.*, 2009). In other words, Hispanics have the highest prevalence of NAFLD, hepatic steatosis, and elevated aminotransferase levels, followed by non-Hispanic Whites, with the lowest rate reported in African Americans (Vernon *et al.*, 2011). Recent genotyping evidence has further advanced our understanding of the epidemiology of NAFLD. More specifically, familial clustering of NAFLD suggests the possible role of a common genetic variant, rs738409 G allele in *PNPLA3* gene, which encodes adiponutrin (Kotronen *et al.*, 2009). This genetic variant is associated with increased quantitative measures of hepatic fat content ($p=0.01$) and serum

aspartate aminotransferase concentrations ($p=2.0e-3$) independent of age, sex and BMI (Kotronen *et al.*, 2009). Recent studies indicate that variations in the TM6SF2 gene may act as a potential "master regulator" of metabolic syndrome outcomes, determining not only risk of advanced liver disease, but also risks of other co-morbidities (Hardy *et al.*, 2015). Interestingly, the variant rs58542926 confers protection against CVD at the expense of an increased risk of NAFLD (Pirola *et al.*, 2015).

Drivers for the progression of NAFLD to NASH

As outlined, much of the population is at risk of developing NAFLD as a result of a rise in obesity. The clinical challenge for hepatologists is how best to stratify patients with NAFLD, especially patients who progress to NASH, and who are at greatest risk of liver related complications (Anstee, 2011). The sections that follow will focus on the modifiable factors that contribute to disease progression, i.e. diet, immunity/inflammation, gut microbiota, and hepatocellular cell death.

Dietary Drivers. At the heart of the obesity epidemic is consumption of high caloric diets rich in refined sugars and lipids, which result in an increase in adiposity and weight gain. Both obesity and metabolic syndrome exacerbate the

progression of NAFLD to NASH through a number of mechanisms. The most well studied mechanism is that of dietary lipids, long chain polyunsaturated fatty acids (PUFAs). Long chain PUFAs are essential; the human body relies on these lipid molecules for growth and development. Importantly, these molecules cannot be produced on their own due to the lack of desaturation enzymes necessary for their biosynthesis. However, in the diets of NAFLD and NASH patients, an excess of saturated (SFA) and n-6 polyunsaturated fatty acids (PUFA) as well as reduced n-3 PUFA levels are observed (Valenzuela *et al.*, 2011). Speaking generally, these lipids have a detrimental effect on many metabolic parameters; however, an administration of n-3 PUFA and monounsaturated fatty acids (MUFA) ameliorates that effect. Free cholesterol and phospholipid levels are also altered in human NAFLD. As experimental models indicate, NASH-like liver injury may be induced by accumulation of free cholesterol and the shift in the n-6/n-3 PUFAs ratio. These lipid levels are candidate targets for the prevention of the NAFLD progression to NASH (Miura *et al.*, 2013). In preventive trials, favorable results are achieved using statins, ezetimibe, a non-statin drug that lowers plasma cholesterol levels, and n-3 PUFAs; however, their ability to reverse already established NASH, is limited (Takahashi *et al.*, 2015).

The n-6/n-3 PUFAs ratio and NAFLD seems to be connected through the role of PUFAs in systemic inflammation. One of the important classes of mediators and regulators of inflammation, eicosanoids, are a byproduct of these 20-carbon PUFAs. Inflamed tissues typically contain a higher proportion of n-6 PUFAs, especially arachidonic acid (AA) (20:4n-6) (Calder, 2006). The n-3 PUFAs such as α -linolenic acid, eicosapentaenoic acid and docosahexaenoic acid, have important anti-inflammatory properties and are inhibitors of hepatic glycolysis and lipogenesis (Wree *et al.*, 2013). Dentin and colleagues demonstrated *in vivo* and *in vitro* mice that these PUFAs suppress carbohydrate-responsive element binding protein (ChREBP) activity by increasing ChREBP mRNA decay and by altering ChREBP translocation from the cytosol to the nucleus (Dentin *et al.*, 2005). ChREBP is a key transcription factor responsible for regulation of the glycolytic genes and genes involved in lipogenesis, including those encoding L-pyruvate kinase (L-PK) and fatty acid synthase (FAS) (Dentin *et al.*, 2005).

It is evident now that the n-6/n-3 PUFAs ratio plays a role in multiple stages of NAFLD. In brief, a diet with an excess of carbohydrates and fats leads to an increase in oxidative stress in the liver due to increased oxidation of fatty acids and subsequent generation of Reactive Oxygen Species (ROS) and resultant redox imbalance. This, in turn, leads to the development of insulin

resistance along with hyperinsulinemia throughout the body, while the depletion of n-3 PUFAs contributes to systemic inflammation. Steatosis can develop in the liver through a multitude of pathways: 1) IR-dependent higher peripheral lipolysis and fatty acid mobilization to the liver; 2) n-3 LCPUFA depletion leading to substantial enhancement in hepatic SREBP-1c/PPAR- α ratio that favors *de novo* lipogenesis over fatty acid oxidation; and 3) hyperinsulinemia-induced up-regulation of liver lipogenic factor PPAR- γ (Valenzuela *et al.*, 2011).

In addition to PUFAs, another important contributor to the establishment and the progression of NAFLD is the consumption of refined sugars, more specifically high fructose corn syrup (HFCS), which is commonly found in everyday sweets (Marshall *et al.*, 1957; Tappy *et al.*, 2010). Based on data collected from the NHANES 1999-2004 study, HFCS consumption had continuously increased over a 30-year period (1970-2006) and accounted for 42% of total caloric sweetener consumption in 1999-2004 versus 16% in 1977 (Tappy *et al.*, 2010).

Importantly, consumption of fructose and glucose show different effects. Hepatic glucose metabolism is regulated by phosphofructokinase, which is inhibited by ATP and citrate when energy is high, thus limiting hepatic uptake of dietary glucose and production of *de novo* lipogenesis substrates. The metabolism of dietary fructose is independent of energy status, resulting in

unregulated hepatic fructose uptake and increased lipogenesis (Stanhope KL et al. 2009). In one the of studies, the consumption of fructose-sweetened beverages as opposed to glucose-sweetened beverages for 10 weeks increased *de novo* lipogenesis, promoted dyslipidemia, decreased insulin sensitivity, and increased visceral adiposity in overweight/obese humans (Stanhope *et al.*, 2009).

The clinical manifestation of increased fructose consumption can be seen in NAFLD patients. In a cross-sectional study of 427 adult NAFLD patients from the NASH Clinical Research Network (CRN), increased fructose consumption was associated with fibrosis severity in patients with NAFLD (Abdelmalek *et al.*, 2010). Furthermore, studies have shown that subjects with NAFLD have a significantly, about two-fold greater, intake of sweetened beverages as evidenced by their food diaries (Ouyang *et al.*, 2008). In the years to come, further supporting evidence linking excessive consumption of dietary fructose to the progression of NAFLD is likely to be seen.

Immunity and Inflammation-Related Drivers. The first line of defense against pathogens (antigens) is humans' innate immunity that quickly responds to tissue injury and infection and eliminates the pathogens in a non-specific manner. A variety of cells are involved: dendritic cells and macrophages that

function in tissues; circulating phagocytes and granulocytes (i.e. neutrophils, monocytes and eosinophils) and lymphocytes (i.e. B lymphocytes, T lymphocytes and Natural Killer cells (NK)). The actions of these cells and events to follow can be described as an inflammatory response. The aforementioned cell types recognize pathogen invasion or cell damage using their intracellular (endosomal) or surface-expressed pattern recognition receptors (PRRs) (Newton *et al.*, 2012). The receptors detect, either directly or indirectly, pathogen-associated molecular patterns (PAMPs), such as microbial nucleic acid, lipoproteins (i.e. LPS), and carbohydrates, or damage-associated molecular patterns (DAMPs) released from injured cells (Newton *et al.*, 2012). The principle functions of PRRs are opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways and induction of apoptosis (Medzhitov, 2001). One of the best-characterized PRR family members is the Toll Like receptors (TLRs) responsible for sensing invading pathogens (Takeuchi, 2010). TLRs are comprised of type I transmembrane receptors, which are characterized by an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/IL-1 receptor (TIR) domain (Medzhitov, 2001).

TLR4 serves as the receptor for gram-negative bacterial lipopolysaccharide (LPS) inducer of inflammatory pathway (Medzhitov, 2008).

The recognition of LPS by TLR4 involves several accessory molecules and signal transduction pathways that rely on a cytoplasmic Toll/interleukin (IL) 1 receptor (TIR) domain and serves as the docking site for TIR-containing cytoplasmic adaptor proteins (Newton *et al.*, 2012). This sets the stage for a cascade of downstream signaling leading to activation of the nuclear factor kappa B (NF-κB) pathway, a master regulator of immune and inflammatory processes (Napetschnig *et al.*, 2013).

In 2006, Shi and colleagues demonstrated *in vitro* and *in vivo* that TLR4 can function as a “sensor” for endogenous lipids that may contribute to the pathogenesis of lipid-induced insulin resistance (Shi *et al.* 2006) by inducing pro-inflammatory cytokine expression in macrophages, in adipocytes, and in the liver (Shi *et al.* 2006).

The term ‘meta-inflammation’ (i.e. metabolic inflammatory state) is defined as low-grade, chronic inflammation orchestrated by metabolic cells in response to excess nutrients and energy (Gregor *et al.*, 2011). The mechanism and pathways that initiate the meta-inflammatory signals in obesity was recently reported by Saberi and colleagues and tied back to TLR4. They performed bone marrow transplantation (BMT) of Tlr4^{LPS-del} or control C57Bl/10J donor cells into irradiated wild-type C57Bl/6 recipient mice to generate hepatopoietic cell-specific Tlr4 deletion mutant (BMT-Tlr4^{-/-}) and control (BMT-WT) mice

(Saber *et al.*, 2009). Tlr4 knockout hematopoietic cells prevented high fat diet (HFD)/obesity-induced hyperinsulinemia, hyperglycemia, and ameliorated insulin resistance in liver and adipose in these mice (Saber *et al.*, 2009). The improved insulin action in adipose tissue and in the liver was in concordance with reduced macrophage infiltration of adipose tissue, as well as reduced expression of pro-inflammatory cytokines, such as TNF- α , in the respective tissues (Saber *et al.*, 2009).

More recently, evidence for tissue/organ specific role of TLR4 in diet-induced obesity and associated metabolic abnormalities have been presented. A study of 2 mouse models that are deficient in either hepatocyte (TLR4^{LKO}) or myeloid cell (TLR4 ^{Δ m Φ}) TLR4 displayed disparate results in ameliorating high fat diet (HFD)-induced insulin resistance (Jia *et al.*, 2014). Inactivation of hepatocyte TLR4 resulted in significantly reduced pro-inflammatory markers in the liver and enhanced hepatic insulin sensitivity in obese TLR^{LKO} mice (Jia *et al.*, 2014). The authors emphasize that the advantage of performing this type of gene elimination study is that it is taking place in a fundamentally different setting compared to the whole body knock out, hepatocyte specific null, and bone marrow transplant based models.

In a study of TLR4 mutant mice, Rivera *et al.* demonstrated the importance of TLR4 signaling and a direct link between TLR4 and Kupffer cells

in pathogenesis of non-alcoholic steatohepatitis (NASH) (Rivera CA *et al.*, 2007). Mice were fed a methionine and choline deficient diet (MCDD), which results in hepatic microvascular dysfunction and pronounced pathological changes associated with NASH within 3-4 weeks. Kupffer cell depletion was achieved by treating C57BL/6 mice with clodronate liposomes (200 μ l of 1 mg/ml suspension), dosage previously shown to selectively deplete Kupffer cells and some splenic macrophages within 24 hours. They observed that histological evidence was typical of steatohepatitis, portal endotoxemia, and enhanced TLR4 expression in wild type mice fed MCDD. However, markers of injury and fat accumulation were significantly lower in TLR4 mutant mice. Kupffer cell depletion studies showed no histological evidence of NASH and also, in this model, an increase in TLR4 expression was prevented. This was one of the first hallmark studies to implicate the role of TLR4 dependent inflammatory response and fibrogenesis in Kupffer cells.

Role of NOD-Like Receptors (NLR) Inflammasome system. In 2002, a group from Switzerland led by Fabio Martinon was the first to describe a molecular platform triggering activation of inflammatory caspases and termed it “Inflammasome” (Martinon *et al.*, 2002). Inflammasomes are multi-protein complexes that sense PAMPs and DAMPs, intracellular danger signals, via

NOD-like receptors (NLRs), which are members of the pattern recognition receptor family (Szabo et al., 2012). NLRs contain a C-terminal leucine-rich repeat (LRR) domain that plays a role in the recognition of ligands, a central NACHT domain that is responsible for oligomerization and dNTPase activity, and an N-terminal CARD, pyrin (PYD), BIR (baculoviral inhibitory repeat), or acidic transactivation domain (Szabo et al., 2012). NLRs form a complex with the effector molecule, pro-caspase-1, with or without the contribution of an adaptor molecule, such as the apoptosis-associated speck like CARD domain containing proteins (ASC) (Szabo et al., 2012). Inflammasome activation is thought to be a 2-step process: the signals from TLR activation upregulate inflammasome expression and inflammasome ligand induced activation (Szabo et al., 2012). Activation leads to auto-activation of the 45 kDa inactive pro-caspase-1 precursor into p20 and p10 subunits that form the active caspase-1, resulting in the cleavage of pro-IL-1 β and/or IL-18 pro-inflammatory cytokines, a central regulator of inflammation that binds to the IL-1 receptor (IL-1R), exerting a wide range of biological effects (Szabo et al., 2012).

Role of the Gut Microbiome. Microbiota is the intestinal microbial community thought to consist of 10^{14} micro-organisms, while the term “dysbiosis” refers to an imbalance in this community of microbes (Mehal, 2013).

The imbalance in this large community of microorganisms, which collectively encode 3-4 million genes, can lead to metabolic endotoxaemia (Mehal, 2013). The fairly new field of microbiomics has been linking alterations in gut microflora to many different health complications such as inflammatory bowel disease (IBD), cancer, cardiovascular morbidities and obesity (Ray, 2012). Metabolic endotoxemia refers to modest concentrations of circulating bacterial LPS in response to non-infectious stimuli (Cox *et al.*, 2015). The entry of LPS in circulation is mediated by chylomicron fractions and has been described to occur by passive diffusion across an intestinal mucosa where tight junction integrity has been compromised, changes in intestinal permeability, and absorption across the intestinal mucosa (Cox *et al.*, 2015).

Blood supply leaving the gut empties directly into the portal veins of the liver exposing liver tissue to gut-derived endotoxins (i.e. endotoxemia) (Rivera *et al.*, 2007). In response to these insults, Kupffer cells (tissue macrophage) are activated. Kupffer cells are responsible for swift containment and clearance of endogenous/exogenous immunoreactive material, recognizing them as non-self and harmful (Baffy, 2009). Activated Kupffer cells are enabled to (1) launch biochemical attacks and initiate interactions with hepatocytes and other liver cells by releasing a variety of biologically active mediators including cytokines, chemokines, eicosanoids, proteolytic enzymes, reactive oxygen species (ROS),

and nitric oxide; (2) recruit and retain non-resident cellular players to the liver such as neutrophils, natural killer (NK) T lymphocytes and blood monocyte-derived macrophages by expressing adhesion molecules and secreting chemokines; (3) engulf, ingest, and eliminate solid particles, including microorganisms, apoptotic cells; and (4) contributing to adaptive immunity by processing and presenting antigens to attract cytotoxic and regulatory T cells (Baffy, 2009).

Henao-Mejia and colleagues provided evidence that modulation of the intestinal microbiota through multiple inflammasome components is a critical determinant of NAFLD/NASH progression as well as multiple other aspects of metabolic syndrome such as weight gain and glucose homeostasis (Henao-Mejia *et al.*, 2012). Their set of experiments highlights a complex and cooperative effect of two sensing protein families, NLRs and TLRs, in disease progression. In particular, nucleotide-binding oligomerization domain-like receptor protein 3 inflammasome (NLR3) is involved in the caspase-1 activation and caspase-1-mediated IL-1 β and IL-18 release (Henao-Mejia *et al.*, 2012). Furthermore, genetic inflammasome deficiency-associated dysbiosis determines the high concentration of bacterial products (PAMPs) in portal blood, introducing further tissue damage to the liver and making it prone to steatosis by a high-fat diet (Henao-Mejia *et al.*, 2012).

Role of Hepatocellular Death in Progression of NAFLD/NASH

New breakthroughs in the area of hepatocellular death as a consequence of cell injury (i.e. FFA lipotoxicity, oxidative stress, unfolded protein response, adipokine/cytokine effects, mitochondrial injury, and inflammation) have identified complex crosstalk and biochemical cooperation resulting in different types of cell death. Cell death involves different pathways (apoptosis, autophagy, necroptosis, pyroptosis and necrosis) and can arise in the form of programmed and non-programmed cell death (Wree *et al.*, 2013). Hepatocyte cell death can manifest itself in two forms histologically 1) necrotic death, which results in hepatocyte becoming swollen (ballooning) and losing metabolic function, and 2) apoptosis, which results in nuclear and cytoplasmic fragmentation (Tiniakos *et al.*, 2010). Necrosis is a metabolically inactive set of events leading to karyolysis, rupture of the cytoplasmic membrane associated with ATP depletion and is commonly associated with an inflammatory cell reaction (Tiniakos *et al.*, 2010). Apoptosis on the other hand is a highly regulated, metabolically active form of cell death. Apoptosis is further classified as extrinsic (i.e. involving cell death ligands) or intrinsic (i.e. lysosomal, ER, and mitochondrial injury) (Tiniakos *et al.*, 2010). Both pathways eventually lead to the final common step of apoptosis, activation of intracellular proteases in the

form of caspases 3 and caspase 7. Effectors caspase 3 and 7 are known to cleave several cellular substrates, including the epithelial-specific intermediate filament proteins, cytokeratin-18 (CK-18). During apoptosis, CK-18 is cleaved at 2 sites into 3 fragments and ultimately released by apoptotic cells. More specifically, cleavage at Asp396 generates a neo-epitope recognized by monoclonal antibody, M30. Hepatocytes are mainly composed of CK-18 and liver disease studies have demonstrated an increased number of apoptotic cells compared to normal liver sections suggesting the role of caspase activation in the pathogenesis of various liver diseases (Bantel *et al.*, 2001). In 2006, Wieckowska and colleagues were the first to demonstrate hepatocyte caspase activation in the blood of human subjects with NASH (Wieckowska *et al.*, 2008). Plasma CK-18 levels were markedly increased in patients with NASH compared to patients with simple steatosis or normal liver biopsies (Wieckowska *et al.* 2008). CK-18 fragment levels independently predicted NASH and receiver operating characteristic (ROC) curve analysis showed specificity of 99%, a sensitivity of 85.7%, and positive and negative predictive values of 99.9% and 85.7%, respectively, for the diagnosis of NASH (Wieckowska *et al.*, 2008). Subsequent studies performed in Dr. Baranova's laboratory led to the assembly of a novel diagnostic marker panel for obesity related NASH (Younossi ZM *et al.* 2008) that could be predicted by a combination of serum adiponectin,

resistin and cleaved CK-18 (M30) with a sensitivity of 95.4% , specificity of 70.21%, and AUC of 0.908 ($p < 10^{-4}$) (Younossi *et al.*, 2008).

Studies aimed at quantifying hepatocyte apoptosis in NASH show a strong correlation between disease severity and increased levels of apoptosis in patients with NASH compared to disease free controls (Feldstein *et al.*, 2003). Correlation analysis between the number of TUNEL-positive cells and the independent grading of fibrosis in NASH show hepatocyte death to be significantly higher in patients with advanced (stage 3-4) fibrosis compared to patients with early (stage 1-2) fibrosis (Feldstein *et al.*, 2003). Semi-quantitative analysis of immune-staining for the Fas cell surface death receptor (Fas) of NASH samples, compared with simple steatosis and normal control samples, reveal Fas-staining scores to be significantly higher in patients with NASH compared to control groups (Feldstein *et al.*, 2003). An increase in Fas protein staining suggests an overexpression of Fas and implicates its potential role in the mechanism of extrinsic hepatocyte cell death.

Role of Obesity Related Low Grade Chronic Inflammation in Progression of NAFLD/NASH

Obesity is a pro-inflammatory state whereby enlarged (hypertrophied) adipocytes respond to their stressed environment by working in concert with

adipose tissue-resident immune cells (lymphocytes and macrophages) to increase release of proinflammatory cytokines and adipokines (Makki *et al.*, 2013). Research in the 1990s led to a major breakthrough showing that white adipose tissue (WAT) was not inert but a metabolically active organ releasing proinflammatory cytokine tumor necrosis factor alpha (TNF- α) (Hotamisligil *et al.* 1993). Since then, the list of adipocyte secreted inflammatory mediators (adipokines) has increased over the years and suggests the missing link between obesity and associated comorbidities (IR, MS, and NAFLD) (Baranova *et al.*, 2007; Waki *et al.*, 2007).

Adipokines exert both anti-inflammatory and inflammatory effects along with other biological roles in autocrine and paracrine; or systemically, to influence energy homeostasis and immunity (Baranova *et al.*, 2007, Makki *et al.*, 2013). Several specific adipokines have been clinically linked to the development and/or progression of NAFLD to NASH including adiponectin, resistin, leptin, and TNF α (Baranova *et al.*, 2007). Recently, Wolf *et al.* have reported very interesting results profiling the expression of 48 different adipokines in human hepatic, visceral and subcutaneous adipose tissue (SAT) in a cohort of obese NAFLD patients (Wolfs *et al.*, 2015). They observed the hepatic expression of several pro-inflammatory adipokines (TNF- α , IFG1, IL1RN, PAI-1 and CXCL10) to correlate with features of NAFLD and most

notably, they showed that the expression of leptin, chemerin, and ANGPT2 in visceral adipose tissue (VAT) is associated within the spectrum of NAFLD (Wolfs *et al.*, 2015). This study further highlights the role of adipose tissue-induced systemic inflammation in the pathogenesis of NAFLD.

The effects of pro-inflammatory cytokine and adipokines released by adipocytes may be a reaction to local hypoxic conditions caused by hypertrophy and adipocyte redox dysfunction (Page *et al.*, 2010; Jankovic *et al.*, 2015). *In vitro* studies of cultured adipocytes exposed to hypoxic conditions resulted in an increase in pro-inflammatory adipokines (IL-6, leptin and monocyte migration inhibitory factor, with a decrease in anti-proinflammatory adiponectin (Wang *et al.*, 2007). Chronic inflammatory and hypoxic milieu of tissue can lead to activation of tissue fibrosis, altering normal tissue structure and function. Recently, Henninger *et al.* investigated the presence of adipocyte hypertrophy and gene expression patterns in subcutaneous adipose tissue of first-degree relatives (healthy, non-obese) with known genetic predisposition for type 2 diabetes (Henninger *et al.* 2014). They successfully showed adipocyte hypertrophy to be accompanied by increased inflammatory markers (TNF- α , TLR4, IL1B, IL1RN, IL6, IL10, IL13, CCL2 and CD68) and Wnt-signal activation involved in adipose tissue differentiation (Henninger *et al.*, 2014). It would be beneficial to perform a similar study of the morbidly obese healthy and un-

healthy subjects to better assess the exacerbating effect of increased adiposity on adipokine gene expression. This study provides further evidence to corroborate previous findings of the role of adipocyte size and consequential local hypoxia and pro-inflammatory adipokine release (Skurk *et al.*, 2007; Bays *et al.*, 2008).

Interestingly, there seems to be a resurgence of clinical data first published in the 1980s in the field of obesity research, which shows huge individual variability in the risk for metabolic and clinical morbidities associated with obesity, which has led to new classifications of the obesity state (Andres, 1980; Sims, 1982; Brochu *et al.*, 2001; Primeau *et al.*, 2011). Accordingly, a unique subset of obese individuals seems to be protected and more resistant to the development of metabolic abnormalities associated with obesity, termed 'metabolically healthy but obese' (MHO) (Primeau *et al.*, 2011). MHO individuals display favorable metabolic profiles characterized by high levels of insulin sensitivity, lack of hypertension, as well as favorable lipid, inflammation, hormonal, liver enzyme and immunity profiles (Primeau *et al.*, 2011). Recently, Aung *et al.* examined the risk of developing cardiovascular disease (CVD) and type 2 diabetes mellitus (DM) in the metabolically healthy obese (MHO) and the metabolically unhealthy normal weight (MUH-NW) individuals. They successfully showed that both MHO and MUH-NW individuals have increased

risk for CVD and DM (Aung *et al.*, 2014). Additionally, data from the *Prospective Pizarra Study* conducted by Soriguer *et al.*, showed the metabolically unhealthy obese to be at high risk of developing DM after 11 years of follow-up and MHO subjects to have lower risk of developing DM compared to metabolically unhealthy obese subjects (Soriguer *et al.*, 2013). These results suggest that MHO subjects are an intriguing clinical phenomenon and it further raises questions regarding the physiological mechanisms involved. For example, if obesity does not determine metabolic health status, then what does?

Recently, research in the area of translational endocrinology suggests inflammation to determine metabolic health status in obese and non-obese adults (Phillips *et al.*, 2013). A cross sectional study of 2047 men and women, both obese (defined as ≥ 30 kg/m²) and non-obese (< 30 kg/m²), showed MHO and metabolically unhealthy obese (MUH) subjects have lower levels of complement component 3, C-reactive protein, TNF- α , IL-6, and plasminogen activator inhibitor-1, and higher adiponectin levels compared to their metabolically unhealthy counterparts (Phillips *et al.*, 2013). They successfully show that favorable inflammatory status is positively associated with metabolic health in obese and non-obese individuals, and that inflammatory state is a better predictor of metabolic health regardless of BMI. These findings can have

major public health implications and clinical significance with respect to patient screening and stratification where appropriate therapy or intervention can be employed.

In free radical biology, oxidative stress (OS) refers to the imbalance between the prooxidant and antioxidant levels in favor of a prooxidant state in cells and tissues (Kalyanaraman, 2013). Prooxidant species (free radicals, reactive oxygen species (ROS), reactive nitrogen intermediates (RNI)) cause modification and damage to DNA, proteins and lipids. There are numerous intracellular sources of ROS such as an imbalance between pro and antioxidant levels and/or antioxidant enzymes (superoxide dismutase and glutathione peroxidase), mitochondrial oxidants and NADPH oxidases (i.e. NOX enzymes) (Holmstrom *et al.*, 2014). Mitochondria are considered to be the largest contributor of ROS because they generate ATP in an oxygen-dependent manner, during which the flow of electrons down the respiratory chain culminates in reduction of molecular oxygen to water (Holmstrom *et al.*, 2014). However, throughout this process molecular oxygen can also undergo a one-electron reduction to generate a reactive superoxide anion (O_2^-) (Murphy *et al.*, 2009). Normally, cells use oxygen to produce ATP and water without any toxic by-products (superoxide anion) but this can lead to mitochondrial dysfunction. Another important intracellular source of ROS production is the family of

NADPH oxidases (NOX enzymes) (Holmstrom *et al.*, 2014). In the context of host defense systems (neutrophils), studies show the activation of various inflammatory mediators and production of large amount of ROS defends against foreign microorganisms (Holmstrom *et al.*, 2014). Animal studies by Furukawa S *et al.* show increased production of ROS in adipose tissue of obese mice, accompanied by augmented expression of NADPH oxidase and decreased expression of antioxidative enzymes (Furukawa *et al.*, 2004). Recently, the potential of redox imbalance in adipose tissue as an initiator of adipocyte dysfunction in obesity has been reported (Jankovic *et al.*, 2015).

In NAFLD, increased mitochondrial fatty acid oxidation (mtFAO) plays a significant role in curtailing fat accumulation in hepatocytes, but this compensatory mechanism produces secondary oxidative stress (Begrache *et al.*, 2006). Increased hepatic mtFAO has been found in patients with obesity-induced NAFLD, especially patients with the severe form of the disease (NASH) (Begrache *et al.*, 2013). ROS is known to activate NF- κ B signaling, inducing the synthesis of tumor necrosis factor- α (TNF- α), which is released by Kupffer cells in the liver (Yin *et al.*, 2001; Begrache *et al.*, 2006).

Histological Diagnostic Criteria and Scoring Systems for NAFLD/NASH

In 1980, Ludwig and colleagues were the first to describe non-alcoholic steatohepatitis as a poorly understood and hitherto unnamed liver disease that histologically mimics alcoholic hepatitis and tend to progress to cirrhosis (Ludwig *et al.*, 1980). In this seminal study, liver biopsies of 20 moderately obese patients with non-alcoholic steatohepatitis of unknown etiology were histologically examined. The specimens were characterized by the presence of striking fatty changes with evidence of lobular hepatitis, focal necrosis with mixed inflammatory infiltrates and the presence of Mallory bodies (Ludwig *et al.*, 1980). Furthermore, evidence of fibrosis was observed and cirrhosis was diagnosed in biopsy tissues of 3 patients (Ludwig *et al.*, 1980). Naturally, there has been an evolution in improvements to histological criteria used to diagnose NAFLD/NASH. Liver biopsy was the foundation technique used in 1980 by Ludwig and colleagues and still remains the 'gold standard' for confirming or excluding the diagnosis of NASH in patients (Tiniakos *et al.*, 2010). As of 2009, according to the American Association for the Study of Liver Diseases (AASLD), liver biopsy currently has three major roles: (1) diagnosis, (2) assessment of prognosis (disease staging), and/or (3) assistance in making therapeutic management decisions (Rockey *et al.*, 2008).

The histological hallmark of NAFLD in both adults and children is steatosis. This histology is the manifestation of intracytoplasmic lipids deposited in the form of triglyceride (TG) within hepatocytes (Tiniakos *et al.*, 2010). The TG in the hepatocytes can manifest itself in different forms: (a) large droplets of macro-vesicular fat that fill the cytoplasm, displacing the remaining contents of the cell and nucleus peripherally, (b) mixed large and small lipid droplets, which can easily be delineated, or (c) rarely, in addition to macro-vesicular steatosis, foci of hepatocytes with true micro-vesicular steatosis (Tiniakos *et al.*, 2010).

The minimum histological criterion for the diagnosis of steatosis is the presence of fat in more than 5% of hepatocytes (Tiniakos *et al.*, 2010). Semi-quantitative methods for the histological assessment of steatosis are based on routine laboratory stains, exposing the lipid droplets as large white spots that occur as a result of vacuolation during tissue processing (Tiniakos *et al.*, 2010). Semi-quantitative methods for the assessment of steatosis follow the acinar architecture and refer to the percentage of liver parenchyma occupied by steatotic hepatocytes: 0-33% (or 0-5%, 5-33%), 33-66% or more than 66% (Tiniakos *et al.*, 2010). Chalasani *et al.* have conducted studies examining the relationship of the severity and zonal location of steatosis to the presence of NASH and to other histological features that define NASH in adult patients with NAFLD (Chalasani *et al.*, 2008). They observed that increasing levels of

steatosis severity were positively associated with lobular inflammation ($p < 1.0 \times 10^{-4}$), zone-3 fibrosis ($p < 1.0 \times 10^{-3}$), and definite steatohepatitis ($p = 0.02$), suggesting that patients with severe steatosis are at high risk for developing NASH (Chalasani *et al.*, 2008).

Liver biopsy

In adults, the minimal histologic criteria for the diagnosis of adult NASH include the presence of steatosis, hepatocellular injury in the form of ballooning, and lobular inflammation, typically predominant in zone 3 (Tiniakos *et al.*, 2010). Hepatocellular injury can manifest itself in the form of ballooning, apoptosis and lytic necrosis. Hepatocellular ballooning refers to enlarged hepatocytes, with rarefied cytoplasm, that may have a reticulated appearance or contain Mallory-Denk bodies, which are dense, ropy, eosinophilic, and intracytoplasmic perinuclear inclusions (Tiniakos *et al.*, 2010). The presence of inflammatory markers can be observed both in lobules and portals. Lobular inflammation is considered to be mild, consisting of small foci of inflammatory cell infiltrates composed of lymphocytes, eosinophils, and Kupffer cell aggregates in the form of microgranulomas (Tiniakos *et al.*, 2010). Portal inflammation in NAFLD takes the form of chronic mononuclear cell infiltrates with varying degrees of portal inflammation observed in the NASH spectrum (Tiniakos *et al.*, 2010). Evidence

from research conducted by the Non-alcoholic Steatohepatitis Clinical Research Network (NASH CRN) suggests increased portal chronic inflammation to be associated with many clinical and pathologic features of progressive NAFLD in both adults and children (Brunt *et al.*, 2009).

These consequences of hepatocyte injury can further lead to liver fibrosis, the underlying mechanism for hepatic insufficiency and for most clinical complication of end-stage liver disease (Moreira, 2007). Over the past decades there has been an evolution in our understanding of the development and consequences of fibrosis. Fibrosis is known to be part of a dynamic process of continuous extracellular matrix (ECM) remodeling in the setting of chronic hepatocyte injury, which leads to an excessive accumulation of several extracellular proteins, proteoglycan, and carbohydrates (Moreira, 2007). Histologically, fibrosis has a characteristic “chicken wire” pattern in adult patients with non-cirrhotic NASH and is perisinusoidal/pericellular and usually first observed in acinar zone 3 (Tiniakos *et al.*, 2010). Progressive fibrosis can lead to bridging (central-portal, portal-portal, or central-central) fibrosis and ultimately to cirrhosis (Tiniakos *et al.*, 2010). It is important to mention that in addition to these histologic features, there are other features that a trained pathologist can identify under a microscope (Table 1).

Table 1. Histologic features and description related to NAFLD/NASH. *Adapted from (Tiniakos et al., 2010)*

Histologic Lesions	Description
Iron deposition	With variable degrees and distribution within hepatocytes and/or the cells of the reticulo-endothelial system. The hormonal regulator, hepcidin, is under investigation in obesity and NASH
Ductular reactions	The presence of hyperplastic ductular structures accompanied by varying amounts of inflammation and connective tissue at the portal tract interface is thought to arise from hepatic progenitor cells. Possible pathway for progressive fibrosis.
Megamitochondria (giant mitochondria)	Intracellular, round or cigar-shaped eosinophilic structures, commonly observed in hepatocytes with microvesicular steatosis. Electron microscopy reveals that mitochondria in NAFLD may show paracrystalline inclusions, and loss of cristae. Significance in NAFLD not known.
Glycogenated hepatocyte nuclei	Vacuolated and may be seen in clusters within the parenchyma. When located within periportal hepatocytes, they are considered by some investigators to be characteristic of NASH or diabetes.

Semi quantitative NAFLD activity scoring system (NAS)

The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) sponsored the NASH Clinical Research Network (CRN) which proposed and validated a scoring system for the clinical trials in NAFLD and NASH cohorts (Tiniakos *et al.*, 2010). This scoring system takes into account the disease spectrum by assigning disease activity scores (NAS), which are feature based and composed of individual scores for steatosis, lobular

inflammation, and hepatocellular ballooning, Table 2 (Tiniakos *et al.*, 2010). In addition, the staging system for fibrosis was expanded to include the pediatric pattern of portal fibrosis (stage 1c); and the initial perisinusoidal fibrosis in zone 3 was further divided into delicate and dense (stages 1a and 1b, respectively) (Tiniakos *et al.*, 2010).

Table 2. NASH Clinical Research Network Scoring System for NAFLD (Adapted from Tiniakos *et al.*, 2010).

Steatosis Grade		Lobular Inflammation		Hepatocellular Ballooning		Fibrosis Score	
Degree	Description	Degree	Description (20x optical field)	Degree	Description	Degree	Description (zone 3 perisinusoidal)
0	<5% Fat	0	None	0	None	0	None
1	5-33% Fat	1	<2 foci	1	Mild; few	1a	Mild (delicate)
2	34-66% Fat	2	2-4 foci	2	Moderate/marked; many	1b	Moderate (dense)
3	>66% Fat	3	>4 foci	N/A		1c	Portal/periportal only
						2	Portal/periportal
						3	Bridging fibrosis
						4	Cirrhosis

Pharmacological Treatments for NASH

Presently, there is no single evidence-based pharmacological treatment for NAFLD. Available treatment options are geared toward alleviating the risk factors and symptoms associated with NAFLD, such as obesity, lack of activity, insulin resistance and cardiovascular risks. A complete list of clinical symptoms and laboratory features are listed in Table 3. Treatments for NASH may include

improving insulin sensitivity by reducing caloric intake and increasing caloric expenditure; bariatric surgery in morbidly obese patients; medication with thiazolidinediones and metformin, as well as antifibrotic and antioxidant agents such as pentoxifylline and vitamin E (LaBrecque *et al.*, 2012).

Table 3. Clinical and laboratory features of NAFLD (Adapted from Tilg *et al.*, 2005)

Symptoms	Asymptomatic (50-100% of patients)
	Uncommon: fatigue, malaise, right upper quadrant pain
Signs	Hepatomegaly (up to 75%)
Laboratory Features	<p>Normal to 4 fold increase in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, usually ALT>AST except in advanced fibrosis/cirrhosis (up to 50% in patients with simple steatosis and in up to 75% of patients with NASH).</p> <p>Elevated γ-glutamyltransferase levels (50-60%) of patients).</p> <p>Elevated alkaline phosphatase levels (30% of patients).</p> <p>Elevated ferritin levels (50-60% of patients).</p> <p>Normal levels for bilirubin, albumin and prothrombin time.</p> <p>Low titer antinuclear antibodies (<1:320) (in up to a third of patients).</p> <p>Elevated C-reactive levels (% unknown).</p>

CHAPTER 2

Vitamin D

The production of vitamin D₃ in the skin is a non-enzymatic process. The reaction can be broken down into two steps (photoconversion and thermoconversion). In photoconversion, solar ultraviolet B photons with an energy range between 290 and 315 nm of radiation initiate photolysis of pro-vitamin D₃ 7-dehydrocholesterol (7-DHC) to pre-vitamin D₃ (MacLaughlin *et al.*, 1982). This photochemical process occurs in the plasma membrane of skin cells. Pre-vitamin D₃ is thermodynamically unstable; therefore, it rapidly undergoes a rearrangement of double bonds, transforming it into the more stable form, *s-trans*, *s-cis* pre-vitamin D₃ (Tian *et al.*, 1995). This conversion is quite efficient with approximately 50% of pre-vitamin D₃ converting to vitamin D₃ and entering the circulatory system with the help of transport proteins, within two hours of sun exposure (Tia *et al.*, 2010). In the following sections, this process will be discussed in greater detail.

Photo-conversion of 7-Dehydrocholesterol (7-DHC) to Pre-vitamin D₃

The first step in the photoconversion of pre-vitamin D₃ in the skin occurs when 7-DHC absorbs UVB (290-315 nm) of radiation, causing a bond cleavage between carbon 9 and carbon 10 and an isomerization of 5,7-diene to form the *s-cis*, *s-cis*-pre-vitamin D₃. The steric interaction between the C ring and the carbon 19 methyl group of *s-cis*, *s-cis*-pre-vitamin D₃ leads to generation of an energetically less stable molecule. To achieve stability, a rotation around the carbon 5/carbon 6 single bond occurs. This results in an energetically more stable *s-trans*, *s-cis*-pre-vitamin D₃ (Holick *et al.*, 1995). Alternatively, the unstable *s-cis*, *s-cis*-pre-vitamin D₃ conformer can form vitamin D₃ as a result of an intramolecular rearrangement of its hydrogen atoms.

7-dehydrocholesterol (7-DHC) is present in all layers of human skin, with 65% of 7-DHC per unit area found in the epidermis and the remaining 35% in the dermis (Holick *et al.*, 1981). Hence, the epidermal layers have the highest potential for pre-vitamin D₃ production. Indeed, over 95% of pre-vitamin D₃ production occurs in the epidermis (Holick *et al.* 1981). For the photolysis to take place, light radiation levels in the range of 290-315 nm must penetrate the layers of skin so that a state of maximum energy is attained. In 1982, MacLaughlin *et al.* determined the pathway for pre-vitamin D₃ production from

7-DHC in human skin after exposure to narrow-band radiation or simulated solar radiation and reported that the optimum wavelengths for the production of pre-vitamin D₃ was between 295-300 nm of radiation (MacLaughlin *et al.*, 1982). Once pre-vitamin D₃ is synthesized in the skin, it can either undergo a photoconversion to lumisterol, tachysterol, or back to 7-DHC, or undergo a thermal isomerization to vitamin D₃ (Holick *et al.*, 1981).

Subcellular localization studies of 7-DHC and pre-vitamin D₃ in human epidermal tissue revealed that most 7-DHC and pre-vitamin D₃ reside in the membrane fraction, while only 20% are found in the cytosol (Tia *et al.*, 2010). A majority of 7-DHC molecules got entrapped in the plasma membrane because of its 3 β -hydroxyl group's hydrophobic attraction to the polar head groups of membrane fatty acids, while its non-polar rings and side chain associating with the non-polar fatty acid tail hydrophobic by van der Waals interactions (Tia *et al.* 2010). When 7-DHC in the skin's plasma membrane is exposed to UVB radiation, the thermodynamically less favorable conformation of the *s-cis*, *s-cis*-pre-vitamin D₃ is stabilized through the hydrophobic and hydrophilic interactions with the bilayer lipid membrane fatty acids (Holick, 2010).

Thermal Conversion of Pre-vitamin D₃ to Vitamin D₃

The second and final step in the synthesis of vitamin D₃ is the thermo-isomerization of pre-vitamin D₃ in human skin. To date, there is no evidence for an enzymatic process in the skin that can convert pre-vitamin D₃ to vitamin D₃. Earlier studies by Havinga *et al.* demonstrated that this process cannot be affected by acids, bases, catalysts, or inhibitors of radical chain processes. Nor it is affected by the lack of an intermediate during this thermo-isomerization (Havinga *et al.*, 1973), leading to the conclusion that the reaction is an intramolecular concerted process involving a [1,7] sigmatropic hydrogen rearrangement on the opposite side of a plane (antarafacially) are on the same of side of a plane (suprafacially) (Havinga *et al.* 1973), where antarafacial arrangements are more favorable than suprafacial arrangements due to steric hindrance. Much of the experimental information about the pre-vitamin D₃ thermo-isomerization was obtained from *in vitro* experiments (Tian *et al.*, 1993) with organic solvents assumed to be the same as those present in human skin. Kinetic and thermodynamic studies of the thermo-isomerization of pre-vitamin D₃ to vitamin D₃ in human skin compared to those performed in an organic solvent revealed that both the equilibrium of the reaction is in favor of vitamin D₃ production in human skin (equilibrium constant K at 37°C = 11.44) compared to hexane ($K=6.15$). The rate of the reaction was increased by more than ten-fold

in human skin ($T_{1/2}$ at 37°C = 2.5 hours) when compared to hexane ($T_{1/2}$ = 30 hours) (Tian *et al.*, 1993). In terms of thermodynamics, the enthalpy (ΔH°) change for the reaction was $\Delta H^\circ = -21.58 \text{ kJ mol}^{-1}$ in human skin and $\Delta H^\circ = -15.60$ in hexane. In sum, the activation energy for both the forward and reverse reactions (rate constants and equilibrium constant) were lower in human skin as compared to hexane, highlighting the efficiency of skin cells in the formation of vitamin D₃ (Tian *et al.*, 1993).

Factors Influencing Regulation of Vitamin D₃ Synthesis by Sunlight

In 1967, W. Farnsworth Loomis was the first to propose the theory that human pigmentation evolved to play a critically important role in regulating the cutaneous photosynthesis of vitamin D₃. His rationale was that human melanin pigmentation evolved from darker to lighter in northern climates to mediate efficient photosynthesis of pre-vitamin D₃. This hypothesis points to the conditions of insufficient exposure to sun; people with lesser amounts of melanin production (Caucasian) may have an advantage over those of African descent. On the other hand, in Southern latitudes, a selection for darker skin made sure that up to 95 percent of the ultraviolet light is prevented from reaching the deeper layers of the skin where vitamin D is synthesized. In

addition to skin pigmentation, other key factors that influence the production of vitamin D synthesis include geographic location, relative thickness of the ozone (O_3) layer, and seasonal variation.

Geographic location, or more specifically, latitude has a major influence on the production of vitamin D synthesis. As one moves away from the equator and toward the poles, ultraviolet (UV) levels decrease. At the equator, the solar zenith angle (SZA) is the smallest, resulting in higher UV levels. Conversely, at higher latitudes, the SZA increases, which results in longer path for solar radiation. This means that latitude impacts vitamin D effective radiation. When all other parameters including ozone, altitude, and aerosol pollution are kept constant, seasonal variations are noted at the equator (0° latitude) (Kimlin, 2008).

When the latitude increases to 40° , a significant annual variability of the UV radiation, and therefore vitamin D production, becomes noticeable. During the winter months of December and January in Japan, there is virtually no vitamin D effective UV radiation. At the latitude of 90° , this problem exacerbates further, with no vitamin D effective radiation produced for 8 months of the year. When 40° and 90° latitudes are compared, a 7 fold difference in the maximum vitamin D producing UV amounts is noted during the summer months at 40°

latitude. These levels exceed the summer values at the equator (0° latitude) (Kimlin, 2008).

Ozone (O₃) is created when free oxygen atoms in the atmosphere are generated by splitting the diatomic oxygen (O₂) molecules by short ultraviolet (UV) waves and then combined with existing O₂ molecules. The ozone layer absorbs UV (primarily UVB) waves as they pass through. It is located roughly 10 kilometers (km) above the Earth within the stratosphere. One of the major consequences of this process is thermal energy production. Hence, the stratosphere has a slightly higher temperature than the top of the troposphere. This phenomenon is responsible for modern day climate change.

Bioactivation of Vitamin D to “Hormone D”

In higher vertebrate animals, the vitamin D₃ hormone is essential. Recently, this molecule received a lot of attention, with an increasing number of peer-reviewed articles published on the subject. For example, in 1975 there were approximately 100 manuscripts published that included the term *Vitamin D* in the title or abstract, while by 2007, the number of *Vitamin D*-related publications ballooned to greater than 1400 articles per year (Norman, 2008). In 2014, more than 4600 manuscripts were published on the subject.

It is important to note that the precursor to the hormonally active form, the skin-derived vitamin D₃, has no known intrinsic biological activity. In fact, it does not even circulate for long in the bloodstream, as it is either immediately sequestered in adipose tissue or enters the liver and, subsequently, the kidneys for further metabolism to become the biologically active hormone 1 α , 25 dihydroxyvitamin D₃ in a multi-enzymatic process. The steps of activation, including 25-hydroxylation, 1 α -hydroxylation and 24-hydroxylation, involve cytochrome p450 mixed-function oxidases (CYPs).

Vitamin D metabolizing hydroxylases (CYPs) are classified into two main subtypes based upon their subcellular location. These enzymes are located either in the endoplasmic reticulum (ER) or in the mitochondria. Neither microsomal nor mitochondrial CYP subtypes function alone but are components of the respective electron transport chains. The electron donor for the ER enzymes is the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent P450 reductase. The electron donor chain for the mitochondrial enzymes is comprised of ferredoxin and ferredoxin reductase. All of the vitamin D related CYPs catalyze single or multiple hydroxylation reactions on specific carbons of the vitamin D substrate using a transient Fe-O intermediate.

Hepatic 25-Hydroxylation of Vitamin D₃ to 25(OH)D₃

The first step in the functional metabolism of vitamin D, namely 25-hydroxylation, occurs in the liver (Suda *et al.*, 1969). In the past, there has been a discussion as to whether 25-hydroxylation is carried out by one enzyme or multiple enzymes and whether the cytochrome P450 enzyme is found in the mitochondria or microsomal fractions of the liver (Jones, 2010). In 1979, Madhok and DeLuca reported that a rat liver microsomal system requires NADPH, molecular oxygen, a flavoprotein, and a cytochrome P450 to achieve 25-hydroxylation of vitamin D₃. Furthermore, work by Fukushima *et al.* suggested existence of two 25-hydroxylase enzyme activities: a high affinity, low capacity type (microsomal, CYP2R1) and a low-affinity, high-capacity type (mitochondrial, CYP27A1).

The mitochondrial enzyme CYP27A was the first to be cloned. This enzyme is well conserved across species and is having two separate functions; it also expresses 27-hydroxylase activity toward cholesterol and related sterols in two separate bile acid biosynthetic pathways (Norlin *et al.*, 2003). The much-elusive microsomal CYP2R1 was identified by preparing a cDNA library from hepatic mRNA of sterol 27-hydroxylase-deficient mice. This cDNA library was screened with a ligand activation assay to identify an evolutionary conserved

microsomal cytochrome P450 (CYP2R1) with vitamin D 25-hydroxylase activity. Expression analysis of CYP2R1 in cells led to the transcriptional activation of the vitamin D receptor when vitamin D₃ was added to the medium. Furthermore, thin-layered chromatography and radio-immunoassays indicated that the product of CYP2R1 enzymatic activity was 25-hydroxyvitamin D₃. Thus, CYP2R1 became a strong candidate to be a key player in the microsomal 25-hydroxylation of vitamin D₃. The CYP2R1 enzyme, comprised of 501 amino acids, has been cloned from mouse and human tissue; then, by quantitative real-time PCR, its expression was characterized as specific for the liver and the testes (Cheng *et al.*, 2003).

Formation of Steroid Hormone 1 α , 25 Dihydroxyvitamin D₃ (1 α .25 (OH)₂ D₃)

The circulating levels of 25(OH)D₃ are commonly used as a measure of vitamin D status in the human body. However, a final bioactive form of this hormone is produced by yet another enzyme, 25-hydroxyvitamin D₃-1 α -hydroxylase. This tightly regulated step involves the introduction of a 1 α -hydroxyl group in the A ring of 25(OH)D₃ and the creation of the hormone 1 α , 25 Dihydroxyvitamin D₃ --all taking place in the kidneys (Cheng *et al.*, 2003). Unlike 25-hydroxylation, there seems to be only one cytochrome P450 enzyme

responsible for 25-hydroxyvitamin D₃ 1 α -hydroxylase activity and that is CYP27B1. This enzyme was sequenced and cloned by four different groups (Fu et al., 1997; Shinki et al., 1997; Takeyama et al., 1997; St-Arnaud et al., 2000).

The complexities of vitamin D biosynthesis have been corroborated by clinical observations. Patients with chronic kidney disease progressively lose 1 α -hydroxylase activity and gradually deplete their circulating levels of 1 α ,25 dihydroxyvitamin D₃ (1,25(OH)₂D₃) over the five-stage history of their disease. If left unsupplemented, these patients go on to develop renal osteodystrophy caused by a deficiency of 1,25(OH)₂D₃, and secondary hyperthyroidism (Jones, 2010).

Transport of Vitamin D Metabolite in Circulation: Role of Vitamin D Binding Protein (DBP)

Lipophilic steroid hormones and sterols are commonly transported through the bloodstream while being bound to plasma proteins (i.e. albumin and lipoproteins). This is largely due to their low aqueous solubility. Given the large number of plasma protein types and their sheer volume, some fraction of lipophilic hormones is always transported by one or another non-specific ligand carrier. However, there is also a specific mechanism for the transport of steroid

hormones and sterols including corticosteroid-binding globulin (CBG) (glucocorticoids, mineralocorticoids), vitamin A (retinol)-binding protein, sex hormone-binding globulin (SHBG) (estrogens, androgens), thyroid hormone-binding globulin and vitamin D-binding protein (DBP) (Chun, 2014).

A specific carrier for vitamin D transport, so-called Vitamin D-binding protein (DBP) is a multifunctional molecule capable of binding and transport of vitamin D metabolites, sequestering globular actin, and interacting with fatty acids and playing a number of prominent roles in the immune system and host defense (White et al., 2000).

Synthesis of DBP

Vitamin D binding protein (DBP) is produced mainly by the liver. In the human genome, the DBP-encoding gene is located on chromosome 4q11-q13 (Cooke *et al.*, 1986) in close proximity to serum albumin and α -fetoprotein encoding genes (Harper *et al.*, 1983). A comparative genomic analysis indicates that DBP is the oldest member of the Group-specific component of the serum (Gc globulin) family, whereas albumin (*ALB*), α -fetoprotein (*AFP*) and afamin (*AFM*) are more recent members (Bouillon, 2011). The proximal

promoter of the *DBP* gene contains three hepatocyte nuclear factor 1 (HNF-1) binding sites that serve as targets for liver specific HNF-1 α and HNF-1 β transcription factors (Song *et al.*, 1998). Before secretion into the bloodstream, DBP undergoes post translational modification, in particular, the cleavage of the N-terminal 16 amino acid leader and the glycosylation results in a protein of variable molecular weight of 52-59 kDa (Svasti *et al.*, 1979). In patients with liver disease, the levels of DBP production are reduced (Dusso, 2005).

Role DBP in Vitamin D Metabolites Transport and Diffusion

Vitamin D binding protein (DBP) is the major transport protein for more than 95-99% of 25(OH)D₃, whereas albumin and lipoproteins, despite their much higher serum concentration in comparison with DBP (650 μ M for albumin versus 5 μ M for DBP), non-specifically contribute to the transport of vitamin D metabolites to a substantially lesser degree (Bikle *et al.*, 1986). The measured affinity for 25(OH)D₃ binding to DBP ($7 \times 10^8 \text{ M}^{-1}$) compared to 25(OH)D₃ affinity for ALB ($6 \times 10^5 \text{ M}^{-1}$) is much higher (Bikle *et al.*, 1986). Similar trends are observed in the affinity of DBP to the active form of vitamin D, 1,25(OH)₂ D₃ ($4 \times 10^7 \text{ M}^{-1}$) as compared to the affinity of DBP to the same compound ($5.4 \times 10^5 \text{ M}^{-1}$) (Bikle *et al.*, 1985).

“Free Hormone” Hypothesis: Bound, Free and Bioavailable 25(OH)D₃

The relative high affinity and specificity of DBP for vitamin D metabolites in circulation result in almost 99% of vitamin D metabolites being bound to DBP. The relative molar abundance of DBP (650 μM) to 25(OH)D₃ (5 μM) in circulation, results in very a small fraction $\sim 0.1\%$ of 25(OH)D₃ being unbound or “free” (Chun, 2014). It is important to note that highly lipophilic molecules, i.e. vitamin D metabolites, have the potential to rapidly and passively diffuse across cell membranes (Chun, 2014). Vitamin D bound to DBP is thereby largely excluded from direct cellular entry. The bound fraction of the hormone plays an essential a role as a circulatory reservoir of the hormone for local tissue delivery.

The “free hormone” hypothesis describes the biological activity of a given hormone being affected by its unbound or “free” rather than protein-bound concentration in the plasma (Mendel, 1989). This “free hormone” hypothesis has been considered the universal mechanism for cellular uptake of steroid hormones due to the “sticky” (lipophilic) nature of these molecules. A slight modification to the “free hormone” hypothesis led to a different interpretation of the unbound, or bioavailable 25(OH)D₃ that refers to all the circulating

25(OH)D₃ that is not bound to DBP, plus the fraction bound to albumin (Chun, 2014).

Notably, while the “free hormone” hypothesis mathematically explains the biological activity of most steroid hormones, it does have its limitations. For instance, the high capacity/high affinity binding of DBP to 25(OH)D₃ was established. Hence, the free 25(OH)D₃ concentrations in serum are extremely low and one could question whether the free diffusion of such low 25(OH)D₃ is sufficient to produce the microgram amounts of 1,25(OH)₂D₃ produced in the kidney (Bouillon, 2011). Obviously, a mechanism that allows active accumulation of 25(OH)D₃ in the kidneys shall be in place. Indeed, a cell surface receptor for DPB, megalin/gp330, a member of the LDL-receptor family of clathrin-coated pit-associated receptors, was discovered recently. It is abundant on the brush border surface of the proximal tubular epithelium of the kidney (White *et al.*, 2001), hence, after binding with DBP, it is being internalized through endocytosis in proximal tubules and delivers DPB along with its cargo to the kidneys (Christensen *et al.*, 2001).

However, vitamin D is produced internally not only in kidneys, but also in other target tissues. Outside the kidney, megalin is expressed by the placenta, mammary glands and parathyroid glands; all these organs also are known to

express 1 α -hydroxylase, hence, it is likely that active form of vitamin D may also be produced there after similar internalization (Lundgren *et al.*, 1997). Other studies have shown that three other proteins, cubulin (Christensen *et al.*, 2001), α -fetoprotein (α -FP) and albumin (McLeod *et al.*, 1989) work in conjunction with megalin to facilitate the renal processing of DBP.

Another limitation of the “free hormone” hypothesis is that the concentrations of free 1,25(OH) $_2$ D $_3$ in serum are even lower than that for 25(OH)D $_3$, at approximately 10 $^{-13}$ M, which is much less than the apparent concentrations normally quoted for binding to the VDR (dissociation constant (Kd) = approximately 10 $^{-10}$ M).

Genomic and Non-Genomic Actions of Steroid Hormone 1,25(OH) $_2$ D $_3$

The vitamin D endocrine actions can be categorized into three biological processes (Metabolism, Transport, and Transcription), involving three types of proteins (Enzymes, Binding Protein, and Receptors). Furthermore, the biologic actions of 1,25(OH) $_2$ D $_3$, may be dependent on either genomic or non-genomic effects of this hormone in target tissues. In 1984, Nemere I et al. were the first to provide evidence for the “rapid” or non-genomic effects of 1,25(OH) $_2$ D $_3$ on

calcium transport in a model of transcalcitachia, the rapid hormonal stimulation of intestinal calcium absorption observed in vascularly perfused duodena of normal vitamin D replete chicks (Nemere *et al.*, 1984). They were able to demonstrate that adding 130 pM of 1,25 (OH)₂D₃ to the perfusate resulted in a significant increase of labeled calcium transport from the lumen to the vascular effluent. It was the first study to suggest that 1,25(OH)₂D₃ is capable of producing rapid “unidirectional” calcium transport responses implicating the role of cell membrane. Initially, it was thought that the non-genomic effects of vitamin D are mediated through its interaction with a novel protein. However, the membrane bound receptor turned to be the classic Vitamin D Receptor (VDR) bound to caveolae, a flask shaped membrane invaginations rich in sphingolipids and cholesterol (Huhtakangas *et al.*, 2004).

Over the years, numerous studies have reported evidence for the presence of “rapid” cytoplasmic responses mediated by 1,25(OH)₂D₃ in various cells and organs, Table 4 below (Mizwicki *et al.*, 2009).

Table 4. Summary of rapid responses mediated by 1,25(OH)₂D₃. Adapted from (Mizwicki et al., 2009).

Cells, Organs/Systems	Rapid Response Reported
Intestine	Rapid transport of intestinal calcium; PKC activation; PLC activation; MAPK activation
Colon	PKC activation
Adipocytes	Calcium signaling
Endothelial (primary rat aorta cells)	Vascular smooth muscle cell migration
Osteoblasts (ROS17/2.8 cells)	Calcium channel opening; Chlorine channel opening
Chondrocytes	PKC activation; PLA ₂ activation
Liver	PKC activation; MAPK activation
Muscle	PKC activation; calcium signaling
Promyelocytic (NB4 cells)	Cell differentiation
Leukemia cells	MAPK activation
Keratinocytes	Alteration of PKC subcellular distribution; Sphingomyelin hydrolysis; Src activation; Raf activation; prevention of skin cancer
Pancreatic β cells	Calcium signaling; Insulin secretion
Heart ventricular myocytes	Increased rate of contraction and relaxation
Fibroblasts	Calcium signaling
Parathyroid cells	Phospholipid metabolism
Lipid bilayer	PKC activation

In 1994, Beno and colleagues successfully demonstrated that 1,25(OH)₂D₃ induces nuclear signaling via stimulating mitogen-activated protein kinase (MAPK) by a protein kinase C (PKC)-dependent pathway in hepatic Ito cells (Beno *et al.*, 1995). Interestingly, when they experimented with dominant negative Raf/MAPK transfections, the nuclear signaling induced by 1,25(OH)₂D₃ still proceeded by a pathway independent of Raf induction (Beno *et al.*, 1995).

Genomic effects of steroid hormone 1,25(OH)₂D₃ are mediated through interactions with its cognate receptor, Vitamin D receptor (VDR).



Figure 2. Diagram of the functional domains of Vitamin D receptor (VDR).

The nuclear receptor vitamin D (VDR) belongs to a transcription factor superfamily, members of which have the unique property to be directly activated by small hydrophobic (lipophilic) molecules (Tuoresmaki *et al.*, 2014). They are

considered to be one of the largest families of metazoan transcription factors characterized by the presence of a highly conserved DNA-binding domain (DBD) and a structurally conserved ligand binding domain (LBD) (Mangelsdorf *et al.*, 1995). The nuclear receptor superfamily has 48 human members, of which only 12, including VDR, are classical endocrine receptors (Evans *et al.*, 2014; Mangelsdorf *et al.*, 1995). The human VDR gene is located in the *q* arm of the chromosome 12 (12q13-14) region (Szpirer *et al.*, 1991). In fact, the two most important players in the vitamin D endocrine system, VDR and 1 α -hydroxylase, both map close to each other in the *q* arm of chromosome 12, 12q13.11 and 12q14.1, respectively (MacDonald *et al.*, 2011). The human VDR gene consists of 14 exons, eight of them are coding (2-9), while six more exons (1a-1f) cover the GC-rich regulatory region (Jehan *et al.*, 2007). Exon 2 encodes the two known translation initiation codons, the first of which encodes a receptor protein of 427 amino acids (MacDonald *et al.*, 2011). In addition, exon 2 contains sequences for the first zinc finger of the DNA binding domain while exon 3 encodes the second zinc finger of the domain. The C-terminal ligand-binding domain is encoded by exons 8 and 9.

The human VDR mRNA transcript consists of 4,605 bp and contains a 115 bp noncoding leader sequence, a 1,281 bp open reading frame, and 3,209 bp of 3' noncoding sequence (MacDonald *et al.*, 2011). Vitamin D receptor

(VDR) cDNA sequence comparison shows a striking similarity with other members of the superfamily of nuclear receptors for steroid and thyroid hormones, specifically in the DNA-binding domain (DBD) of these receptors (MacDonald *et al.*, 2011). The DBD is responsible for high-affinity interactions with specific DNA sequence elements; it consists of 70 amino acids rich in cysteine, lysine, and arginine residues (MacDonald *et al.*, 2011). The specificity of these nuclear receptors for their designated ligands is defined by their C-terminal ligand binding domains (LBD).

Vitamin D receptor (VDR) mediates genomic signaling of 1,25 (OH)₂D₃ steroid hormone

The mechanism of VDR mediated gene expression requires well-coordinated cascades of reactions set in motion by ligand binding. In the basal state, the VDR and RXR heterodimer are held together by weak association and scan the genomic DNA in a non-specific manner. In the ligand free-state, the VDR-RXR heterodimer interact with transcription factor II (TFII) and co-repressor that bind to the AF-2 domain of both VDR and RXR. This leads to the recruitment of histone deacetylases (HDACs) and results in a repressed state of gene expression within the cognate locus.

Mutational analysis reveals that binding of 1,25 (OH)₂D₃ to the ligand binding pocket of VDR results in conformational change in the position of helix 12 at the C-terminus of VDR, bringing it to the “closed” position, where it can serve as part of a platform for coactivator binding (Jurutka *et al.*, 1997; Haussler *et al.*, 2011). In addition, a much stronger heterodimerization of VDR and RXR is established after subsequent hormone-dependent phosphorylation events, including these facilitated by casein kinase II (CK2) that acts on human VDR serine 208 (Jurutka *et al.* 1993; Jurutka *et al.*, 1996). These events ultimately lead to the dissociation of the co-repressor/HDAC complex and promote the recruitment of co-activator complex [i.e. SRC-1, CBP/p300, nuclear receptor coactivator-62 (NCoA-62)] and chromatin remodeling complex. An important note here is that the recruitment of these co-activators/co-modulators requires DNA looping to facilitate contact between comodulators tethered to enhancer elements and the transcriptional start site (Haussler *et al.*, 2011).

The steroid receptor coactivator 1 (SRC-1) possess histone acetyl transferase (HAT) activity and interacts with the AF2 domain of steroid receptors, including VDR, in a ligand dependent manner. The SRC/p160 coactivators have been reported to recruit CBP (CREB binding protein), which also has HAT activity, resulting in an assembly of multisubunit complex (McKenna *et al.*, 1999; Christakos *et al.*, 2003). Nuclear coactivator-62 (NcoA-

62) is an AF-2 independent coactivator of VDR that has HAT activity, but acts by forming a ternary complex with VDR and SRC coactivators to result in a synergistic effect on VDR mediated transcription (Zhang *et al.*, 2001; Zhang *et al.*, 2003). Early transcriptional targets of 1,25(OH)₂D₃ participate in adhesion, growth regulation, angiogenesis, actin cytoskeleton regulation, hexose transport, inflammation and immunomodulation, apoptosis, endocytosis and cell signaling (Goeman *et al.*, 2014).

CHAPTER 3

Vitamin D Deficiency and NAFLD/NASH

Introduction

NAFLD, an increasingly common condition affecting about 70% of obese and overweight individuals is now being recognized as a major cause of liver-related morbidity and mortality (Hamaguchi *et al.*, 2005). The pathological picture of NAFLD encompasses a spectrum of liver injury ranging from simple hepatic steatosis to more severe manifestations, including NASH, which can progress to fibrosis, cirrhosis, and ultimately, liver failure (Hanley *et al.*, 2005). Studies have reported frequent association of metabolic syndrome and diabetes in patients with NASH, which can progress to NAFLD (Kim *et al.*, 2003). Metabolic syndrome, an umbrella term, is characterized by an array of metabolic perturbations including type 2 diabetes, insulin resistance, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), cardiovascular disease, hypertension, hyperlipidemia and obesity (Shoelson *et al.*, 2007).

Vitamin D is a steroid hormone involved in the intestinal absorption of calcium and the regulation of calcium homeostasis. There are two different

forms of vitamin D: D₃ and D₂, although these are highly similar in structure, D₂ is a synthetic product, typically absorbed via fortified food. Serum 25-hydroxyvitamin D₃ is produced by the hydroxylation of vitamin D₃ (cholecalciferol) in the liver and is the major circulating metabolite of vitamin D. 25(OH)-vitamin D is widely accepted as the best indicator of circulating vitamin D status in patients.

Recent evidence suggest that vitamin D deficiency may have a potential role in inflammatory processes, and there is a an ever increasing body of literature indicating that low vitamin D levels may play an instrumental role in the development of both hypertension and metabolic syndrome (MS) (Reis JP *et al.*, 2008; Chiu *et al.*, 2004; Martins *et al.*, 2007; Ford *et al.*, 2005). Furthermore, reports from randomized, placebo-controlled trials also suggest that vitamin D supplementation effectively reduces obesity related comorbidities, such as insulin resistance and the spectrum of conditions within metabolic syndrome (MS), further solidifying the relationship between vitamin D and obesity (von Hurst PR *et al.*, 2010; Nagpal J *et al.*, 2009).

Pertinent to NAFLD and NASH, a significant inverse relationship between serum vitamin D levels and unexplained elevation in ALT were recently observed in the NHANES III cohort (Liangpunsakul *et al.*, 2011). The correlation between inflammatory response and vitamin D levels seems to be a

plausible connection that may underline the involvement of vitamin D in chronic liver disease. Consequently, the examination of vitamin D levels in patients with NASH and various degrees of liver involvement, may lead to a better understanding of the relationship between chronic liver disease and vitamin D. In addition, if vitamin D is found to be directly involved in the pathogenesis and progression of NASH, it may prove to be a reliable and non-invasive biomarker of the severity of the disease and its propensity for progression.

This study profiles serum samples of 210 morbidly obese bariatric patients with NAFLD/NASH for the following markers: 25 OH-vitamin D; Vitamin D Binding Protein (VDB); Intact Parathyroid Hormone (1-84, PTH) and Cytokeratin 18 (M30). Correlation analysis was conducted for these biomarkers and a set of clinical parameters routinely measured in NAFLD patients, including histological scores of inflammation and fibrosis of their liver biopsies.

Materials and Methods

This study was conducted in collaboration with the Beatty Liver and Obesity Research Program at Fairfax INOVA Hospital (Falls Church, VA). Samples (serum and solid tissue biopsies) from morbidly obese patients undergoing bariatric surgery had been collected by the Center from patients histologically confirmed NAFLD, and a repository of specimens was assembled.

This repository was made accessible for IRB approved research, and included liver and adipose tissues as well as fasting serum samples gathered at the time of liver biopsy, all de-identified to protect patient identity and stored at -80°C. Liver histology analysis was performed by a single hepatopathologist, Dr. Zachary Goodman. Stage and grade were assigned for each sample for steatosis, NASH, and degrees of fibrosis. The NASH patients included the following subgroups of patients: NASH with zero to minimal fibrosis=Grade 0 and 1 fibrosis; NASH with Mild fibrosis= Grade 2 fibrosis; NASH with Severe Fibrosis= Grades 3 and 4 fibrosis (may include bridging fibrosis or cirrhosis). A control group with liver steatosis and minimal non-specific inflammation or no inflammation in the liver (NAFLD Non-NASH) was included. Sixty microliters of each serum sample was used to test for vitamin D levels. Each sample was run in duplicate. Resultant data was subjected to non-parametric statistical tests. Correlations of the measured analyte values to a variety of clinical parameters, histological scores of inflammation and fibrosis in liver biopsies were assessed.

The majority of patients were morbidly obese ($BMI \geq 40$). For each patient, extensive clinical and demographic data, which included information on drug and alcohol use, the presence of diabetes mellitus, hypertension, or hyperlipidemia were collected. Each patient underwent a physical examination in which height, weight, hip and waist measurements were obtained. Patients

with excessive alcohol use (≥ 10 g/d), other etiologies of liver disease (i.e., Viral or Auto-immune) and those receiving treatment with PPAR- γ agonists were excluded. Vitamin D deficiency status was assessed, defined as >30 ng/ml. Table 5 outlines the descriptive statistics of the cohort. Patients who were on vitamin D supplements were identified by questionnaires. Pathologic diagnosis and stages of NAFLD disease were assessed for each patient. This study protocol was approved by the Institutional Review Board (IRB) of Fairfax INOVA Hospital.

Histopathology

Liver tissues were histologically processed, specifically formalin fixed, to preserve the quality of the tissue and routinely sectioned for slide preparation and stained with hematoxylin-eosin (H&E) and Masson trichrome. H&E stained slides were used to assess stages of steatosis, which were graded as an estimate of the percentage of tissue occupied by fat vacuoles (as a result of vacuolation) as follows: 0 = none, 1 = $<5\%$, 2 = 6-33%, 3 = 34-66%, 4 = $>66\%$. For this study, histological NASH was defined to include the following: steatosis, ballooning degeneration and lobular inflammation with or without pericellular fibrosis, and the presence of Mallory-Denk bodies.

Masson's trichrome stain was applied to slides to assess the level of fibrosis for each sample. The application of this stain imparts a blue color to collagen against a background of hepatocytes and other structures. Staining highlights the presence and distribution of reactive fibrosis that resulted from liver injury. Portal fibrosis and interlobular fibrosis were staged as follows: 0 = none, 1 = fibrosis, 2 = mild, and 3 = advanced, for each sample.

Quantification of Serum Biomarkers

A panel of biomarkers including 25(OH)-vitamin D, (Alpco Immunoassays, Salem NH); Vitamin D Binding Protein (DBP) (Quantikine, Minneapolis, MN); Parathyroid Hormone (1-84, PTH, (Alpco Diagnostics, Salem, NH); and apoptotic marker cytokerating-18 (M30) (Alpco Immunoassays, Salem, NH) were selected for this study. Serum levels of these markers were measured by Enzyme Linked Immunosorbent Assay (ELISA) or Enzyme Immunoassays (EIA), following the manufacturer's protocol. In principle, these assays employ enzyme immunoassay techniques where an antibody, specific to the biomarker of interest (antigen), is pre-coated onto a microplate. Standards, controls and samples are pipetted into the wells with the appropriate number of replicates. Biomarkers of interest found in the serum of patients become bound to the immobilized antibody. After several wash steps

removing unbound substances, a secondary monoclonal antibody, specific to the biomarker of interest (antigen), is added to the wells. Subsequently, a secondary, enzyme-coupled antibody is applied and for quantification. A chromogenic substrate for the enzyme yields a visible color change or fluorescence, indicating the presence of a biomarker (antigen). Quantitative and qualitative measurements are then obtained from the colorimetric absorbance reading (range 405 nm - 630 nm) measured according to each biomarker/ELISA manufacturer's recommendations. The main purpose of recording at different wavelength settings is to correct against any wavelengths detected in blank wells. Gen6 software was used to produce concentration curves based on the respective standard curves. An average of the concentration is calculated for each replicate sample, with standard deviations and confidence coefficients percentages (%CV) for each set.

Statistical Analysis:

Developing NAFLD Predictive Models

The statistical approach used for this study followed that used in previously published work (Younossi ZM et al. 2011). The complete NAFLD cohort was divided into sub-cohorts according to the following diagnostic comparisons: (a) patients with NASH were compared to those with Non-NASH;

(b) Patients with any liver fibrosis were compared to those without fibrosis; and
(c) patients with advanced liver fibrosis were compared to those with non to minimal fibrosis.

All three comparison groups was further analyzed separately. Specifically, for each of these comparisons, means and variances of each clinical, demographic, and laboratory parameters were calculated for each of the two corresponding sub-cohorts. Then, two-sample statistical tests were performed for each parameter in each comparison separately. Comparisons between the sub-cohorts were made with Mann-Whitney tests for continuous variables, and using chi-square homogeneity tests for categorical variables. Two-tailed hypotheses were tested, and p values not exceeding 0.05 were considered significant (unless noted otherwise).

The main endpoint of the study is to examine the performance of a newly constructed marker profile from combinations of clinical and laboratory parameters to predict the presence of any fibrosis, advanced fibrosis, and NASH in patients with NAFLD. Regression models were developed using the stepwise bi-directional selection for each comparison. For regression model design, the bi-directional selection procedure started with a complete model that contained all the predictors potentially associated with dependent variables (i.e. NASH, fibrosis and advanced fibrosis), and obtained the most improvement in

data fitting was possible with the addition or elimination of any predictor. In regression models, the predictor variables were the clinical and laboratory parameters, and the dependent variables was the presence of any fibrosis, advanced fibrosis, or NASH. To meet the applicability criteria for regression models, data transformation for non-normally distributed variables were used. At each step of model design, including variable selection and model training, the resulting regression models with the highest coefficient of determination was selected for future correctly predicted outcomes by these models. The stability of the variables selection process and model design were verified at each step in a series tenfold cross validation (10-CV) experiments. In those experiments, the complete cohort was partitioned into ten subsamples and, of the ten subsamples used exactly once as the validation data. The predictive power of the developed models, namely, the sensitivity, specificity, positive/negative predictive values, and the area under the ROC-curve (AUC) with 95% confidence intervals (CIs), were measured.

Results

Demographic, clinical, laboratory and histological data and the outcomes of group statistical comparisons for patients with and without NASH are listed in Table 7. No histological attribute was statistically different in patients with NASH

as compared to those without NASH, except for hepatic fibrosis, which was significantly more prevalent in NASH patients ($p < 0.015$). NASH patients were, on average, slightly older than those without NASH (Table 5). Caucasian ethnicity and male gender were more prevalent in the cohort with NASH; this cohort was also enriched by various co-morbid conditions, including diabetes type II, hyperlipidemia and hypertension. Proportions of people receiving or not receiving Vitamin D supplementation in NASH and non-NASH cohorts were same.

Table 5. Demographic, clinical, and laboratory data for patients with and without NASH. Entries are counts for discrete measures with percentages of group total given in parentheses or mean \pm S.D. for continuous measures. p -values of ≤ 0.05 were considered significant and shown in bold. NS: Non-significant.

	NASH	no NASH	<i>p-values</i>	<i>Entire cohort</i>
N	94	116		210
Prevalence (%)	44.76	55.24		
Fibrosis	53 (56.4%)	7 (6.1%)	1.6e-15	60 (28.8%)
White	77 (81.9%)	74 (63.8%)	3.7e-3	151 (71.9%)
Black	7 (7.4%)	25 (21.6%)	4.6e-3	32 (15.2%)
Hispanic	5 (5.3%)	2 (1.7%)	0.14	7 (3.3%)
Female	58 (61.7%)	96 (83.5%)	3.7e-4	154 (73.7%)
Diabetes type II	37 (39.4%)	30 (26.5%)	0.04	67 (32.4%)
Hyperlipidemia	47 (51.1%)	40 (36.4%)	0.03	87 (43.1%)
Hypertension	55 (59.1%)	50 (44.6%)	0.03	105 (51.2%)
Age, years	47.87 \pm 10.71	42.07 \pm 11.46	2.6e-4	44.68 \pm 11.47
ALT (U/L)	47.17 \pm 36.30	26.47 \pm 16.61	2.1e-08	35.77 \pm 29.09
AST (U/L)	41.57 \pm 62.00	20.97 \pm 10.39	1.4e-11	30.29 \pm 43.51

BMI	46.07 +/- 13.77	46.20 +/- 9.83	NS	46.145+/- 11.70
Glucose (mg/dL)	112.79 +/- 38.40	105.18 +/- 34.55	NS	108.40 +/- 36.34
HDL (mg/dL)	44.10 +/- 11.66	50.94 +/- 13.77	9.3e-4	47.73 +/- 13.23
LDL (mg/dL)	114.59 +/- 42.61	110.364/- 36.23	NS	112.35 +/- 39.28
M30 (U/L)	279.03 +/- 206.87	165.390+/- 82.39	2.9E-05	216.08+/- 161.01
Platelet count	268.66 +/- 78.60	286.74 +/- 64.34	0.05	279.144+/- 71.04
Total bilirubin(μ mol/l)	0.59 +/- 0.75	0.46 +/- 0.32	8.0e-3	0.52 +/- 0.56
Total cholesterol (mg/dL)	192.24 +/- 40.82	190.03 +/- 37.00	NS	190.99 +/- 38.62
Triglycerides (mg/dL)	163.64 +/- 76.42	148.98 +/- 72.62	NS	155.32 +/- 74.43
25(OH)D3 (ng/ml)	61.49 +/- 40.12	43.96 +/- 28.71	5.1E-05	51.81 +/- 35.30
Weight (kg)	133.13 +/- 45.98	128.32 +/- 31.56	NS	130.47 +/- 38.65

The Vitamin D Metabolism is Substantially Modified in Obese Patients with Non-Alcoholic Fatty Liver Disease (NAFLD).

Vitamin D levels were found to negatively correlate with BMI ($r = -0.28$, $p < 1.0e-4$) and the levels of PTH ($r = -0.30$, $p < 9.7e-5$) and positively correlate with the presence of histologic NASH ($r = 0.26$, $p < 4.0e-4$) and higher stages of fibrosis ($r = 0.16$, $p < 0.03$) (Table 6).

Table 6. Correlation of 25(OH)D₃ serum level with clinical parameters in cohort of NAFLD Patients.

Clinical Parameter	Correlation Coefficient (R)	p-Value
BMI	-0.284 (Negative)	1.0e-4
Parathyroid Hormone (PTH)	-0.298 (Negative)	9.7e-5
NASH	0.26 (Positive)	4.0e-4
Higher Stages of Fibrosis	0.16 (Positive)	0.03

Vitamin D and PTH levels in morbidly obese patients with NAFLD

The levels of PTH were positively correlated with BMI ($r=0.23$, $p<3.0e-3$) and negatively correlated with the levels of ALT ($r=-0.18$, $p<2.5e-3$) and triglycerides ($r=-0.213$, $p<0.01$) (Table 7).

Table 7. Correlation of the serum levels of PTH.

Clinical Parameter	Correlation Coefficient (R)	p-Value
BMI	0.23	3.0e-3
ALT	-0.18	2.5e-3
Triglycerides	-0.21	0.01

Circulating levels of vitamin D and PTH were significantly different when a cut off of 40 was used for BMI [49.56 nmol/L +/- 34.45 vs. 73.12 nmol/L +/- 43.68 ($p\text{-value} = 1.2e-4$) and 81.57 pg/mL +/- 37.05 vs. 57.79 pg/mL +/- 23.54 ($p\text{-value} = 3.0e-3$)]. Pearson's correlation analysis revealed an inverse relationship between vitamin D and PTH levels ($r=-0.39$, $p=1.0e-4$) and BMI ($r=-0.35$, $p=1.0e-4$). On the other hand, there was a significant positive correlation between PTH levels and BMI ($r=0.35$, $p=1.0e-3$). The data indicated that

vitamin D and PTH levels were significantly different in morbidly obese persons, irrespective of liver disease status. The vitamin D, PTH and obesity axis needs to be studied further for potential therapeutic targets (Table 8).

Table 8. Circulating levels of 25(OH)D₃ and Parathyroid Hormone (PTH) in Patients Cohorts with BMI >40 and BMI<40.

Clinical Parameter	BMI>40	BMI<40	p-value
25(OH)D ₃	49.56±34.45	73.12±43.68	1.19e-5
PTH (intact, 1-84)	81.57±37.05	57.79±23.54	2.8e-3

Levels of Vitamin D Binding Protein Correlate with the levels of Triglycerides and Hepatic Ballooning in NAFLD Patients.

The levels of the vitamin D binding protein showed a positive correlation with the levels of triglycerides ($r=0.34$, $p<1.2e-4$) and a negative correlation with the degree of histologic hepatocyte ballooning ($r=-0.23$, $p<0.02$) in the liver biopsies (Table 11). In addition, both vitamin D levels ($p=4.5e-3$) and vitamin D binding protein levels ($p=1.9e-3$) were significantly lower in the non-Caucasian group ($n=135$) within the cohort (Table 9).

Table 9. Correlation of the levels Vitamin D Binding Protein (VDBP) with the levels of triglycerides and the score for Hepatocyte ballooning.

Clinical Parameter	Correlation Coefficient (R)	p-Value
Triglycerides	0.34 (Positive)	p<1.2e-4
Histologic Hepatocyte Ballooning	-0.23 (Negative)	p<0.02

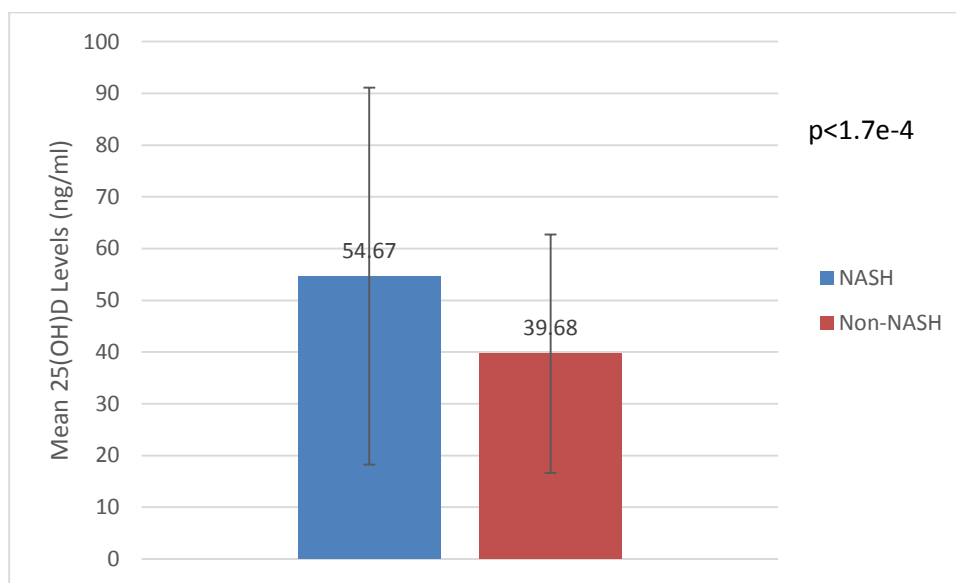


Figure 3. Mean 25(OH)D₃ levels in NASH and Non-NASH patients at BMI>40.

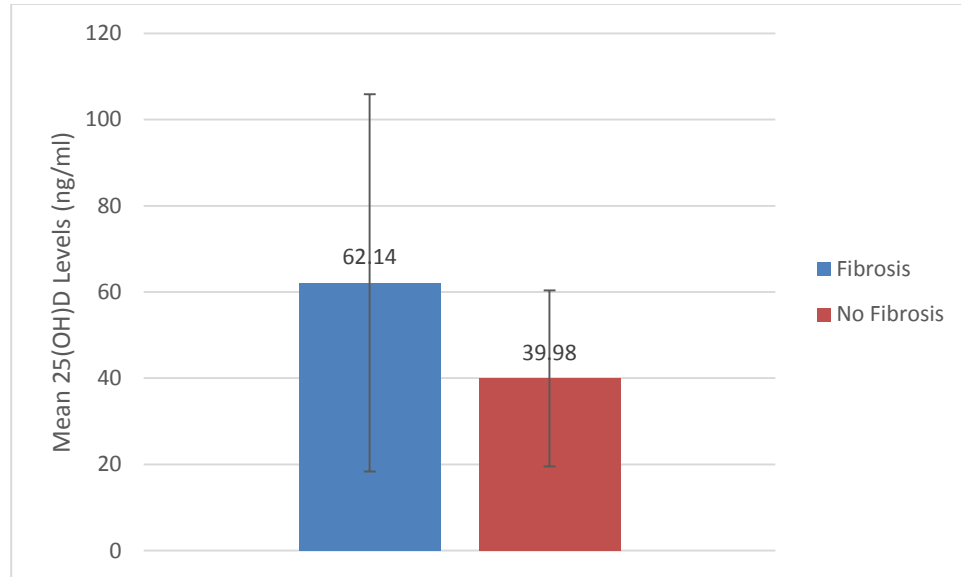


Figure 4. Mean 25(OH)D₃ levels in NASH with and without fibrosis at BMI>40.

Vitamin D levels significantly correlated with marker of apoptosis in patients with non-alcoholic fatty liver disease (NAFLD).

The levels of the apoptosis biomarker M30 also were correlated with both the presence of histologic NASH ($r=0.28$, $p<1.6e-5$) and the presence of fibrosis ($r=0.36$, $p<1.07e-6$), but not with BMI (Table 10).

Table 10. Correlation of the serum levels of the apoptosis biomarker M30.

Clinical Parameter	Correlation Coefficient (R)	p-Value
NASH	0.28 (Positive)	p<1.6e-5
Presence of Fibrosis	0.36 (Positive)	p<1.07e-6

Table 11. Demographic, clinical, and laboratory data for patients with and without vitamin D supplementation. Entries are counts for discrete measures with percentages of group total given in parentheses or mean \pm S.D. for continuous measures. A *p*-value of ≤ 0.05 was considered significant. Significant results are shown in bold text. NS: Non-significant

	Vitamin D Supplementation	No-Vitamin D supplementation	p-values	Entire cohort
N	44	166	NA	210
Prevalence (%)	20.56	79.44	NA	
NASH	19 (43.2%)	75 (45.2%)	NS	94 (44.8%)
Fibrosis	12 (27.3%)	48 (29.1%)	NS	60 (28.7%)
White	33 (75.0%)	121 (71.2%)	NS	154 (72.0%)
Black	7 (15.9%)	25 (14.7%)	NS	32 (15.0%)
Hispanic	0 (0.0%)	7 (4.1%)	NS	7 (3.3%)
Female	30 (68.2%)	127 (75.6%)	NS	157 (74.1%)
Diabetes type II	18 (40.9%)	50 (30.1%)	NS	68 (32.4%)
Hyperlipidemia	16 (36.4%)	73 (45.3%)	NS	89 (43.4%)
Hypertension	19 (44.2%)	88 (53.3%)	NS	107 (51.4%)
Age	46.46 \pm 11.11	44.37 \pm 11.54	NS	44.80 \pm 11.46
ALT (U/L)	29.74 \pm 19.89	37.67 \pm 31.05	NS	35.97 \pm 29.16
AST (U/L)	26.75 \pm 16.85	31.53 \pm 48.22	NS	30.49 \pm 43.36
BMI	44.12 \pm 10.18	46.51 \pm 12.09	NS	46.01 \pm 11.74
Glucose	107.91 \pm 31.62	108.44 \pm 37.59	NS	108.32 \pm

(mg/dL)				36.25
HDL (mg/dL)	49.28 +/- 13.09	47.25 +/- 13.20	NS	47.64 +/- 13.16
LDL (mg/dL)	102.63 +/- 32.91	114.58 +/- 40.04	NS	112.27 +/- 38.95
M30 (U/L)	172.94 +/- 134.39	228.86 +/- 165.37	3.6e-3	217.0 +/- 160.7
Platelet count	271.46 +/- 76.90	280.67 +/- 68.93	NS	278.7 +/- 70.61
Total bilirubin(μ mol/l)	0.63 +/- 1.07	0.49 +/- 0.29	NS	0.52 +/- 0.56
Total cholesterol (mg/dL)	179.22 +/- 34.34	193.96 +/- 38.79	0.04	191.04 +/- 38.31
Triglycerides (mg/dL)	136.03 +/- 57.56	161.07 +/- 78.21	NS	156.23 +/- 75.18
25(OH)D ₃ (ng/ml)	62.32 +/- 45.42	49.22 +/- 31.88	NS	51.92 +/- 35.37
Weight (kg)	127.51 +/- 33.61	130.73 +/- 40.03	NS	130.05 +/- 38.7

Correlation analyses were performed separately for those taking vitamin D supplements (n=44) and for those who were not (n= 166). For the group of patients without vitamin supplementation, vitamin D levels were positively correlated with M30 ($p<4.6e-4$), hyperlipidemia ($p<4.1e-4$), age ($p<3.7e-4$), ALT ($p<1.1e-4$), total bilirubin ($p<8.0e-4$), and AST ($p<1.3e-4$) (Table 14), whereas in the African American population ($p<6.0e-4$), LDL ($p<2.0e-4$), weight ($p<2.7e-4$) and BMI ($p<1.5e-6$) were negatively correlated with vitamin D levels (Table 12).

Table 12. Correlation of serum 25(OH)D₃ levels in Vitamin D supplemented group, n=44).

Clinical Parameter	Correlation Coefficient (R)	p-Value
M30	0.22	4.6e-4
Hyperlipidemia	0.16	4.2e-4
Age	0.16	3.7e-4
ALT	0.26	1.1e-4
Total bilirubin	0.26	8.0e-4
AST	0.25	1.3e-4

Table 13. Correlation of serum 25(OH)D₃ levels in Vitamin D non-supplemented group, n=166).

Clinical Parameter	Correlation Coefficient (R)	p-Value
African American	-0.26	6.0e-4
LDL	-0.21	2.0e-4
Weight	-0.23	2.7e-4
BMI	-0.33	1.5e-6

Vitamin D May Be Protective Against Apoptosis in Patients with Non-Alcoholic Fatty Steatohepatitis (NASH)

NASH patients who were not receiving vitamin D supplementation had higher levels of M30 than non-NASH (295.73 ± 210.77 vs. 172 ± 87.85 mg/ml, $p < 2.8E-05$). In a similar analysis, NAFLD with advanced fibrosis had higher M30 levels regardless of vitamin D supplementation [with Vitamin D (248 vs. 145, $p <$

9.0E-05), without vitamin D (278.85 vs. 206.12, $p < 5.0e-3$]. Vitamin D supplementation seems to have an effect on the levels of apoptosis on histologic NASH but not in the advanced fibrosis cohort. These data suggest that the protective effects of vitamin D may be subject to a systemic damage threshold. Further research is warranted to elucidate the impact of vitamin D on apoptosis in NAFLD.

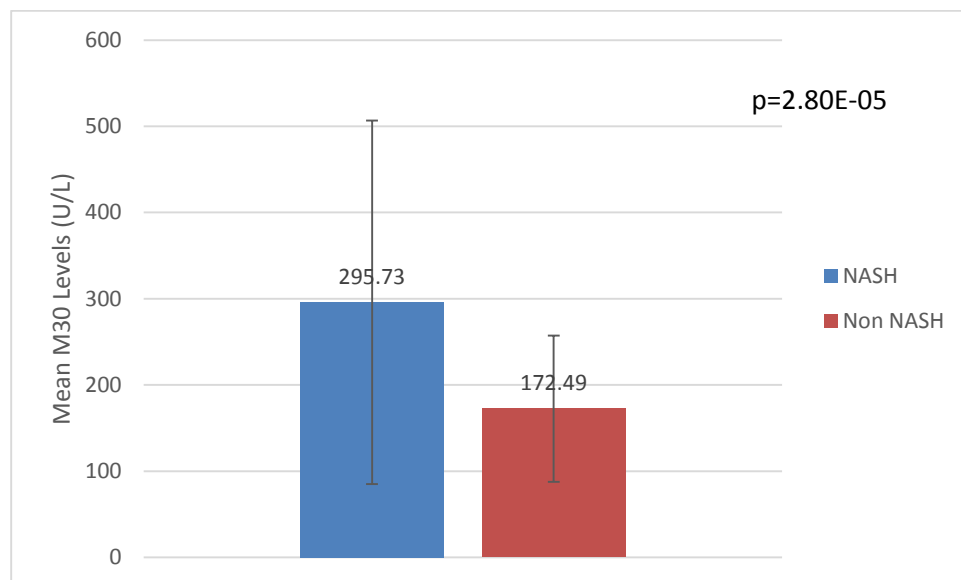


Figure 5. Mean M30 levels in NASH patients with no vitamin D supplementation status.

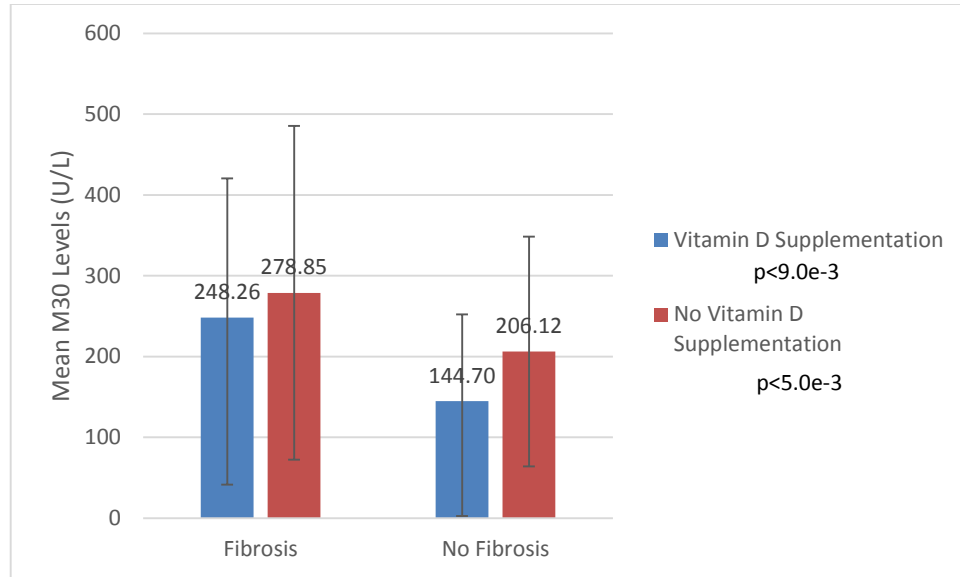


Figure 6. Mean M30 levels in patients with and without fibrosis and vitamin D supplementation status.

Additionally, for the entire NAFLD cohort (NASH subtype and non-NASH subtype), analysis of the trends did not reveal significant changes in serum 25(OH)D₃ concentrations with multivitamin supplementation. However, when NAFLD patients with the lowest serum vitamin D levels were compared, effects of multivitamin supplementation registered as partial corrections of serum 25(OH)D₃ levels. Importantly, in the group of NAFLD patients receiving specific vitamin D supplementation, the effects on 25OHD₃ levels in serum were substantially greater (95+/- 41 nmol/L, $p < 3.6e-3$) as compared to NAFLD

patients who were receiving multivitamin supplementation (49 \pm 29 nmol/L, $p < 0.02$) or no supplementation at all (56 \pm 35 nmol/L, $p < 1.5e-3$) (Table 14).

Table 14. NAFLD patients' supplementation status and effect on 25(OH)D₃ levels. (1ng=0.40nmol/l)

Clinical Parameter	25(OH)D₃ Levels	<i>p</i>-Value
Specific vitamin D supplementation	95 \pm 41 nmol/L (38 ng/ml)	3.6e-3
Multivitamin supplementation	49 \pm 29 nmol/L (19.6 ng/ml)	0.02
No supplementation	56 \pm 35 nmol/L (22.4 ng/ml)	1.5e-3

By multiple regression analysis the following predictive ($p=3.591e-008$) model for NASH was identified: probability of NASH= -0.2637 + 0.1605 (Hyperlipidemia) + 0.0027 [25(OH)D₃] + 0.0009 (M30) + 0.0019 (AST) + 0.0020 (weight), Table 15.

Table 15. Model for the prediction of NASH.

	<i>β</i>-value	\pmS.D.	<i>p</i>-value
(Intercept)	-0.2637	0.1566	9.41e-4
Hyperlipidemia	0.1605	0.0680	1.94e-4
25(OH)D₃	0.0027	0.0010	8.1e-4
M30	0.0009	0.0002	1.0e-4
AST	0.0019	0.0008	1.92e-4
Weight	0.0020	0.0009	3.12e-4

By multiple regression analysis the following predictive ($p=1.0e-5$) model for fibrosis was identified: probability of fibrosis= $-0.1667 + -0.2405$ (Black) + 0.0025 [25(OH)D₃] + 0.0003 (M30) + 0.0019 (AST) + 0.00208 (BMI), Table 16. Black race is a categorical variable and entered as follows: Black, 1=yes, 0=no.

Table 16. Model for the prediction of Fibrosis.

	<i>β-value</i>	<i>±S.D.</i>	<i>p-value</i>
(Intercept)	-0.1667	0.1594	0.297
Black	-0.2405	0.0880	6.9e-4
25(OH)D₃	0.0025	0.0010	0.0102
M30	0.0003	0.0002	0.1387
AST	0.0019	0.0007	0.0124
BMI	0.0051	0.0028	0.0677

Discussion

Vitamin D deficiency is defined by the Institute of Medicine (IOM) as a 25(OH)D₃ level below 20 ng/mL (50 nmol/L) (Holick *et al.*, 2011; Del Valle *et al.*, 2011). Vitamin D insufficiency is defined at serum levels of 25(OH)D₃ between 21-29 ng/ml (1 ng/ml=2.5 nmol/L) and sufficiency as 30-100 ng/ml. The IOM arrived at these definitions in part by studying adult motor vehicle accident victims who had serum and bone biopsies recovered (Priemel *et al.*, 2010). They evaluated 25(OH)D₃ levels and correlated the levels with the presence or absence of increased unmineralized, organic moiety (osteoids) in their bone. This method was considered the gold standard for Vitamin D deficiency and they concluded that in order to prevent vitamin D deficiency osteomalacia in adults the blood level of 25(OH)D₃ should be >20 ng/ml (Priemel *et al.*, 2010). Several other studies by the Endocrine Society have compared serum 25(OH)D₃ with parathyroid hormone (PTH) and suggest that PTH concentrations begin to plateau when 25(OH)D₃ were above 30 ng/ml which can ameliorate secondary hyperparathyroidism (Holick *et al.*, 2011; Kitson *et al.*, 2012). A recent systematic review conducted by Palacios C and Gonzalez L provides a global picture of this pandemic and shows the prevalence of vitamin D deficiency to be a global health problem in all age groups (Palacios *et*

al., 2014). Epidemiologic estimates from the United States population show 25% to 50% of adults suffer from vitamin D deficiency (Iruzubieta *et al.*, 2014).

Within the population as a whole, obese individuals are at a much higher risk of vitamin D deficiency. Increased adiposity (BMI > 30) has been shown to have an inverse relationship to serum 25(OH)D₃ levels and, because obesity is a major risk factor for NAFLD, this further highlights the urgent need to investigate the possible pathological connection between vitamin D deficiency and NAFLD (Wortsman *et al.*, 2000).

Recently, a non-reductionist approach suggested that vitamin D deficiency can be considered a “universal risk factor” for multifactorial and multi-system diseases such as depression, cancer, coronary heart disease, diabetes and NAFLD (de Borst *et al.*, 2011; Byrne *et al.*, 2015). This categorization of vitamin D deficiency may be plausible if one considers the pleotropic effects of vitamin D on various human physiological systems and organs outlined in Table 17 (Kitson *et al.*, 2012).

Table 17. List of pleiotropic (hepatic and extra-hepatic) effects of vitamin D. (Adapted from Kitson *et al.*, 2012).

Target	Action
Hepatic	<p>Inhibits <i>in vitro</i> HCV replication in a dose-dependent manner</p> <p>Supplementation may improve SVR rate in HCV</p> <p>Vitamin D-binding protein is one of 3 metaproteins associated with SVR in HCV</p> <p>Supplementation/phototherapy improves liver histology in preclinical studies of NAFLD and fibrosis</p> <p>Supplementation decreases risk of acute rejection post transplantation</p>
Extra-hepatic	
Mortality	Mortality Supplementation decreases mortality by 7%
Calcium and bone homeostasis	<p>Enhances Ca^{2+} and PO_4 absorption from small intestine</p> <p>Suppresses PTH secretion</p> <p>Induces osteoclast maturation</p>
Pancreas/adipocyte	BMI inversely associated with $25(\text{OH})\text{D}_3$ level

	<p>Normal vitamin D status associated with 67% lower prevalence of metabolic syndrome</p> <p>Activates transcription of insulin gene</p> <p>Supplementation improves insulin sensitivity and lowers risk of developing type 2 diabetes</p>
Innate Immune system	<p>Activates macrophage TLR response to TB infection</p> <p>Hastens sputum culture conversion in pulmonary TB in those with tt <i>TaqI VDR</i> allele</p> <p>Downregulates expression of TLR2, TLR4 and TLR9</p> <p>Necessary for NK cell development and function</p> <p>Enhances NK cell cytotoxicity</p> <p>Promotes tolerant DC phenotype by suppressing DC maturation</p> <p>Enhances secretion of IL10 and decreases secretion of IL12 from DCs</p>
Adaptive Immunity	<p>Activates naive T cells</p> <p>Inhibits proliferation of Th1 lymphocytes</p> <p>Shifts balance to a Th2 phenotype</p> <p>Increases numbers of Treg cells</p> <p>Inhibits Th17 cell development</p> <p>Supplementation decreases risk of developing MS in women and type 1 diabetes in children</p>

Cancer	<p>Higher 25(OH)D₃ levels associated with lower incidence of colorectal adenoma</p> <p>Sunlight exposure associated with reduced risk of NHL</p>
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The general understanding of the connection between vitamin D and NAFLD has been evolving over the years. In 2007, for the first time, Targher *et al.* reported an astonishing association between serum 25(OH)D₃ concentrations and liver histology in patients with NAFLD (Targher *et al.*, 2007). They successfully showed that patients with NAFLD have markedly lower levels of 25(OH)D₃ compared to match controls and can predict histological severity independent of other risk factors (i.e., IR and MS) (Targher *et al.*, 2007). Recently, affirmation of Targher *et al.*'s results was published which also showed vitamin D levels to be inversely related to histological NASH in an obese cohort of children with NAFLD (Nobili *et al.*, 2014,). Epidemiological analysis of the third National Health and Nutrition Examination Survey (NHANES III) data (n=6, 826 fasting subjects) showed an inverse relationship between serum vitamin D levels and unexplained elevation in ALT liver enzymes (Liangpunsakul *et al.*, 2011). Subjects with unexplained elevation of

ALT had significantly lower serum vitamin D levels compared to controls (61.8 +/- 26.0 nmol/l vs. 66.8 +/- 27.1 nmol/l, $P < 0.01$), (Liangpunsakul *et al.*, 2011). Aligning with these studies, are the results of a recent meta-analysis whereby PubMed and EMBASE databases were systematically reviewed for the association between 25(OH)D₃ and NAFLD (Eliades M *et al.* 2013). Using no language restrictions, data were used from 9 cross-sectional and case-control studies. They compared vitamin D levels between NAFLD cases and controls, and also compared those who were vitamin D deficient with their NAFLD status. Their results showed that compared to controls, NAFLD patients had 0.36 ng/mL less of 25(OH)D₃ (95% CI: 0.32, 0.40 ng/mL) and were 1.26 times more likely to be vitamin D deficient (OR 1.26, 95% CI: 1.17, 1.35) (Eliades M *et al.* 2013). Cause and effect of this association is difficult to determine from case-control studies and other limitations as discussed above. However, there is overwhelming evidence supporting the fact that NAFLD patients are more likely to be vitamin D deficient compared to controls.

In order to understand the role of vitamin D in the pathogenesis of NAFLD, one must explore the mechanisms and signaling pathways that link them. The mechanisms by which vitamin D deficiency contributes to the development and/or progression of NAFLD include insulin secretion/resistance (IR), adipose tissue inflammation, hepatic inflammation and fibrosis (Eliades *et*

al., 2015). The table below demonstrates the protective role of vitamin D at different stages of NAFLD and highlights the evidence to date that supports the urgent need to eradicate the pandemic of vitamin D deficiency (Eliades et al., 2015).

Table 18. Possible mechanisms and evidence to support a beneficial role of Vitamin D in NAFLD. (Adapted from Eliades M et al. 2015)

Mechanisms	Evidence
Improvement in insulin secretion and insulin resistance	<ul style="list-style-type: none"> -Presence of VDR in pancreatic beta cells -Expression of 1-α-hydroxylase enzyme in pancreatic beta cells -Impaired insulin secretion response in mice lacking a functional VDR -Transcriptional activation of the human insulin gene by 1,25(OH)₂D₃ -Vitamin D deficiency impairs glucose-mediated insulin secretion from rat pancreatic beta cells <i>in Vitro</i> and <i>in Vivo</i> -Vitamin D enhances insulin responsiveness for glucose transport in muscle cells -Vitamin D up-regulates glucose transporter 4 (GLUT4) translocation and glucose utilization in adipocytes

Improvement in adipose tissue inflammation	<p>-Higher 25(OH)D₃ concentrations were independently associated with higher adiponectin concentrations in a large cohort of men and women</p> <p>-Reduction IL-6 in adipocyte after supplementation of vitamin D in mice fed high fat diets</p> <p>-1,25(OH)₂D₃ treatment in human adipocytes inhibits NF-κB pathway and reduces pro-inflammatory cytokine release</p> <p>-1,25(OH)₂D₃ inhibits macrophage recruitment and increases adiponectin expression in preadipocytes</p>
Improvement in hepatic inflammation	<p>-Vitamin D deficiency causes TLR activation and exacerbates hepatic inflammation</p> <p>-Artificial sunlight therapy in rats reduced liver inflammation and apoptosis</p> <p>-VDR expression on cholangiocytes was inversely correlated with steatosis severity and NAFLD score in NASH patients</p>
Improvement in hepatic fibrosis	<p>-Presence of VDR in hepatic stellate cells (HSC)</p> <p>-Vitamin D treatment suppresses HSC proliferation in cultured HSC from rats</p> <p>-Vitamin D treatment downregulates pro-</p>

	fibrotic marker TIMP-1 and collagen type- I production in cultured HSCs -VDR knockout mice develop spontaneous liver injury with fibrosis
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Vitamin D levels negatively correlate with BMI

In our NASH cohort, 51 (58%) subjects were vitamin D deficient, as defined by serum vitamin D levels lower than 30ng/ml (Table 5). The possible mechanisms that contribute to lower 25(OH)D₃ levels in obese subjects are complex and can involve multiple risk factors such as behavioral (sedentary lifestyle), reduced cutaneous synthesis, reduced intestinal absorption, reduced activation and/or increased catabolism and sequestration of 25(OH)D₃ in adipose tissue (Vanlint, 2013). The most likely reason for low levels of serum 25(OH)D₃ is due to sequestration of 25(OH)D₃ in adipose tissue.

A study conducted by Wortsman *et al.* sought to determine why obese individuals are prone to vitamin D deficiency. They did this by analyzing fat soluble vitamin D (either administered orally or through synthesis by the skin, whole body UV radiation), and determining whether or not obese and non-obese subjects process vitamin D differently (Wortsman *et al.*, 2000). They concluded that the increase in blood vitamin D₃ concentrations was 57% lower

in the obese than in the non-obese subjects, 24 hours after UV-B exposure. These results were contrary to the popular belief that obese subjects have a larger body surface area for UV-B exposure which should result in a higher rate of vitamin D synthesis (higher serum vitamin D) (Wortsman *et al.*, 2000). A plausible explanation for these observations is that with increased adiposity (subcutaneous fat), vitamin D metabolites are sequestered in the obese subjects more so than in the non-obese subjects (Worstman *et al.*, 2000). The decreased bioavailability of vitamin D due to sequestration in adipose tissue can account for the consistent observation by us and others that morbidly obese patients are prone to vitamin D deficiency. It must be mentioned, that while to date there have been few attempts to quantify 25(OH)D₃ in animal adipose tissue (Heaney *et al.*, 2009; Lipkie *et al.*, 2013), not as much is known about this phenomenon in adult human adipose tissue particularly in visceral adipose tissue (VAT). Recently, Piccolo *et al.* has challenged the results of sequestration of vitamin D in adipose tissue by measuring 25(OH)D₃ in subcutaneous white adipose tissue (SWAT). This tissue was obtained from overweight and obese individuals participating in controlled feeding weight loss intervention to determine whether SWAT 25(OH)D₃ levels were altered as a result of weight loss (Piccolo *et al.*, 2013). Correlation analysis showed a positive, significant association between SWAT 25(OH)D₃ concentrations and

serum levels. But both SWAT and serum 25(OH)D₃ concentrations did not significantly change after a 12-week energy restriction and 6% weight loss (Piccolo *et al.*, 2013). These results suggest that SWAT 25(OH)D₃ levels do not likely contribute to serum 25(OH)D₃ concentrations, or their contribution is not taking place in subcutaneous fat.

In a recent multi-national, bi-directional Mendelian Randomization study, which is a genetic approach to infer causality from associations between genetic variants that mimic the influence of a modifiable environmental exposure and the outcome of interest, suggests a possible genomic mechanism (Vimaleswaran *et al.*, 2013). The authors hypothesized that if lower vitamin D level status is causally related to obesity, a genetic variant associated with lower 25(OH)D₃ concentrations should be associated with a higher BMI and *vice versa* (Vimaleswaran *et al.*, 2013). The study included 21 adult cohorts with 42,024 participants and they compared 12 BMI-related single nucleotide polymorphisms (SNPs) and 4 vitamin D-related SNPs by using existing genotyping data as well as performing *de novo* genotyping. Their meta-analysis suggests that higher BMI leads to lower 25(OH)D₃, and any effects of a lower 25(OH)D₃ level resulting in increased BMI, are very minute. This further suggests that BMI reduction is vital for increasing vitamin D but not *vice versa* (Vimaleswaran *et al.*, 2013).

25(OH)D₃ levels negatively correlate with levels of PTH (Parathyroid hormone)

The parathyroid hormone (PTH) is a protein of 84 amino acids and is synthesized as a larger precursor, pre-pro-parathyroid hormone (Silver *et al.*, 2011). Pre-pro-parathyroid has a 25-residue “pre” or a 6-residue “pro” sequence. Along with the short pro-sequence, this signal sequence directs the protein into the secretory pathway. In healthy individuals, regulation of PTH secretion normally occurs *via* a negative feedback action of serum calcium on the parathyroid glands. Intact PTH is biologically active and clears very rapidly from the circulation with a half-life of less than four minutes (Silver *et al.*, 2011). Parathyroid hormone (PTH) regulates serum concentrations of calcium and phosphate, which in turn, regulate the synthesis and secretion of PTH through a feedback mechanism. Vitamin D has an independent effect on calcium and phosphate levels and also participates in a well-defined feedback loop between the active form of vitamin D, 1,25(OH)₂D₃ and PTH (Silver *et al.*, 2011). More specifically, 1,25(OH)₂D₃ decreases transcription of the PTH gene product (Silver *et al.* 1985; Russell *et al.*, 1984; Silver *et al.*, 2011) and consequently decreases PTH secretion (Cantley *et al.*, 1985). *In vivo* studies in rats injected with amounts of 1,25(OH)₂D₃ showed that it did not increase serum calcium and

had a marked decrease in PTH mRNA levels, reaching <4% of control at 48 hours (Okazaki T et al. 1988, Navey-Many T et al. 1990).

Contrary to this study's findings, a cross sectional study investigating the disturbances of PTH and vitamin D levels in chronic liver disease (CLD) reported no significant correlation between PTH and vitamin D (Miroliaee *et al.*, 2010). This contradiction can be explained by the fact that the two respective cohorts of patients were different. Unlike the cohort in this study, the etiologies of liver disease in that cohort ranged from hepatitis B and C infections, autoimmune hepatitis to cryptogenic causes (Miroliaee *et al.*, 2010). Interestingly, they observed low vitamin D levels and liver function insufficiency markers (i.e., coagulopathy, hypoalbuminemia, hyperbilirubinemia, and thrombocytopenia) in the cirrhotic versus non-cirrhotic groups and recommended vitamin D assessment and supplementation in the management of patients with non-cholestatic CLD. This study further highlights the wide range of liver diseases that vitamin D can affect.

The levels of 25(OH)D₃ positively correlate with the presence of histologic NASH and higher stages of fibrosis

In the NASH sub-cohort, vitamin D deficiency, defined by <30ng/ml, positively correlated with the presence of histologic NASH ($r=0.26$, $p<4.0e-4$)

and higher stages of fibrosis ($r=0.16$, $p<0.03$) (Table 6). This suggests that patients with the severe form of the disease would be vitamin D deficient. The inverse relationship between vitamin D levels and NASH histology observed in this study is in agreement with others (Targher *et al.*, 2007; Nobili *et al.*, 2014) and may suggest a linear dose response effect in progression of disease. Recent random clinical trials investigating the effects of vitamin D supplementation on serum aminotransferases, insulin resistance (IR), high-sensitive C-reactive protein (hs-CRP), tumor necrosis factor α (TNF- α), malondialdehyde (MDA), total antioxidant capacity, transforming growth factor β 1 (TGF β 1), and grade of steatosis were assessed pre- and post- intervention. Patients who received vitamin D supplements, when compared to controls, had a median serum 25(OH)D₃ level increase (16.2 vs. 1.6 ng/ml, $p<1.0e-3$) (Sharifi *et al.*, 2014). In addition, a significant decrease in serum MDA and hs-CRP was observed. The authors conclude that vitamin D might serve as an adjunctive therapy to attenuate systemic inflammation and lipid peroxidation in NAFLD (Sharifi *et al.*, 2014).

These results indicate an inverse relationship between vitamin D levels and NASH histology that could also partially be explained by dysregulation of vitamin D metabolism due to severity of NAFLD. The liver plays a key role in

metabolizing vitamin D. Refer to the vitamin D chapter for details, but essentially, vitamin D is converted to 25(OH)D₃ in the liver and to 1,25(OH)₂D₃ in the kidneys. 25-hydroxylation of the liver produces substrate availability of vitamin D and is the choice marker used to assess vitamin D status. As NAFLD progresses to NASH, it can lead to liver dysfunction and normal hepatic functions to become compromised. Reduced hepatic function may lead to decreased 25-hydroxylation of vitamin D and reduced production of vitamin D binding protein (DBP) which can affect circulatory levels.

The progression of fibrosis in NASH is associated with activation of hepatic stellate cells (HSC) responsible for collagen deposition and fibrosis. HSCs are activated, in part, by platelet-derived growth factor (PDGF) through pro-inflammatory cytokines such as TNF- α and IL-6 (Tilg *et al.*, 2010; Tilg *et al.*, 2010). Evidence from animal studies shows that the inhibition of HSC proliferation by vitamin D was associated with anti-fibrotic effects *in vitro* and *in vivo* (Abramovitch S *et al.* 2011). More specifically, the active form of vitamin D, 1,25(OH)₂D₃ suppressed HSC proliferation and cyclin D1 expression by approximately 50% and the tissue inhibitor of metalloproteinase I (TIMP-1) by 60% and led to a 40% downregulation of collagen 1 α 1 expression (Abramovitch *et al.*, 2011). More recent genomic research has revealed a previously

unrecognized mechanism by which vitamin D regulates hepatic fibrogenesis by serving as an “endocrine checkpoint” modulating amelioration of liver fibrosis through wound healing response (Ding *et al.*, 2013). Mechanistically, TGF β 1 signaling causes redistribution of genome-wide VDR binding sites (VDR cistrome) in HSCs, and facilitates VDR binding at SMAD3 pro-fibrotic target genes via TGF β 1-dependent chromatin remodeling (Ding *et al.*, 2013). This suggests that the VDR/SMAD genomic circuitry regulates hepatic fibrogenesis. VDR may serve as an endocrine checkpoint, modulating the wound healing response and serving as a potential therapy for liver fibrosis (Ding *et al.*, 2013).

The levels of 25(OH)D₃ positively correlate with marker of apoptosis (M30)

The next set of results, from the study of the role of 25(OH)D₃ and markers of apoptosis (M30) in patients with a spectrum of NAFLD, are very interesting and important to the quest to identify non-invasive serum biomarkers. There seems to be a positive correlation between serum 25(OH)D₃ levels and serum caspase-cleaved cytokeratin-18 (CK-18) fragment levels. This positive correlation suggests a direct relationship between levels of vitamin D and hepatic cell death. Also, markers of apoptosis (M30) showed to be positively correlated with the presence of histological NASH, suggesting that the sickest patients experience a high rate of hepatocyte cell death due to insults to

the liver. A hallmark of the progression from not-NASH to NASH, with degrees of fibrosis, is hepatocyte cell turnover due to injury in the form of increased apoptosis and decreased regeneration. This cell turnover is the putative “*third hit*”. For a detailed overview of the role of apoptosis in NAFLD, refer to the NAFLD chapter. Briefly, apoptosis is a highly regulated, metabolically active form of cell death and it involves two pathways, the intrinsic and the extrinsic (Singh *et al.*, 2010). Activation of both pathways leads to the final effector caspases to mediate cell death. However, activation of each pathway involves different cell signaling cascades. For instance, intrinsic pathway activation involves mechanisms of cell and membrane stress (lysosomal, ER, and mitochondrial injury); and extrinsic pathway activation involves death ligand (FAS) and their receptors (FASL) and by TNF- α -apoptosis-inducing ligand (TRAIL) (Tiniakos *et al.*, 2010).

Research data on the mechanism of hepatocyte apoptosis in pathogenesis of NAFLD suggest the involvement of death-receptor-mediated apoptosis (Yoon *et al.*, 2002). In a translational study conducted by Feldstein AE *et al.*, they sought to ascertain the occurrence and significance of apoptosis in 34 untreated NASH patients compared to normal subjects (Feldstein *et al.*, 2003). Their data show a significant occurrence of hepatocyte apoptosis in NASH with caspase activation and enhanced Fas protein expression (Feldstein

et al., 2003). The authors propose that activation of the Fas signaling cascade produces pro-apoptotic signals and mitochondrial dysfunction leading to further tissue injury and promotion of disease progression.

In the NASH cohort, hepatocyte injury is defined as individual and/or cumulative effects of free fatty acid (FFA) lipotoxicity, oxidative stress, adipokine/cytokine effects, inflammation and hepatocellular death (Tiniakos *et al.*, 2010). In 2006, Wieckowska *et al.* were the first to quantify hepatocyte apoptosis by measuring caspase-3-generated cytokeratin-18 fragments (M30) in plasma from patients (n=44) with NAFLD and correlate it with histological severity (Wieckowska *et al.*, 2006). Serum M30 levels were significantly increased in patients with NASH, compared to patients with simple steatosis. Furthermore, M30 levels independently predicted NASH with a receiver operating characteristic curve (ROC), showing a specificity of 99.9%, a sensitivity of 85%, and positive and negative predictive values of 99.9% and 85.7%, respectively, for diagnosis of NASH (Wieckowska A *et al.* 2006). In concordance with these results, Feldstien *et al.*, using a different assay, TUNEL and immunohistochemistry, showed that hepatocyte apoptosis is significantly increased in patients with NASH and correlates with disease severity (Feldstein *et al.* 2003). Currently, M30 ELISA assay is the most widely validated, non-

invasive and reliable biomarker used to distinguish those patients with NASH and to rule out NASH in other patients.

The role of vitamin D in apoptosis seems to involve alteration in content of various bcl-2- family, apoptotic regulatory proteins and affects the levels of pro-apoptotic (*bax*, *bak*) and/or anti-apoptotic (*bcl-2*, *bcl-XL*) proteins, thereby tipping the balance toward apoptosis rather than cell survival (Samuel *et al.*, 2008). Overwhelming data from cancer research suggest a protective role of vitamin D against cancer cell proliferation. For example, studies of glioma, melanoma and breast cancer cells show vitamin D initiates apoptosis and, in colorectal cancer, the transcription factor SNAIL reduces the expression of vitamin D receptors resulting in the progression of colon cancer cells (Lai *et al.*, 2013). McGuire *et al.* demonstrated that vitamin D induces apoptosis in squamous cell carcinoma via a unique mechanism involving selective caspase-dependent MEK cleavage and up-regulation of MEKK-1 (McGuire *et al.* 2001).

However, there are also contradictory research data to suggest vitamin D has an anti-apoptotic effect on cells. In a rat liver allograft study, vitamin D was shown to regulate hepatocyte cell death by significantly inhibiting apoptosis by increasing Bcl-2 and Bcl-xL (anti-apoptosis) levels while Bax and caspase-3 (pro-apoptosis) levels significantly decreased (Zhang *et al.*, 2007).

Furthermore, they report inhibition of Fas/Fas ligand pathway expression, highlighting the protective (anti-apoptotic) role of vitamin D in hepatocyte apoptosis (Zhang *et al.*, 2007). Similar observations of vitamin D have been reported in *in vitro* studies of human osteoblasts showing a significant inhibition of caspase-8 cleavage in conjunction with a decrease in the expression of the pro-apoptotic protein Bax, with a significant increase in the expression of anti-apoptotic protein Bcl-2 (Duque *et al.*, 2004). Vitamin D also inhibits apoptosis through vitamin D-regulated gene, kinase suppressor of Ras-2 (hKSR-2) gene, which harbors a vitamin D response element involved in cell survival and differentiation in various cell types (Wang X *et al.* 2008). In this study, vitamin D demonstrates its anti-apoptotic activity by inducing up-regulation of hKSR-2 in HL60 leukemia cells and could possibly serve as a new target for novel therapies of acute myelogenous leukemia (AML) (Wang *et al.*, 2008).

Considering the pleiotropic effects of vitamin D on apoptosis, these results suggest a linear relationship between 25(OH)D₃ and apoptotic marker M30 and implicates vitamin D to be a key player in the pathogenesis of NAFLD. While it cannot be inferred from this type of cross-sectional translational study that this is the mechanism of pathogenesis, it is reasonable to propose that vitamin D plays a protective role in the progression of NAFLD to NASH, and exhibits its protective role through using both its pro-apoptotic and/or anti-

apoptotic abilities differently and in different tissue/cell types. To further test this hypothesis, a separate analysis was performed to see if vitamin D supplementation status affects apoptosis levels by comparing those in this study's cohort who were receiving vitamin D or multivitamin supplementation and those who were not receiving supplementation, Table 12 and 13. NASH patients who were not receiving vitamin D supplementation had higher levels of M30 than non-NASH patients (296 vs. 172, $p < 3.0 \times 10^{-5}$). In a similar analysis, NAFLD with advanced fibrosis showed higher M30 levels regardless of vitamin D supplementation [with vitamin D (248 vs. 145, $p < 9.0 \times 10^{-3}$), without vitamin D (279 vs. 206, $p < 5.0 \times 10^{-3}$)]. Vitamin D supplementation seems to have an effect on the levels of apoptosis on histologic NASH patients, but not on those with advanced fibrosis. These data suggest that the protective effects of vitamin D may be subject to a systemic damage threshold and adiposity. Correcting for deficient vitamin D levels through supplementation may be used as adjunctive therapy to take advantage of its wide range of protective properties (i.e. anti-inflammatory, anti-fibrotic, regulation of cell turnover, and immunogenicity) to manage NAFLD. A recent randomized double blind clinical trial was the first study to show that vitamin D supplementation improved serum 25(OH)D₃ concentrations, and that it was associated with at least a 1.5 fold change in the expression of 291 genes (Hosseini-nezhad A et al. 2013). Seventeen of these

genes are vitamin D regulated, and new candidate vitamin D response elements (VDRE) are involved in transcriptional regulation, stress response and immune function (Hosseini-Nezhad *et al.* 2013).

Unfortunately, while supplementation can correct vitamin D levels in majority of the patients, a sub-group of the population does not respond to supplementation, calling into question the dose response aspect of vitamin D supplementation on serum 25(OH)D₃. In a separate analysis, an investigation into the effects of supplementation was done by targeting the most vitamin D deficient, morbidly obese NAFLD patients. Analysis of the trends reveals partial changes in serum 25(OH)D₃ concentrations in patients who were on a multivitamin supplement regimen, Table 16. Interestingly, in a group of patients receiving specific vitamin D supplementation, effects on 25(OH)D₃ levels in serum were greater as compared to NAFLD patients who were receiving multivitamin supplementation or non-supplementation. These data clearly show that vitamin D deficiency in morbidly obese NAFLD patients cannot be corrected with multivitamin supplementation, but can be corrected with specific vitamin D supplementation. A recent longitudinal study examined the effect of vitamin D supplementation on serum 25(OH)D₃ in thin and obese women and showed women with BMI < 25 kg/m² develop much higher levels of serum 25(OH)D₃ after vitamin D supplementation compared to those with BMI>25

kg/m² (Gallagher *et al.* 2013). The different response curves observed based on BMI can be due to the dilution effect, suggesting that elevation in serum 25(OH)D₃ in thin individuals could be a function of smaller physiological volume, and response curves observed in obese patients are a function of adequate intake (Gallagher *et al.* 2013). In fact, studies recommend vitamin D supplementation dosage be 2 or 3 times higher for obese patients and 1.5 times higher for overweight individuals relative to those of normal weight (Ekwaru JP *et al.* 2014). However, other factors might also be at play such as an increase in adiposity, vitamin D transport proteins, enzymes involved in vitamin D metabolism or other factors like genotype variation.

The levels of the vitamin D binding protein (DBP) positively correlate with the levels of triglycerides, and negatively - with the degree of histologic hepatocyte ballooning in liver biopsies.

Unlike most vitamin D studies, which regrettably fail to account for the role of DBP in their analysis and interpretation, this study considers DBP levels to obtain a comprehensive picture of the “vitamin D-axis” and the relationship between the role of the “vitamin D-axis” and NAFLD. Vitamin D binding protein

(DBP) is a multifunctional, highly expressed, polymorphic serum protein with an array of physiologic functions mediating inflammatory and immunomodulatory responses. DBP binds to and transports 25(OH)D₃, acts as the scavenger for monomeric G-actin, activates macrophages and acts as a chemotaxin for phagocytic cells (White et al., 2000).

Vitamin D binding protein (DBP) is multifaceted with multiple functions involved in a wide range of human pathophysiology. Recently, Moon *et al.* investigated the possible role of DBP in the pathogenesis of Alzheimer's disease (AD). They demonstrated that DBP levels are increased in cerebrospinal fluid of AD patients and is directly associated with amyloid β (A β) peptide, which is responsible for AD related brain damage in AD models (Moon *et al.* 2013). DBP decreased A β -induced synaptic loss in the hippocampus and rescued memory deficits in mice (Moon *et al.*, 2013). DBP exhibits a protective/preventative effect attenuating the harmful effects of A β in AD models and their results suggest a promising therapeutic strategy for AD (Moon *et al.* 2013). More recently, studies of regulation of DBP during early and late phase reaction of allergic asthma showed DBP concentration levels to be significantly elevated 24 hours after allergen exposure (Bratke *et al.*, 2014). Patients who suffer from acute intermittent porphyria (AIP), an autosomal dominant metabolic

disease caused by deficiency of hepatic porphobilinogen deaminase (PBGD), causing life threatening neurovisceral symptoms, have increased glycosylated-DBP levels (Serrano-Mendioroz *et al.* 2015). Authors propose that the high levels of glycosylated DBP observed could reflect the activation of the intracellular unfolded protein response caused by incorrect folding of the mutated PBGD protein in AIP patients (Serrano-Mendioroz *et al.* 2015).

The levels of DBP were shown to negatively correlate with the degree of histologic hepatocyte ballooning in liver biopsies of NAFLD patients, Table 11. Ballooning degeneration is a form of liver parenchymal cell death and a common feature of inflammatory NASH. These results suggest that DBP levels are altered with respect to disease severity in this study's patient cohort. Different physiologic (*obesity*) and pathologic (*liver dysfunction*) conditions can affect DBP levels (Yousefzadeh *et al.*, 2014), which in turn affect a wide array of systems which can contribute to disease progression. Because these patients are morbidly obese and suffer from NAFLD, one must consider the possible mechanisms that involve obesity and liver dysfunction on DBP levels. A study of obese women compared to normal weight women showed obese women have significantly higher concentrations of serum DBP (320 µg/mL +/- 121) compared to lean women (266 +/- 104 µg/mL) (Karlsson *et al.* 2014). However,

the increased levels of DBP in obese women did not result in higher levels of 25(OH)D₃ levels. In fact, obese women had a 20.1 nmol/L lower mean 25(OH)D₃ concentration compared to lean women (Karlsson *et al.* 2014). While their study included mainly women of normal weight and Caucasian women, results from this study show DBP levels to be significantly lower in the non-Caucasian (African American, Hispanic and Middle Eastern) group. Non-Caucasian women are 10 times more likely to have low serum 25(OH)D₃ levels than Caucasians (Yao S *et al.* 2013) and 25(OH)D₃ levels inversely associate with markers of adiposity (Young *et al.* 2009). Normal liver function is required for production of 25(OH) D₃ and for synthesis of DBP. A study of serum 25(OH)D₃ and DBP concentrations in over 200 patients undergoing liver transplantation showed both 25(OH)D₃ and DBP concentrations to increase substantially 3 months following transplantation (Reese *et al.*, 2012). In concordance with this study's results, other studies have also reported changes in DBP concentrations linked to the pathology of advanced disease and hepatic failure (Stokes *et al.*, 2013).

There is overwhelming scientific evidence to exploit the diverse properties of DBP as a preventative and/or therapeutic agent affecting several pathways involved in the pathogenesis of NAFLD. An area of interest which warrants further investigation is the role of DBP in tissue damage due to injury,

infection or inflammation, which are hallmarks of NASH. More specifically, exploiting the ability of DBP to sequester actin production and inflammation can serve as a biomarker for disease management and/or a target for therapy. Extracellular over production of actin has been shown to be associated with hepatic necrosis (Gomme *et al.*, 2004). Intervention in improving DBP concentrations can serve a protective role against disease progression.

Future studies should explore the relationship between vitamin D and adipocyte gene expression. In particular, the expression of vitamin D related genes in adipocytes should be quantified and correlated with serum levels identified in this study and others. A set of vitamin D responsive genes involved in the inflammatory processes (INF- γ , TLR2, and TNF- α) and/or vitamin D metabolism related genes such as cytochrome p450s, *CYP27A1* (25-hydroxylase) or *CYP27B1* (1- α -hydroxylase) be selected for expression analysis. Real-time PCR analysis of this kind can further elucidate the complex “*vitamin D-obesity*” axis, potential role in pathogenesis of NAFLD/NASH and explain results reported in this observational translational research.

CHAPTER 4

Gastric Peptides and Inflammatory Cytokines in Obesity Related NAFLD.

Introduction

Progressive obesity is often associated with increased dysregulation of appetite control. Gastric tissue is well known to be the principal source of small peptides, such as ghrelin and obestatin that have central roles in both appetite stimulation and appetite suppression. Ghrelin and obestatin also have metabolic consequences, exhibiting effects on processes such as lipogenesis, adipogenesis and adipose tissue energy production. These peptides have even been associated with metabolic diseases like type II diabetes mellitus, demonstrating regulatory activity on glucose and insulin secretion. Ghrelin and obestatin also have some relationship to inflammation, both in response and effect. Ghrelin regulates immune function through reducing pro-inflammatory cytokine production as well as neutrophil infiltration. Additionally, ghrelin and obestatin concentrations have been shown to increase in response to local inflammation. Obesity, itself, is a condition of chronic systemic inflammation. As visceral adiposity increases, so too does the total concentration of circulating inflammatory cytokines, such as IL-6, MCP-1/CCL2, or TNF α . The central question being investigated here is whether the gastric peptides Ghrelin and

Obestatin participate in the pathogenesis of obesity related NAFLD, while noting if serum levels of Ghrelin and Obestatin can serve as an effective non-invasive biomarker for disease progression.

The central hypothesis of this study is that circulating levels of inflammatory adipokines influence the relative concentrations of appetite stimulating and suppressing peptides secreted by gastric tissue. To support this hypothesis, a three step strategy was employed. First, serum concentrations of inflammatory cytokines were correlated to serum concentrations of acylated and non-acylated ghrelin, as well as obestatin. Second, the association of both *GHRL* gene products and inflammatory cytokines with weight loss was evaluated. Finally, concentrations of obestatin and ghrelin isoforms were correlated with clinical and demographic data to establish a link between gut hormone expression and metabolic diseases associated with obesity.

Background:

Ghrelin is one of the first gastric peptides to be isolated from rat stomachs and identified to be an agonistic endogenous ligand for the growth hormone secretagogue receptor 1a (GHSR1a), a G-protein-coupled receptor (Kojima M et al. 1999). The name Ghrelin is derived from the protoindoeuropean language, in which “ghre” means growth and ‘relín’ means

release (Massadi *et al.*, 2014). The pre-pro-ghrelin gene, located on the *p* arm of chromosome 3 (3p26-p25), encodes the ghrelin-obestatin pre-protein that is cleaved to yield two peptides, ghrelin and a 23- amino- acid small peptide, obestatin.

Ghrelin undergoes a rare post-translational modification whereby the third serine residue undergoes O-octanoylation, involving medium chain fatty acid octanoate to covalently bind this third serine (Kojima *et al.*, 1999). Several products of ghrelin are produced, of which, the most metabolically relevant are acyl-ghrelin (AG) and desacyl ghrelin (DAG), comprised of 28 amino acids each (Kojima *et al.*, 1999; Massadi *et al.*, 2014). The structure of the octanoylated peptide hormone, ghrelin, is only known up to the level of its primary structure; identifying an active core of residues 1-5 or 1-4 including octanoyl-Ser3 as necessary for receptor activation (Kukul, 2008). Ghrelin is a multifunctional peptide modulating numerous physiologic systems and organs. Early research by Mark Heiman and Mattias Tschop indicated that ghrelin acts in the brains of rodents, regulating food intake, body weight, adiposity and glucose metabolism (Tschop *et al.* 2000). To date, central and peripheral actions of ghrelin have been described in numerous organ systems (Figure 7).

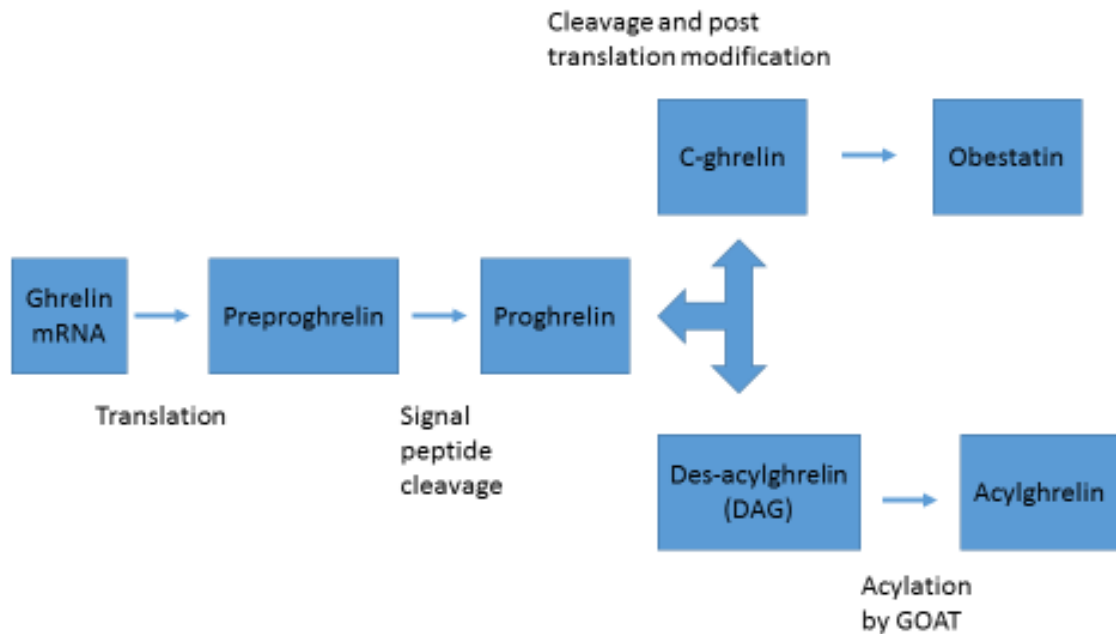


Figure 7. Products of the GHRL gene. The most common terminal products of the GHRL gene, des-acyl-ghrelin, obestatin, and acyl-ghrelin or ghrelin are represented in brown.

The next milestone in ghrelin research came in 2008, when the laboratories of Michael S. Brown and Joseph L. Goldstein from the University of Texas Southwestern Medical Center, reported the identification of the enzyme responsible for this rare post-modification of ghrelin, termed "Ghrelin O-Acyltransferase" (GOAT) (Yang *et al.* 2008). They clearly demonstrated that GOAT and des-acyl ghrelin are sufficient to recapitulate the production of acyl modified ghrelin in cells; ghrelin and GOAT share similar tissue expression profiles in both humans and mice, with highest GOAT expression in pancreas

and gastric tissues of humans and mice (Yang *et al.* 2008). The identification of the GOAT enzyme has led to major breakthroughs in the physiological effects of acyl-modification of ghrelin (Muller *et al.*, 2015).

The initial identification of ghrelin as a gastric peptide by Masayasu Kojima, Kenji Kangawa and Hiroshi Hosoda required some luck along with passion for their research (Kojima *et al.*, 1999). In their 30 years of research, searching for novel unknown endogenous peptides, they were the only group to change their target tissue to stomach samples in order to discover ghrelin (Kojima *et al.*, 2013). Hence, they made the initial designation of ghrelin to be a gastric peptide. Since then, the expression distribution of GOAT in several human tissues has been reported. RT-PCR analysis using primers for the MBOAT4 gene shows GOAT mRNA expression to have widespread distribution in human tissues (Lim *et al.*, 2011). These findings further implicate the pleiotropic involvement of the ghrelin-GOAT system in the pathogenesis of various physiological disorders.

Recently, the ghrelin system has been linked to energy and lipid metabolism, IR, inflammation and apoptosis, which are all hallmarks of obesity related NAFLD. Several studies have reported the ghrelin gene products to be involved in inflammatory processes (Mafra *et al.*, 2011; Stengel *et al.*, 2010; Sung *et al.* 2011, Wu *et al.* 2008), especially in the local inflammatory response

(Koca *et al.* 2008). These properties of ghrelin are particularly relevant to obesity and the associated low grade, chronic systemic inflammation (i.e. Meta-inflammation) orchestrated by metabolic cells in response to excess nutrients and energy (Gregor *et al.*, 2011). Increased adiposity leads to an IR state in adipose tissue, liver and muscle tissues, and is a strong risk factor for the development of diabetes and NAFLD (Olefsky *et al.*, 2010). The IR state induced in these target tissues results in reduced insulin-stimulated glucose uptake in muscle, impaired suppression of glucose output in the liver, and increased fatty acid release from adipose tissue (Schenk *et al.*, 2008). In addition, in obese individuals, an expansion of the visceral adipose tissue promotes an increase in the total amount of circulating inflammatory cytokines and chemokines, such as MCP-1/CCL2, interleukin (IL)-6, IL-1 β , macrophage migratory inhibitory factor (MIF), and TNF- α , released by both adipocytes and associated macrophages (Wang *et al.*, 2008; Strohacker *et al.*, 2010; Sun *et al.*, 2009; Tateya *et al.*, 2010).

In 2013, Li *et al* were the first to demonstrate the protective role of ghrelin in an animal model using Sprague-Dawley rats with induced NAFLD. They successfully showed that treatment with ghrelin improved liver injury measurements such as histological changes, including necrosis, inflammation foci, elevated serum enzyme levels (ALT and AST), dysregulated hepatic lipid

metabolism, increased formation of oxidative stress, lipid peroxidation markers, and up-regulated levels of pro-inflammatory cytokines and apoptotic cells (Li *et al.*, 2013). Real time PCR and ELISA experiments showed that, after 8 weeks of NAFLD induction, the expression of inflammatory cytokines (TNF- α and IL-6) was significantly up-regulated at both mRNA and protein levels. Upon administration of ghrelin, both mRNA and protein levels of TNF- α and IL-6 were reduced without disturbing their basal expressions ($p < 0.01$) (Li *et al.*, 2013). Results of this study are in concordance with other studies showing therapeutic function in several inflammatory diseases in rodent models, including Sepsis (Dufour *et al.*, 2010), intestinal ischemia/reperfusion injury (Wu R *et al.* 2008), pancreatic disease (Kasimay *et al.*, 2006), cardiovascular disease (Huang *et al.*, 2009), and gastrointestinal disease (Gonzalez-Rey *et al.*, 2006).

In addition to ghrelin, GOAT has been implicated to possibly play a role in NAFLD. GOAT is the only enzyme discovered to transfer carboxylic-acid moieties from the cytosol to the luminal side of the endoplasmic reticulum (ER) site where pro-ghrelin is Acylated (Gualillo O *et al.* 2008). The acylation of ghrelin by GOAT is the key to its activity and can serve as a potential therapeutic target in NAFLD (Gualillo *et al.*, 2008; Yang *et al.*, 2008). The hypothesis being that inhibiting GOAT activity can lead to decreased levels of acylation and increased levels of des-acyl-ghrelin (DAG), altering the

physiological role of ghrelin in the nervous system and in local injury (Zhang *et al.*, 2015; Ariyasu *et al.*, 2005; Iwakura *et al.*, 2005). The ghrelin-GOAT system's most exciting potential is its role in cytokine mediated inflammation, a hallmark of progressive NAFLD and NASH. The mechanism by which the ghrelin-GOAT system ameliorates inflammatory responses has been demonstrated in both *in vitro* and *in vivo* studies (Waseem *et al.*, 2008, Gonzalez-Rey *et al.*, 2006; Li WG *et al.*, 2004). Waseem *et al.* demonstrated *in vitro* that the therapeutic role of exogenous ghrelin in production of pro-inflammatory and anti-inflammatory cytokines in lipopolysaccharide (LPS) activated murine based macrophages. Ghrelin treatment resulted in a decrease in LPS-induced NF κ B activation and exogenous ghrelin significantly augmented the release of the anti-inflammatory cytokine IL-10 in a dose and time-dependent manner from LPS stimulated murine macrophages (Waseem *et al.*, 2008). Similar anti-inflammatory properties of ghrelin were reported using human umbilical vein endothelial cells (HUVECs), along with a reduction in endotoxin-induced cytokine production *in vivo* (Li *et al.*, 2004). Interestingly, they also reported that Des-acyl ghrelin (DAG) had no effect on TNF- α induced cytokine production, further giving credence to the importance of ghrelin acylation by GOAT (Li *et al.*, 2004). Finally, the hepato-protective role of ghrelin was recently evaluated on a carbon tetrachloride (CCL₄)-induced acute liver

injury in a rat model (Cetin *et al.*, 2011). Ghrelin treatment reduced plasma and liver oxidative stress markers and increased antioxidant enzyme activity in the liver tissue leading to improved histological changes (Cetin *et al.*, 2011).

The focus of this research was to investigate the interplay between obesity related chronic systemic inflammation (i.e. Meta-inflammation), ghrelin gene products in morbidly obese individuals undergoing bariatric surgery. This study assesses the potential connections between NAFLD, circulating inflammatory cytokines, the serum concentrations of the ghrelin genetic products. I hypothesized that the activity of the ghrelin gene products would be modulated by inflammatory cytokines and reflect the severity of NAFLD.

Methods

This study included obese patients who were scheduled for bariatric surgery (average BMI=48.9). After informed consent, fasting serum samples were obtained prior to surgery, processed, and immediately frozen at -80°C. For each patient, relevant clinical and demographic data were collected. Pre-surgical laboratory tests including fasting glucose, serum aminotransferases (alanine aminotransferase-ALT and aspartate aminotransferase-AST), and lipid panel were available. During surgery, a liver biopsy was also performed to

asses for NAFLD. These samples and corresponding clinical data have been de-identified in compliance with HIPAA regulations and this study was approved by Inova Institutional Review Board (IRB).

Histopathology

Histopathological assessment of liver biopsies was performed by an experienced hepatopathologist (Zach Goodman). According to the pathologic protocols, patients were divided into histologically proven NASH and non-NASH subtype of NAFLD. These groups were matched for clinical and demographic variables (Table 21). For this study, histologic NASH was defined as steatosis, ballooning degeneration and lobular inflammation with or without pericellular fibrosis, and/or Mallory-Denk bodies.

Measurement of Serum Cytokines, Obestatin, and Acylated (AG) and Des-acylated (DAG) Ghrelin

Serum obestatin concentrations were measured by competitive enzyme immunoassay (ALPCO Diagnostics, Salem, NH, USA) as per manufacturer's instructions. Acylated and des-acylated ghrelin forms were measured by sandwich enzyme immunometric assays (ALPCO Diagnostics) following the manufacturer's instructions.

Serum concentrations of inflammatory cytokines were assessed in these patients using a BioPlex multiplex 17-plex cytokine assay. This assay simultaneously measures serum concentrations of IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, IFN- γ , MCP-1/CCL2, MIP-1 β /CCL4, and TNF- α were profiled in the serum using the Bio-Plex Human Cytokine 17-Plex panel as part of the Bio-Plex Suspension Array System (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol.

Statistical Analysis

Pairwise comparisons of the clinical parameters and serum concentrations of gastric hormones and cytokines were performed between groups of patients using the nonparametric Mann–Whitney test. Associations between the concentrations for pairs of adipokines and cytokines of interest were tested with the use of Spearman correlation coefficients. Additionally, multivariate linear regressions with stepwise variable selection were used to test for significant relations in continuous data with adjustment for possible confounders. Unless otherwise noted, two-tailed hypothesis tests were used and p values < 0.05 were considered significant.

To possible association of adipocytokine levels with steatosis and fibrosis, a series of multiple regression analyses were performed with stepwise

(bi-directional) selection of variables. Bi-directional selection began with a full model that contained the adipocytokine levels as variables and ended when no more improvement of the prediction fitting was achieved with the addition or removal of adipocytokine or clinical predictors. The predictive performance was evaluated for the generated models. Specifically, the sensitivity, specificity, and area under the receiver operating characteristic (ROC) curve with 95% confidence intervals were calculated for each model. All predictive models were cross-validated with the leave-one-out method that determines the accuracy of a learning algorithm (by training it multiple times using all but one of the training set data points). The regression analyses were executed with S-Plus 8.0 statistical package, and the ROC analyses were generated with the MedCalc statistical tool.

Results

Obestatin, Acyl-ghrelin and Des-acyl-ghrelin (DAG) in Patients with NASH

Demographic, clinical, laboratory and histological data and the outcomes of group statistical comparisons for patients with and without NASH are listed in Table 18. This study included 75 patients with biopsy-proven NAFLD. Of these, 41 patients had biopsy-proven NASH (Table 19) with 20 NASH patients having fibrosis stage ≥ 2 (Table 20). Patients with NASH had an approximately two fold higher concentration of circulating DAG than patients with the non-NASH subtype of NAFLD (2.58 vs. 1.24 pg/ml, $P < 0.02$). Levels of DAG were significantly and positively correlated with ALT ($r = 0.29$, $P < 0.02$), AST ($r = 0.26$, $P < 0.03$), fasting glucose ($r = 0.29$, $P < 0.02$), and triglycerides ($r = 0.25$, $P < 0.03$), as well as histologic finding of ballooning degeneration ($r = 0.30$, $P = 0.01$), Mallory-Denk bodies ($r = 0.29$, $P < 0.02$), and portal fibrosis ($r = 0.39$, $P < 1.0e-3$) on the liver biopsies.

Table 19. Clinico-demographic and laboratory data for cohorts of NASH and non-NASH NAFLD in gastric hormone study. All P values > 0.05 were considered not significant (NS)

	NASH	Non-NASH	<i>P Value</i>
N	41	34	
ALT (IU/l)	35.64\pm24.68	25.10\pm12.84	<0.05
AST (IU/l)	29.89\pm21.50	21.03\pm9.27	<0.01

Fasting serum cholesterol (mg/dl)	203.53±41.25	186.20±40.31	NS
Fasting serum triglyceride (mg/dl)	215.32±125.82	154.69±102.09	<0.01
Fasting serum glucose (mg/dl)	125.03±52.03	95.47±23.38	<0.01
Body mass index (BMI)	49.64±10.44	48.19±9.12	NS
Female (%)	26 (70.3%)	25 (78.1%)	NS
Caucasian (%)	28 (66.7%)	20 (58.8%)	NS
Hypertension (%)	22 (61.1%)	19 (59.4%)	NS
Hyperlipidemia (%)	12 (34.3%)	16 (50.0%)	NS
Type 2 DM (%)	11 (30.6%)	10 (31.3%)	NS
Des-acyl-ghrelin (pg/ml)	2.58±5.6	1.24±14.9	<0.02
Acyl-ghrelin (pg/ml)	6.42±15.00	2.85±6.00	NS
Obestatin (ng/ml)	2.98±1.19	3.44±2.26	NS

Interestingly, neither ghrelin nor obestatin showed a significant relationship with histologic NASH; however, both ghrelin and obestatin showed moderate but statistically significant increases in patients with NASH and fibrosis stage ≥ 2 ($N=20$) vs. those NASH patients with fibrosis stage < 2 ($N=22$). In fact, circulating ghrelin concentrations in NASH patients with fibrosis score ≥ 2 were almost double the concentration of NASH patients with a fibrosis stage < 2 (8.73 vs. 4.22 pg/ml, $P<0.04$).

Table 20. Clinico-demographic and laboratory data for cohorts of advanced fibrosis and undetected/mild fibrosis.

	NASH w fibrosis score ≥ 2	NASH w fibrosis score < 2	<i>P</i> Value
N	20	22	
ALT (IU/l)	39.73 \pm 34.16	32.71 \pm 15.08	NS
AST (IU/l)	37.87\pm30.69	24.19\pm8.23	<0.05
Fasting serum cholesterol (mg/dl)	195.07 \pm 45.32	209.45 \pm 38.22	NS
Fasting serum triglyceride (mg/dl)	207.14 \pm 83.44	221.05 \pm 150.49	NS
Fasting serum glucose (mg/dl)	125.29 \pm 47.85	124.85 \pm 55.99	NS
Body mass index (BMI)	51.55 \pm 14.20	48.29 \pm 6.69	NS
Female (%)	11 (73.3%)	15 (68.2%)	NS
Caucasian (%)	10 (71.4%)	18 (81.8%)	NS
Hypertension (%)	8 (60.0%)	13 (61.9%)	NS
Hyperlipidemia (%)	5 (33.3%)	7 (35.0%)	NS
Type 2 DM (%)	6 (40.0%)	5 (23.8%)	NS
Des-acyl-ghrelin (pg/ml)	4.98 \pm 5.93	3.95 \pm 5.37	NS
Acyl-ghrelin (pg/ml)	8.73\pm18.66	4.22\pm10.45	<0.05
Obestatin (pg/ml)	3.46\pm1.41	2.54\pm0.73	<0.05

Obestatin levels also increased from 2.54 pg/ml in NASH patients with low fibrosis scores (fibrosis stage < 2) to 3.46 pg/ml in NASH patients with fibrosis stage ≥ 2 ($P < 0.03$). In multivariate analysis, the model to predict NASH with fibrosis stage ≥ 2 included ghrelin, ALT, AST, and glucose ($P = 1.0e-3$)

(Table 21). Acylated ghrelin levels correlated positively with circulating obestatin ($r=0.33$, $P<3.0e-3$) but negatively with total cholesterol ($r=0.28$, $P<0.03$). Additionally, circulating obestatin levels correlated negatively with the levels of total cholesterol ($r=0.25$, $P<0.05$). Finally, levels of DAG expression were positively correlated with that of acyl-ghrelin ($r=0.34$, $P=3.0e-3$), but not with any cytokine assayed.

Table 21. Best fitting multiple linear regression model ($P<0.001$) predicting NASH with moderate fibrosis (score ≥ 2).

	Regression coefficient β and SE	<i>P</i> values of independent variables
Intercept	-0.2119 ± 0.1423	0.14
Ghrelin (pg/ml)	0.0100 ± 0.0037	$9.2e-4$
ALT (IU/l)	-0.0077 ± 0.0044	0.08
AST (IU/l)	0.0164 ± 0.0052	$2.4e-4$
Glucose	0.0018 ± 0.0011	0.11

Regression coefficient β represents slope estimate \pm standard error of the estimate (SE). Positive and negative regression coefficients indicate the dependence of NASH with moderate fibrosis on levels of soluble serum components.

Inflammatory Cytokines in Obese NAFLD Patients

Of the 17 cytokines measured, only 6 had levels that were higher than the background in a substantial number of patients to allow comparative analysis. These include IL-6, IL-7, IL-8, G-CSF, CCL2/MCP-1, and CCL4/MIP-

1β. Of these cytokines, only IL-7 exhibited statistically significant differential expression. IL-7 exhibited lower concentrations in NAFLD patients with fibrosis stage ≥2 (16.89 vs. 10.68 pg/ml, $P=0.01$) compared to NAFLD patients with simple steatosis. Furthermore, concentrations of IL-7 were also significantly lower in NAFLD patients with type II diabetes compared to those without a diagnosis of type II diabetes (11.78 vs. 6.77 pg/ml, $P=8.0e^{-3}$) (Table 21). When considering just the NASH cohort, expression of IL-7 was almost twice as high in NASH patients without a diagnosis of Type II diabetes (N=25) as compared to patients with NASH and diabetes (N=11) (13.04 vs. 6.77 pg/ml, $P=5.0e^{-3}$).

Acylated ghrelin levels correlated negatively with circulating IL-6 ($r=-0.29$, $P<9.0e^{-3}$) and CCL2/MCP-1 ($r=-0.29$, $P<0.01$) levels. Circulating obestatin levels correlated negatively to CCL/MCP-1 levels ($r=-0.24$, $P=0.05$), but positively with CCL4/MIP-1β ($r=0.26$, $P=0.03$).

Table 22. Clinco-demographic and laboratory data for NAFLD patients with type 2 Diabetes.

	Diabetes	No diabetes diagnosis	<i>P Value</i>
N	21	47	
ALT (IU/l)	32.62±12.92	29.49±23.50	NS
AST (IU/l)	27.43±10.83	24.69±19.87	NS
Fasting serum cholesterol (mg/dl)	184.50±46.92	200.63±38.62	NS
Fasting serum	174.10±109.82	186.12±114.36	NS

triglyceride (mg/dl)			
Fasting serum glucose (mg/dl)	133.85±58.83	100.62±29.98	NS
Body mass index (BMI)	50.16±11.46	48.70±9.17	NS
Female (%)	13 (61.9%)	37 (78.7%)	NS
Caucasian (%)	17 (81.0%)	30 (63.8%)	NS
Hypertension (%)	18 (85.7%)	23 (48.9%)	<0.01
Hyperlipidemia (%)	14 (70.0%)	14 (29.8%)	<0.01
Interleukin-7 (IL-7) pg/ml	11.78±11.5	6.77±6	<1.0e-3

Post-bariatric Surgery Follow-up

The association of GHRL gene products and inflammatory cytokines were assessed in weight loss post-bariatric surgery. A total of 35 patients had post-surgery follow-up data (age 45.9 years, gender 25.7% male, median length of follow-up after surgery 53 weeks, baseline BMI 47.5, BMI on follow-up visit 33.4). There was a resolution of obesity-related conditions during the follow-up period: baseline Type II diabetes (25.7%) vs. type II diabetes on follow-up visit (2.9%) and baseline hyperlipidemia (42.9%) vs. hyperlipidemia on follow-up visit (8.6%). For the entire length of the follow-up, the average weight loss per day for the cohort was 0.22 kg/day. However, the rate of the weight loss was faster during the first 3 months (0.49 kg/day) after surgery (Table 22).

Table 23. Baseline obestatin and the rate of weight loss over time

Follow-up by	Average weight loss (kg/day)	Pearson correlations* and respective <i>P</i> values	Pearson partial correlations* controlled for baseline BMI and respective <i>P</i> values
3 months	0.73	0.81 ($P<0.05$)	0.93 ($P<0.05$)
6 months	0.49	0.61 ($P<0.03$)	0.60 ($P=0.05$)
12 months	0.41	0.59 ($P<0.02$)	0.58 ($P<0.05$)
24 months	0.32	0.57 ($P<5.0e-3$)	0.56 ($P<0.01$)
Full cohort	0.22	0.51 ($P<2.0e-3$)	0.51 ($P<0.01$)

Serum obestatin was the only circulating hormone measured at baseline and significantly correlated with the rate of the weight loss ($r=0.52$, $P=2.0e^{-3}$). The strongest correlation between baseline circulating obestatin and rate of weight loss was seen within the first 3 months post-surgery ($r=0.81$, $P=0.05$). The correlation between the rate of weight loss and baseline obestatin concentration seemed to be weaker by the end of the first 6 months after surgery, but still remained significant ($r=0.61$, $P=0.03$). Pearson partial correlations adjusted for baseline BMI remained generally unchanged.

Discussion

In this study, the circulating concentration of these three molecules and select set of inflammatory cytokines were profiled in a cohort of morbidly obese patients with biopsy-proven NAFLD. This study shows an increase of DAG levels in patients with NASH with or without moderate hepatic fibrosis (stage \geq 2). In fact, DAG positively correlated with several factors such as ALT, AST, fasting glucose, and triglycerides as well as liver biopsy findings of ballooning degeneration, presence of Mallory-Denk bodies, and portal fibrosis. These laboratory and histologic factors are strongly associated with the presence of histologic NASH. This study is the first to postulate a potential role for DAG in the pathogenesis of the progressive form of NAFLD or NASH.

The results of this study are novel but consistent with previous limited research demonstrating DAG to have independent intrinsic activities in various physiological and pathophysiological conditions. Unfortunately, since the discovery of ghrelin, DAG has been considered an inactive product of the ghrelin gene with no receptor activity and only ~300 manuscripts published on the subject according to PubMed as of March of 2012 (Delhanty *et al.* 2013). These results are part of a wave of new and emerging research data to implicate the importance of the ghrelin axis in glucose metabolism, inflammatory processes and hepatic fibrosis.

Animal studies on the effect of ghrelin gene products on insulin secretion show acyl-ghrelin and obestatin to inhibit insulin secretion in a dose-dependent manner. Islet cells and DAG exhibit an inhibitory effect on acyl ghrelin but not on obestatin (Qader *et al.*, 2008). DAG was shown not to affect the secretion of insulin directly, but it may alter insulin secretion by regulating acyl-ghrelin. Furthermore, DAG acts as a potent insulin secretagogue in glucose-stimulated conditions in vitro, only to be blocked by co-administration of exogenous acyl-ghrelin (Gauna *et al.*, 2007). However, contradictory in vivo studies have shown insulin sensitivity to be enhanced by the co-administration of acyl-ghrelin and DAG (Gauna *et al.*, 2004). A major challenge in DAG research is that its cognate receptor has not yet been discovered. In order to objectively study the effects of DAG, GHSR-ablated mice are analyzed and recent genome-wide expression studies of this model revealed DAG to modulate the expression of metabolically vital genes in adipose, muscle and liver tissues (Delhanty *et al.*, 2010). Integrative analysis of these studies suggests positive effects of DAG on glucose metabolism and insulin sensitivity, as well as on adipogenesis and fatty acid uptake in visceral adipose tissue. Interestingly, in the context of obesity, DAG favors decreased adiposity through GHSR-independent suppression of lipogenic genes, while acyl-ghrelin promotes fat accumulation through GHSR-dependent lipid retention (Davies *et al.*, 2009). In vitro studies of the direct

effects of DAG on adipocytes using 3T3-L1 cells showed DAG to stimulate proliferation and significantly stimulate differentiation (Miegyeu *et al.*, 2011). Des-acyl ghrelin (DAG) stimulated adipocytes displaying an increase in fatty acid (FA) uptake, which interestingly can be blocked by the GHSR-1 antagonist (Miegyeu *et al.*, 2011). Transgenic mice overexpressing a ghrelin analog, trp(3)-ghrelin, exhibit normal growth and glucose metabolism in early development but develop insulin resistance and impaired glucose tolerance by their first year (Yamada *et al.*, 2010). Contradictory to these results, Ariyasu *et al.* has shown transgenic mice overexpressing DAG to result in small size phenotype and DAG has not shown any difference compared to control animals as it relates to glucose metabolism and IR (Ariyasu *et al.*, 2005). In addition, fatty acid binding protein-4 (*FABP4*)-ghrelin transgenic mice are resistant to diet-induced obesity and show improvement in glucose metabolism and insulin sensitivity (IR) (Zhang *et al.*, 2008). These studies highlight the importance of further ghrelin research and provide evidence connecting DAG with insulin resistance (IR) and glucose metabolism, which are both important in the pathogenesis of NAFLD and NASH.

This study revealed a much stronger association between elevated DAG expression and the severity of NAFLD than to type II diabetes status. Furthermore, no significant differences in circulating obestatin, DAG, ghrelin, or

even the ratio of acyl-ghrelin to total ghrelin were detected when comparing cohorts with and without a diagnosis of diabetes type II. Instead, the results suggest that the products of the *GHRL* gene may have some direct or indirect role in the pathogenesis of NAFLD. As outlined in detail in the NAFLD chapter, oxidative stress-induced hepatocyte cell death is a key event in the progression from steatosis to NASH, and ghrelin has been shown to have anti-apoptotic activity (Zhang *et al.*, 2011; Chung *et al.*, 2007; Lear *et al.*, 2010). In an in vitro study of H₂O₂-induced apoptosis in H9c2 cells, treatment with ghrelin restored ROS and mitochondrial mediated signaling by decreasing H₂O₂-induced Bax production and caspase-9 activation, with increased Bcl-2 (Zhang *et al.*, 2011). Additionally, NF-κB phosphorylation was significantly inhibited by ghrelin in H₂O₂-treated cells (Zhang *et al.*, 2011). These results suggest ghrelin attenuates the generation of intracellular ROS by reversing mitochondrial dysfunction. In a separate model of apoptosis, oxygen and glucose (OGD)-induced pretreatment of cells with ghrelin, prevented OGD-induced apoptosis by significantly decreasing apoptotic DNA and ROS production (Chung *et al.*, 2007). In fact, Hou *et al.* has successfully demonstrated the inhibitory effects of ghrelin on IL-8 (a chemokine mediator of the inflammatory response) production via NF-κB pathway (Hou *et al.*, 2009). These studies provide ample evidence to support and partially explain the observation of elevated DAG

levels in patients with NASH than in patients with non-NASH, and in NASH patients with fibrosis ≥ 2 (Table 21). Elevated ghrelin levels in this cohort of patients may serve as a compensatory measure to impede progression of steatosis to NASH.

One of the main aims of this study was to examine the relationship between select inflammatory cytokines (IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, IFN- γ , MCP-1/CCL2, MIP-1 β /CCL4, and TNF- α) and the products of the *GHRL* gene in the context of obesity-associated NAFLD. Although little evidence of a direct relationship was found between these inflammatory cytokines, an additional finding relevant to NAFLD was observed. In this study, decreased concentrations of circulating IL-7 in NAFLD patients with type II diabetes or fibrosis was observed. This finding corroborates the previously reported evidence by Dr. Baranova's laboratory that type II diabetes is an independent clinical predictor of fibrosis in NAFLD (Stepanova *et al.*, 2010). Additionally, long-term follow up evaluation of patients with NAFLD and type II diabetes are shown to be at dramatically increased risk for liver related mortality (Rafiq *et al.*, 2009).

This study was the first to implicate IL-7 in the pathogenesis of fibrosis and type II diabetes. IL-7 is 25-kDa glycoprotein that was initially isolated from the bone marrow of stroma cells with various functions related to cell motility

(Goodwin *et al.*, 1989; Jample *et al.*, 1990). Interleukin-7 inhibits TGF- β -induced PKC- δ phosphorylation at Ser-646 and Thr-505 leading to restoration of TGF- β mediated induction of Smad7 and significantly reduces TGF- β mediated collagen production (Zhang *et al.*, 2004). Additionally, Yamanaka *et al.*, has shown IL-7 to stimulate either the expression or the activity of SMAD7 that protect against fibrosis through inhibition of extra-cellular matrix (ECM) production (Yamanaka *et al.*, 2006). In general, the presence of sufficient IL-7 primarily suppresses the activity of molecules involved in the pathogenesis of insulin resistance and diabetes, while simultaneously stimulating the expression of primarily anti-fibrogenic molecules (Figure 8).

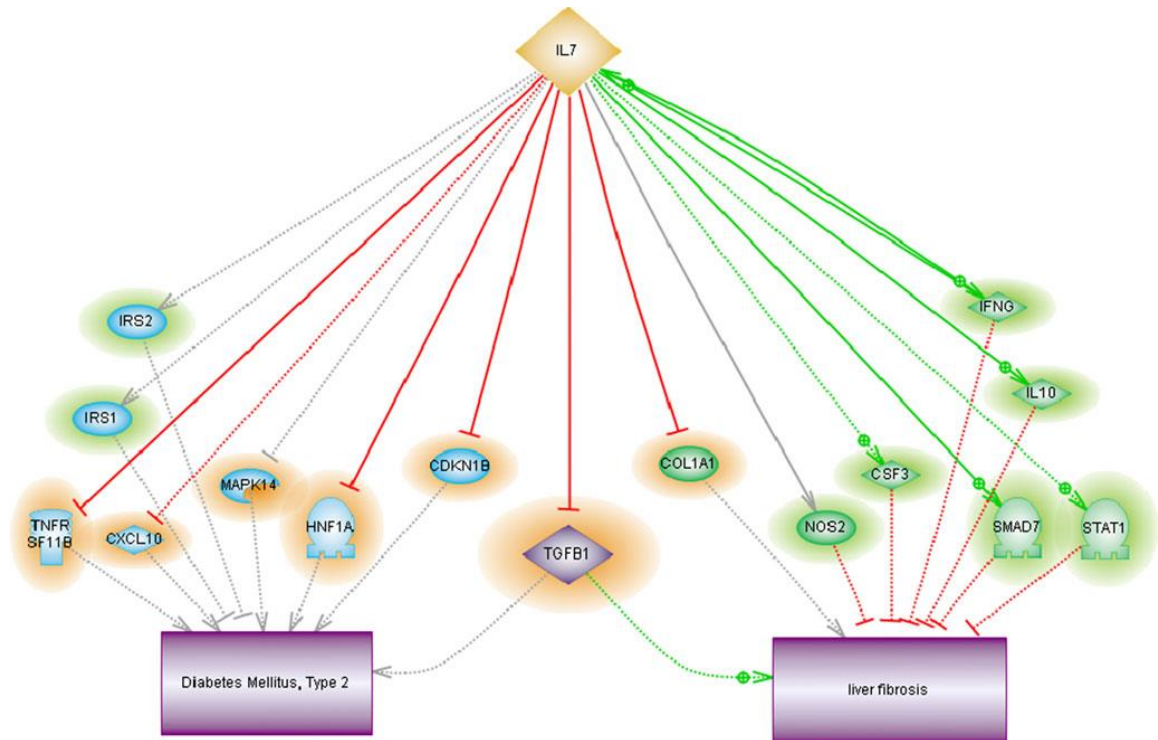


Figure 8. Low circulating IL-7 can may increase susceptibility to type 2 diabetes or hepatic fibrosis by a number of mechanisms including and allowing increased activity of pathogenic molecules and/or failure to stimulate the expression of molecules capable of suppressing the development of diabetes type 2 and/or liver fibrosis.

Based on these findings one can hypothesize that in the morbidly obese patients with low grade systemic inflammation (meta-inflammation), the lack of inhibition of the molecules contributing to diabetes and fibrosis might elicit substantially stronger detrimental effects as compared to lean individuals. These effects could be further exacerbated in morbidly obese patients with NAFLD/NASH and chronic inflammation. Collectively, it can be suggested that

administration of IL-7 or improving IL-7 circulatory levels can be a novel therapeutic strategy in preventing low-grade systemic inflammation-induced fibrosis in NAFLD/NASH.

Finally, the study of patients following post-bariatric surgery showed a relatively strong correlation between baseline circulating obestatin and rate of weight loss. These results indicate that obestatin might participate in the metabolic and behavioral underpinning of weight loss. Nevertheless, it would be premature to put forth a physiological hypothesis explaining these intriguing observations without additional confirmatory research.

In summary, this research study is the first to implicate the products of the *GHRL* gene as being potentially significant in the pathogenesis of NASH. Ghrelin, DAG and obestatin are associated with NASH and associated fibrosis. Although the expression of these molecules could not be directly correlated with circulating levels of inflammatory cytokines, one of the markers studied, IL-7, did display an expression profile that may be important to the study of NAFLD. This study provides further evidence of a paradigm shift in DAG research and warrants further investigation to elucidate the mechanisms involved in the pathogenesis of a silent killer of morbidly obese people inflicted with NAFLD.

CHAPTER 5

α -Melanin Stimulating Hormone (α -MSH) and Melanin Concentrating Hormone (MCH) Expression in Obesity and Obesity Related Non-Alcoholic Fatty Liver Disease (NAFLD)

In humans, melanin is a complex bio-polymer endogenously produced in melanocytes, in retinal pigment cells, and in some specialized cells of the inner ear and the central nervous system (Page, 2011). These molecules are responsible for the pigmentation of skin, hair follicles and play a major photoprotective role by reducing ultraviolet (UV) induced DNA damage (Ishikawa *et al.*, 1984). Epidemiological data indicate an inverse relationship between the incidence of skin cancer and skin pigmentation. Incidence of skin cancer is markedly lower in individuals who have higher melanin (dark pigment) concentrations than their counterparts who have low melanin concentrations (fair skin) (Halder *et al.*, 1995; Gilchrest *et al.*, 1999; Kvam *et al.*, 2003; Bishop *et al.*, 2005). While it is not the focus of my research, it should be mentioned here that melanin related hormones (α -Melanin Stimulating hormone (α -MSH) and Melanin Concentrating hormone (MCH) have pleiotropic affects that go beyond skin pigmentation such as energy homeostasis, orexigenic/anorexigenic properties and a role in social behavior (mood and emotion), signal transduction, and pathogen destruction (Shi *et al.*, 2004; Raposo *et al.*, 2007).

In nature, melanin exists in two major copolymers eumelanin and pheomelanin, responsible for brown/black and yellow/red pigment respectively, but eumelanin is the most widely studied for its anti ROS activity (Sarna, 1992; Nofsinger *et al.*, 2002; Page, 2010). Melanin is synthesized in epidermal epithelia sequestered within specialized membrane-bound organelles called melanosomes, which help protect the newly synthesized melanin from cytosol and membranous organelles and from oxidative attack and degradation (Raposo *et al.*, 2007).

The primary signaling pathway leading to melanin production in melanocytes involves extracellular ligand α -MSH which is a product of the proopiomelanocortin (POMC) gene, binding to its cognate cell surface Melanocortin receptor 1 (i.e. MC1R) (Page, 2011). The melanocortin receptor family belongs to the G-protein-coupled receptor superfamily which, upon activation, leads to elevated cyclic adenosine monophosphate (cAMP) levels via stimulation of adenylate cyclase. Cyclic AMP activates cAMP-dependent protein kinase, which phosphorylates the cAMP responsive, element-binding protein (CREB) family of transcription factors in the nucleus. CREB binds cAMP response elements in the promoter of the gene encoding microphthalmia-associated transcription factor (MITF), which initiates the expression of melanogenic enzymes such as the melanosome membrane-spanning proteins,

tyrosinase (TYR) and the TYR-related proteins, TRP-1 and TRP-2 (Page, 2011). Tyrosinase is the key enzyme in melanogenesis which oxidizes tyrosine to produce the melanin precursor, dopaquinone, which further can be processed into eumelanin in the absence of thiols, or pheomelanin in the presence of thiols (Page, 2011, Ito *et al.*, 2003).

In 2009, research studies conducted by Manpreet Randhawa, under Dr. Baranova's direction, revealed for the first time the presence of ectopic synthesis of melanin in human adipose tissue (Randhawa *et al.*, 2009). Successful confirmation of the expression of melanogenesis-related mRNAs and proteins in human adipose tissue and were further expressed at much higher levels in samples from obese individuals using real-time PCR and immunohistochemical staining. Melanin was shown to be present in human adipose tissue by Fontana-Masson staining and by permanganate degradation of melanin coupled with liquid chromatography/ultraviolet/mass spectrometry. In addition to these studies, previous analysis of the transcriptome of visceral adipose tissue of morbidly obese individuals revealed several melanogenesis related genes (Baranova *et al.*, 2005). This intriguing evidence naturally led to further inquiries into the possible implications of the role of melanin in adipose tissue and in obesity overall. The ectopic synthesis of melanin in obese adipose tissue may serve as a compensatory mechanism that utilizes its anti-

inflammatory and its oxidative damage absorbing properties. With the progression of obesity and an increase of the cellular fat deposition, adipocytes become more exposed to endogenous apoptotic signal, especially ROS. To counteract pro-apoptotic ROS effects, the adipocytes in turn may ectopically activate the genetic program of melanogenesis, thus neutralizing excessive ROS. Adipocyte melanin would also suppress the secretion of pro-inflammatory background in obese subjects and alleviating the metabolic syndrome. High levels of polymorphisms in human genes regulating melanin biosynthesis may account for the highly individual melanogenic responses of adipocytes and for the difference in an individual's propensity to develop secondary complications of obesity.

If the hypothesis linking the production of the melanin to the suppression of the pro-inflammatory effects of the accumulation of the visceral fat is confirmed, its potential impacts on the global health could be quite significant. The molecular compounds stimulating melanin biosynthesis, a number of the synthetic agonists of α -MSH receptors, have already been proven safe in human clinical trials for therapeutic tanning. These compounds need to be further tested as the preventive medications aimed at curtailing the development of devastating metabolic complications in obese and overweight populations.

Materials and Methods

Fasting serum samples, liver biopsies as well as clinical and laboratory data were collected from biopsy-proven NAFLD patients (N=76). All liver biopsies were interpreted by a single hepatopathologist. Fasting serum samples were profiled for 17 soluble inflammatory cytokines (IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, IFN- γ , MCP-1/CCL2, MIP-1 β /CCL4, and TNF- α) using the Bio-Plex Human Cytokine 17-Plex panel as part of the Bio-Plex Suspension Array System (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol.

In addition, two melanogenesis-related hormones, α -melanocyte stimulating hormone (α -MSH) and melanin concentrating hormone (MCH) (Phoenix Pharmaceuticals, CA) were measured by sandwich enzyme immunoassays following manufacturers' protocol. For these molecules, serum levels were compared between patients with and without histologic NASH and patients with and without hepatic fibrosis using Mann-Whitney tests. Regression models were generated by stepwise bi-directional selection using serum levels of soluble molecules as predictors of NASH and hepatic fibrosis.

Statistical Analysis

The statistical approach for this study followed that used in previously published study by Younossi Z et al. In summary, the entire study cohort was divided into sub-cohorts according to the following diagnostic comparisons: (1) those with NASH were compared to those without NASH; (2) those with any hepatic fibrosis were compared to those with no fibrosis; and (3) those with advanced hepatic fibrosis were compared to with minimal to no fibrosis. Within each sub-cohort, group statistical comparisons were conducted for all demographic, clinical, laboratory, and histological parameters. All continuous variables were tested for normality by the by the Shapiro Wilk test; a p-value ≤ 0.05 was considered significant and thus indicative that the data comparisons between groups were made by two-tailed, two-sample t-test assuming separate variances, and for non-normally distributed data comparisons between groups were made by Mann-Whitney U test. For categorical variables, group comparisons were made using the Pearson chi-square test for homogeneity.

In addition, multiple linear regression using stepwise bidirectional selection was performed to identify predictive models for the diagnostic outcome of NASH, any hepatic fibrosis, and advanced hepatic fibrosis as previously described in Younossi Z et al.

Results

Demographic, clinical, laboratory and histological data and the outcomes of group statistical comparisons for patients with and without NASH are listed in Table 23. This study included 76 patients with biopsy-proven NAFLD. Of these, 32% of patients had biopsy-proven NASH (Table 24)

Table 24. Clinico-demographic and laboratory data for cohort of morbidly obese bariatric patients with histologically confirmed NAFLD and NASH diagnoses.

N	76
BMI	45±12
Age, years	47±11.5
ALT (IU/l)	37.5±67.65
AST (IU/l)	37.4±32.5
NASH	32%

Circulating levels of MCH and α -MSH displayed strong positive correlation ($r=0.77$, $P\leq 5.30E^{-08}$). Concentrations of α -MSH showed small but statistically significant positive correlations with IL-6 ($r=0.36$, $P<0.05$), and Kupfer cell hypertrophy ($r=0.39$, $P<0.015$) and Portal inflammation ($r=0.30$, $P<0.05$), Table 25.

Table 25. Correlation coefficient of α -MSH levels and related parameters.

	α-MSH R Value	P-Value ≤ 0.05
MCH levels (ng/ml)	0.77	5.30E-08
Kupffer Cell Hypertrophy	0.39	0.01
IL-6 (pg/ml)	0.36	0.03
IL-17 (pg/ml)	0.27	0.05
Portal Inflammation	0.30	0.05

Circulating levels of MCH showed a positive correlations with IL-6 ($r=0.33$, $P<0.04$), and Kupfer cell inflammation ($r=0.35$, $P<0.03$), Table 26. Circulating levels of MCH and α -MSH significantly correlate with markers of inflammation and may participate in the pathogenesis of NAFLD.

Table 26. Correlation coefficients of MCH levels and related parameters.

	MCH R-Values	P-Value
α -MSH Levels (ng/ml)	0.77	5.30E-08
Kupffer Cell Hypertrophy	0.35	0.03
IL-6 (pg/ml)	0.33	0.04
IL-17 (pg/ml)	0.27	0.05
IL-13 (pg/ml)	0.24	0.05

Circulating levels of the ratio of α -MSH and MCH (α -MSH/MCH) showed a positive correlation with IL-17 ($r=0.27$, $P<0.05$), and IL-13 ($r=0.24$, $P<0.05$), Table 27.

Table 27. Correlation coefficients of α -MSH/MCH ratio.

	MSH/MCH R-Values	P-Value
IL-17 (pg/ml)	0.27	0.05
IL-13 (pg/ml)	0.24	0.05

Correlation coefficient of α -MSH displayed a positive correlation with AST levels ($r=0.81$, $P<1.0e-3$), MCH levels ($r=0.77$, $P<1.0e-3$), and degree of hepatic lymphocyte infiltration ($r=0.46$, $P<0.46$), Table 28.

Table 28. Correlation coefficient of α -MSH and related parameters in NASH cohort.

	α-MSH R-Values	P-Value ≤ 0.05
AST Levels (IU/l)	0.81	$<1.0e-3$
MCH Levels (ng/ml)	0.77	$<1.0e-3$
Degree of hepatic lymphocyte infiltration	0.46	<0.46

Correlation coefficient of MCH displayed a positive correlation with AST levels ($r=0.70$, $P<1.0e-3$), and degree of hepatic lymphocyte infiltration ($r=0.49$, $P<1.0e-3$), Table 29.

Table 29. Correlation coefficient of MCH and related parameters in NASH cohort.

	MCH R-Values	<i>P</i>-Value ≤ 0.05
AST Levels (IU/l)	0.70	$<1.0e-3$
Degree of hepatic lymphocyte infiltration	0.49	$<1.0e-3$

Comparison of correlation coefficients of α -MSH and MCH with disease parameters are displayed in Figure 9. Both α -MSH and MCH positively correlated with (Kupfer cell hypertrophy, Portal inflammation, Lymphoplasmacytic inflammation, Diabetes and CCL2). However, both α -MSH and MCH displayed a negative correlation with (Cirrhosis, Mallory Bodies, Pericellular Fibrosis and Bridging Fibrosis). Interestingly, α -MSH and MCH correlated uniquely with respect to steatosis as outlined in the figure below.

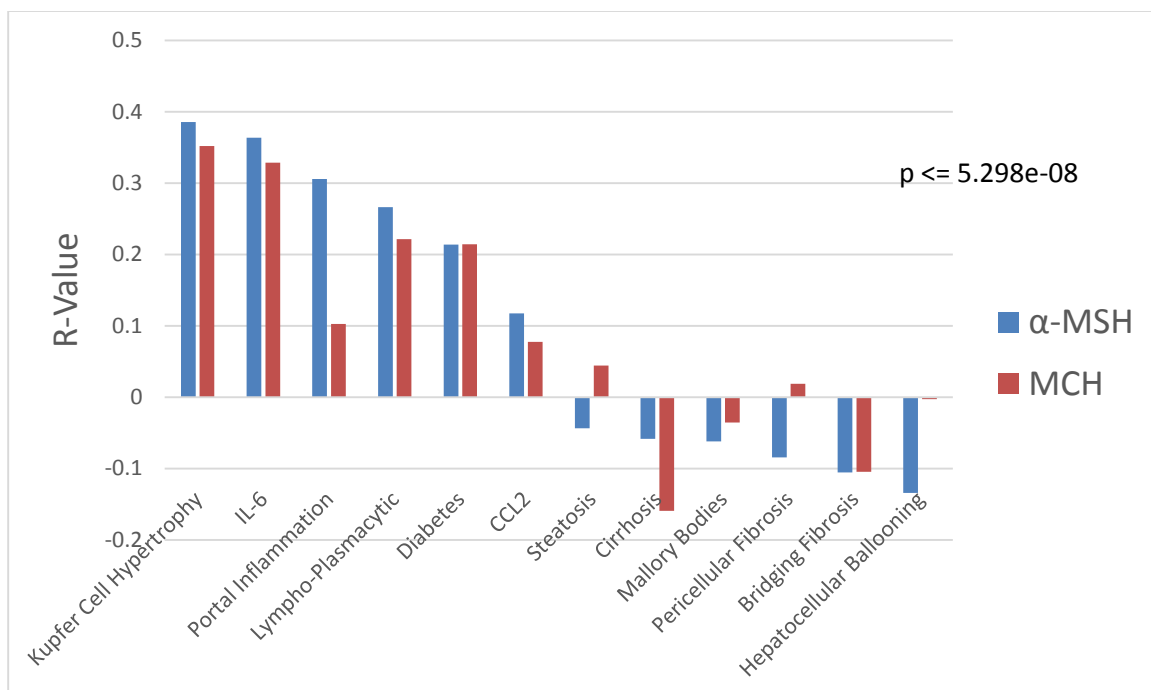


Figure 9. Comparison of correlation coefficients of α-MSH and MCH with disease parameters.

By multiple regression the following predictive ($p=0.01$) model for fibrosis was identified: probability of fibrosis = $0.87 + (-0.12)[\text{MCH}] + (0.12)[\alpha\text{-MSH}] + (-0.46)[\text{IL-4}] + (0.48)[\text{High MCP1}] + (0.35)[\text{High IL8}]$. The standard deviation and p-value associated with each beta value are shown in Table 30.

Table 30. Regression analysis of predictive model of fibrosis.

	<i>β-value</i>	<i>±S.D.</i>	<i>p-value</i>
(Intercept)	0.87	0.22	2.0e-4
MCH (ng/ml)	-0.12	0.06	0.05
α-MSH (ng/ml)	0.12	0.06	0.08
IL4 (pg/ml)	-0.46	0.19	0.01
High MCP1 (pg/ml)	0.48	0.21	0.02
High IL8 (pg/ml)	0.35	0.19	0.06

Discussion

The main goal of this study was to investigate a novel hypothesis, whether adipose tissue of morbidly obese activate melanogenesis and release α-MSH and MCH hormones to counteract the chronic low grade inflammatory state of morbid obesity in patients with NAFLD. α-MSH and MCH exert profound autocrine/paracrine effects including potent protective and anti-inflammatory effects in cells expressing melanocortin receptors (MCRs) with the key tyrosinase (TYR) enzyme activity. The melanin biosynthesis pathway functions in adipose tissue and is hyperactive in visceral fat samples of morbidly obese individuals (Randhawa *et al.*, 2009). This study is the first to measure α-MSH and MCH levels in serum of morbidly obese bariatric NAFLD patients and correlate these values with disease parameters to be used as a novel biomarker for patient stratification.

Interestingly, α -MSH and MCH levels positively correlated with Kupfer cell hypertrophy, liver portal inflammation and inflammatory cytokines IL-6, IL-13 and IL-17, Table 25 and 2). These results are consistent with those reported in animal and human models. Increased release of pro-inflammatory IL-6 was observed from mesenteric adipocytes of high-fat compared with standard diet-fed mice (Wueest S et al. 2012). Accordingly, IL-6 has been reported to be released two to three times in adipose tissue of obese human subjects (Fried SK et al. 1998). Additionally, IL-6 levels were higher in portal veins compared with radial artery of obese individuals (Fontana L et al. 2007). If our hypothesis holds true, naturally a negative correlation between α -MSH and MCH should be observed in relation to inflammatory cytokines. While studies of serum levels of α -MSH and MCH are limited in morbidly obese human subjects, the positive correlation observed in this study might be explained by an early response to stressed liver (Kupfer cell hypertrophy and portal inflammation) and response to these physiological states in the form of increased levels of cytokines, IL-6, IL-13 and IL-17. When serum levels of α -MSH and MCH were assessed in the sickest of patients (NASH cohort), a positive correlation between these hormones and degree of hepatic lymphocyte infiltration was observed. This suggests that serum α -MSH and MCH levels respond according to immunologic/inflammatory response.

The most powerful data that buttress the potential anti-inflammatory properties of α -MSH and MCH came when comparison of correlation coefficients were made with disease parameters, Figure 9. α -MSH and MCH displayed a negative correlation with worsening features of NAFLD including hepatocellular ballooning, degrees of fibrosis and cirrhosis. These results suggest that low levels of serum α -MSH and MCH can be expected with advanced disease stage and might suggest the lack the compensatory mechanism to ameliorate such stressed state as hypothesized.

By multiple regression analysis a predictive model ($p=0.01$) for fibrosis was identified to include serum levels of α -MSH, MCH, IL-4, high MCP1 and high IL-8. The purpose of developing such a model is to utilize analytics of statistical significance to predict future behavior of fibrosis in patients with similar marker profile. While this predictive model is based on this specific cohort of patients, future studies should focus on testing this model with different datasets to better evaluate its predictive power.

Chapter 6

CONCLUSIONS

This dissertation encompasses three studies investigating the role of various hormonal biomarkers for their possible involvement in the pathogenesis of NAFLD/NASH in a cohort of morbidly obese bariatric patients.

NAFLD is an increasingly common condition affecting about 70% of obese individuals and is now considered a major cause of liver-related morbidity and mortality. NAFLD encompasses a spectrum of hepatic pathologies with excessive accumulation of fat in the liver in patients consuming less than 20 grams of alcohol per day. NAFLD related liver injury ranges from simple hepatic steatosis to the more severe form of the disease including NASH, which can progress to fibrosis, cirrhosis, and ultimately organ failure. NAFLD is most commonly associated with metabolic syndrome (MS) and diabetes; the prevalence of NAFLD has increased steadily with the global epidemic of obesity. Currently, the only reliable modality for diagnosing NASH and liver fibrosis is an invasive liver biopsy, which has its limitations and risks. Therefore, a non-invasive clinical and laboratory parameter-based method is needed for detecting and stratifying patients with NAFLD and those at risk for disease progression.

The first study of this dissertation explores the role of vitamin D deficiency in NAFLD/NASH patients. Recent evidence suggests that vitamin D deficiency may have a potential role in inflammatory processes and that vitamin D levels may play an instrumental role in the comorbidities of NAFLD such as hypertension and metabolic syndrome (MS). According to the recent NHANES III study, patients with NAFLD and NASH showed an inverse relationship between serum vitamin D levels and unexplained elevations in ALT liver enzymes. The examination of vitamin D levels in patients with NASH and various degrees of liver involvement may lead to a better understanding of the relationship between chronic liver disease and vitamin D status.

This study was the first to profile serum samples of morbidly obese bariatric patients for vitamin D axis-related hormones [25 (OH) D; Vitamin D Binding protein (VDBP); Intact Parathyroid Hormone (1-84)]. Data from this study shows vitamin D metabolism to be altered in morbidly obese patients with NAFLD. Patients with the severe form of the disease (NASH) were found to be vitamin D deficient, as defined by serum vitamin D levels lower than 30ng/ml. Additionally, 25(OH)D levels significantly correlate with markers of apoptosis and results suggest 25(OH)D has a protective role against apoptosis in patients with NASH.

The second study described herein involves the role of small gastric peptides, Ghrelin and Obestatin, and inflammatory cytokines in obesity related NAFLD patients. Traditionally, Ghrelin and Obestatin have been shown to play central roles in both appetite stimulation and appetite suppression. The changes in the levels of Ghrelin and Obestatin also have metabolic consequences, as they influence lipogenesis, adipogenesis and adipose tissue energy production as well as glucose and insulin secretion. These peptides play role in pathogenesis of MS and type II diabetes.

This study was the first to explore the role of Ghrelin and Obestatin with obesity-related inflammation in morbidly obese patients with NAFLD. The central question being investigated here is whether the gastric peptides Ghrelin and Obestatin participate in the pathogenesis of obesity-related NAFLD. This study implicates the *GHRL* gene products (acylated ghrelin (AG), non-acylated ghrelin (DAG), and Obestatin) as being potentially significant in the pathogenesis of NASH and NASH-associated fibrosis. While the expression of these small peptides did not correlate with circulating levels of a majority of inflammatory cytokines, one important marker studied, IL-7, displayed an expression profile that may be important in the pathogenesis of NAFLD development.

The third study reported herein investigates the levels of circulatory melanin related α -Melanocyte Stimulating Hormone (α -MSH) and Melanin Concentrating Hormone (MCH) in morbidly obese patients with obesity related conditions (i.e. NAFLD and obesity-induced low-grade chronic inflammation). In humans, melanin is produced in melanocytes and a few other specialized cells of the body. Melanin is known for its antioxidant and anti-inflammatory properties that scavenge reactive oxygen species. The research led by Dr. Baronova has been the first to demonstrate that the melanin biosynthesis pathway is functional in adipose tissue of morbidly obese subjects. Melanin biosynthesis is regulated by melanogenic peptides α -MSH and MCH.

The focus of this study was to investigate the interplay between obesity-related chronic systemic inflammation and melanin-related hormones. This study is the first to measure α -MSH and MCH levels in serum of morbidly obese bariatric NAFLD patients and correlate these values with disease parameters. α -MSH and MCH levels were positively correlated with clinical (Kupfer cell hypertrophy and liver portal inflammation) and laboratory parameters (inflammatory cytokines IL-6, IL-13 and IL-17). This correlation might be due to an early response (Kupfer cell hypertrophy and portal inflammation) of a stressed liver. Conversely, α -MSH and MCH levels displayed a negative correlation with features of advanced NAFLD (late response) including

hepatocellular ballooning as well as degrees of fibrosis and cirrhosis. Our data suggest that low levels of serum α -MSH and MCH should be expected with advanced disease stage and might suggest the developed deficiency of the compensatory mechanism.

In conclusion, this dissertation gives a diverse holistic view of hormonal imbalance in the complex condition NAFLD/NASH that often accompanies morbidly obesity. Contrary to popular hierarchy of study designs, observational studies of this kind (cohort) are capable of providing high impact insights into pathophysiology of chronic diseases.

REFERENCES

- Abdelmalek, M.F., Suzuki, A., Guy, C., Unalp-Arida, A., Colvin, R., Johnson, R.J., Diehl, A.M. 2010. Nonalcoholic Steatohepatitis Clinical Research Network. Increased fructose consumption is associated with fibrosis severity in patients with nonalcoholic fatty liver disease. *Hepatology*, **51**(6): 1961-1971.
- Abramovitch, S., Dahan-Bachar, L., Sharvit, E., Weisman, Y., Ben Tov, A., Brazowski, E. Reif, S. 2011. Vitamin D inhibits proliferation and profibrotic marker expression in hepatic stellate cells and decreases thioacetamide-induced liver fibrosis in rats. *Gut*, **60**: 1728-1737.
- Andres, R. 1980. Effect of obesity on total mortality. *Int J Obes*, **4**: 381-386.
- Anstee, Q.M., McPherson, S. Day, C.P. 2011. How big a problem is non-alcoholic fatty liver disease? *BMJ*, **343**: 1-5.
- Ariyasu, H., Takaya, K., Iwakura, H., Hosoda, H., Akamizu, T., Arai, Y., Kangawa, K., Nakao, K. 2005. Transgenic mice overexpressing des-acyl ghrelin show small phenotype. *Endocrinology*, **146**: 355-364.
- Ariyasu, H., Takaya, K., Iwakura, H., Hosoda, H., Akamizu, T., Arai, Y., Kangawa, K., Nakao, K. 2005. Transgenic mice overexpressing des-acyl ghrelin show small phenotype. *Endocrinology*, **146**(1): 355-64.
- Aung, K., Lorenzo, C., Hinojosa, M.A., Haffner, S.M. 2014. Risk of Developing Diabetes and Cardiovascular Disease in Metabolically Unhealthy Normal-Weight and Metabolically Healthy Obese Individuals. *J Clin Endocrinol Metab*, **99**(2): 462-468.
- Baffy, G. 2009. Kupffer cells in non-alcoholic fatty liver disease: The emerging view. *Journal of Hepatology*, **51**: 212-223.
- Bantel, H., Ruck, P., Gregor, M., Schulze-Osthoff, K. 2001. Detection of elevated caspase activation and early apoptosis in liver disease. *European Journal of Cell Biology*, **80**: 230-239.

- Baranova, A., Biredinc, A., Younossi, Z.M. 2007. Adipokines in Nonalcoholic Fatty Liver Disease. In G. Fantuzzi and C. Braunschweig (Eds). *Nutrition and Health: Adipose Tissue and Adipocytokines in Health and Disease* (pp. 291-307) New York, NY: Humana Press, Inc.
- Baranova, A., Collantes, R., Gowder, S.J., Elariny, H., Schlauch, K., Younoszai, A., King, S., Randhawa, M., Pusulury, S., Alsheddi, T., Ong, J.P., Martin, L.M., Chandhoke, V., Younossi, Z.M. 2005. Obesity-related differential gene expression in the visceral adipose tissue. *Obes. Surg.*, **6**: 758-765.
- Bays, H.E., Gonzalez-Campoy, J.M., Bray, G.A., Kitabchi, A.E., Bergman, D.A., Schorr, A.B., Rodbard, H.W., Henry, R.R. 2008. Pathogenic potential of adipose tissue and metabolic consequences of adipocyte hypertrophy and increased visceral adiposity. *Expert Rev. Cardiovasc. Ther.*, **6**(3): 343-368.
- Begriche, K., Igoudjil, A., Pessayre, D., Fromenty, B. 2006. Mitochondrial dysfunction in NASH: Causes, consequences and possible means to prevent it. *Mitochondrion.*, **6**(1): 1-28.
- Begriche, K., Massart, J., Robin, M.A., Bonnet, F., Fromenty, B. 2013. Mitochondrial adaptations and dysfunction in nonalcoholic fatty liver disease. *Hepatology*, **58**(4): 1497-1507.
- Beno, D.W., Brady, L.M., Bissoneette, M., Davis, B.H. 1995. Protein kinase C and mitogen-activated protein kinase are required for 1,25-dihydroxyvitamin D₃-stimulated Egr induction. *J Biol Chem*, **270**(8): 3642-3647.
- Bikle, D.D., Gee, E., Halloran, B., Kowalski, M.A., Ryzen, E., Haddad, J.G. 1986. Assessment of the free fraction of 25-hydroxyvitamin D in serum and its regulation by albumin and the vitamin D-binding protein. *J Clin Endocrinol Metab*, **63**(4): 954–959.
- Bikle, D.D., Siiteri, P.K., Ryzen, E., Haddad, J.G. 1985. Serum protein binding of 1,25-dihydroxyvitamin D: a reevaluation by direct measurement of free metabolite levels. *J Clin Endocrinol Metab*, **61**(5): 969–975.
- Bishop, J.A.N., Bishop, D.T. 2005. The genetics of susceptibility to cutaneous melanoma. *Drugs Today (Barc)*, **41**(3): 193-203.

- Bouillon, R. 2011. The vitamin D binding protein DBP. In Feldman, D., Pike, J.W., Adams, J.S. (Eds.), *Vitamin D* (pp. 57-72). Elsevier, New York.
- Bratke, K., Wendt, A., Garbe, K., Kuepper, M., Julius, P., Lommatzsch, and Virchow, J.C. 2014. Vitamin D binding protein and vitamin D in human allergen induced endobronchial inflammation. *Clinical and Experimental Immunology*, **177**: 366-372.
- Brochu, M., Tchernof, A., Dionne, I.J., Sites, C.K., Eltabbakh, G.H., Sims, E.A., Poehlman, E.T. 2001. What are the physical characteristics associated with a normal metabolic profile despite a high level of obesity in postmenopausal women? *J Clin Endocrinol Metab.*, **86**(3): 1020-1025.
- Brunt, E.M., Kleiner, D.E., Wilson, L.A., Unalp, A., Behling, C.E., Lavine, J.E., Neuschwander-Tetri, B.A., and the NASH Clinical Research Network. 2009. Portal chronic inflammation in nonalcoholic fatty liver disease (NAFLD): A histologic marker of advanced NAFLD-Clinicopathologic correlations from the nonalcoholic steatohepatitis clinical research network. *Hepatology*, **49**: 809-820.
- Byrne, C.D., Targher, G. 2015. NAFLD: A multisystem disease. *Journal of Hepatology*, **62**: S47-S64.
- Calder, P.C. 2006. N-3 Polyunsaturated fatty acids, inflammation, and inflammatory disease. *Am J Clin Nutr*, **83**(suppl):1505S-1519S.
- Cantley, L.K., Russell, J., Lettieri, D., Sherwood, L.M. 1985. 1, 25 dihydroxyvitamin D₃ suppresses parathyroid hormone secretion from bovine parathyroid cells in tissue culture. *Endocrinology* **117**: 2114-2119.
- Cetin, E., Kanbur, M., Cetin, N., Eraslan, G., Atasever, A. 2011. Hepatoprotective effect of ghrelin on carbon tetrachloride-induced acute liver injury in rats. *Regul Pept.* **171**(1-3): 1-5.
- Chalasani, N., Wilson, L., Kleiner, D.E., Cummings, O.W., Brunt, E.M., Unalp, A. 2008. Relationship of steatosis grade and zonal location to histological features of

- steatohepatitis in adult patients with non-alcoholic fatty liver disease. *J Hepitol.*, **48**(5): 829-834.
- Chen, Z.W., Chen, L.Y., Dai, H.L., Chen, J.H., Fang, L.Z. 2008. Relationship between alanine aminotransferase levels and metabolic syndrome in nonalcoholic fatty liver disease. *J Zhejiang Univ Sci B*, **9**: 616–622.
- Cheng, J.B., Motola, D.L., Mangelsdorf, D.J., Russell, D.W. 2003. Deorphanization of cytochrome P450 2R1: a microsomal vitamin D 25-hydroxylase. *J. Biol. Chem*, **278**: 38084-38093.
- Chiu, K.C., Chu, A., Go, V. L. W., Saad, M. F. 2004. Hypovitaminosis D is associated with insulin resistance and β cell dysfunction, *American Journal of Clinical Nutrition*, **79**(5): 820–825.
- Christakos, S., Dhawan, P., Liu, Y., Xiaorong, P., Porta, A. 2003. New insights into the mechanisms of vitamin D action. *Journal of Cellular Biochemistry*, **88**: 695-705.
- Christensen, E.I., Birn, H. 2001. Megalin and cubilin: synergistic endocytic receptors in renal proximal tubule. *Am J Physiol Renal Physiol.*, **280**: F562-F573.
- Chun, R.F., Percy, B.E., Orwoll, E.S., Nielson, C.M., Adams, J.S., Hewison, M. 2014. Vitamin D and DBP: The free hormone hypothesis revisited. *Journal of Steroid Biochemistry & Molecular Biology*, **144PA**: 132–137.
- Chung, H., Kim, E., Lee, D.H., Seo, S., Ju, S., Lee, D., Kim, H., Park, S. 2007. Ghrelin Inhibits Apoptosis in Hypothalamic Neuronal Cells during Oxygen-Glucose Deprivation. *Endocrinology* **148**(1):148-159.
- Cooke, N.E., David, E.V., 1985. Serum vitamin D-binding protein is a third member of the albumin and alpha fetoprotein gene family. *J Clin Invest*, **76**(6): 2420–2424.
- Cooke, N.E., Willard, H.F., David, E.V., George, D.L. 1986. Direct regional assignment of the gene for vitamin-D binding-protein (GC-globulin) to human-chromosome 4q11-q13 and identification of an associated DNA polymorphism. *Human Genet*, **73**: 225-229.

- Cornier, M.A., Dabelea, D., Hernandez, T.L., Lindstrom, R.C., Steig, A.J., et al. 2008. The metabolic syndrome. *Endocr Rev.*, **29**: 777–822.
- Cox, A.J., West, N.P., Cripps, A.W. 2015. Obesity, inflammation, and gut microbiota. *Lancet Diabetes Endocrinol.*, **3**: 207-215.
- Davies, J.S., Kotokorpi, P., Eccles, S.R., Barnes, S.K., Tokarczuk, P.F., Allen, S.K., Whitworth, H.S., Guschina, I.A., Evans, B.A.J., Mode, A., Zigman, J.M., Wells, T. 2009. Ghrelin induces abdominal obesity via GHS-R-Dependent lipid retention. *Mol Endocrinol.*, **23**(6): 914-924.
- Day, C.P., James, O.F. 1998. Steatohepatitis. *Gastroenterology*, **114**(4): 842-845.
- de Borst, M.H., de Boer, R.A., Stolk, R.P., Slaets, J.P., Wolffenbuttel, B.H., Navis, G. 2011. Vitamin D deficiency: Universal risk factor for multifactorial disease? *Current Drug Targets*, **12**: 97-106.
- Del Valle, Heather B., et al., (Eds.) *Dietary Reference Intakes for Calcium and Vitamin D*. National Academies Press, 2011.
- Delhanty, P.J., Neggers, S.J., van der Lely, A. (2013). Des-Acyl Ghrelin: A metabolically active peptide. In A. Benso, F. Casanueva, E. Ghigo, A. Granata (Eds.), *The Ghrelin System: Endocrine Development Vol 25*. (pp. 112-121) Basel: Karger.
- Delhanty, P.J.K., Dun, Y., Visser, J.A., van Kerkwijk, A., Huisman, M., van Ijcken, W.F.J., Swagemakers, S., Smith, R.G., Themmen, A.P.N., van der Lely, A.J. 2010. Unacylated ghrelin rapidly modulates lipogenic and insulin signaling pathway gene expression in metabolically active tissues of GHSR deleted mice. *PLoS ONE*, **5**(7) e11749.
- Dentin, R., Benhamed, F., Pegorier, J.P., Fougère, F., Viollet, B., Vaulont, S., Girard, J., Postic, C. 2005. Polyunsaturated fatty acids suppress glycolytic and lipogenic genes through the inhibition of ChREBP nuclear protein translocation. *J. Clin. Invest.*, **115**: 2843-2854.
- Ding, N., Yu, R.T., Subramaniam, N., Sherman, M.H., Wilson, C., Rao, R., Leblanc, M., Coulter, S., He, M., Scott, C., Lau, S.L., Atkins, A.R., Barish, G.D., Gunton,

- J.E., Liddle, C., Downes, M. Evans, R.M. 2013. A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell*, **153**: 601-613.
- Dufour, J., and Clavien, P. (2010). *Signaling Pathways in Liver Diseases* (p. 425). Berlin Heidelberg: Springer-Verlag.
- Duque, G., Abdaimi, K.E., Hendersen, J.E., Lomri, A., Kremer, R. 2004. Vitamin D inhibits Fas ligand-induced apoptosis in human osteoblasts by regulating components of both the mitochondrial and Fas-related pathways. *Bone*, **35**: 57-64.
- Dusso, A.S., Brown, A.J., Slatopolsky, E. 2005. Vitamin D. *AJP-Renal*, **289**: 8-28.
- Ekwaru, J.P., Zwicker, J.D., Holick, M.F., Giovannucci, E., Veugelers, P.J. 2014. The importance of body weight for the dose response relationship of oral vitamin D supplementation and serum 25-Hydroxyvitamin D in healthy volunteers. *PLoS ONE*, 9(11): e111265.
- Eliades, M., Spyrou, E. 2015. Vitamin D: A new player in non-alcoholic fatty liver disease? *World J Gastroenterol*, **21**(6): 1718-1727.
- Eliades, M., Spyrou, E., Agrawal, N., Lazo, M., Brancati, F.L., Potter, J.J., Koteish, A.A., Clark, J.M., Guallar, E., Hernaez, R. 2013. Meta-analysis: Vitamin D and non-alcoholic fatty liver disease. *Ailment Pharmacol Ther*, **38**: 246-254.
- Evans, R.M., Mangelsdorf, D.J. 2014. Nuclear receptors, RXR, and the Big Bang. *Cell*, **157**: 255-266.
- Feldstein, A.E., Canbay, A., Angulo, P., Tanai, M., Burgart, L.J., Lindor, K.D., Gores, G.J. 2003. Hepatocyte Apoptosis and Fas Expression Are Prominent Features of Human Nonalcoholic steatohepatitis. *Gastroenterology*, **125**: 437-443.
- Feldstein, A.E., Canbay, A., Angulo, P., Tanai, M., Burgart, L.J., Lindor, K.D., Gores, G.J. 2003. Hepatocyte apoptosis and Fas expression are prominent features of human nonalcoholic steatohepatitis. *Gasteroenterology*, **125**: 437-443.
- Fontana, L., Eagon, J.C., Trujillo, M.E., Scherer, P.E., Klein, S. 2007. Visceral fat adipokine secretion is associated with systemic inflammation in obese humans. *Diabetes*, **56**: 1010–1013.

- Ford, E.S., Ajani, U.A., McGuire, L.C., Liu, S. 2005. Concentrations of serum vitamin D and the metabolic syndrome among U.S. adults, *Diabetes Care*, **28**(5): 1228–1230.
- Fried, SK., Bunkin, DA., Greenberg, AS. 1998. Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *J Clin Endocrinol Metab*; **83**: 847–850.
- Fu, G.K., Lin, D., Zhang, M.Y., Bikle, D.D., Shackleton, C.H., Miller, W.L., Portale, A.A. 1997. Cloning of human 25-hydroxyvitamin D-1 α -hydroxylase and mutations causing vitamin D-dependent rickets type 1. *Mol. Endocrinol*, **11**: 1961–1970.
- Fukushima, M., Suzuki, Y., Tohira, Y., Nishii, Y., Suzuki, M., Sasaki, S., Suda, T. 1976. 25-Hydroxylation of 1 α -hydroxyvitamin D3 in vivo and in the perfused rat liver. *FEBS Letters*, **65**: 211–214.
- Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima, M., Matsuda, M., Shimomura, L. 2004. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin. Invest.*, **114**:1752-1761.
- Gallagher, C.J., Yalamanchili, V., Smith, L.M. 2013. The effect of vitamin D supplementation on serum 25OHD in thin and obese women. *Journal of Steroid Biochemistry & Molecular Biology*, **136**: 195-200.
- Gilchrest, B.A., Eller, M.S., Geller, A.C., Yaar, M. 1999. The pathogenesis of melanoma induced by ultraviolet radiation. *New Engl. J. Med.*, 340(17): 1341-1348.
- Goeman, F., De Nicola, F., D'Onorio De Meo, P., Pallocca, M., Elmi, B., Castrignano, T., Pesole, G., Strano, S., Blandino, G., Fanciulli, M., Muti, P. 2014. VDR primary targets by genome-wide transcriptional profiling. *J Steroid Biochem Mol Biol.*, **143**: 348-356.
- Gomme, P.T., Bertolini, J. 2004. Therapeutic potential of vitamin D-binding protein. *TRENDS in Biotechnology*, **22**(7): 340-345.

- Gonzalez-Rey, E., Chorny, A., Delgado, M. 2006. Therapeutic action of ghrelin in a mouse model of colitis. *Gastroenterology*, **130**: 1707-1720.
- Goodwin, R.G., Lupton, S., Schmierer, A., Hjerrild, K.J., Jerzy, R., Clevenger, W., Gillis, S., Cosman, D., Namen, A.E. 1989. Human interleukin 7: molecular cloning and growth factor activity on human and murine B-lineage cells. *Proc Natl Acad Sci USA*, **86**(1): 302-306.
- Gregor, M.F., Hotamisligil, G.S. 2011. Inflammatory mechanisms in obesity. *Annu. Rev. Immunol.*, **29**: 415-445.
- Gregor, M.F., Hotamisligil, G.S. 2011. Inflammatory Mechanisms in Obesity. *Annual Review of Immunology*, **29**: 415-445.
- Gualillo, O., Lago, F., Dieguez, C. 2008. Introducing GOAT: a target for obesity and anti-diabetic drugs? *Trends in Pharmacological Sciences*, **29**(8): 398-401.
- Guana, C., Kiewiet, R.M., Janssen, J.A.M., van de Zande, B., Delhanty, P.J.D., Ghigo, E., Hofland, L.J., Themmen, A.P.N., van der Lely, A.J. 2007. Unacylated ghrelin acts as a potent insulin secretagogue in glucose-stimulated conditions. *Am J Physiol Endocrinol Metab.*, **293**: E697-E704.
- Guana, C., Meyler, F.M., Janssen, J.A.M., Delhanty, P.J.D., Abribat, T., van Koetsveld, P., Hofland, L.J., Broglio, F., Ghigo, E., van der Lely, A.J. 2004. Administration of Acylated Ghrelin Reduces Insulin Sensitivity, Whereas the Combination of Acylated Plus Unacylated Ghrelin Strongly Improves Insulin Sensitivity. *The J Clinical Endocrinology & Metabolism*, **89**(10): 5035-5042.
- Guerrero, R., Vega, G.L., Grundy, S.M., Browning, J.D. 2009. Ethnic differences in hepatic steatosis: An insulin resistance paradox? *Hepatology*, **49**: 791–801.
- Halder, R.M., Bridgeman-Shah, S. 1995. Skin cancer in African Americans. *Cancer*, **75**(S2): 667-673.
- Hamaguchi, M., Kojima, T., Takeda, N., et al. 2005. The Metabolic Syndrome as a Predictor of Nonalcoholic Fatty Liver Disease. *Annals of Internal Medicine*, **143**: 722-728.

- Hanley, A.J., Williams, K., Festa, A., et al. 2005. Liver markers and development of the metabolic syndrome. *Diabetes*, **54**: 3140-3147.
- Hardy, T., Anstee, Q.M., Day, C.P. 2015. Nonalcoholic fatty liver disease: new treatments. *Curr Opin Gastroenterol.*, **31**(3): 175-183.
- Harper, M.E., Dugaiczky, A. 1983. Linkage of the evolutionarily-related serum-albumin and alpha-fetoprotein genes within q11-22 of human chromosome-4. *Am. J. Human Genet.*, **35**: 565-572.
- Haussler, M.R., Whitfield, G.K., Haussler, C.A., Hsieh, J.C., and Jurutka, P.W. (2011). Nuclear Vitamin D Receptor: Natural Ligands, Molecular Structure-Function, and Transcriptional Control of Vital Genes. *Vitamin D*, David Feldman. (pp. 137-170) San Francisco: Elsevier, Inc.
- Havinga, E. 1973. Vitamin D, example and challenge. *Experientia*, **29**: 1181-1193.
- Heaney, R.P., Horst, R.L., Cullen, D.M., Armas, L.A. 2009. Vitamin D₃ distribution and status in the body. *J Am Coll Nutr.*, **28**(3): 252-256.
- Henao-Mejia, J., Elinav, E., Jin, C., Hao, L., Mehal, W.Z., Strowig, T., Thaiss, C.A., Kau, A.L., Eisenbarth, S.C., Jurczak, J.M., Camporez, J.P., Shulman, G.I., Gordon, J.I. 2012. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature*, **482**: 179-186.
- Henninger, A.M.J., Eliasson, B., Jenndahl, L.E., Hammarstedt, A. 2014. Adipocyte hypertrophy, inflammation and fibrosis characterize subcutaneous adipose tissue of healthy, non-obese subjects predisposed to type 2 diabetes. *PLoS ONE*, **9**(8).
- Holick, M.F., Binkley, N.C., Bischoff-Ferrari, H.A., Gordon, C.M., Hanley, D.A., Heaney, R.P., Murad, M.H., Weaver, C.M. 2011. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab*, **96**(7): 1911-1930.
- Holick, M.F., Binkley, N.C., Bischoff-Ferrari, H.A., Gordon, C.M., Hanley, D.A., Heaney, R.P., Murad, M.H., Weaver, C.M. 2011. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab*, **96**(7): 1911-1930.

- Holick, M.F., MacLaughlin, J.A., Doppelt, S.H. 1981. Regulation of cutaneous previtamin D₃ photosynthesis in man: Skin pigment is not an essential regulator. *Science*, **211**: 590-593.
- Holick, M.F., Tian, X.Q., Allen, M. 1995. Evolutionary importance for the membrane enhancement of the production of vitamin D₃ in the skin of poikilothermic animals. *Proc. Natl. Acad Sci*, **92**: 3124-3126.
- Holmstrom, K.M., Finkel, T. 2014. Cellular mechanisms and physiological consequences of redox-dependent signaling. *Nature Reviews Molecular Cell Biology*, **15**, 411-421.
- Hosseini-Nezhad, A., Spira, A., Holick, M.F. 2013. Influence of vitamin D status and vitamin D₃ supplementation on genome wide expression of white blood cells: A randomized double blind clinical trial. *PLoS one*, **8**(3): e58725.
- Hotamisligil, G.S., Shargill, N.S., Spiegelman, B.M. 1993. Adipose expression of tumor necrosis factor- α : direct role in obesity-linked insulin resistance. *Science*, **259**(5091): 87-91.
- Hou, Y., An, J., Hu, X.R., Sun, B.B., Lin, J., Xu, D., Wang, T., Wen, F.Q. 2009. Ghrelin inhibits interleukin-8 production induced by hydrogen peroxide in A549 cells via NF- κ B pathway. *International Immunopharmacology*, **9**(1):120-126.
- Huang, C.X., Yuan, M.J., Huang, H., Wu, G., Liu, Y., Yu, S.B., Li, H.T., Wang, T., 2009. Ghrelin inhibits post-infarct myocardial remodeling and improves cardiac function through anti-inflammation effect. *Peptides*, **30**: 2286–2291.
- Huhtakangas, J.A., Olivera, C.J., Bishop, J.E., Zanello, L.P., Norman, A.W. 2004. The vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1,25(OH)₂-vitamin D₃ in vivo and in vitro. *Mol Endocrinol*, **18**: 2660-2671.
- Iruzubieta, P., Teran, A., Crespo, J., Fabrega, E. 2014. Vitamin D deficiency in chronic liver disease. *World J of Hepatol*, **6**(12): 901-915.
- Ishikawa, T., Kodama, K., Matsumoto, J., Takayama, S. 1984. Photoprotective role of epidermal melanin granules against ultraviolet damage and DNA repair in guinea pig skin. *Cancer Res.*, **44**(11): 5195-5199.

- Ito, S., Wakamatsu, K. 2003. Quantitative Analysis of Eumelanin and Pheomelanin in Humans, Mice, and Other animals: a Comparative Review. *Pigment Cell Res.*, **16**: 523-531.
- Iwakura, H., Hosoda, K., Son, C., Fujikura, J., Tomita, T., Noguchi, M., Ariyansu, H., Kojima, M., Itoh, H., Toyokuni, S., Kangawa, K., Nakao, K. 2005. Analysis of rat insulin II promotor-ghrelin transgenic mice and rat glucagon promoter-ghrelin transgenic mice. *J Biol Chem*, **280**: 15247-15256.
- Jampel, H.D., Roche, N., Stark, W.J., Roberts, A.B. 1990. Transforming growth factor- β in human aqueous humor. *Current Eye Res.*, **9**: 963-969.
- Jankovic, A., Korac, A., Buzadzic, B., Otasevic, V., Stancic, A., Daiber, A., Korac, B. 2015. Redox implications in adipose tissue (dys)function-A new look at old acquaintances. *Redox Biology*, **6**: 19-32.
- Jehan, F., d'Alesio, A., Garabedian, M. 2007. Exons and functional regions of the human vitamin D receptor gene around and within the main 1a promoter are well conserved among mammals. *Journal of Steroid Biochemistry & Molecular Biology*, **103**: 361-367.
- Jia, L., Vianna, C., Fukuda, M., Berglund, E., Liu, C., Tao, C., Sun, K., Liu, T., Harper, M., Lee, C.E., Lee, S., Scherer, P.E., Elmquist, J.K. 2014. Hepatocyte toll-like receptor 4 regulates obesity-induced inflammation and insulin resistance. *Nat. Comm.*, **5** (3878): 1-25.
- Jones G. 2010. Metabolism and Catabolism of Vitamin D, Its Metabolites and Clinically Relevant analogs. In M. Holick (Ed.), *Nutrition and Health: Vitamin D Physiology, Molecular Biology, and Clinical Applications*. (pp.99-134). New York, NY: Humana Press, Inc.
- Jurutka, P.W., Hsieh, J.C., MacDonald, P.N., Terpening, C.M., Haussler, C.A., Haussler, M.R., Whitfield, G.K. 1993. Phosphorylation of serine 208 in the human vitamin D receptor: the predominant amino acid phosphorylated by casein kinase II, in vitro, and identification as a significant phosphorylation site in intact cells, *J. Biol. Chem*, **268**: 6791-6800.
- Jurutka, P.W., Hsieh, J.C., Nakajima, S., Haussler, C.A., Whitfield, G.K., Haussler, M.R. 1996. Human vitamin D receptor phosphorylation by casein kinase II at

ser-208 potentiates transcriptional activation. *Proc. Natl. Acad. Sci. USA*, **93**: 3519-3524.

Jurutka, P.W., Hsieh, J.C., Remus, L.S., Whitfield, G.K., Thompson, P.D., Haussler, C.A. et al. 1997. Mutations in the 1,25-dihydroxyvitamin D₃ receptor identifying C-terminal amino acids required for transcriptional activation that are functionally dissociated from hormone binding, heterodimeric DNA binding and interaction with basal transcription factor IIB, in vitro. *J. Biol. Chem.*, **272**: 14592-14599.

Kalyanaraman, B. 2013. Teaching the basics of redox biology to medical and graduate students: Oxidants, antioxidants and disease mechanisms. *Redox Biology*, **1**: 244-257.

Karlsson, T., Andersson, L., Hussain, A., Bosaeus, M., Jansson, N., Osmanovic, A., Hulthen, L., Holmang, A., Larsson, I. 2014. Lower vitamin D status in obese compared with normal weight women despite higher vitamin D intake in early pregnancy. *Clinical Nutrition*, **34**(5): 892-898.

Kasimay, O., Iseri, O.S., Barlas, A., Bangir, D., Yeg'en, C., Arbak, S., Yeg'en, B.C., 2006. Ghrelin ameliorates pancreaticobiliary inflammation and associated remote organ injury in rats. *Hepatol. Res.*, **36**: 11–19.

Kim, S., Kim, T. 2003. Benchmarks - Selection of optimal internal controls for gene expression profiling of liver disease. *Biotechniques*, **35**: 456-458.

Kimlin, M.G. 2008. Geographic location and vitamin D synthesis. *Molecular Aspects of Medicine*, **29**: 453-461.

Kitson, M.T., Roberts, S.K. 2012. D-Livering the message: The importance of vitamin D status in chronic liver disease. *Journal of Hepatology*, **57**: 897-909.

Koca, S.S., Ozgen, M., Aydin, S., et al. 2008. Ghrelin and obestatin levels in rheumatoid arthritis. *Inflammation*, **31**(5): 329–335.

Kojima, M., and Kangawa, K. (2013). Ghrelin discovery: A decade after. *Endocrine Development Vol 25*. (pp. 1-4) Basel: Karger.

- Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., Kangawa, K. 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*, **402** (6762): 656-660.
- Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., Kangawa, K. 1999. Ghrelin is a growth-hormone releasing acylated peptide from stomach. *Nature*. 402(6762): 656-660.
- Kukul, A. 2008. The structure of ghrelin. *Vitamins and Hormones*, **77**: 1-12.
- Kvam, E., Dahle, J. 2003. Pigmented Melanocytes Are Protected Against Ultraviolet-A-Induced Membrane Damage. *The J of Invest. Dermatology*, **121**: 564-569.
- LaBrecque, D., Abbas, Z., Anania, F., Ferenci, P., Ghafoor, K. A., Goh, K.I., Hamid, S.S., Isakov, V., Lizarzabal, M., Mojica Pernaranda, M., Rivera Ramos, J.F., Sarin, S., Stimac, D., Thomson, A.B., Umar, M., Krabshuis, J., LeMair, A. 2014. WGO Global Guidelines: Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *J Clin Gastroenterol*. **48**(6): 467-473.
- Lai, Y.H., & Fang, T.C. (2013). The pleiotropic effect of vitamin D. *ISRN nephrology*, 2013.
- Lear, P.V., Iglesias, M.J., Feijoo-Bandin, S., Rodriguez-Penas, D., Mosquera-Leal, A., Garcia-Rua, V., Gualillo, O., Ghe, C., Arnoletti, E., Muccioli, G., Dieguez, C., Gonzalez-Juanatey, J.R., Lago, F. 2010. Des-acyl ghrelin has specific binding sites and different metabolic effects from ghrelin in cardiomyocytes. *Endocrinology*, **151**(7): 3286-3298.
- Li, W.G., Gavrilu, D., Liu, X., Wang, L., Gunnlaugsson, S., Stoll, L.L., McCormick, M.L., Sigmund, C.D., Tang, C., Weintraub, N.L. 2004. Ghrelin inhibits proinflammatory responses and nuclear factor-kB activation in human endothelial cells. *Circulation*, **109**(18): 2221-2226.
- Li, Y., Hai, J., Li, L., Chen, X., Cao, M., Zhang, Q. 2013. Administration of ghrelin improves inflammation, oxidative stress, and apoptosis during and after non-alcoholic fatty liver disease development. *Endocrine*. **43**(2):376-386.
- Liangpunsakul, S., Chalasani, N. 2011. Serum vitamin D concentrations and unexplained elevation in ALT among US Adults. *Dig Dis Sci*, **56**(7): 2124-2129.

- Liangpunsakul, S., Chalasani, N. 2011. Serum vitamin D concentrations and unexplained elevation in ALT among US adults. *Dig Dis Sci.*, **56**(7): 2124-2129.
- Lim, C.T., Kola, B., Grossman, A., Korbonits, M. 2011. The expression of ghrelin O-acyl transferase (GOAT) in human tissues. *Endocrine Journal*, **58** (8): 707-710.
- Lipkie, T.E., Janasch, A., Cooper, B.R., Hohman, E.E., Weaver, C.M., Ferruzzi, M.G. 2013. Quantification of vitamin D and 25-hydroxyvitamin D in soft tissues by liquid chromatography-tandem mass spectrometry. *J Chromatogr B*, **932**: 6-11.
- Loomba, R., Sanyal, A.J. 2013. The Global NAFLD epidemic. *Nat. Rev. Gastroenterol. Hepatol*, **10**: 686-690.
- Loomis, F.W. 1967. Skin-pigment regulation of vitamin D biosynthesis in man, *Science*, **157**: 501-506.
- Ludwig, J., Viggiano, T.R., McGill, D.B., Oh, B.J. 1980. Nonalcoholic steatohepatitis: Mayo Clinic experience with a hitherto unnamed disease. *Mayo Clin Proc.*, **55**(7): 434-438.
- Lundgren, S., Carling, T., Hjalml, G., Juhlin, C., Rastad, J., Pihlgren, U., Rask, L., Akerstrom, G., Hellman, P. 1997. Tissue Distribution of Human gp330/Megalin, a Putative Ca²⁺ sensing Protein. *J Histochem Cytochem*, **45**: 383-392.
- MacDonald, P.N. and Dowd, D.R. (2011). The Molecular Biology of Biology of the Vitamin D Receptor. In M.F. Holick (Ed.), *Nutrition and Health: Vitamin D* (pp.135-152). Boston: Humana Press, Inc.
- MacLaughlin, J.A., Anderson, R.R., Holick, M.F. 1982. Spectral character of sunlight modulates photosynthesis of previtamin D₃ and its photoisomers in human skin. *Science*, **216**(4549): 1001-1003.
- Madhok, T.C., Deluca, H.F. 1979. Characteristics of the rat liver microsomal enzyme system converting cholecalciferol to 25-hydroxycholecalciferol. Evidence for participation of cytochrome P-450. *Biochem. J.*, **184**: 491–499.
- Mafra, D., Farage, N.E., Lobo, J.C., et al. 2011. Relationship between total ghrelin and inflammation in hemodialysis patients. *Peptides*, **32**(2): 358–361.

- Makki, K., Froguel, P., Wolowczuk, I. 2013. Adipose tissue in obesity-related inflammation and insulin resistance: Cells, cytokines, and chemokines. *ISRN Inflammation*, **2013**: Article ID 139239, 1-12.
- Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., Evans, R.M. 1995. The nuclear receptor superfamily: the second decade. *Cell*, **83**: 835-839.
- Marshall, R.O., Kooi, E.R. 1957. Enzymatic conversion of D-glucose to D-fructose. *Science*, **125**: 648–649.
- Martinon, F., Burns, K., Tschopp, J. 2002. The Inflammasome: A molecular platform triggering activation of inflammatory caspases and processing of proIL- β . *Molecular Cell*, **10**: 417-426.
- Martins, D.M., Wolf, M., Pan, D., Zadshir, A., Tareen, N., Thadhani, R., Felsenfeld, A., Levine, B., Mehrotra, R., Norris, K. 2007. Prevalence of cardiovascular risk factors and the serum levels of 25-hydroxyvitamin D in the United States: data from the Third National Health and Nutrition Examination Survey, *Archives of Internal Medicine*, **167**(11): 1159–1165.
- Massadi, O.A., Lear, P.V., Muller, T.D., Lopez, M., Dieguez Tschop, M.H., Nogueiras, R. 2014. Review of novel aspects of the regulation of ghrelin secretion. *Current Drug Metabolism*, **15**: 398-413.
- McCullough, A.J. 2004. The clinical features, diagnosis and natural history of nonalcoholic fatty liver disease. *Clin. Liver Dis.*, **8**: 521–533.
- McGuire, T.F., Trump, D.L., Johnson, C.S. 2001. Vitamin D₃-induced Apoptosis of Murine Squamous cell Carcinoma Cells. *The Journal of Biological Chemistry*, **276**(28): 26365-26373.
- Mckenna, N.J., Xu, J., Nawaz, Z., Tsai, S.Y., Tsai, M.J., O'Malley, B.W. 1999. Nuclear receptor coactivators: Multiple enzymes, multiple complexes, multiple functions. *J Steroid Biochem Mol Biol*, **69**: 3-12.
- McLeod, J.F., Cooke, N.E. 1989. The Vitamin D-binding protein, α -Fetoprotein, albumin multigene family: Detection of transcripts in multiple tissues. *The J Biological Chemistry*, **264**(36): 21760-21769.

- Medzhitov R. 2001. Toll-like receptors and innate immunity. *Nat. Rev.*, **1**: 135-145.
- Medzhitov, R. 2008. Origin and physiological roles of inflammation. *Nature*, **454**: 428-435.
- Mehal, W.Z. 2013. The Gordian Knot of dysbiosis, obesity and NAFLD. *Nat. Rev. Gastroenterol. Hepatol.*, **10**: 637-644.
- Mendel, C.M. 1989. The Free Hormone Hypothesis: A physiologically Based Mathematical Model. *Endocrine Reviews*, **10**(3): 232-274.
- Miegunu, P., Pierre, D.S., Broglio, F., Cianflone, K. 2011. Effects of desacyl ghrelin, obestatin and related peptides on triglyceride storage, metabolism and GHSR signaling in 3T3-L1 adipocytes. *Journal of Cellular Biochemistry*, **112**:704-714.
- Miroliaee, A., Nasiri-Toosi, M., Khalilzadeh, O., Esteghamati, A., Abdollahi, A., Mazloumi, M. 2010. Disturbances of parathyroid hormone-vitamin D axis in non-cholestatic chronic liver disease: a cross-sectional study. *Hepatol. Int.*, **4**: 634-640.
- Miura, K., Yang, L., van Rooijen, N., Brenner, D.A., Ohnishi, H., Seki, E. 2013. Toll-like receptor 2 and palmitic acid cooperatively contribute to the development of nonalcoholic steatohepatitis through inflammasome activation in mice. *Hepatology*, **57**(2): 577-589.
- Mizwicki, M.T., Norman, A. 2009. The vitamin D sterol vitamin D receptor ensemble model offers unique insights into both genomic and rapid response signaling. *Science Signaling*, **2**(75): 1-14.
- Moon, M., Song, H., Hong, H.J., Nam, D.W., Cha, M.Y., Oh, M.S., Yu, J., Ryu, H., Mook-Jung, I. 2013. Vitamin D binding protein interacts with A β and suppresses A β -mediated pathology. *Cell Death and Differentiation*, **20**: 630-638.
- Moreira, R.K. 2007. Hepatic stellate cells and liver fibrosis. *Arch Pathol Lab Med.*, **131**: 1728-1734.
- Muller, T.D., Nogueiras, R., Andermann, M.L., Andrews, Z.B., Anker, S.D., Argente, J., Batterham, R.L., Beniot, S.C., Bowers, C.Y., Broglio, F., Casanueva, F.F., D'Alessio, D., Depoortere, I., Geliebter Chigo, E., Cole, P.A., Cowley, M.,

- Cummings, D.E., Dagher, A., Diano, S., Dickson, S.L., Dieguez, C., Granata, R., Grill, H.J., Grove, K., Habegger, K.M., Heppner, K., Heiman, M.L., Holsen, L., Holst, B., Inui, A., Jansson, J.O., Kirchner, H., Korbonits, M., LaFerrere, B., LeRoux, C.W., Lopez, M., Morin, S., Nakazato, M., Nass, R., Perez-Tilve, D., Pfluger, P.T., Schwartz, T.W., Seeley, R.J., Sleeman, M., Sun, Y., Sussel, L., Tong, J., Thorner, M.O., van der Lely, A.J., van der Ploeg, L.H.T., Zigman, J.M., Kojima, M., Kangawa, K., Smith, R.G., Horvath, T., Tschop, M.H. 2015. Ghrelin. *Molecular Metabolism*, **4**: 437-460.
- Murphy, M.P. 2009. How mitochondria produce reactive oxygen species. *Biochem. J.*, **417**: 1-13.
- Nagpal, J., Pande, J.N., Bhartia, A. 2009. A double-blind, randomized, placebo-controlled trial of the short-term effect of vitamin D supplementation on insulin sensitivity in apparently healthy, middle-aged, centrally obese men. *Diabetic Medicine*, **26**(1): 19–27.
- Napetschnig, J., Wu, H. 2013. Molecular basis of NF- κ B signaling. *Annu. Rev. Biophys.*, **42**: 443-468.
- Navey-Many, T., Marx, R., Keshet, E., Pike, J.W., Silver, J. 1990. Regulation of 1,25-dihydroxyvitamin D₃ receptor gene expression by 1,25-dihydroxyvitamin D₃ in the parathyroid in vivo. *J. Clin. Invest.*, **86**: 1968-1975.
- Nemere, I., Yoshimoto, Y., Norman, A.W. 1984. Calcium transport in perfused duodena from normal chicks: enhancement within fourteen minutes of exposure to 1,25-dihydroxyvitamin D₃. *Endocrinology*, **115**(4): 1476-1483.
- Newton, K., Dixit, V.M. 2012. Signaling in innate immunity and inflammation. *Cold Spring Harb Perspect Biol*, **4**: 1-19.
- Nobili, V., Giorgio, V., Liccardo, D., Bedogni, G., Morino, G., Alisi, A., Cianfarani, S. 2014. Vitamin D levels and liver histological alterations in children with nonalcoholic fatty liver disease. *European Journal of Endocrinology*, **170**: 547-553.
- Nofsinger, J.B., Liu, Y., Simon, J.D. 2002. Aggregation of eumelanin mitigates photogeneration of reactive oxygen species. *Free Radic Biol. Med.*, **32**: 720-730.

- Norlin, M., von Bahr, S., Bjorkhem, I., Wikvall, K. 2003. On the substrate specificity of human CYP27A1: implications for bile acid and cholesterol formation. *J. Lipid Res.*, **44**: 1515-1522.
- Norman, A.W. 2008. From Vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. *Am. J Clin. Nutr.*, **88** (suppl): 491S-499S.
- Okazaki, T., Igarashi, T., Kronenberg, H.M. 1988. 5'-Flanking region of the parathyroid hormone gene mediates negative regulation by 1,25-(OH)₂ vitamin D₃. *J. Biol. Chem.*, **263**(5): 2203-2208.
- Olefsky, J.M., Glass, C.K. 2010. Macrophages, inflammation, and insulin resistance. *Annual Review of Physiology*, **72**: 219-246.
- Ouyang, X., Cirillo, P., Sautin, Y., McCall, S., Bruchette, J.L., Diehl, A.M., Johnson, R.J., Abdelmalek, M.F. 2008. Fructose consumption as a risk factor for non-alcoholic fatty liver disease. *J Hepatol.*, **48**(6): 993-999.
- Page, S., Chandhoke, V., Baranova, A. 2011. Melanin and melanogenesis in adipose tissue: possible mechanisms for abating oxidative stress and inflammation? *Obesity Reviews*, **12**(5): e21-e31.
- Palacios, C., Gonzalez, L. 2014. Is vitamin D deficiency a major global public health problem? *Journal of Steroid Biochemistry & Molecular Biology* **144**: 138-145.
- Phillips, C.M., Perry, I.J. 2013. Does Inflammation Determine Metabolic Health Status in Obese and Nonobese Adults? *J Clin Endocrinol Metab*, **98**(10): E1610-1619.
- Piccolo, B.D., Dolnikowski, G., Seyoum, E., Thomas, A.P., Gertz, E.R., Souza, E.C., Woodhouse, L.R., Newman, J.W., Keim, N.L., Adams, S.H., Van Loan, M.D. 2013. Association between subcutaneous white adipose tissue and serum 25-Hydroxyvitamin D in overweight and obese adults. *Nutrients*, **5**: 3352-3366.
- Pirola, C.J., Scian, R., Gianotti, T.F., Dopazo, H., Rohr, C., Martino, J.S., Castano, G.O., Sookoian, S. 2015. Epigenetic modifications in biology of nonalcoholic fatty liver disease. *Medicine*, **94**(36): 1-10.

- Priemel, M., von Domarus, C., Klatte, T.O., Kessler, S., Schlie, J., Meier, S., Proksch, N., Pastor, F., Netter, C., Streichert, T., Puschel, K., Amling, M. 2010. Bone mineralization defects and vitamin D deficiency: Histomorphometric analysis of iliac crest bone biopsies and circulating 25-hydroxyvitamin D in 675 patients. *J Bone Miner Res*, **25**: 305-312.
- Primeau, V., Coderre, L., Karelis, A.D., Brochu, M., Lavoie, M.E., Messier, V., Sladek, R., Rabasa-Lhoret, R. 2011. *Int J Obes (Lond)*, **35**(7): 971-981.
- Qader, S.S., Hakanson, R., Rehfeld, J.F., Lundquist, I., Salehi, A. 2008. Proghrelin-derived peptides influence the secretion of insulin, glucagon, pancreatic polypeptide and somatostatin: A study on isolated islets from mouse and rat pancreas. *Regulatory Peptides*, **146**: 230-237.
- Rafiq, N., Bai, C., Fang, Y., Srishord, M., McCullough, A., Gramlich, T., Younossi, Z.M. 2009. Long-term follow-up of patients with nonalcoholic fatty liver disease. *Clin Gastroenterol Hepatol.*, **7**(2):234-238.
- Randhawa, M., Huff, T., Valencia, J.C., Younossi, Z., Chandhoke, V., Hearing, V.J., Baranova, A. 2009. Evidence for the ectopic synthesis of melanin in human adipose tissue. *FASEB J.*, **23**: 835-843.
- Raposo, G., Marks, M.S. 2007. Melanosomes-dark organelles enlighten endosomal membrane transport. *Nature Reviews Molecular Cell Biology*, **8**: 786-797.
- Ray, K. 2012. Married to our gut microbiota. *Nat. Rev. Gastroenterol. Hepatol.*, **9**: 555.
- Reese, P.P., Bloom, R.D., Feldman, H.I., Huverserian, A., Thomasson, A., Shults, K., Hamano, T., Goral, S., Shaked, A., Olthoff, K., Rickels, M.R., Bleicher, M., Leonard, M.B. 2012. Changes in vitamin D binding protein and vitamin D concentrations associated with liver transplantation. *Liver Int.*, **32**(2): 287-296.
- Reis, J.P., von Mühlen, D., Miller, III, E.R. 2008. Relation of 25-hydroxyvitamin D and parathyroid hormone levels with metabolic syndrome among US adults. *European Journal of Endocrinology*, **159**(1): 41–48.
- Rivera, C.A., Adegboyega, P., van Rooijen, N., Tagalicud, A., Allman, M., Wallace, M. 2007. Toll-like receptor 4 signaling and Kupffer cells play pivotal roles in the

- pathogenesis of non-alcoholic steatohepatitis. *Journal of Hepatology*, **47**: 571-579.
- Rockey, D.C., Caldwell, S.H., Goodman, Z.D., Nelson, R.C., Smith, A.D. 2009 Liver Biopsy. *Hepatology*, **49**(3): 1017-1044.
- Russell, J., Silver, J., Sherwood, L.M. 1984. The effects of calcium and vitamin D metabolites on cytoplasmic mRNA coding for parathyroid hormone in isolated parathyroid cells. *Trans. Assoc. Am. Physicians*, **97**: 296-303.
- Sabeti, M., Woods, N.B., de Luca, C., Schenk, S., Lu, J.C., et al. 2009. Hematopoietic cell-specific deletion of Toll-like receptor 4 ameliorates hepatic and adipose tissue insulin resistance in high-fat-fed mice. *Cell Metab.*, **10**:419–429.
- Samuel, S., Sitrin, MD. 2008. Vitamin D's role in cell proliferation and differentiation. *Nutr Rev.*, 66(10 Suppl 2):S116-S1124.
- Sarna, T. 1992. Properties and function of the ocular melanin-a photobiophysical view. *J Photochem Photobiol.*, **12**: 215-258.
- Schenk, S., Sabeti, M., Olefsky, J.M. 2008. Insulin sensitivity: modulation by nutrients and inflammation. *J. Clin. Investig*, **118**: 2992-3002.
- Serrano-Mendioroz, I., Samperdo, A., Mora, M.I., Mauleon, I., Segura, V., Enriquez de Salamanca, R., Harper, P., Sardh, E., Corrales, F.J., Fontanellas, A. 2015. Vitamin D-binding protein as a biomarker of active disease in acute intermittent porphyria. *J Proteomics*, **127**(Pt B):377-85.
- Sharifi, N., Amani, R., Hajiani, E., Cheraghian, B. 2014. Does vitamin D improve liver enzymes, oxidative stress, and inflammatory biomarkers in adults with non-alcoholic fatty liver disease? A randomized clinical trial. *Endocrine*, **47**: 70-80.
- Shi, H., Kokoeva, M.V., Inouye, K., Tzameli, I., Yin, H., Flier, J.S. 2006. TLR4 links innate immunity and fatty acid induced insulin resistance. *J. Clin. Invest.*, **116**: 3015–3025.
- Shi, Y. 2004. Beyond skin color: emerging roles of melanin-concentrating hormone in energy homeostasis and other physiological functions. *Peptides*, **25**(10): 1605-1611.

- Shinki, T., Shimada, H., Wakino, S., Anazawa, H., Hayashi, M., Saruta, T., De-Luca, H.F., Suda, T. 1997. Cloning and expression of rat 25-hydroxyvitamin D3-1alpha-hydroxylase cDNA. *Proc. Natl. Acad. Sci. USA*, **94**: 12920–12925.
- Shoelson, S., Herrero, L., Naaz, A. 2007. Obesity, inflammation, and insulin resistance. *Gastroenterology*, **132**: 2169-2180.
- Silver, J., Naveh-Many, T. 2011. Vitamin D and Parathyroid. *Vitamin D*, Third Edition. DOI: 10.1016/B978-0-12-381978-9.10027-7.
- Silver, J., Russell, J., Sherwood, L.M. 1985. Regulation of calcium and vitamin D metabolites of messenger ribonucleic acid for preproparathyroid hormone in isolated bovine parathyroid cells. *Proc. Natl. Acad. Sci. USA*, **82**: 4270-4273.
- Sims, E.A. (1982). Characterization of the syndromes of obesity. In: B. Brodoff & S. Bleicher (Eds.), *Diabetes Mellitus and Obesity*. (pp.219-226).: Baltimore: Williams & Wilkins.
- Singh, A., Bastian, T.S., Denny, C.E., Varghese, V.I. 2010. Apoptosis-A Review. *Oral & Maxillofacial Pathology Journal (OMPJ)*, **1**(2): ISSN 0976-1225.
- Skurk, T., Alberti-Huber, C., Herder, C., Hauner, H. 2007. Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab*, **92**: 1023-1033.
- Song, Y.H., Ray, K., Liebhaber, S.A., Cooke, N.E., 1998. Vitamin D-binding protein gene transcription is regulated by the relative abundance of hepatocyte nuclear factors 1alpha and 1beta. *J Biol Chem*, **273**(43): 28408–28418.
- Soriguer, F., Gutierrez-Repiso, C., Rubio-Martin, E., Garcia-Fuentes, E., Almaraz, M.C., Colomo, N., de Antonio, I.E., de Adana, M.S.R., Chavez, F.J., Morcillo, S., Valdes, S., Rojo-Martinez, G. 2013. Metabolically Healthy but Obese, a Matter of Time? Findings From the Prospective Pizarra Study. *J Clin Endocrinol Metab*, **98**: 2318-2325.
- St-Arnaud, R., Arabian, A., Travers, R., Barletta, F., Raval-Pandya, M., Chapin, K., Depovere, J., Mathieu, C., Christakos, S., Demay, M.B., Glorieux, F.H. 2000. Deficient mineralization of intramembranous bone in vitamin D-24-hydroxylase-

ablated mice is due to elevated 1,25-dihydroxyvitamin D and not to the absence of 24, 25-dihydroxyvitamin D. *Endocrinology*, **141**: 2658–2666.

- Stanhope, K.L., Schwarz, J.M., Keim, N.L., Griffen, S.C., Bremer, A.A., Graham, J.L., Hatcher, B., Cox, C.L., Dyachenko, A., Zhang, W., McGaham, J.P., Seibert, A., Krauss, R.M., Chiu, S., Schaefer, E.J., Ai, M., Otokozawa, S., Nakajima, K., Nakano, T., Beysen, C., Hellerstein, M.K., Berglund, L., Havel, P.J. 2009. Consuming fructose-sweetened, not glucose-sweetened, beverages increases visceral adiposity and lipids and decreases insulin sensitivity in overweight/obese humans. *J. Clin. Invest.*, **119**: 1322-1334.
- Stengel, A., Goebel, M., Wang, L., et al. 2010. Lipopolysaccharide differentially decreases plasma acyl and desacyl ghrelin levels in rats: potential role of the circulating ghrelin-acylating enzyme GOAT. *Peptides*, **31**(9): 1689–1696.
- Stepanova, M., Aquino, R., Alsheddi, A., Gupta, R., Fang, Y., Younossi, Z. 2010. Clinical predictors of fibrosis in patients with chronic liver disease. *Ailment Pharmacol Ther*, **31**(10): 1085-1094.
- Stokes, C.S., Volmer, D.A., Grunhage, F., Lammert, F. 2013. Vitamin D in chronic liver disease. *Liver International*, **33**: 338-352.
- Strohacker, K., McFarlin, B.K. 2010. Influence of obesity, physical inactivity, and weight cycling on chronic inflammation. *Front. Biosci (Elite Ed)*, **2**: 98–104.
- Suda, T., DeLuca, H.F., Schnoes, H.K., Blunt, J.W. 1969. Isolation and identification of 25-hydroxyergocalciferol. *Biochemistry*, **8**: 3515-3520.
- Sun, J., Xu, Y., Dai, Z., et al. 2009. Intermittent high glucose stimulate MCP-I, IL-18, and PAI-1, but inhibit adiponectin expression and secretion in adipocytes dependent of ROS. *Cell Biochem Biophys*, **55**(3): 173–180.
- Sung, E.Z., Da Silva, N.F., Goodyear, S.J., et al. 2011. Ghrelin promotes nuclear factor kappa-B activation in a human B-lymphocyte cell line. *Mol Biol Rep*, **38**(8):4833-4838.
- Svasti, J., Kurosky, A., Bennett, A., Bowman, B.H. 1979. Molecular basis for the three major forms of human serum vitamin D binding protein (group specific component). *Biochemistry*, **18**(8): 1611–1617.

- Szabo, G., Csak, T. 2012. Inflammasomes in liver diseases. *Journal of Hepatology*, **57**: 642-654.
- Szpirer, J., Szpirer, C., Riviere, M. et al. 1991. The Sp1 transcription factor gene (*SP1*) and the 1,25-dihydroxyvitamin D₃ receptor gene (*VDR*) are colocalized on human chromosome arm 12q and rat chromosome 7. *Genomics*, **11**:168–173.
- Tai, C.C., Lu, Z., Holick, M.F. 2010. Photobiology of Vitamin D. In M. Holick (Ed.), *Nutrition and Health: Vitamin D Physiology, Molecular Biology, and Clinical Applications*. (pp.35-60). New York, NY: Humana Press, Inc.
- Takahashi, Y., Sugimoto, K., Inui, H., Fukusato, T. 2015. Current pharmacological therapies for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World J Gastroenterol.*, **21**(13): 3777-3785.
- Takeuchi, O., Akira, S. 2010. Pattern recognition receptors and inflammation. *Cell*, **140**: 805-820.
- Takeyama, K., Kitanaka, S., Sato, T., Kobori, M., Yanagisawa, J., and Kato, S. 1997. 25-Hydroxyvitamin D₃ 1 α -hydroxylase and vitamin D synthesis. *Science*, **277**: 1827–1830.
- Tappy, L., Le, K.A. 2010. Metabolic effects of fructose and the worldwide increases in obesity. *Physiol Rev*, **90**: 23-46.
- Targher, G., Bertolini, L., Scala, L., Cigolini, M., Zenari L, Falezza G and Acaro, G. 2007. Associations between serum 25-hydroxyvitamin D₃ concentrations and liver histology in patients with non-alcoholic fatty liver disease. *Nutrition, Metabolism & Cardiovascular Diseases*, **17**: 517-524.
- Tateya, S., Tamori, Y., Kawaguchi, T., et al. 2010. An increase in the circulating concentration of monocyte chemoattractant protein-1 elicits systemic insulin resistance irrespective of adipose tissue inflammation in mice. *Endocrinology*, **151**(3): 971–979.
- Tian, X.Q., Chen, T.C., Matsuoka, L.Y., Wortsman, J., Holick. M.F. 1993. Kinetic and thermodynamic studies of the conversion of previtamin D₃ to vitamin D₃ in human skin. *J Biol Chem*, **268**: 14888-14892.

- Tian, X.Q., Holick, M.F. 1995. Catalyzed Thermal Isomerization between Previtamin D₃ and Vitamin D₃ via β -Cyclodextrin Complexation. *The Journal of Biological Chemistry*, **270** (15): 8706-8711.
- Tilg, H. 2010. The role of cytokines in non-alcoholic fatty liver disease. *Dig. Dis.*, **28**:179-185.
- Tilg, H., Kaser, A. 2005. Treatment strategies in nonalcoholic fatty liver disease. *Nature Clinical Practice Gastroenterology and Hepatology*. **2**(3): 148-155.
- Tilg, H., Moschen, A.R. 2010. Evolution of inflammation in nonalcoholic fatty liver disease: The multiple parallel hits hypothesis. *Hepatology*, **52**(5): 1836-1846.
- Tiniakos, D.G., Vos, M.B., Brunt, E.M. 2010. Nonalcoholic fatty liver disease: Pathology and pathogenesis. *Annu. Rev. Pathol. Mech. Dis.*, **5**:145-171.
- Tschop, M., Smiley, D.L., Heiman, M.L. 2000. Ghrelin induces adiposity in rodents. *Nature*, **407**(6806): 908-913.
- Tuoresmaki, P., Vaisanen, S., Neme, A., Heikkinen, S., Carlberg, C. 2014. Patterns of Genome-Wide VDR Locations. *PLOS 1*, **9**(4): 1-11.
- Valenzuela, R., Videla, L.A. 2011. The importance of the long-chain polyunsaturated fatty acids n-6/n-3 ratio in development of non-alcoholic fatty liver associated with obesity. *Food Funct.*, **2**: 644-648.
- Vanlint, S. 2013. Vitamin D and obesity. *Nutrients*, **5**: 949-956.
- Vernon, G., Baranova, A., Younossi, Z.M. 2011. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment Pharmacol Ther*, **34**: 274-285.
- Vimaleswaran, K.S., Berry, D.J., Lu, C., Tikkanen, E., Pilz, S., Hiraki, L.T., Cooper, J.D., Dastani, Z., Li, R., Houston, D.K., Wood, A.R., Michaelsson, K., Vandenput, L., Zgaga, L., Yerges-Armstrong, L.M., McCarthy, M.I., Dupuis, J., Kaakinen, M., Kleber, M.E., Jameson, K., Arden, N., Raitakari, O., Viikari, J., Lohman, K.K., Ferrucci, L., Melhus, H., Ingelsson, E., Byberg, L., Lind, L., Lorentzon, M., Salomaa, V., Campbell, H., Dunlop, M., Mitchell, B.D., Herzig, K.H., Pouta, A., Hartikainen, L., the Genetic Investigation of Anthropometric

- Traits (GIANT) consortium, Streeten, E.A., Theodoratou, E., Jula, A., Wareham, N.J., Ohlsson, C., Frayling, T.M., Kritchevsky, S.B., Spector, T.D., Richards, J.B., Lehtimäki, T., Ouwehand, W.H., Kraft, P., Cooper, C., März, W., Power, C., Loos, R.J.F., Wang, T.J., Jarvelin, M.R., Whittaker, J.C., Hingorani, A.D., Hyppönen, E. 2013. Causal relationship between obesity and vitamin D status: Bi-Directional Mendelian randomization analysis of multiple cohorts. *PLoS Med*, **10**(2): e1001383.
- von Hurst, P.R., Stonehouse, W., Coad, J. 2010. Vitamin D supplementation reduces insulin resistance in South Asian women living in New Zealand who are insulin resistant and vitamin D deficient—a randomised, placebo-controlled trial. *British Journal of Nutrition*, 103(4): 549–555.
- Waki, H., Tontonoz, P. 2007. Endocrine Functions of Adipose Tissue. *Annu. Rev. Pathol. Mech. Dis.*, **2**: 31-56.
- Wang, B., Wood, I.S., Trayhurn, P. 2007. Dysregulation of the expression and secretion of inflammation-related adipokine by hypoxia in human adipocytes. *Pflügers Arch*, **455**: 479-492.
- Wang, P., Mariman, E., Renes, J., Keijer, J. 2008. The secretory function of adipocytes in the physiology of white adipose tissue. *J. Cell Physiol*, **216**: 3-13.
- Wang, X., Patel, R., Studzinski, G.P. 2008. hKSR-2, a vitamin D-regulated gene, inhibits apoptosis in arabinocytosine-treated HL60 leukemia cells. *Mol. Cancer Ther.*, **7**(9): 2798-2806.
- Waseem, T., Duxbury, M., Ito, H., Ashley, S.W., Robinson, M.K. 2008. Exogenous ghrelin modulates release of pro-inflammatory and anti-inflammatory cytokines in LPS-stimulated macrophages through distinct signaling pathways. *Surgery*, **143**: 334-342.
- Weston, S.R., Leyden, W., Murphy, R., Bass, N.M., Bell, B.P., et al. 2005. Racial and ethnic distribution of nonalcoholic fatty liver in persons with newly diagnosed chronic liver disease. *Hepatology*, **41**:372–379.
- White, P., Cooke, N. 2000. The multifunctional properties and characteristics of vitamin D-binding protein. *TEM*, **11**(8): 320-327.

- White, P., Cooke, N. 2000. The Multifunctional Properties and Characteristics of Vitamin D binding Protein. *Trends in Endocrinology & Metabolism*, **11**(8): 320-327.
- Wieckowska, A., Zein, N.N., Yerian, L.M., Lopez, R., McCullough, A.J., Feldstein, A.E. 2006. *In Vivo* assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. *Hepatology*, **44**(1): 27-33.
- Wolfs, M.G.M., Gruben, N., Rensen, S.S., Verdam, F.J., Greve, J.W., Driessen, A., Wijmenga, C., Buurman, W.A., Franke, L., Scheja, L., Koonen, D.P.Y., Shiri-Sverdlov, R., van Haeften, T.W., Hofker, M.H., Fu, J. 2015. Determining the association between adipokines expression in multiple tissues and phenotypic features on non-alcoholic fatty liver disease in obesity. *Nutrition & Diabetes*, **5**(2): e146.
- Worstman, J., Matsuoko, L.Y., Chen, T.C., Lu, Z., Holick, M.F. 2000. Decreased bioavailability of vitamin D in obesity. *Am J Clin Nutr*, **72**: 690-693.
- Wortsman, J., Matsuoka, L.Y., Chen, T.C., Lu, Z., Holick, M.F. 2000. Decreased bioavailability of vitamin D in obesity. *Am J Clin Nutr.*, **72**(3): 690-693.
- Wree, A., Broderick, L., Canbay, A., Hoffman, H.M., Feldstein, A.E. 2013. From NAFLD to NASH to cirrhosis-new insights into disease mechanisms. *Nat. Rev. Gastroenterol. Hepatol.*, **10**: 627-636.
- Wu, R., Dong, W., Ji, Y., et al. 2008. Orexigenic hormone ghrelin attenuates local and remote organ injury after intestinal ischemia–reperfusion. *PLoS One*, **3**(4): e2026.
- Wuest, S., Yang, X., Liu, J., Schoenle, E.J., Konrad, D. 2012. Inverse regulation of basal lipolysis in perigonadal and mesenteric fat depots in mice. *Am J Physiol Endocrinol Metab* **302**: E153–E160.
- Yamada, G., Ariyasu, H., Iwakura, H., Hosoda, H., Akamizu, T., Nakao, K., Kangawa, K. 2010. Generation of transgenic mice overexpressing a ghrelin analog. *Endocrinology*, **151**: 5935-5940.

- Yamanaka, O., Saika, S., Ikeda, K., Miyazaki, K., Ohnishi, Y., Ooshima, A. 2006. Interleukin-7 modulates extracellular matrix production and TGF- β signaling in cultured human subconjunctival fibroblast. *Current Eye Research*, **31**: 491-499.
- Yang, J., Brown, M.S., Liang, G., Grishin, N.V., Goldstien, J.L. 2008. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell*, **132**: 387-396.
- Yang, J., Zhao, T.J., Goldstein, J.L., Brown, M.S. 2008. Inhibition of ghrelin O-acyltransferase (GOAT) by octanoylated pentapeptides. *Proc Natl Acad Sci USA*, **105**(31): 10750-10755.
- Yao, S., Ambrosone, C.B., 2013. Associations between vitamin D deficiency and risk of aggressive breast cancer in African-American women. *J Steroid Biochem Mol Biol*. 136:337-341.
- Yin, M., Gabele, E., Wheeler, M.D., Conner, H., Bradford, B.U., Dikalova, A., Rusyn, I., Mason, R., Thruman, R.G. 2001. Alcohol-induced free radicals in mice: direct toxicants or signaling molecules? *Hepatology*, **34**: 935-942.
- Yoon, J.H., Gores, G.J. 2002. Death receptor-mediated apoptosis and the liver. *J of Hepatol*, **37**: 400-410.
- Young, K.A., Engelman, C.D., Langefeld, C.D., Hairston, K.G., Haffner, S.M., Bryer-Ash, M., Norris, J.M. 2009. Association of Plasma Vitamin D Levels with Adiposity in Hispanic and African Americans. *J. Clin. Endocrinol. Metab.*, **94**(9): 3306-3313.
- Younossi, Z.M., Jarrar, M., Nugent, C., Randhawa, M., Afendy, M., Stepanova, M., Rafiq, N., Goodman, Z., Chandhoke, V., Baranova, A. 2008. A novel diagnostic biomarker panel for obesity-related Nonalcoholic Steatohepatitis (NASH). *Obes. Surg.*, **18**:1430-1437.
- Yousefzadeh, P., Shapses, S.A., Wang, X. 2014. Vitamin D Binding Protein Impact on 25-hydroxyvitamin D Levels under Different Physiologic and Pathologic Conditions. *International Journal of Endocrinology*, **2014**:981581.

- Zhang, A., Wang, Y., Xie, H., Zheng, S. 2007. Calcitriol inhibits hepatocyte apoptosis in rat allograft by regulating apoptosis-associated genes. *International Immunopharmacology*, **7**: 1122-1128.
- Zhang, C., Baudino, T.A., Dowd, D.R., Tokumaru, H., Wang, W., MacDonald, P.N. 2001. Ternary complexes and cooperative interplay between NcoA-62/Ski-interacting protein and steroid receptor coactivators in vitamin D receptor-mediated transcription. *J Biol Chem.*, **276**: 40614-40620.
- Zhang, C., Dowd, D.R., Staal, A. Gu, C., Lian, J.B., van Wijnen, A.J. et al. 2003. Nuclear coactivator-62 kDa/Ski-interacting protein is a nuclear matrix-associated coactivator that may couple vitamin D receptor-mediated transcription and RNA splicing. *J. Biol. Chem.*, **278**: 35325-35336.
- Zhang, L., Keane, M.P., Zhu, L.X., Sharma, S., Rozengurt, E., Strieter, R.M., Dubinett, S.M., Huang, M. 2004. Interleukin-7 and transforming growth factor- β play counter regulatory roles in protein kinase C- δ -dependent control of fibroblast collagen synthesis in pulmonary fibrosis. *J Biol Chem.*, **279**(27): 28315-28319.
- Zhang, Q., Huang, W., Lv, X., Yang, Y. 2011. Ghrelin protects H9c2 cells from hydrogen peroxide-induced apoptosis through NF- κ B and mitochondria-mediated signaling. *European Journal of Pharmacology*, **654**: 142-149.
- Zhang, S.R., Fan, X.M. 2015. Ghrelin-ghrelin O-acyltransferase system in the pathogenesis of nonalcoholic fatty liver disease. *World J Gastroenterol*, **21**(11): 3214-3222.
- Zhang, W., Chai, B., Li, J.Y., Wang, H., Mulholland, M.W. 2008. Effect of des-acyl ghrelin on adiposity and glucose metabolism. *Endocrinology*, **149**(9): 4710-4716.

BIOGRAPHY

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