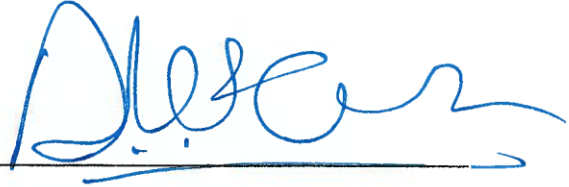




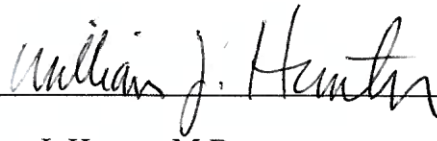
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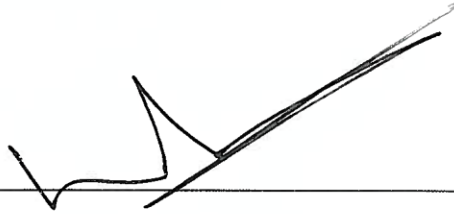
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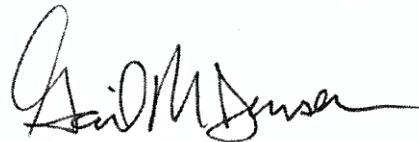
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**PKC- $\delta$  and INSULIN RESISTANCE in NON-ALCOHOLIC FATTY LIVER  
DISEASE: EFFECT of VITAMIN D**

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By

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A THESIS

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## ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) due to excess ectopic fat deposit in the liver of nonalcoholic is a broad spectrum of liver diseases encompassing simple liver steatosis to more complicated and morbid steatohepatitis (NASH) that can lead to cirrhosis and even to hepatocellular carcinoma. Its prevalence in the Western World is about 30%. Insulin resistance dominates the pathogenesis of NAFLD and its complications. Serine phosphorylation of insulin receptor substrate (IRS) has been one of the most common pathways in the establishment of insulin resistance (IR). Novel Protein kinase C (nPKC), is solely activated by diacylglycerol (DAG). Diacylglycerol has been found to be increased in the liver during NAFLD. Several studies have demonstrated a link between vitamin D deficiency, high fructose diet, and NAFLD in human and rodents. The specific aim of this study is to investigate and characterize the isotype of nPKCs involved in the inhibition of IRS-1 pathway using a swine model and to evaluate the effect of vitamin D status on the phenomenon. The Yucatan micro-swine were divided into four experimental groups based on the diet: (i) high cholesterol fed vitamin D-deficient (DEF), (ii) high cholesterol fed vitamin D-sufficient (SUF), high cholesterol fed and vitamin D-supplemented (SUP), and (iv) high cholesterol and high fructose-fed and vitamin D-sufficient (HCHF). We found that DEF and HCHF swine developed IR and presented histological features of NAFLD. HCHF swine were prone to liver fibrosis. PKC- $\delta$  was the most relevant novel PKC associated with the pathogenesis of IR and NAFLD. PKC- $\delta$  was up-regulated in the liver of both DEF and HCHF swine. PKC- $\delta$  was associated with the degradation of IRS-1

in the liver of the DEF swine but not in the liver of the SUF or SUP swine. Even though there were an IR and up-regulation of PKC- $\delta$  in the liver of HCHF swine, it was neither associated with the IRS-1 degradation nor with the co-localization of the PKC- $\delta$  and p-IRS-1.

These findings suggest that the high cholesterol diet alone does not cause IR or NAFLD in swine; it is either associated with high fructose or with vitamin D deficiency. The combination of high cholesterol and high fructose diet induces IR, NAFLD, and NASH in vitamin D-sufficient swine but not through the degradation of IRS-1.

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## LIST OF ABBREVIATIONS

ACS	Acyl CoA synthetase
AFL	Alcoholic fatty liver

AFLD	Alcoholic fatty liver disease
Akt-1	Alpha serine/threonine-protein kinase
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
CAP	Catabolite Activator Protein
CO <sub>2</sub>	Carbon dioxide
CoA	Coenzyme-A
CPT-1	Carnitine palmitoyltransferase I
CT-Scan	Computerized tomography scanning
DAG	Diacylglycerol
DEF	Vitamin-D deficient and high cholesterol fed swine
dL	Deciliter
ECM	Extracellular Matrix
ER	Endoplasmic reticulum
FA	Fatty acid

FATP/FAT	Fatty Acid transporter protein
FFA	Free fatty acid
Fruc	Fructose
G	Gram
GGT	Gamma-glutamyl transferase
Glu	Glucose
GLUT	Glucose transporter
GS	Glycogen synthase
GSK3	Glycogen synthase kinase-3
HCHF	Vitamin-D sufficient and high cholesterol and high fructose fed swine
HOMA-IR	Homeostasis Assessment -insulin resistance
IKK	Inhibitor Kappa Beta kinase
IR	Insulin resistance
IRS-1	Insulin receptor substrate-1
JAK	Janus kinase
JNK	Jun N-terminal Kinase
Kcal	Kilocalories

kD	Kilo Dalton
Kg	Kilograms
LD	Lipid droplet
MAG	Monoacyl glycerol
MetS	Metabolic syndrome
MGL	Monoacyl glycerol lipase
mL	milliliter
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic Steatohepatitis
nPKC	Novel Protein Kinase C
p-	Phosphorylated
PDFF	Proton density far fraction
PKC- $\alpha$ , $\beta$ , $\gamma$ , $\delta$ , $\epsilon$ , $\zeta$ , $\eta$ , $\theta$ , $\iota$ , $\lambda$	PKC, alpha, beta, gamma, delta, epsilon, zeta, eta, theta, iota, lambda
PLC	Phospholipase C
PNPLA3	Patatin-like phospholipase domain-containing

protein 3

PPAR	peroxisome proliferator-activated receptors
RER	Rough Endoplasmic Reticulum
ROS	Reactive oxygen species
SER	Smooth endoplasmic reticulum
SOCS	Suppressor of cytokine signaling
SREBP-c	Sterol regulatory element-binding protein cleavage-activating protein
STAT-3	Signal transducer and activator of transcription 3
SUF	Vitamin-D sufficient and high cholesterol fed swine
SUP	Vitamin-D supplemented and high cholesterol fed swine
T2DM	Type-2 diabetes mellitus
TAG	Triacylglycerol
TZD	Thiazolidinedione
US	Ultrasound
VDR	Vitamin D (1,25- Dihydroxyvitamin D3)

Receptor

Vit D

Vitamin D

## **1. Introduction**

The chronic fat buildup in human liver of individuals, who do not drink alcohol excessively, has lately shifted from mere and benign fatty liver to more complex liver disease listed under a new nosological term of Non-alcoholic Fatty Liver Disease (NAFLD) (1, 2). NAFLD represents a spectrum of liver diseases ranging from the simple fatty liver, now termed Nonalcoholic fatty liver (NFL) (3-5), asymptomatic and benign, to steatohepatitis also called nonalcoholic steatohepatitis (6), which carries a considerable potential to progress to liver fibrosis, cirrhosis and even to hepatocellular carcinoma (7). The prevalence of NAFLD is 20-30 % worldwide (8), higher in the western world, even expected to reach 50% by 2030 (9). The high-calorie diet and increased sedentary lifestyle in the population are major contributing factors in the pathogenesis of obesity, diabetes and NAFLD (10). NASH, now, surpasses Hepatitis C as the most common indication for liver transplantation (11, 12). NAFLD has become a major public health concern in the USA for the considerable toll it exacts on liver-related morbidity and mortality; its association with steadily growing prevalence of obesity and diabetes, and its complications to deadly liver disease. Despite the growing interest in the disease, NAFLD is at emerging stage of knowledge regarding its pathogenesis, clinical manifestations, management, and prognosis. Due to its strong association with components of metabolic syndrome (MS) (13, 14), NAFLD is considered a hepatic manifestation of MS. Insulin resistance (IR) is the hallmark of the pathogenesis of the disease (13, 15, 16). The systemic IR is found to be associated with NAFLD in more than 90% of cases. Hepatic insulin resistance is always present in NASH (17).

Studies are yet to determine the precise mechanism by which the hepatic IR occurs and how it causes the accumulation of fat in hepatocytes. The mechanisms of the progression from fatty liver to NASH still need to be elucidated. Notably, the absence of reliable biomarkers for the early diagnosis to apply adequate prevention and early treatment remains elusive. To my knowledge, there is no proper treatment protocol for NAFLD (18). The management of NAFLD utilizes therapeutics of other related diseases especially that of the MS (19-22).

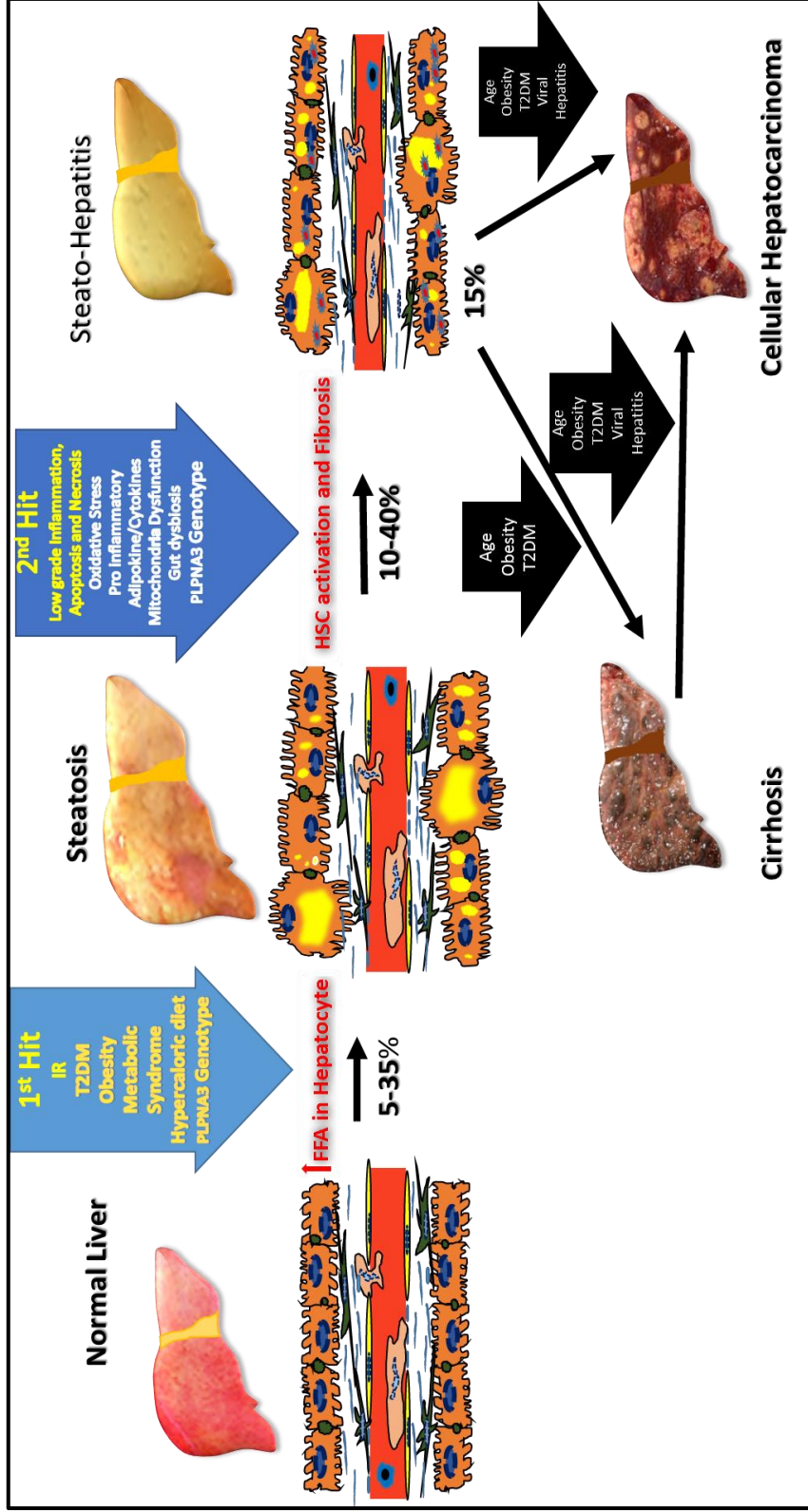
One of the primary mechanisms by which the IR occurs is through the phosphorylation of the serine residue (23) of insulin receptor substrate (IRS) (24) that hinders the insulin downstream pathway. There is a high pool of free fatty acids (FFA) (25) in the presence NAFLD. FFA metabolizes into diacylglycerol (DAG) (26), which is a specific activator of novel protein kinases C (nPKC). However, the most relevant nPKC involved in the inhibition of IRS-1 pathway is yet to be found. It has been established that the westernized diet, rich in fat and sucrose, plays a role in the pathogenesis of NAFLD and vitamin D deficiency is a major contributor to insulin resistance (27, 28). It becomes interesting to investigate the mechanistic approach of NAFLD in regard of the pathogenesis and therapeutics using a humanoid model. The previous findings were on rodents and fishes. In this project, we chose swine, which anatomically and physiologically are similar to human and are also large enough. We did this to study IR establishment in the underlying conditions that lead to NAFLD and to examine the expression and effect of most relevant nPKC on IRS in the pathogenesis of NAFLD in correlation with the diet and vitamin D status.

## **1.1 Definition of NAFL**

NAFLD, also called liver metabolic steatosis (29), is the presence of ectopic triglyceride deposit in more 5% of hepatocytes of an individual who has no history or less than 20 grams of alcohol a day consumption and other steatogenic drugs, or has liver involved hereditary disease (30, 31). The steatosis can be found using liver biopsy or imaging techniques (32). NAFLD represents a spectrum of liver conditions ranging from simple steatosis also termed fatty liver to steatohepatitis marked by lobar and portal inflammation and fibrosis, which can progress to liver cirrhosis and cellular hepatocarcinoma. Thus, NAFLD has been thought to be the cause of cryptogenic cirrhosis (33). This concept was lately questioned because if known NAFLD progresses to cirrhosis, it is no longer cryptogenic (3). The liver steatosis is marked histologically by the presence in hepatocytes of multiple microvacuoles or one large vacuole occupying the cytoplasm and pushing the nucleus against the membrane. Hepatocytes enlargement may be present with pathognomonic hepatocyte ballooning. Liver lobular inflammation foci made of Kupffer cells and Pit cells are regularly associated with the steatosis (3). In fully established NASH the steatosis is in most part in the form of microvesicles with more lobular inflammation and fibrosis in a form "chicken wire." Malory Denk hyaline corps also can be observed. NASH development is evidenced in hepatocyte injury, apoptosis which an inflammatory environment is leading to immunologic reactions with the production of inflammatory cytokines with reparation process in the form of collagen production and deposition in sinusoids. Furthermore, vascular remodeling may progress to liver cirrhosis (34).

NAFLD is pathologically similar to alcoholic fatty liver (AFL) disease except for the fact that the latter occurs in known alcohol abuse patients, most of whom are lean or cachectic. AFL is also associated with higher ALT and GGT than NAFLD. Malory Denk bodies are also more frequent in AFL than NAFLD. Hepatitis derived from AFL is histologically similar to NAFLD complication, but with faster progression and severity (35). The pathogenesis of AFL is mainly caused by ethanol-induced oxidative stress that induces damage to the hepatocyte. The catabolism of alcohol produces acetate by two enzymatic ways. The dehydrogenases produce acetate and induce steatosis while the oxidases produce free radicals. Acetaldehyde causes most injury to the hepatocytes (36, 37). Mitochondrial damage is due to oxidative stress and the depletion of antioxidant by free radicals and the induction of endoplasmic reticulum lipid peroxidation, which render the liver vulnerable. The progression of AFL to cirrhosis deriving from alcoholic hepatitis is driven by toxic adduct products metabolized from acetaldehydes that trigger immune reactions after it has been bound to proteins. This results in stellate cell activation, collagen deposit in liver, intracellular accumulation of lipids, proteins and electrolytes. The result is a loss of cytokeratin (CK8/18), causing the formation of Malory Denk bodies like inclusions (38). CYP2E1 is a microsomal ethanol –oxidizing enzyme that plays a key role in alcohol-related hepatic oxidative stress, with the production of hydrogen peroxide ( $H_2O_2$ ) and superoxide anions ( $O_2^-$ ) with lipid peroxidation. CYP2E1 is also found to be responsible for ubiquitin proteasome system (UPS) inhibition, which compromises the removal of harmful proteins

known as aggresomes. Aggresomes hinder their elimination by autophagy; hence the formation of Mallory-Denk bodies (39-43).



**Figure 1: NAFLD spectrum and spontaneous progression:** The first hit led by IR induces an imbalance in inflow and outflow of fat that accumulates in the liver to cause NAFL or liver steatosis, which in turns under 2<sup>nd</sup> hit dominated active feeding loop of oxidative stress and inflammation causes 10-40% of NAFL to progress to steatohepatitis or NASH. In 15% of cases, NASH can develop to cellular hepatocellular carcinoma via liver cirrhosis or directly.

## **1.2 Epidemiology of NAFLD**

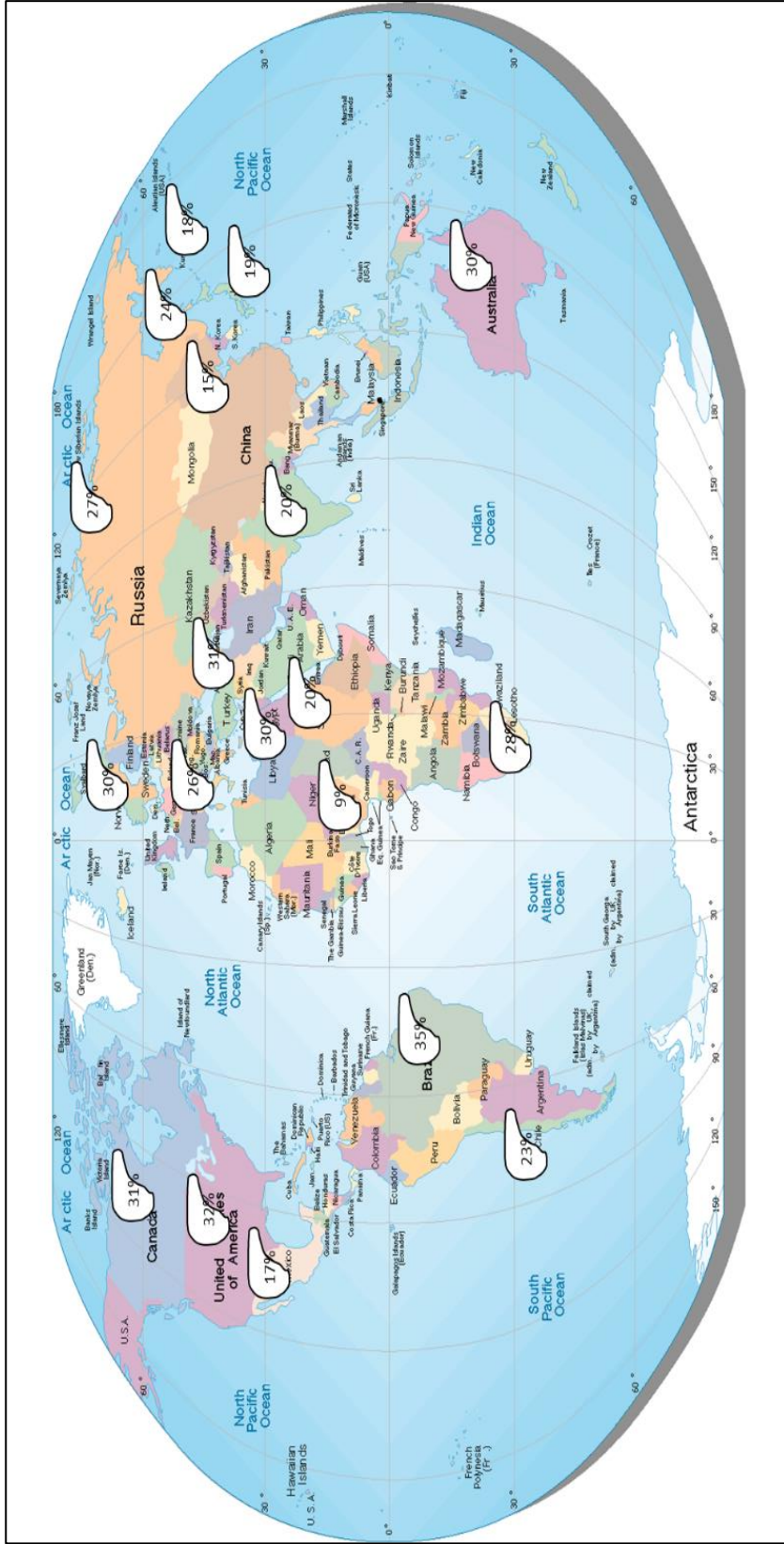
It is a challenge to agree on the prevalence of this common disease because the means of diagnostics differs from one study to the other. In fact, consensus on the diagnostic criteria remains to be reached (44). Liver biopsy remains the gold standard for the diagnostic, and it is very invasive. Liver ultrasounds imaging is the closest to the biopsy in term of sensitivity and specificity, but it remains unavailable in the rural area especially in Asia and Africa (45). The quality and capability of ultrasound devices are not the same. Other challenges are related to the fact that the disease is in most part asymptomatic making it difficult to develop screening criteria (46-48). It is noteworthy that the prevalence keeps increasing worldwide in relation with growing incidence of obesity (9, 49, 50).

US, Canada, Europe Liver NAFLD has become the most common cause of abnormal liver test (3, 9, 51). In proper context, the use of ultrasound cannot diagnose steatosis lesser than 30%. Also, blood tests are not specific at all to the disease. The computerized tomography scanning (CT-Scan) of the liver, in some concordant studies, showed evidence that the illness is very common (52). Proton magnetic resonance spectroscopy has also been used in Hong Kong general population (53).

Overall, the prevalence of NAFLD ranges between 7 to 37% according to means of the evaluation. The populations in Western World using ultrasounds or CT scan imaging found a prevalence of 20-37% (3, 54). Browning, 2004 found with proton magnetic resonance spectroscopy that in America, liver steatosis is present in 45% of Hispanic, 33% of White and 24% of Blacks (55). Studies using autopsies found

steatohepatitis in 18,6% of obese patient and 2.2% in a lean patient (56), and in India, Amarapurkar reported that 16% of adult autopsied had fatty liver (57). In the United States the most common cause of transaminases elevation has shifted to NASH. In Africa and Asia, ultrasound was the most use means of the diagnostic with lower prevalence lower in the rural area. The worldwide reported prevalence over time is shown in Fig.2. In the US the prevalence is the highest in Hispanic, followed by non-Hispanic, white Asians and the Blacks (58).

A Recent meta-analysis conducted by Xu *et Al* 2015 found that Patatin-like phospholipase domain- containing 3 (PNPLA3 rs738409 ) polymorphism strongly associated with NAFLD across races (59). PNPLA3 gene is located on chromosome 22 and encodes for adiponutrin (60) or acyl-glycerol-O-acetyl transferase that catalyzes TGA hydrolysis. Metabolic Syndrome, as well as diabetic patients known, develop NAFLD. Obese children are most affected by NAFLD worldwide. NAFLD represents the most common liver disease in adolescent and often associated with T2DM or MetS.



**Figure 2: Worldwide prevalence reported of NAFLD**, using various diagnostic methods such as autopsies, biopsies, ultrasound, magnetic resonance imaging different prevalence were found in diverse studies worldwide. Western world studies show closer prevalence, which is between 26 and 31% while in Asia and Africa, it is more spread (9-30%). (Map adapted from [www.inbdb.com](http://www.inbdb.com))

### **1.3 Liver Anatomy and Physiology**

The liver represents the second largest organ and the largest gland in the body. It is located at upper right quadrant of the abdomen and divided by deep grooves into four lobes: two large right and left and two small: quadrate and caudate. Capsule Glisson covers the whole parenchyma except a small part that is covered by peritoneum and gallbladder. Histologically, the parenchyma comprises hepatocyte, blood vessels, and bile duct and the stroma consists of connective tissue, capsule, trabeculae and reticular network. The parenchyma is organized in classic hepatic lobules, which are the structural unit of the organ, are roughly hexagonal in shape. The borders consist of connective tissue and angles also called the portal triads are marked by portal venule, hepatic arteriole, and hepatic bile ductule. The center of the lobule lodges the central vein. Hepatic acinus or acinus of Rappaport is a functional unit of the liver. It is diamond in shape and defined as concentric regions of the parenchyma surrounding a hepatic arteriole in the center. It defines three zones according to their closeness to hepatic arteriole the oxygenated blood supplier. Thus zone 1 is the closest to the blood to arteriole while zone 3 is adjacent to the hepatic terminal vein and zone 2 is in between. The portal lobule emphasizes the liver exocrine function of the bile drainage view the parenchyma to the hepatic ductules located in the portal triad. It is conceived as an imaginary line, triangular in shape drawn joining three adjacent central veins. Plates of hepatocytes consist of two anastomosing cell rows that are surrounded by blood sinusoids while bile canaliculus is drawn between the rows. A network of reticular fibers supports the

structure. Perisinusoid space is occupied by space of Disse that lies between the basal surfaces of hepatocytes and the endothelium cell and Kupffer cells.

Hepatocytes are liver parenchymal cells and account for 80-90% of the liver mass and 65% of cell number of a normal liver. They are polyhedral cells containing one or more spherical nuclei in the center having well-developed nucleoli. They have very abundant eosinophilic cytoplasm with basophilic rough endothelial reticulum (RER), SER, several mitochondria, lysosomes and mature Golgi apparatus with glycogen granules and lipid droplet. Their vascular pole is involved in endocrine function (blood-hepatocyte exchange) while the biliary pole is in the exocrine function (bile secretion). Adjacent hepatocytes delimit intercellular gaps within the hepatic plates, which are called bile canaliculi. Their walls are made of consecutive hepatocytes macula adherens tight junctions.

Kupffer cells are non parenchymal resident macrophages, thus part of the mononuclear phagocytic system. They represent (15%) of liver cells population. They are derived from monocytes, sessile, and lying within the sinusoids lumen and trapped between the endothelial cells. They contain bean shaped nucleus and have pseudopodial cytoplasm. They contain numerous vacuoles. They are involved in removal of damaged, senile or dead red blood cells (RBCs). They also act as antigen presenting cell in adaptive immunity.

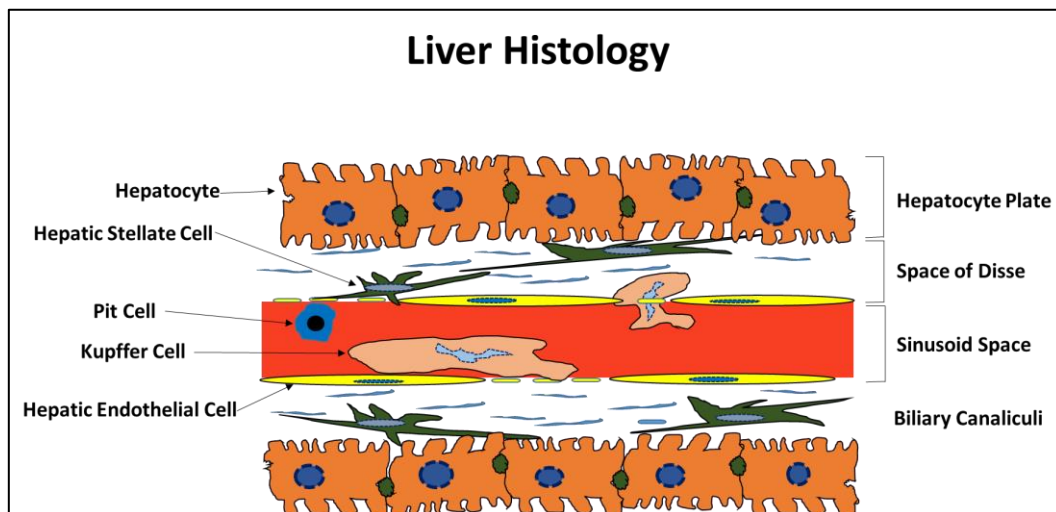
Endothelial cells are also sessile cells of the liver that constitute the sinusoid wall. They form fenestrated linings with an undiaphragmated opening that are together to form mesh plates separate by cytoplasmic extensions. There is no basement

membrane beneath the endothelial cell, which allows a free exchange, but at the limited level since liver endothelium sieve has only 10% of fenestration.

Hepatic stellate cells or Ito cells or fat storing cell are resident cells located in space of Disse, with cytoplasmic process trespassing the endothelial wall through its fenestrae. Cytoplasmic fat droplets contain vitamin A. Their size and number depend on the physiological activity of the cells. The droplets can with the membrane or without. Stellate cells are preferably positioned in the periportal area. In cell culture, they have been shown to develop into myofibroblast –like cells with increased extracellular matrix (ECM) synthesis with decreased of the fat droplets. Pit cells present in sinusoids on the top of the endothelial cell. They are believed to be movable and contain large azurophilic granules. They can be found adhering to the endothelial lining of the Kupffer cells. They may be the hepatic natural killer. They possess same morphology as agranular lymphocyte with polarized cytoplasm rich in Golgi apparatus and multivesicular bodies.

Liver function is very complex. It is an exocrine gland producing and excreting bile and endocrine gland storing glucose in the form of glycogen and releasing glucose as needed by glycogenolysis. It is also the place of metabolism of plasma protein such as albumin and coagulation factors. It metabolizes lipid and cholesterol. The liver also serves as the storage organ for lipid soluble vitamins (A, D, K). It is involved in the metabolism of most nutrients and the detoxification of the organism. It is also responsible for the production of hormones, the phagocytosis of debris and bacteria. It stores some minerals such as iron copper. It metabolizes lipoproteins, ferritin and other growth and coagulation factors. Bile production is

one the particular function that is known to the liver that is part of the detoxification of the body, which occurs in smooth endoplasmic reticulum, by oxidation, conjugation, and conversation of molecules. The hepatic artery carries oxygenated blood, and the portal vein feeds blood rich in nutrients from the digestive system including the pancreas and the spleen. The blood flow goes through hepatic sinusoids through the lobules go out of the liver via the central vein which the terminal hepatic venule. The central veins empty themselves in hepatic vein, which in turn joins the inferior vena cava.



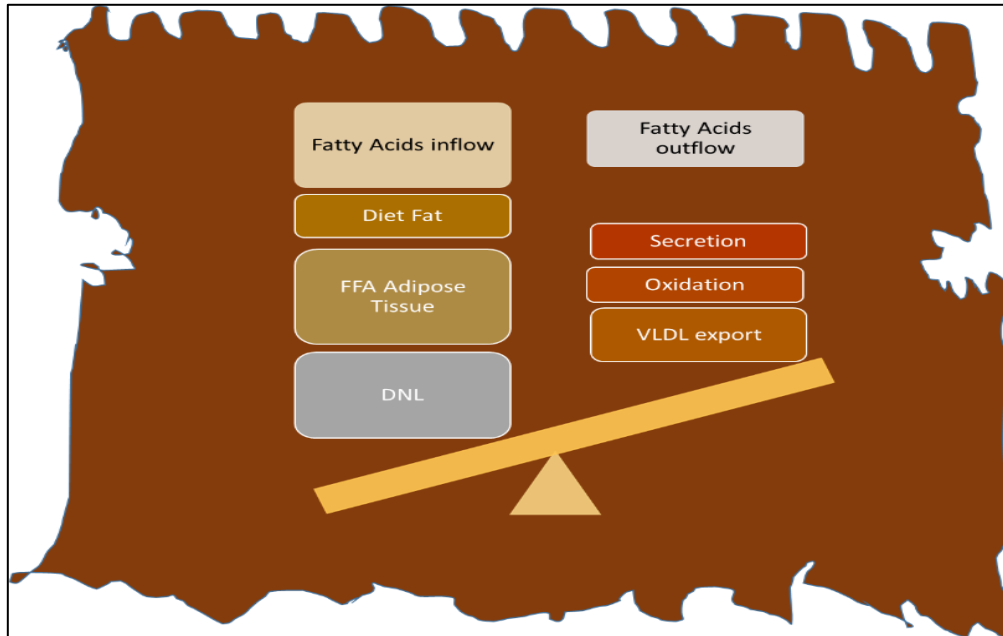
**Figure 3: Basic liver histology diagram:** Hepatocyte plate formed by consecutive attachment of hepatocytes to one another by stable macula adherens tight junctions. The consecutive junctions create the biliary canaliculi. There is a proximity between the blood in the sinusoids and hepatocytes. Endothelial cells streak the sinusoids form a discontinuous endothelium that has wide gaps between cells and lacks a basement membrane and is therefore very permeable and allows maximal contact between the blood and the hepatocytes, which absorb materials from it and secrete materials into it. Hepatocytes secrete bile into biliary canaliculi that are defined by tight junctions between hepatocytes. Bile flows through these narrow tubes toward the bile duct. Kupffer cells are the resident macrophages of the liver and are typically found within the lumen of the sinusoids also in space Disse. These cells are bathed in blood from the sinusoids. The sinusoids have a fenestrated endothelium and do not have basement membrane space of Disse separates them from the hepatocytes.

## 1.4 Pathogenesis of NAFLD

### 1.4.1 Fatty Acid flow (Fig. 4)

Fatty acid (FA) flow imbalance through the liver represents the key process through which triglycerides accumulate in liver(61). In the normal state, diet delivers about 5% of liver FA while 60% come from extrahepatic non-esterified fatty acids (62) and the rest from *de novo* lipogenesis (DNL) (30, 63). It has been found that obesity, IR, T2DM individual has higher levels of circulating FA compared to the general population (64). Hyperglycemia and hyperinsulinemia produce FA synthesis from glucose and at same time block  $\beta$ -oxidation causing FA to produce triacylglycerol (TAG). This reaction helps to protect the hepatocyte from deleterious injury resulting from long chain fatty acid that results from adipose tissue lipolysis. Increased FA flux in the liver also contributes to mitochondrial dysfunction by impairing oxidative phosphorylation cycle through adenosine di-phosphate (ADP) and other anti-oxidants depletion and with increased reactive oxygen species (ROS) production. In fact, the increase FA flux starts by rendering insulin ineffective when it decreases the pool of long-chain polyunsaturated fatty acid (65) leading to IR. It has been found that peroxisome-activated receptor- $\alpha$  (66) is deactivated while sterol regulatory element binding protein-1c (SREBP-c) is activated, and all together cause intra -hepatocytes accumulation of triglycerides and liver steatosis. Later as oxidative stress increases, it causes an up-regulation of ethanol-inducible form of cytochrome P450 (CYP2E1) which adds to Kupffer cells activation and leucocyte infiltration to induce the expression pro-inflammatory cytokines such as NADPH oxidase in phagocyte cells

(67), nuclear factor- $\kappa$ B (NF- $\kappa$ B) activating protein-1 (AP-1), tumor necrosis factor  $\alpha$  (TN- $\alpha$ ) and interleukin-1 (IL-1). These cytokines have been found contributing to the progression of the liver steatosis to NASH.



**Figure 4: Imbalance of fatty acids flow in the liver leading to NAFLD.** FFA flow to the liver is provided by fat consumption, adipose tissue release, and DNL. In the normal state this inflow of FFA is balanced by in and out flow through secretion intestinal tract through bile, oxidation in hepatocytes and VLDL export. FFA accumulates in the liver when the inflow becomes overwhelmingly increase compared to outflow.

#### 1.4.2 Insulin Resistance (Fig.5)

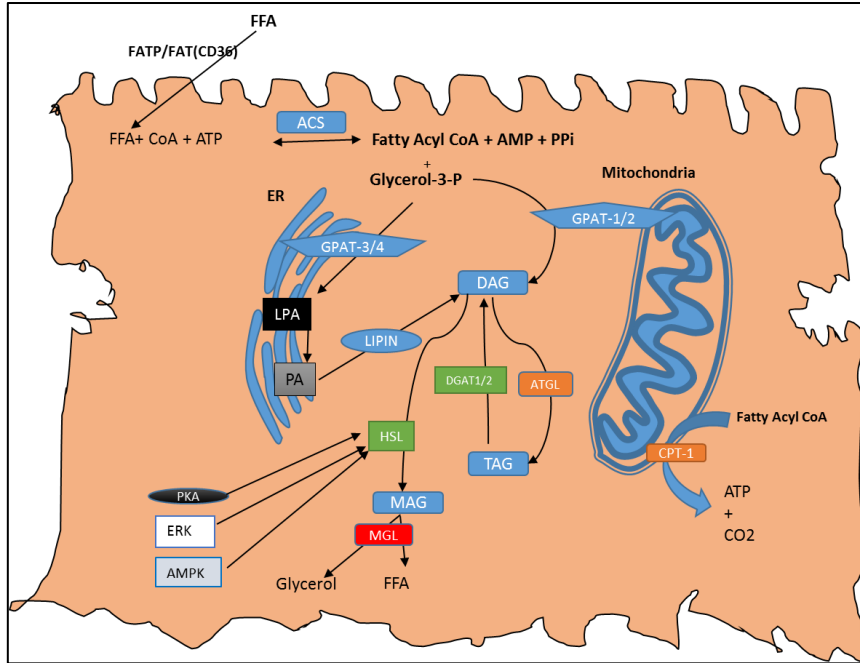
Among the features of metabolic syndrome that correlate with NAFLD and its complications, insulin resistance (6) represents the most common and the strongest. In fact, it is believed that IR is at the beginning of the pathogenesis of NAFLD (28). The first hit of the two hits theory is solely linked to a lasting IR (68), while in multiple hits, IR is always present along with other forms of hepatocyte injuries. Also in obesity, T2DM or dyslipidemia, IR is always present. In fact,

overwhelming bodies of evidence showed that IR is consistently involved in the pathogenesis of NAFLD in obese (70, 71) as well as in lean individuals (72). Interestingly patients with genetic predispositions to NAFLD such as patatin-like phospholipase (59, 73, 74) or diacylglycerol acetyl transferase (75-77) gene mutation or hypobetalipoproteinemia (78-80) have shown to have a normal insulin sensitivity. This suggests that NAFLD does not induce IR.

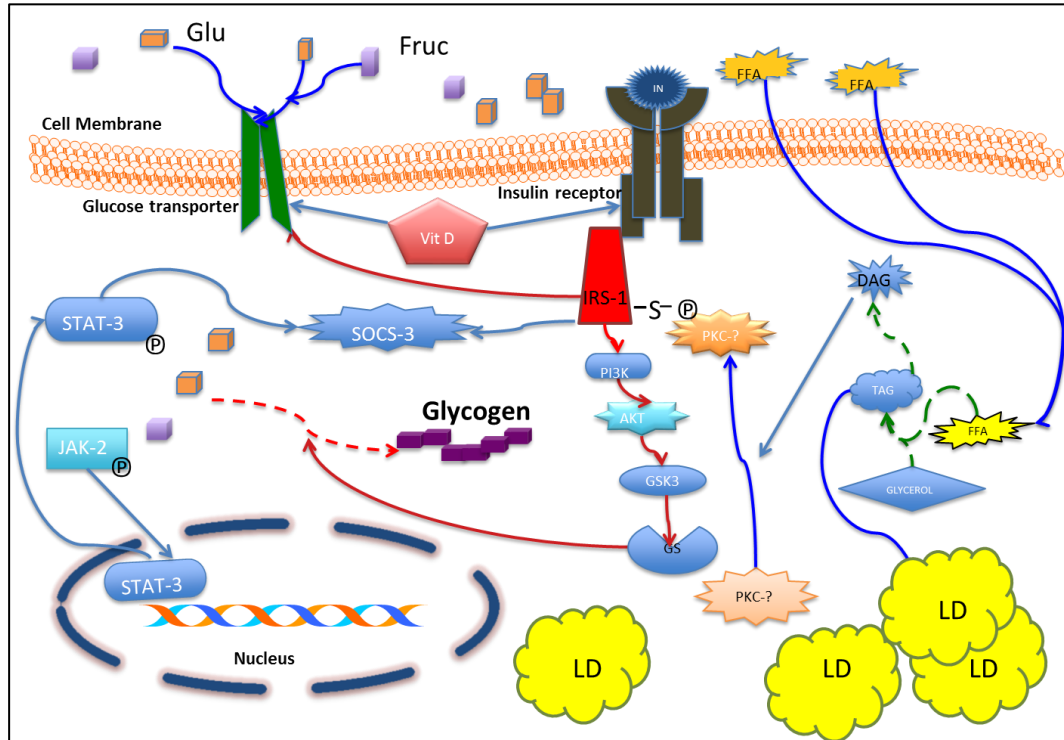
In NAFLD pathogenesis, systemic and hepatic IR are found to play a role.

Systemic IR involvement in NAFLD is through the muscular inability to store glucose in the form of glycogen so increases the glycemia that in turn increase the flow of glucose to the liver. Also, IR causes fat tissue to release FFA from adipocyte lipolysis and inhibition of lipogenesis. The high flow of FFA and glucose to liver fuel the triglyceride droplet building-up in the hepatocyte (81, 82). In fact, FFA released from adipose tissue represents the most important source FFA coming to liver. In the case of adipose tissue IR (83) accessed by the increase of its index calculated with the following formula  $\text{Adipo-IR}(i) = \text{plasma free fatty acids [FFA]} \times \text{insulin [FPI]} \text{ concentration}$  (82)(84), blood FFA increases. It has been found that increase of adipose-IR is associated with liver injury treatment targeting adipo-IR such as visceral fat burn and thiazolidinedione use can improve NAFLD (82).

Liver, as well as muscle ectopic fat deposit, was considered as a defense mechanism against lipotoxicity inherent to FFA(85, 86). And when this mechanism in the liver is overwhelmed, FFA begins to induce reaction involving inflammation and oxidative stress that lead to NASH and its subsequent cirrhosis (87, 88).



**Figure 5: Mechanisms leading to DAG increase in hepatocyte leading to triglyceride accumulation and NAFLD:** Circulating FFA enters the hepatocyte passively through FATP/FAT (CD36). Once intracellular it is acylated under the action of ACS into fatty acyl-CoA. Fatty Acyl-CoA undergoes double esterification by glycerol catalyzed GPAT 1/2 in the mitochondrial membrane to form DAG or under successively GPAT 3/4, LPA and PA to form DAG through ER.



**Figure 6: Insulin resistance in underlying mechanism of NAFLD:** Glycemic increase trigger production and secretion of insulin by pancreatic beta cells. Once in the liver, insulin attaches to its receptor to provoke glucose uptake from the bloodstream and its storage in the form of glycogen. This process is compromised in the pathogenesis of insulin resistance and NAFLD. First, the increase in the circulating fatty acid induce increases of intracellular TAG that produces DAG, which activates and translocates nPKCs from the cytosol to the membrane. Once in the vicinity of IRS-1, nPKC causes serine phosphorylation of IRS-1 to induce its inhibition and degradation. The insulin-stimulated glucose transport and storage via the PI3K-Akt dependent pathway is compromised. Glut2 translocation and glucose uptake are inhibited, and glycogen synthetase activity is also inhibited to produce glycogen. Second, the high flow of glucose and fructose produces FFA through de novo lipogenesis (DNL) also dietary, and FFA released for adipose tissues. The increased accumulation of FFA forms droplets of triglyceride that enlarge as the disease progresses. The excess of glucose is excreted back into the blood stream through Glut2 feeding the hyperglycemia found in insulin resistance.

### 1.5 Fructose in NAFLD

Fructose is one of the three monosaccharides along with glucose and galactose that are absorbed into the bloodstream after digestion. Fructose has a metabolic and hemostatic distinctiveness that makes it unwelcome in the whole body especially

the liver. Fructose was initially less than 10% in the human diet, derived then from fruits and root vegetables. Since the development of sugar industry intake of dietary fructose has increased drastically (89, 90). In westernized diet fructose source is primary from sucrose and high corn fructose syrup (HFCS). While both sucrose and HFCS have 50% of fructose composition HFCS is sweeter. Also to the hedonic reward, fructose contributes in the browning of the food and its preservation.

Fructose becomes very attractive for the food industry to the point that it seems impossible to find today a processed food that does not have fructose product added to it. The liver is the sole organ to metabolize fructose to produce energy, lipid, and waste in the form of acetaldehyde (91). In fact, through the effect of fructose kinase, most parts of ingested fructose are transformed in vivo into fructose-1-phosphate. The fructose-1-phosphate produces glyceraldehyde, which turns to pyruvate and enters the mitochondria and the Krebs cycle. It produces ATP as well as CO<sub>2</sub> with citrate, which through citrate shuttle comes back to the cytosol to participate in *de novo* lipogenesis (DNL) contributor of intrahepatic lipid droplet. Another metabolite of fructose is dihydroacetone-P, which together with glyceraldehyde make fructose-1,6-bis-phosphate that in turn to fructose-6-P and then to xylulose-5-phosphate. Xylulose-5-phosphate is an activator of protein phosphatase-2 A (PP2A) that activates the carbohydrate response element binding protein (ChREBP) for the transcription of ATP-citrate lyase (ACL), Acetyl-CoA carboxylase (ACC) and fatty acid synthetase (FAS) to enhance the production of VLDL and triglycerides (92, 93). Also, the fructose phosphorylation causes depletion of ATP and production IMP and then uric acid that blocks the production

of nitric oxide (NO) to cause hypertension (HTN) hypertension(89, 94, 95). Also xylulose-5-phosphate up-regulates Janus-N-kinase (JNK) to induce inflammation and serine phosphorylation of IRS-1 to cause insulin resistance. All put together Lustig et Al demonstrated fructose acts as “alcohol without the buzz”(96).

## **1.6 Vitamin D in NAFLD**

Deficiency in vitamin D has been linked to metabolic syndrome features and especially to T2DM (74, 97). It has been found Vit-D level negatively correlates with insulin resistance, oxidative stress and inflammation (98). Children with low Vit-D level have been found to have the propensity to develop obesity, IR and early in life T2DM while the supplementation in Vit-D provides protection to children against these mentioned conditions (99, 100). NAFLD is recognized as the liver manifestation of metabolic syndrome. There is a growing body of evidence showing that blood Vit-D level independently correlates with hepatic triglyceride contain. In fact, Jablonski *et al.* found that the development and progression of NAFLD are associated with vitamin D deficiency when they accessed the incidence of NAFLD in randomized matched patients. (101).

## **1.7 Clinical Aspects**

### **1.7.1 Clinical Diagnosis**

It is illusive to rely on clinical examination to make NAFLD diagnostic since the majority of patients are asymptomatic. In rare cases where there are symptoms associated with the disease, they are nonspecific. Most patient complaints are tiredness, right upper quadrant discomfort, anorexia, weight loss, and sleep disturbance. Only at stage NASH can be found jaundice, itching, edema, ascites, or

mental confusion. Signs found during the physical examination are hepatomegaly, signs of portal hypertension, and cirrhosis. Some associations need to be investigated during NAFLD suspicion. Young women can present polycystic ovarian syndrome (102, 103) or obese patients can also have obstructive sleep apnea or psoriasis (104-106). In the absence of clear association with the feature of Met S, apolipoprotein B, and lysosomal acid lipase or partial deficiency should be suspected (107). The examination should be extended to the cardiovascular disease manifestations since they have strong association with NAFLD (108, 109).

### **1.7.2 Blood Testing**

Currently, there are no specific biomarkers available for NAFLD (47, 51, 110). In case where symptoms and signs suggest the diagnostic considerations need to be made to eliminate other liver diseases that present similarity with NAFLD. Testing should eliminate other chronic liver diseases such as alcohol abuse, hemochromatosis, Wilson disease, autoimmune hepatitis, viral hepatitis, and drug-induced hepatitis. AFLD is distinguished from NAFLD by the fact the patient drinks daily more than 20g for women and 30g men of ethanol. Once suspected before the finding of components of metabolic syndrome the evaluation must include personal and family history, blood pressure, exploration of blood glucose hemostasis, blood lipids and liver function test that will include transaminases, platelets counts, albumin level, and gamma-glutamyl-transferase (GGT).

There is a middle elevation of liver transaminases about two to five-fold the normal level. It has been found that ALT is most of the time increased than AST in simple steatosis which is opposite in what is found in AFLD (111). AST come later in

advanced form to increase significantly like in AFLD hepatitis. Ferritin can also be increased in NAFLD, and it is associated with an advanced form of the disease.

Other things must be to evaluate insulin resistance status of the individual using HOMA-IR calculated or through glucose tolerance test.

### **1.7.3 Imaging diagnostic**

Ultrasounds of the liver have been the most used form of imaging in the diagnosis of NAFLD. In fact, most, epidemiologic studies of NAFLD were reported using ultrasounds (112, 113). It is based on quantitative evaluation of controlled attenuation parameter (CAP) (114, 115), which measure the loss of energy of sound particles when going through the liver parenchyma. The presence of fat is noticed by greater attenuation of energy than more attenuation signifies more fat presence in the liver (116).

Magnetic Resonance Imaging uses the proton density fat fraction (PDFF) as quantitative biomarkers for liver fat (117). PDFF needs Magnetic Resonance Spectroscopy (118) to determine the liver triglyceride concentration. MRS is only available in a research center, which makes it difficult to be used as means of general practices despite its accuracy in diagnosis and monitoring of liver steatosis (119).

### **1.7.4 Liver Biopsy**

Liver biopsy is the gold standard for the diagnostic and provides vital information for the disease management and prognosis (34). It is the key exam that determines the stage of the disease and gives details that other diagnosis tools can never find. It

provides data about hepatocytes and location of inflammation and in fibrosis in case they are present. The grading and staging histological features of NAFLD (Table 1) was achieved through biopsies. It is very invasive to the patient due to risks such as bleeding and infection. Since one of the consequences of hepatic disease is the depletion of coagulation factors, physicians have to be careful when performing a liver biopsy on NASH patient. Also, the risks of missing the lesions are high. Biopsies performed by knowledgeable specialists may result in different grading score suggesting the importance of repetition of the biopsies in case of dubious findings (120).

**Table 1: Grading and staging histological features of NAFLD and NASH**

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<b>Scores</b>
<b>NAFLD Activity Score (NAS; 0–8)*</b>
• <b>Steatosis (0: &lt;5%; 1: 5–33%; 2: 33–66%; and 3: &gt;66%)</b>
• <b>Lobular inflammation, foci per 20 magnification (0: not present; 1: &lt;2; 2: 2–4; and 3: &gt;4)</b>
• <b>Ballooning (0: not present; 1: few; and 2: prominent ballooning)</b>
<b>NAFLD Fibrosis Score (0–4)*</b>
• <b>1a: delicate zone 3 psf‡</b>
• <b>1b: dense zone 3 psf‡</b>
• <b>1c: portal only</b>
• <b>2: zone 3 plus portal or periportal</b>
• <b>3: bridging (c–c, c–p and p–p)</b>
• <b>4: cirrhosis</b>
<b>Fatty Liver Algorithm§</b>
• <b>Steatosis (0–3)</b>
• <b>Activity (ballooning and lobular inflammation)</b>
• <b>Ballooning (0–2)</b>
• <b>Lobular inflammation (0–2)</b>
• <b>Fibrosis (similar to the fibrosis score of the NASH CRN above)</b>

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c–c, central-to-central; c–p, central-to-portal; CRN, Clinical Research Network; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; p–p, portal-to-portal; psf, perisinusoidal fibrosis. \*National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) NASH CRN: Pathology Committee NAFLD Scoring System for Clinical Trials. Although the NAFLD Activity Score is known to closely correlate with a diagnosis of NASH, it was created for clinical trials to assess changes in the components and has been shown to be associated with liver tests (alanine aminotransferase and aspartate aminotransferase), whereas the pathologist’s diagnosis of NASH associates with features of insulin resistance. The pathologist is encouraged to make a separate, pattern-based diagnosis in addition to scoring the lesions. ‡Delicate psf requires trichrome staining, whereas dense psf can be visualized on a haematoxylin and eosin stain first. §A primary difference between the European algorithm and the NASH CRN is that the former score was derived to establish a diagnosis of NASH. Steatosis of >1, activity of >2 (with both ballooning and lobular inflammation of >1) equals NASH (Adapted from) (3)

## **1.8 Management**

Today there is no approved drug treatment for NASH. Most of the therapeutic strategy to prevent or cure NASH relies on life style modification and use of drugs pertaining to the management of MetS features.

### **1.8.1 Lifestyle modification**

Lifestyle change is the key management first step. The implementation of the change to healthier eating habits, physical exercises, avoidance of alcohol consumption must start the treatment. Weight loss should be pursued in an obese patient at all stage of the disease. This step also helps alleviate risks accompanying cardiovascular condition that concurs so often with NAFLD. Numerous studies showed that physical exercise and healthy diet improved the histological features of NAFLD even of NASH stage (121, 122).

### **1.8.2 Drug therapy**

#### **1.8.2.1 Therapeutic trials**

Pharmacological therapy utilizes diabetes medications, and other Met S features treatment such as dyslipidemia, hypertension. The ultimate goal of treatment is to reduce the oversupply of fatty acids to liver, oxidative stress and inflammation and the subsequent cell injuries and death. In fact lipogenesis inhibitors such as obeticholic acid, an inhibitor of DNL through the pathway of SREBP-1 C, have been tested with mild improvement of NASH histological features but associated with a significant amount of side effects of pruritus and dyslipidemia (123). Exenatide, an analog of glucagon-like peptide 1 (124) or gliptin a dipeptidyl peptidase 4 inhibitor to decrease hepatic DNL is on the trail (125, 126).

### **1.8.2.2 Blood sugar lowering medications**

In the quest of decreasing lipolysis in adipose tissue production of triglycerides into IR, PPAR $\gamma$  ligands thiazolidinedione (TZDs or glitazones) have been used to improve NAFLD. In fact, pioglitazone has been shown to increase insulin sensitivity of adipocytes and mend NASH progression (127, 128). The risks this therapy exposes the patient to weight gain, hypertriglyceridemia, bladder cancer, osteoporosis, and psoriasis.

Another insulin sensitizing molecule used in NASH is metformin that enhances insulin utilization by the liver. Even though randomized clinical trial has not shown metformin reduces liver transaminases in children, it is still used as an adjuvant in NASH treatment because its advantage of weight loss and prevention of malignancies (129).

### **1.8.2.3 Blood Cholesterol-lowering medications**

Atorvastatin administration to a patient with NASH and dyslipidemia has shown to lower LDL, HDL, and triglyceridemia and improve liver histology. The challenge faced is that high transaminases constitute a concern for statin administration (130).

## **1.9 Prognosis**

Morbidity in NAFLD at the steatosis level is primary link to other elements of metabolic Syndrome. The liver continues assuming all its regular functions afford mentioned of metabolism and detoxification of the body. The cardiovascular impairments and infection due to diabetes complications lead patients to premature death. It is not yet well determined how long it takes for NAFL to progress to NASH even though factors of the progression has been described (Fig.1).

Inflammation is the most common factor found to lead the progression. It initiates and develops the liver fibrosis and subsequently causes NASH (131). NASH has significant potential to develop to cirrhosis and liver failure that will require a liver transplant in the absence of which death can follow (44, 132).

As liver biopsy still the gold standard for the diagnosis and it very invasive, it remains tough to treat NAFLD preventively. The diagnosis may be suggested in presence hepatomegaly in the patient long history of metabolic syndrome. After which liver biopsy can be performed to find the all the histological features for the adequate therapeutic course. Liver cirrhosis and hepatocellular carcinoma may complicate NASH and lead to death. Fibrosis is linked to poor outcome (133, 134).

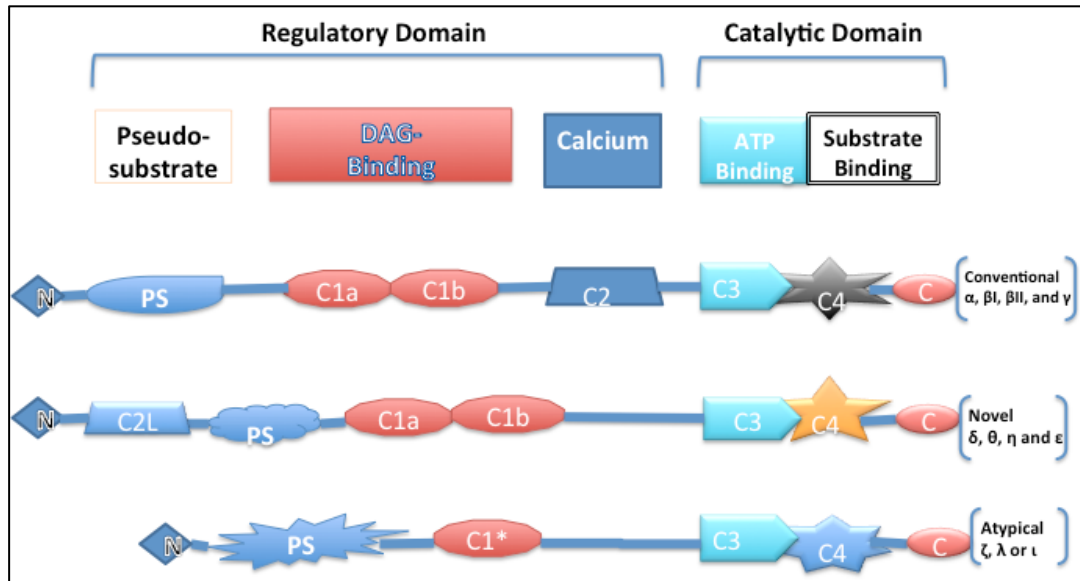
Gut-derived endotoxins flux to the liver is increased due to the intestinal permeability caused by ethanol. These lipopolysaccharides and peptidoglycans induced an activation of pathogen-associated molecule (PAMP) like toll-like receptor 4 (135) on Kupffer cell promote inflammation and production of tumor necrosis factor  $\alpha$  triggers collagen deposit in liver sinusoid (136)

### **1.10 Novel Protein Kinase C**

Novel protein kinases C (nPKC) form with classic or conventional PKC (cPKC) and atypical PKC (aPKC) the whole group PKC. This subdivision is based on their regulatory domain (Fig 7.). In fact, nPKC are the only group that solely activated by diacylglycerol (DAG) make them a special target in milieu where there is an increase of triacylglycerol (TAG) production (Fig.7)

Diacylglycerol is the only the potent activator of novel PKCs. During the accumulation of fatty acids in the hepatocyte, the synthesis of triglycerides is up-

regulated. An intermediate product of this synthesis is diacylglycerol. In fact from glycolysis, glycerol-3 phosphate (G-3-P) is produced from dihydroxyacetone phosphate in hepatocyte which. G-3-P will be twice acylated with two acyl co-enzyme A to form lysophosphatidic acid and then phosphatidic acid. The dephosphorylation of phosphatidic acid yields DAG, which is a potent activator of protein kinase C. DAG then produces triacylglycerol or triglyceride by the action of diglyceride acyltransferase adding a third FA to the glycerol backbone. Because it is an important substrate of TAG, DAG tends to be up-regulated in presence increased glycolysis activity. DAG also functions as second messenger signaling lipid. When phosphatidylinositol 4,5-biphosphate (PIP<sub>2</sub>) is hydrolyzed by phospholipase C (PLC), it produces DAG and inositol triphosphate (IP<sub>3</sub>). This reaction occurs mainly in vicinity of the membrane since PLC is a membrane-bound enzyme. And since DAG is hydrophobic it remains within the plasma membrane while IP<sub>3</sub> diffuses in the cytosol. This position of DAG causes it to deploy its activation function primarily in the closeness of the plasma membrane. The activation of nPKCs produces a change in their structural conformation and their translocation to the membrane where most of their effectors are located. In the pathogenesis of IR is has being found nPKCs are implicated in serine phosphorylation of insulin receptor substrate (IRS) to inhibit its action or cause its degradation. Thus they reduce overall activity of IRS-1. The most relevant pathway and its activation in inducing IR remain elusive.



**Figure 7: Protein kinases C structure:** Three subtypes of PKCs differ from one another based on their regulatory domain. Conventional PKC ( $\alpha$ ,  $\beta$ I,  $\beta$ II and  $\gamma$ ) are activated by both calcium and diacylglycerol. DAG activates novel PKC exclusively. Ca and DAG don't activate atypical PKCs.

## **2 Research**

### **2.1 Specific Aims and Hypothesis, Approach and Innovation**

Even though NAFLD is rampant and NASH very deleterious, there is no specific medical treatment for it owing to the fact all the therapeutic strategy to manage the diseases emanate from the adaption of other conditions related to NAFLD such MetS, T2DM, and dyslipidemia. The aim of our research project is delve in the pathogenesis mechanistic approach of the NAFLD to discover what can be corrected specifically to treat the condition. Since IR is almost linked to the development of all forms of NAFLD and their progression and the serine phosphorylation, IRS is a major way to initiate IR. And nPKC that is solely activated by DAG was shown to play a role in the serine phosphorylation inhibition IRS-1. And synthesis of DAG a metabolite of triglyceride is increased in NAFLD. The specific aim of this project is to find the most relevant nPKC in the IR mechanism leading NAFLD in correlation with diet and vitamin D status. A humanoid model has been considered in our approach to rendering our finding applicable to human. Swine fed with equicaloric-westernized diet has been chosen in our project in order to emulate western world lifestyle. As vitamin D level has been found to be inversely associated with MetS features and also demonstrated anti-inflammatory properties, we evaluated nPKC phosphorylation according to the diet and vitamin status of swine. This has never been done before in our knowledge. We hypothesized that nPKCs would be activated in the presence of increased DAG to serine phosphorylate IRS-1 whether to inhibit its action or to

degrade it. Also, we predicted that vitamin D would prevent IRS serine phosphorylation by nPKCs.

## **2.2 Methods**

### **2.2.1 Research Design**

Animals and treatments: type Micro Yucatan miniature swine were purchased from Sinclair Bio resources (Windham MA). They were cared for in Creighton Animal Resources facility and were raised according to NIH and USDA guidelines. The protocol of animal research #0930 was approved and monitored by Creighton University Institutional Animal Care and Use Council. We used Female Yucatan™ miniature swine fed a control diet (Harlan Teklad Miniswine diet, Madison WI), other fed a special high cholesterol diet (Gupta et al., 2012) other on special high cholesterol and high fructose diet. The high cholesterol diet (HC) consisted of 37.2% corn (8.5% protein), 23.5% soybean meal (44% protein), 20% chocolate mix, 5% alfalfa, 4% cholesterol, 4% peanut oil, 1.5% sodium cholate, and 1% lard; with 52.8% of the kilocalories from carbohydrates and 23.1% of the kilocalories from fat. The high cholesterol-high fructose diet-HCHF form Harlan Laboratories was designed to replicate the high-fat diet of the western world. It is composed of fructose (230g/Kg), casein (195g/Kg), sucrose (127.5g/Kg), cholesterol (40g/Kg), maltodextrin (90 g/Kg), lard (110 g/Kg), coconut oil (110 g/Kg), sodium cholate (15 g/Kg), cellulose (26 g/Kg), hydrogenated vegetable shortening (55 g/Kg), and soybean oil (10 g/Kg); with 18-20% of the kilocalories from fructose and 45% of the kilocalories from fat. The diets were isocaloric with each swine receiving approximately 2700-4500 Kcal/day. Originally, the HCHF swine were to be fed the

HCHF diet for at least 26 weeks (6 months), to increase the probability of developing metabolic syndrome, and the HC swine for 26 weeks as comparison. One of the HCHF swine was euthanized after 22 weeks of the diet because of health factors affecting its quality of life. The Institutional Animal Care and Use Committee of Creighton University recommended euthanasia. Each dietary group of swine were fed their respective diets for at least 26 weeks. In addition to the diet the HC group were sub divided into three groups according to vitamin D regimen to achieve vitamin D deficiency, sufficiency or supplementation. HFCH swine were kept vitamin D sufficient. Control swine were fed on standard chow diet with no intervention in regard to their vitamin D status.

Blood and liver specimen were collected from every swine the day of their euthanasia.

## **2.2.2 Techniques**

### **2.2.2.1 Hematoxylin and eosin (HE), and Masson Trichrome**

#### **Staining**

HE and Masson trichrome staining were performed on 5 $\mu$  formalin fixed and paraffin embedded (FFPE) of the swine liver tissues. We used Thermo Scientific and Richard Allan Scientific Masson Trichrome Kit (Kalamazoo, MI) according to manufacturers' protocols.

### **2.2.2.2 Oil red O staining**

All staining was conducted on frozen liver samples from swine from various vitamin D status and diets, using Oil Red "O" staining kit from NovaUltra Oil Red O Stain Kit IHC World: IW-3008 according to the manufacture's protocol. Briefly,

we cut frozen liver sections at eight to 10mm thick mounted on slide and air dried them for 60 min and fixed them in formalin for 10 min, and briefly washed them with distilled water 1-10 minutes. We dried them again for another 60 min and proceeded with pre-staining and then staining with Oil Red O for 10 minutes. We rinsed the slides with differentiation solution and lightly stained nuclei with alum hematoxylin for 30 seconds. We rinsed with running tap water for 2 minutes and mounted with glycerin jelly.

### **2.2.2.3 Immunohistochemistry**

All experiments were performed on formalin fixed and paraffin embedded liver sample sectioned at 5µm thick. Liver tissue sections on the slides were deparaffinized in xylene, rehydrated in ethanol, and rinsed in distilled water. We used antigen retrieval (DAKO Target Retrieval solution of Carpenteria, CA) warmed 95°C for 20 minutes. Then, slides were cooled down and rinsed in 1xPBS. We blocked endogenous peroxidases with 3% H<sub>2</sub>O<sub>2</sub> for 20 minutes and rinsed the slides in 1xPBS. We used serum from a VECTASTAIN™ ABC kit containing serum rabbit IgG to incubate the sections for 1 hour at room temperature. We drained the slides and incubated the sections with primary antibody (Table 1) for 1 hour at room temp. We the slides rinsed in 1xPBS, and added the secondary antibody from the VECTASTAIN™ ABC kit and incubated on slides for 2 hours at room temp. We rinsed the slides in 1xPBS, and the VECTASTAIN™ ABC-HRP (horseradish peroxidase) and incubated for 30 minutes at room temperature. The slides were then rinsed with 1xPBS and DAB (3,3'-diaminobenzidine) from Vector Laboratories was added to the slides for 2 to 10 minutes until the brown color of

the DAB started to appear. After the DAB had developed sufficiently, we washed slides and briefly stained them in hematoxylin. We rinsed and then dehydrated them in ethanol and cleared them in xylene. Tissue sections were examined with a Nikon Eclipse Ci microscope and images were photographed with a Nikon DS-L3 camera. These images were captured using the Nikon DS-L3 software.

#### **2.2.2.4 Immunofluorescence**

The snap frozen fresh liver tissues in liquid nitrogen were embedded in OCT compound in cryomolds. We cut the blocks in sections of 4-8 um thick mounted them on superfrost plus slides. We warmed slides at room temperature for 30 minutes and fixed the sections in ice-cold acetone for 5 minutes air-dried them for 30 minutes. After washing them in 1XPBS, we incubated the section with normal donkey serum from Vector Laboratories for 2 hours at room temperature. We added the primary antibody (Table) overnight at 4°C. To access the co-localization, we mixed the primary antibodies of the proteins of interest from a different source and added the mixture to the samples. The next day we rinsed the slides three times in 1XPBS and added the Invitrogen Alexa-FluorR for 2 hours at room temperature or in the case of double staining we added the Alexa-Fluor mixture against the primary antibodies accordingly. We washed the slide in PBS and mounted them with DAPI from Vector Laboratories. We viewed the tissue sections on Olympus BX-51 epi-fluorescent microscope and images were photographed with an Olympus DP71 camera. We captured the images using Olympus DP Controller software with the exposure set to SFL-Auto.

#### **2.2.2.5 Western blotting**

Snap frozen whole liver tissue samples were used in our experiment. We obtained the protein from tissue lysate according to the following procedure. Once we cut about 5 mg of piece of tissue, we added 300uL

Quantification of the total protein lysate was done using Bradford assay.

Electrophoresis of the proteins was done using 10% SDS-PAGE. After the band were transferred to nitrocellulose membrane. The membrane was blocked overnight at 4°C with blocking solution made of 1X TBS, pH 7.6, 0.1% Tween20, and 5% w/v of nonfat dry milk. The membranes were incubated with primary antibody diluted in blocking solution (Table 2) for 2 hours at room temperature after which they were washed with PBS X3 for 5 minutes, and the HRP-conjugated secondary antibodies were added for and 1 hour at room temperature (Table 3). Membranes were washed in PBS X3 for 5 minutes, and HRP activity was detected by incubating the membrane in chemiluminescence solution (Bio-Rad, Hercules, California) for a minute. The time for the exposure was adjusted to keep the integrated optical densities within a linear and non-saturated range. Densitometric assessments and analysis were done using Image J software.

**Table 2: List of primaries antibodies used for antigens detections**

Antibody	Source	Class	Manufacture	Dilution		
				Western blotting	IHC	IF
<b>Anti-PKC-<math>\delta</math> (C-17)</b>	Goat	Polyclonal	Santa-Cruz sc-213-G	1/1000	1/100	1/100
<b>Anti-PKC-<math>\epsilon</math></b>	Rabbit	Polyclonal	Thermo Fisher Scientific PA5-13745	1/1000	1/100	1/100
<b>Anti-PKC-<math>\theta</math></b>	Rabbit	Polyclonal	Thermo Fisher Scientific PA5-13746	1/1000	1/500	1/100
<b>Anti-IRS-1</b>	Mouse	Polyclonal	Thermo Fisher Scientific PA5-20137	1/1000	N	N
<b>Anti-p-IRS (Ser 307)</b>	Rabbit	Polyclonal	Santa-Cruz sc-33956	N	N	1/500
<b>Anti-Akt1/2/3 (H-136)</b>	Rabbit	Polyclonal	Santa-Cruz sc-8312	1/1000	1/100	1/100
<b>Anti-p-Akt-1 (5.Ser473)</b>	Mouse	Monoclonal	Santa-Cruz sc-293125	1/1000	N	N
<b>Anti-SOCS-3</b>	Rabbit	Polyclonal	Santa-Cruz sc-9023	1/1000	N	N
<b>Anti-<math>\beta</math>-Actin (C4) HRP</b>	Mouse	Monoclonal	Santa-Cruz sc-47778	1/1000	N	N

HRP: Horseradish Peroxidase

PKC- $\delta$ : Protein kinase C delta

PKC- $\epsilon$ : Protein kinase C epsilon

PKC- $\theta$ : Protein kinase C theta

IRS-1: Insulin receptor substrate 1

SOCS-3: Suppressor of Cytokine Signaling -3

**Table 3: List of secondary antibodies used for antigens detection**

Antibody	Source	Detection System	Manufacture	Dilution used		
				Western blotting	IHC	IF
<b>Anti-mouse IgG Antibody</b>	Rabbit	Conjugated HRP	Novus Biologicals (A14)	1//5000	1/500	N
<b>Anti-Rabbit IgG Antibody</b>	Goat	Conjugated HRP	Novus Biologicals (P38)	1//5000	1/500	N
<b>Anti Goat IgG Antibody</b>	Rabbit	Conjugated HRP	Novus Biologicals (P28)	1//5000	1/500	N
<b>Anti-Goat IgG Antibody</b>	Donkey	Alexa Fluor (488)	Invitrogen (A11055)	N	N	1/1000
<b>Anti-Rabbit IgG Antibody</b>	Goat	Alexa Fluor (594)	Invitrogen (A11072)	N	N	1/1000

IgG: Immunoglobulin G

N: Not reported

HRP: Horseradish Peroxidase

### 2.2.2.6 Real-time Polymerase Chain Reaction

Total RNA was isolated from frozen liver tissue with Trizol reagent (Sigma) according to manufacturer's instructions. Nanodrop (Thermo-Scientific, Rockford, IL) was used to quantify yield RNA. First strand cDNA were synthesized using: Improm II reverse transcription kit from Promega, Madison, WI, and oligo dT primers. Real-time qPCR was performed using SYBR Green Master Mix and RT-PCR system (CFX96; BioRad Laboratories, Hercules, CA). The primers used were according to Table 3.

**Table 3: Primers Used for RT-PCR**

Genes	Direction	Primers (5'->3')
<b>nPKC Delta</b>	Forward	TGGGGTCTCAACAAGCAAGG
	Reverse	CTGCCACAGTGGTCACAGAA
<b>nPKC Epsilon</b>	Forward	TCAAGTTTGCACTTGCGTGG
	Reverse	CTCTGGCCACTGTTGGTGAT
<b>nPKC Theta</b>	Forward	TGAGGAGTTCTGTGCCAACG
	Reverse	CAAAGTGGTCGTAGCCCAGG
<b>GAPDH</b>	Forward	GCCTCCAAGGAGTAAGAGCC
	Reverse	AGGAGATGCTCGGTGTGTTG

nPKC: XM\_005669644.1 Sus scrofa protein kinase C, delta

nPKC: XM\_005662579.1 Sus scrofa protein kinase C, Epsilon

nPKC: XM\_005660026.1 Sus scrofa protein kinase C, Theta

GAPDH: NM\_001206359.1 Sus scrofa Glyceraldehyde-3-phosphate dehydrogenase

### 2.2.3 Statistical Analysis

The values that we reported are all expressed as mean  $\pm$  SEM (standard error of mean) from 3 to 5 independent experiments. Their analyses were done using

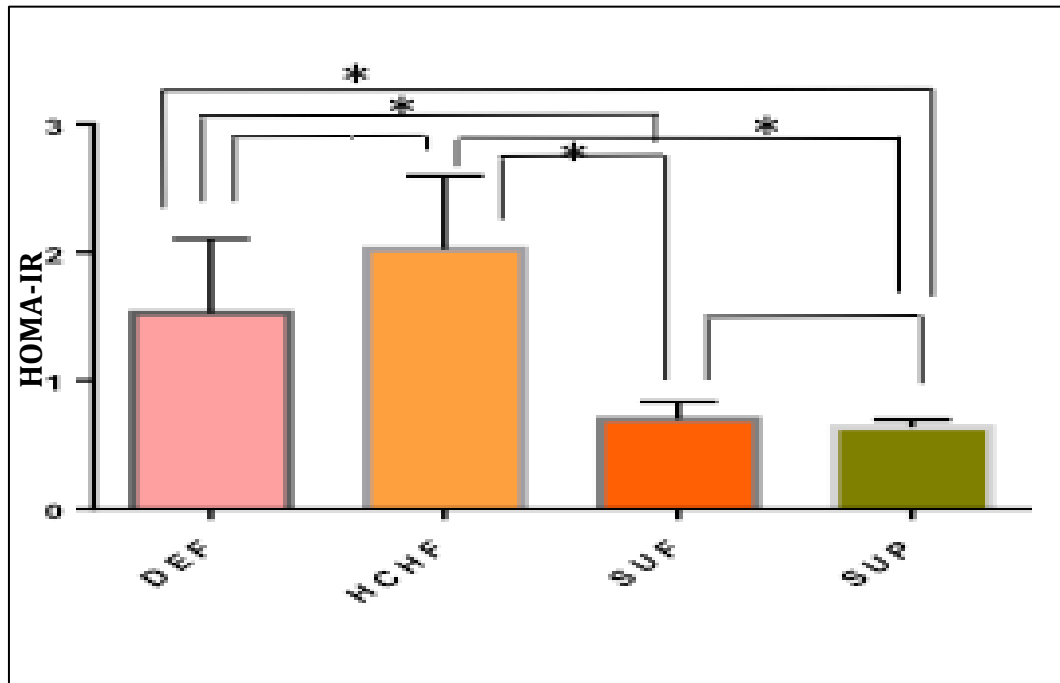
SPSS/PC+ for Windows (v.15.0 Chicago, IL, USA). Differences between groups were tested using Student's t-test or One-way between-subjects ANOVA analysis for parametric dependent variables with all tests assumptions verified, otherwise we considered the non-parametric analysis using Mann-Whitney U test or Kruskal-Wallis test. The consequent Tukey's post hoc test was used to determine means differences that were statistically significant. P-values  $< 0.05$  indicated a statistically significant difference between means.

## 2.3 Results

### 2.3.1 Effect of the diet

The diet has achieved the goal of the vitamin D status according to the results of levels of circulatory vitamin D, 25-hydroxyvitamin D<sub>3</sub> (25[OH] D<sub>3</sub>) in the serum of the swine after six months of the diet. There was a significant increase of 25[OH]D<sub>3</sub> level in Vit-D supplemented diet swine (mean value =  $47.6 \pm 13.8$  ng/dL) compared to respectively Vit-D sufficient swine (mean value =  $21.35 \pm 5.75$  ng/dL,  $p < 0.05$ , means difference = 26.25), high cholesterol and fructose swine (mean value =  $22.32 \pm 4.25$  ng/dL  $p < 0.05$ , means differences = 25.27), and Vit-D deficient swine (mean value =  $6.52 \pm 0.8$  ng/dl  $p < 0.05$  means difference = 41.08). Vit-D sufficient swine and high cholesterol and fructose swine had respectively significantly higher serum 25[OH] D<sub>3</sub> compared to Vit-D deficient swine ( $p < 0.05$ , means differences respectively = 14.83 and 15.80). Vit-D sufficient, high cholesterol High fructose swine group had similar serum 25[OH] D<sub>3</sub> ( $p > 0.05$  means difference = 0.97)

To access the insulin resistance, we calculated the homeostasis model assessment for IR (HOMA-IR) using the formula  $\text{HOMA-IR} = \text{Glycaemia (mg/dl)} * \text{Insulinemia (UI/u)} / 405$ . Glycaemia and insulinemia were obtained from the blood collected from the swine the day of their sacrifice. There was a significant increase in HOMA-IR values of DEF and HCHF compared to SUF and SUP while respectively DEF and HCHF, and SUF and SUP differences were not significant (Fig 8).



**Figure 8: Swine HOMA-IR at the day of euthanasia:** DEF (Vit-D deficient) and HCHF (high cholesterol and high fructose) compared to SUF (Vit-D sufficient) and SUP (Vit-D supplemented) while respectively DEF and HCHF, and SUF and SUP differences were not significant. (\*) Statistically significant  $p < 0.05$ . HOMA, homeostasis model assessment for IR, insulin resistance

### 2.3.2 Liver Histological aspect

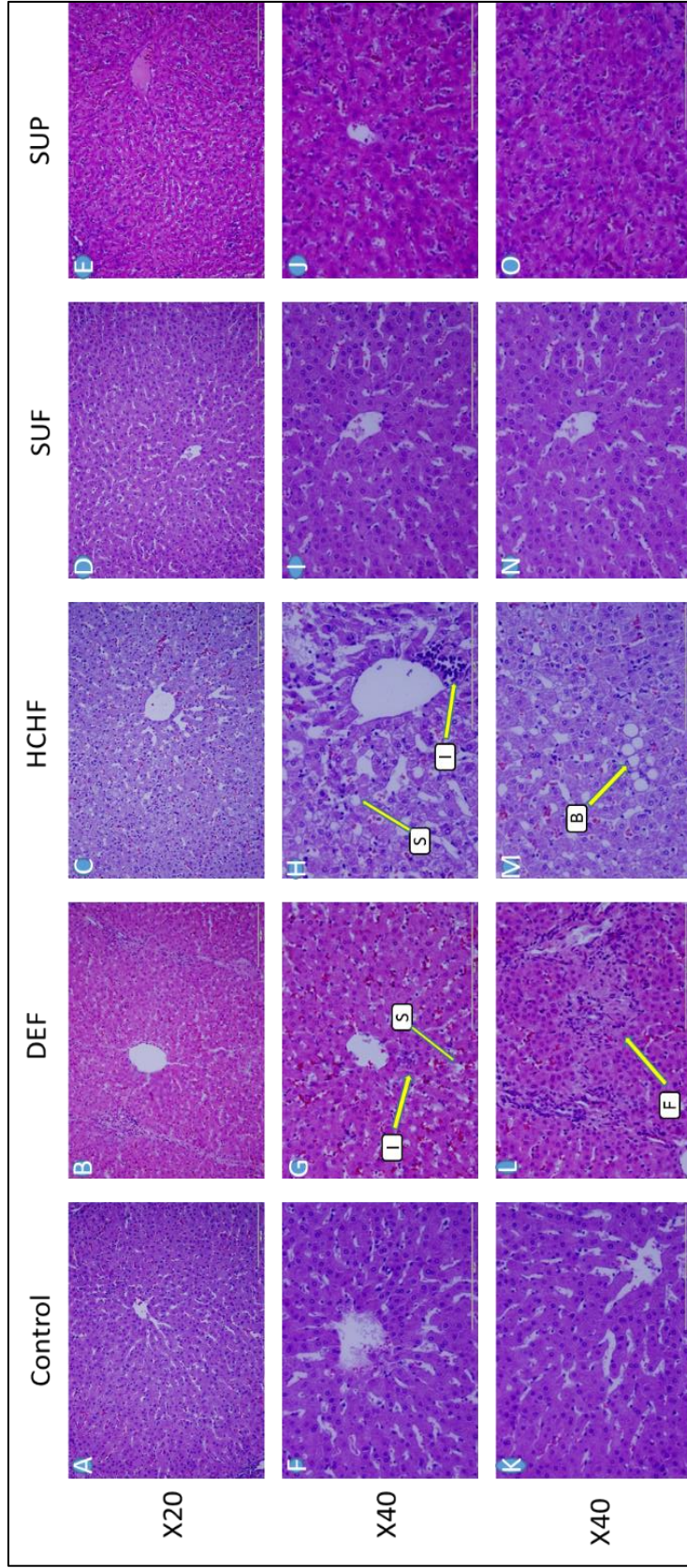
To evaluate the effect of the diet and vitamin D status on the liver of the swine HE, oil red O and trichrome staining were performed on liver tissue for formalin fixed paraffin embedded or liver frozen sections from various groups of swine. Three to 4 animals have been considered for analysis.

Hematoxylin-Eosin (HE) staining (Fig.9) was conducted seeking for the presence of signs of NAFLD and possible grading according to NAFLD activity score (NAS) and NAFLD fibrosis score (NFS) (table 1). It showed presence of steatosis and fibrosis in DEF: NAS (NAFLD Activity Score) S=1, I=1 and B= 0 Total NAS

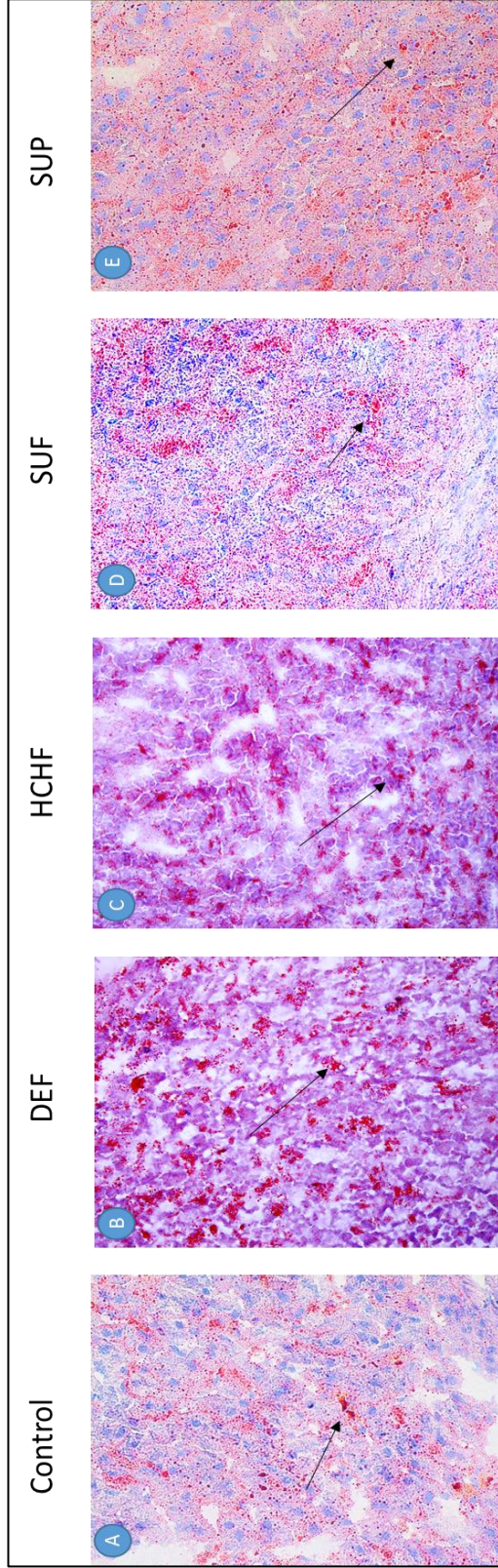
= 2/8 and NFS (NAFLD fibrosis Score= 1c. HCHF has shown NAS- S=1, I= 2, B=1 total NAS= 4/8. SUF and SUP had normal histology total NAS=0/8.

Oil red O staining (Fig 10) was performed to evaluate and compare liver tissue lipid concentration. It showed an increased accumulation of fat in the liver of DEF and HCHF compared to SUF, SUP, and the control.

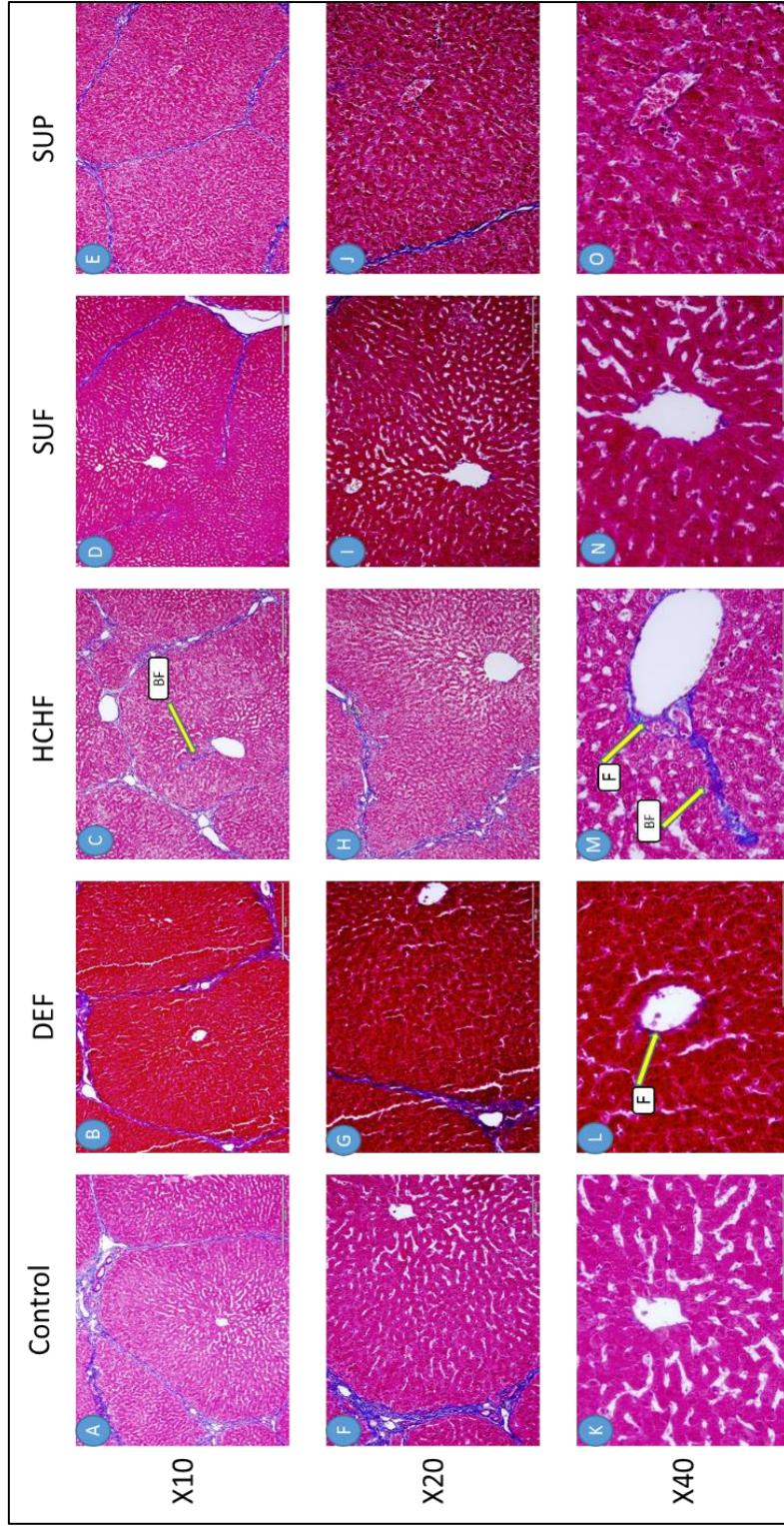
To elucidate the fibrosis state of the liver tissue, Masson trichrome staining was conducted to evaluate and compare the location and intensity of fibrosis (Fig11). It showed the presence of fibrosis in DEF, NFS= 1c and HCHF, NSF=3, compared to SUF and SUP which had an NFS = 0.



**Figure 9: Hematoxylin-Eosin (HE) stain** showed presence of steatosis and fibrosis in **B, G and L** DEF (Vit-D deficient and high cholesterol fed swine): NAS (NAFLD Activity Score) S=1, I=1 and B=0 Total NAS = 2/8 and NFS (NAFLD Fibrosis Score) = 1c and **C, H and M** HCHF (Vit-D sufficient, and high cholesterol and high fructose-fed swine) has shown NAS- S=1, I=2, B=1 total NAS= 4/8 . **D, I, N** for SUF (Vit-D sufficient and high cholesterol fed swine) and **E, J and O** for SUP (Vit-D supplemented and high cholesterol fed swine) had normal histology total NAS=0/8. Box S; steatosis, box I; lobular inflammation and box B; hepatocyte ballooning total NAS= 0/8



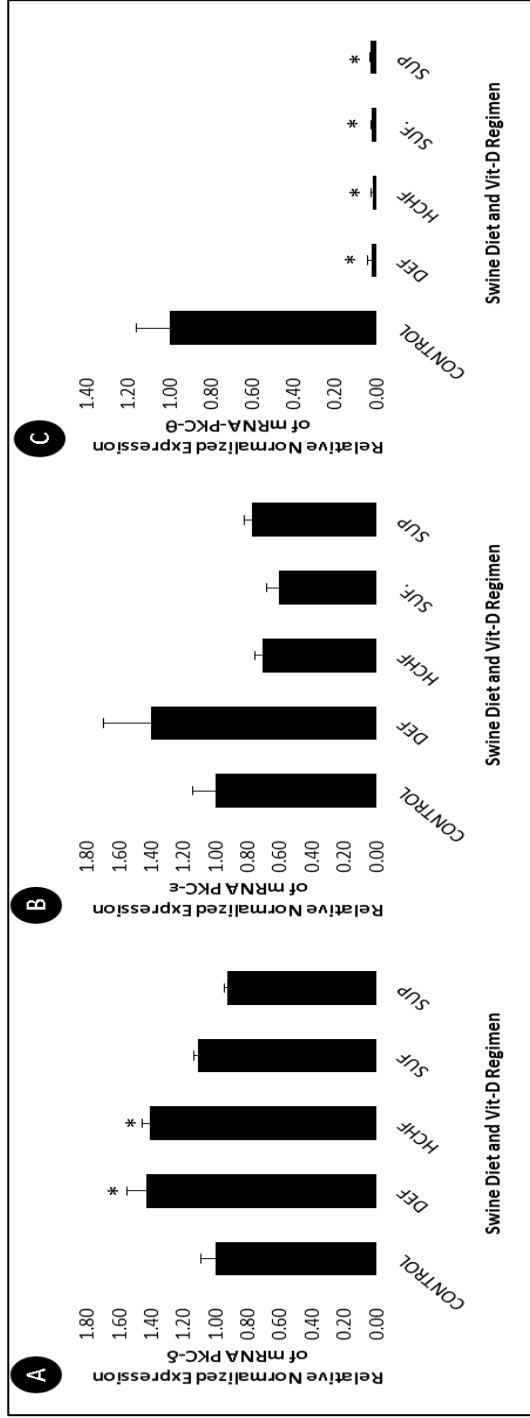
**Figure 10: Oil Red O Staining showed increased accumulation of fat on liver of DEF (Vit-D deficient and high cholesterol fed swine) and HCHF (Vit-D sufficient, and high cholesterol and high fructose-fed swine) compared to SUF (Vit-D sufficient and high cholesterol fed swine) and SUP (Vit-D supplemented and high cholesterol fed swine). Arrow= fat droplet**



**Figure 11: Masson Trichrome Staining showing fibrosis in B, G and L; DEF (Vit-D deficient and high cholesterol fed swine) NFS= 1c NAFLD, C, H and M; HCHF (Vit-D sufficient, and high cholesterol and high fructose fed swine) NSF=3, compared to D, I and N; SUF ( Vit-D sufficient and high cholesterol fed swine) and SUP (Vit-D supplemented and high cholesterol fed swine) NFS= 0. Box F; fibrosis, box BF: Bridging fibrosis. NFS: NAFLD Fibrosis Score**

### **2.3.3 Novel PKC expression**

To find which novel PKC is more relevant in liver IR and steatosis we performed real-time quantitative polymerase chain reaction on RNA extracted for liver tissue from swine of various groups (Fig 12). The result showed a constant upregulation of PKC- $\delta$  in DEF and HCHF compared to the SUF and SUP and the control. PKC- $\epsilon$  expression was inconsistent through experiments and groups of swine. There was no significant difference between means of relative normalized expression ( $p>0.05$ ). PKC- $\theta$  was down regulated in all treated groups: (DEF, HCHF, SUF, and SUP) compared to control, but in inconsistent manner between experiments and swine groups.

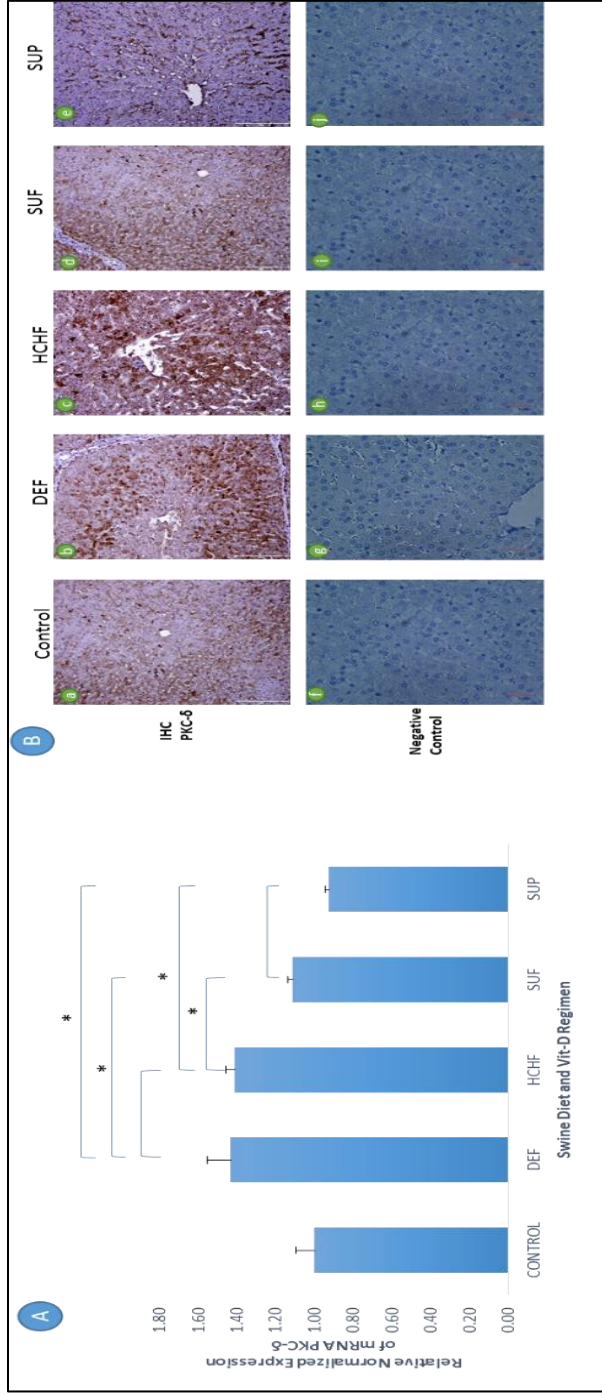


**Figure 12: RT-PCR of m-RNA expression in the liver** performed on extracted RNA from swine liver tissues of different groups (n=3). Groups are DEF (Vit-D deficient and high cholesterol fed swine), HCHF (Vit-D sufficient, and high cholesterol and high fructose fed swine), SUF (Vit-D sufficient and high cholesterol fed swine), SUP (Vit-D supplemented and high cholesterol fed swine), and the control. PKC; protein kinase C, data are reported as means of relative normalized expression of mRNA to GAPDH  $\pm$  SEM, \* :  $p < 0.05$  compared to the control.

#### **2.3.4 PKC delta expression**

Since PKC-delta was the most prevalent the NAFLD in our experiment, we conducted and immunohistochemistry liver tissues to evaluate the expression PKC- $\delta$  in the liver of various groups of swine (Fig 13). RT- qPCR performed on extracted mRNA from livers of control showed a significant increase in both DEF and HCHF compared to SUF and SUP.

Immunohistochemistry of PKC- $\delta$  was performed on frozen sections swine liver. It showed an up-regulation in PKC- $\delta$  expression in DEF and HCHF compared to SUF and SUP.



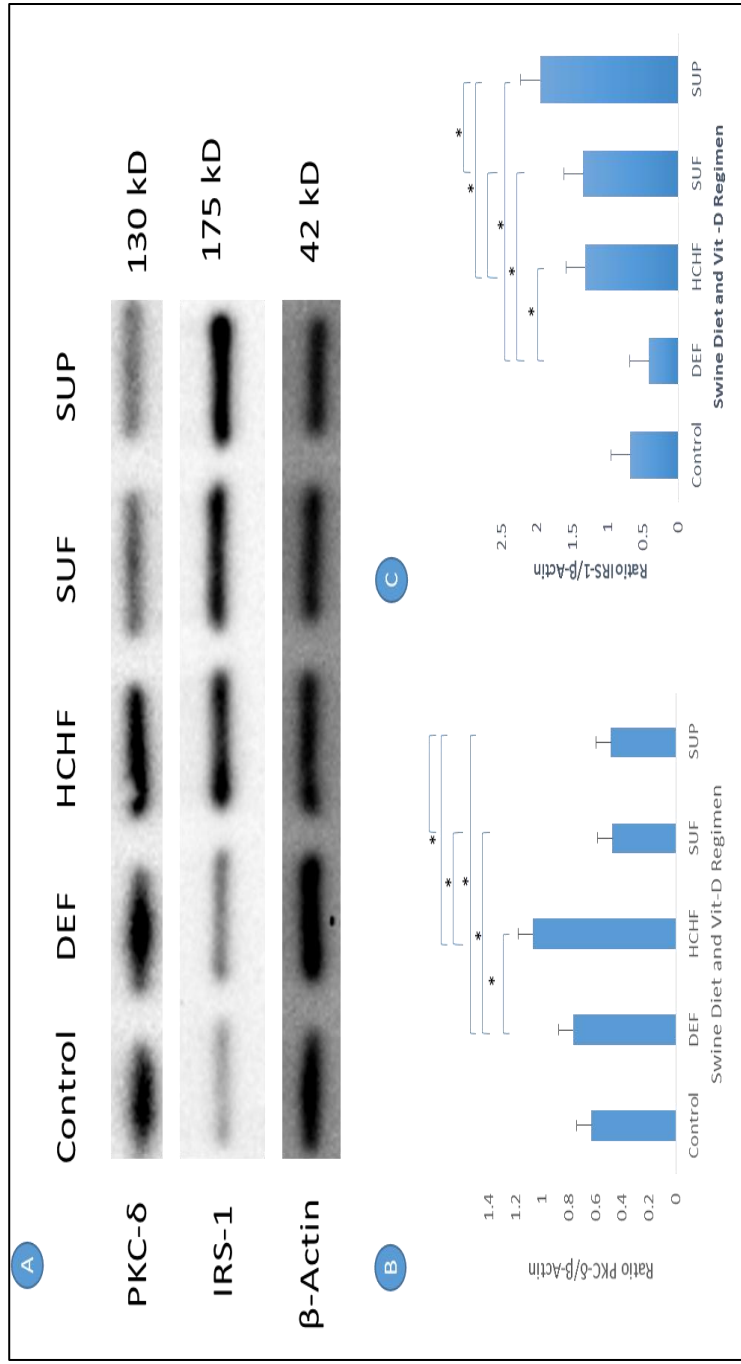
**Figure 13: PKC- $\delta$  is consistently upregulated in the liver with higher HOMA-IR.** A, RT- qPCR performed on extracted mRNA from livers of control, DEF (Vit-D deficient and high cholesterol fed swine), HCHF (Vit-D sufficient, and high cholesterol and high fructose-fed swine), SUF ( Vit-D sufficient and high cholesterol fed swine) and SUP (Vit-D supplemented and high cholesterol fed swine). \*  $p \leq 0.05$ . B, Immunohistochemistry of PKC- $\delta$  was performed on formalin fixed paraffin embedded liver sections from swine groups (n=3).

### **2.3.5 PKC delta and IRS-1 signaling**

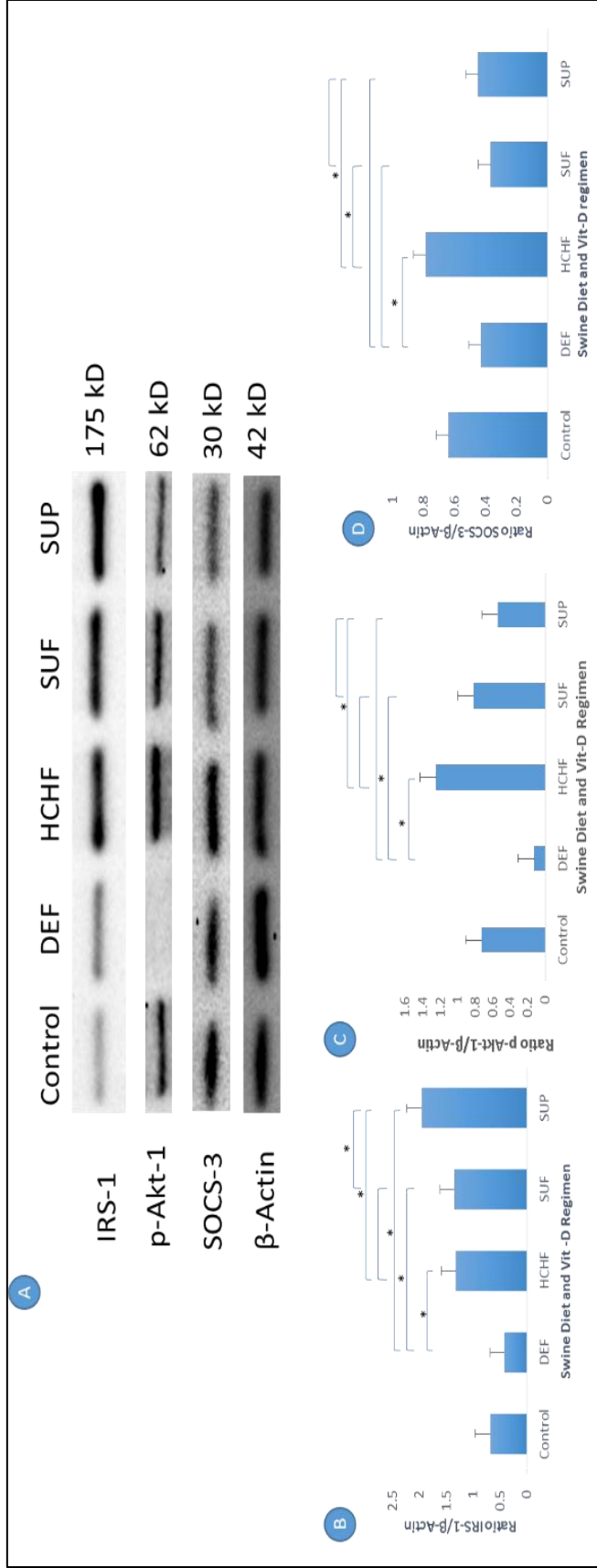
In order to determine the effects of PKC-delta on IRS, we evaluated and compared the expression of PKC- $\delta$  and correlated them with the expression of IRS in proteins obtained from the liver lysate of swine in various groups (Fig 14). The Western blotting of swine liver lysate total protein for PKC- $\delta$  and IRS-1 and semi-quantitative analysis PKC- $\delta$  showed a significant up-regulation in DEF and HCHF compared to SUF and SUP. There was also a significant up-regulation of PKC- $\delta$  in HCHF compared to DEF while its expression was similar in SUF and SUP. Semi-quantitative analysis IRS-1 Western blotting showed a down-regulation of IRS-1 in DEF compared to HCHF, SUF, and SUP. HCHF and SUF were respectively down regulated compared to SUP while the expression of IRS-1 is about the same in HCHF and SUF.

To determine what pathway is involved in the serine phosphorylation of IRS to induce IR with evaluated the expression of p-Akt/PKB and suppressor of cytokine signaling -3 (SOCS-3).

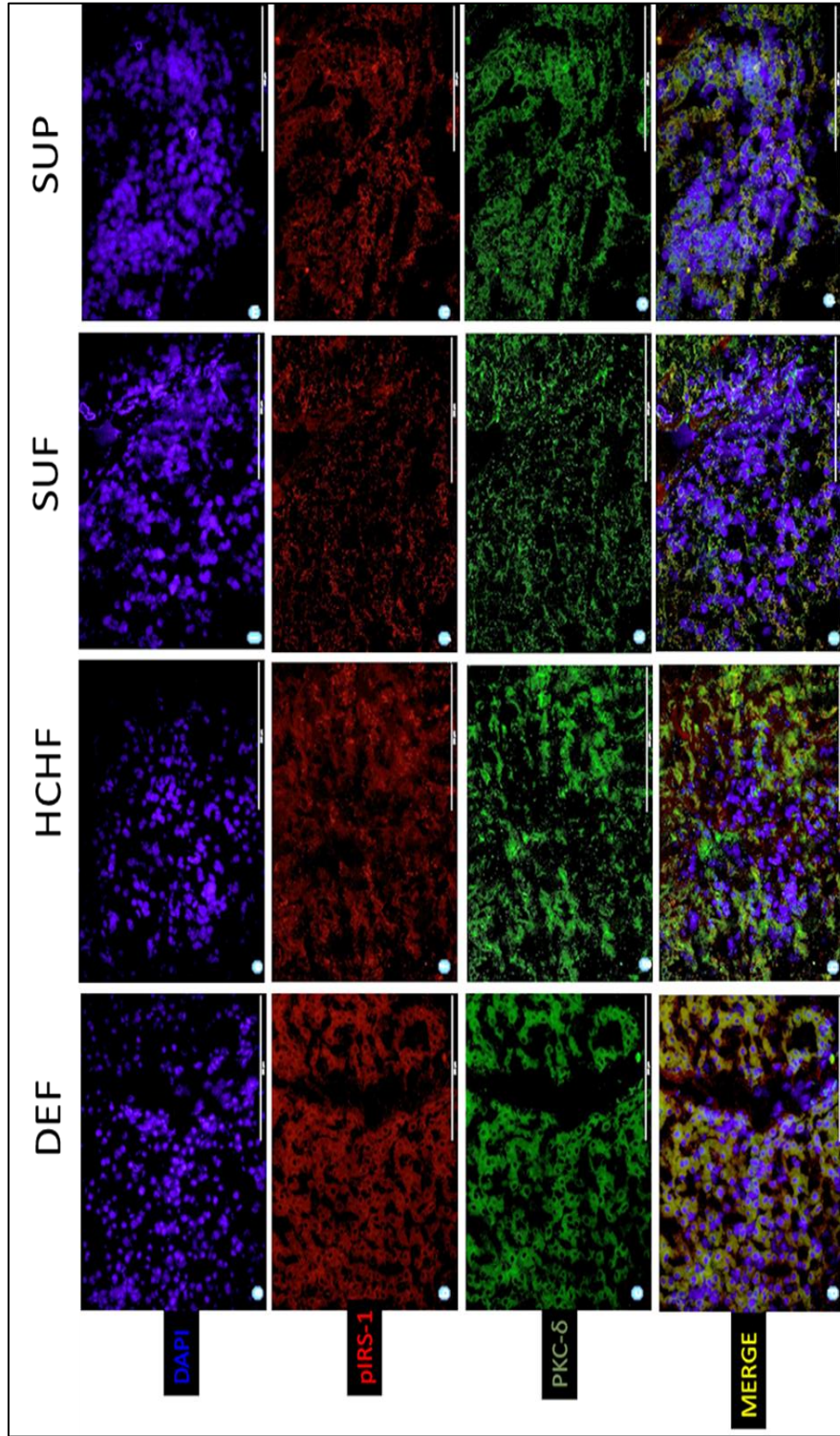
We performed double immunofluorescence staining on liver frozen section to evaluate the expression and co-localization of p-IRS-1 and PKC- $\delta$  to determine the effect of activation of PKC- $\delta$  on IRS (Fig 16). It showed an increased expression of pIRS-1 in DEF and HCHF compared to SUF and SUP. PKC- $\delta$  expression was also increased in DEF and HCHF compared to SUF and SUP. The colocalization of pIRS and PKC- $\delta$  is more shown in DEF compared respectively to HCHF, SUF, and SUP. Even though there is increased expression of pIRS-1 and PKC- $\delta$ , their colocalization is lower compared to DEF.



**Figure 14: A, Western blotting of swine liver lysate total protein for PKC-δ and IRS-1 B, Semi quantitative analysis PKC-δ C, Semi-quantitative analysis IRS-1.** DEF, Vit-D deficient and high cholesterol fed swine, HCHF, Vit-D sufficient, and high cholesterol and high fructose-fed swine, SUF, Vit-D sufficient and high cholesterol fed swine, SUP, Vit-D supplemented and high cholesterol fed swine, PKC-δ, protein kinase C delta, IRS-1, Insulin receptor substrate 1.



**Figure 15: A, Western blotting of swine liver lysate total protein for IRS-1, p-Akt-1 and SOCS-3 with  $\beta$ -Actin for loading control, B, Semi quantitative analysis of IRS-1 C, Semi quantitative analysis of p-Akt-1 D, Semi quantitative analysis of the expression of SOCS-3. DEF, Vit-D deficient and high cholesterol fed swine, HCHF, Vit-D sufficient, and high cholesterol and high fructose-fed swine, SUF, Vit-D sufficient and high cholesterol fed swine, SUP, Vit-D supplemented and high cholesterol fed swine IRS-1, Insulin receptor substrate, p-Akt-1, phosphorylated Akt-1, SOCS-3 signal Suppressor of cytokines signaling. \*,  $p \leq 0.05$ .**



**Figure 16: Double Immunofluorescence** performed on frozen sections of liver from groups of swine (n=3). DEF, Vit-D deficient and high cholesterol fed swine, HCHF, Vit-D sufficient, and high cholesterol and high fructose-fed swine, SUF, Vit-D sufficient and high cholesterol fed swine, and SUP, Vit-D supplemented and high cholesterol fed swine, pIRS, Serine phosphorylated (307) insulin receptor substrate, PKC-δ, protein kinase C delta. DAPI (blue), pIRS-1 (red), PKC-δ (green)

### **3 Discussion**

#### **3.1 Study outcomes and limitations**

This study aim to identify the most relevant novel PKC involved in the serine phosphorylation in IR that leads to NAFLD. We purposefully limited our investigation to novel PKC owing to the fact that DAG solely activates them. We did not evaluate the classic PKC – activated by DAG and Ca<sup>2+</sup> - nor atypical PKC.

#### **3.2 Experimental model**

Most findings in mechanistic and therapeutic approaches of NAFLD have been conducted on rodents and fishes (137). Hence, a humanoid model of swine provides relevant findings for a translational purpose. Swine has anatomical, physiological and dietary similarity to a human. We have used the Yucatan mini-swine because they are relatively easy to handle. They also develop metabolic syndrome actually when they are put on westernized diet (138). We observed when fed with westernized diet; Yucatan mini swine develop MetS as well as NAFLD histological features albeit Neeb *et al.* found that Ossabaw swine develop MetS features faster and more consistently compared to Yucatan swine (139, 140). Methionine choline-deficient (MCD) diet has been commonly used to induce NAFLD in an animal model but fails to induce IR – it also has no parallel with human dietary (141, 142). The data from such experiments are difficult, if not impossible to be translated into medical practice. Our model remedied the issue using cholesterol diet with the addition of fructose in an isocaloric manner, which portrays the western world diet. We found that hyper cholesterol diet alone does not induce IR or features of NAFLD but in Vit D deficient swine only. Only when

fructose is added to the high cholesterol diet, IR and NAFLD even with steatohepatitis may occur despite the sufficient Vit D status. These findings are similar to data of Neeb *et al.*'s in regard of swine, that high-fat diet alone hardly induces IR or NAFLD feature. Other studies also found that high fructose diet alone does not induce IR when consumed in an isocaloric manner, but when it is added to the high-fat diet (89). Fructose and high-fat diet are preeminent inducing IR and NAFLD. Although it may bring crucial data about the effect of fructose rich diet, we did not consider in our research design cases where swine were fed on high fructose diet only, since it does not represent a westernized diet.

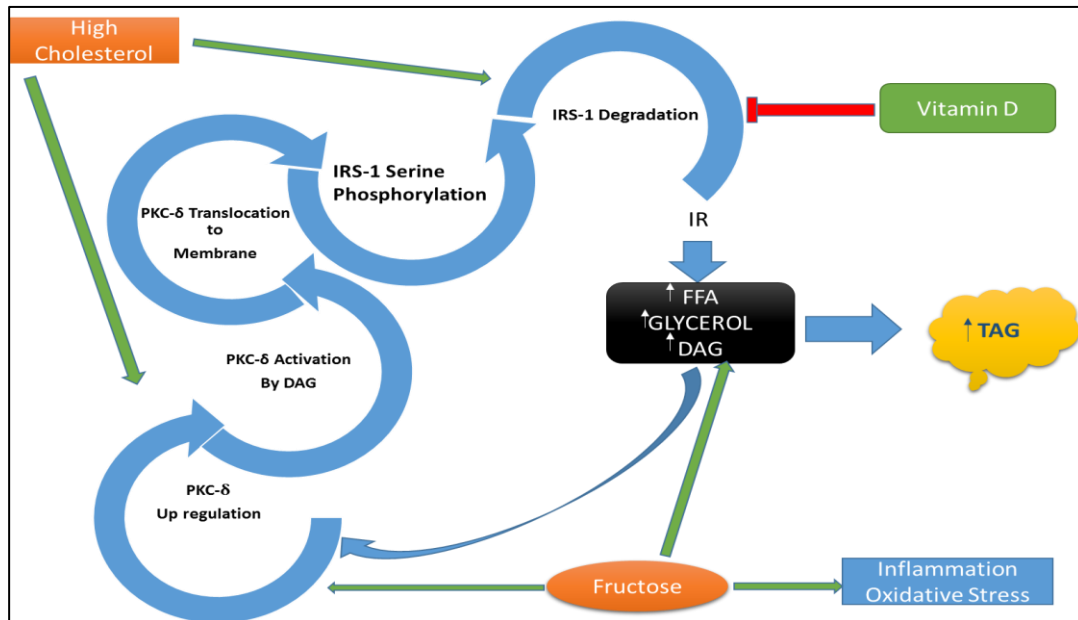
### **3.3 Novel PKC and IRS-1 interaction**

Insulin resistance through serine phosphorylation of IRS-1 is well known (143), but the more relevant isoform nPKCs for this reaction is unknown. Our experiment found that nPKC delta might play that role in the pathogenesis of IR-inducing NAFLD. Notably, we found that PKC delta is up-regulated in IR swine (DEF and HCHF) compared to insulin sensitive ones (SUF and SUP). Interestingly, the up-regulation of PKC delta is associated with down-regulation of IRS-1 in vitamin D deficient swine; but not vit D sufficient regardless of the IR status, suggesting that whether vit D up-regulates IRS-1 or protects against its degradation. Greene *et Al* with rodent fed with MCD found that PKC delta is involved in steatosis but not in fibrosis (142). Swine on high cholesterol diet, with high fructose incorporated diet, still develop IR and NAFLD with fibrosis despite the protective effect of Vit D. This suggests IR occurrence in high fructose diet pass by a different pathway than IRS-1 degradation or it causes its inhibition. Further exploration of the IRS-1

pathway is needed to clarify these findings. It is established that high fructose diet induces a pro-inflammatory environment that can induce IR through the action of cytokines like IKK and JNK that is also found in other studies (89, 144, 145).

#### **4 Conclusion**

Our findings suggest that IR that induces NAFLD might follow the pattern in Fig 17. During high cholesterol diet in Vit D deficient swine, there is an up-regulation an increase of precursor substrate of DAG such as FFA and glycerol. The increase of DAG in the hepatocyte induces the up-regulation and the translocation the cytoplasmic membrane of PKC- $\delta$ . IRS-1 is serine phosphorylated by PKC- $\delta$  owing their colocalization. IRS-1 is probably degraded in the process and end-up incapable to carry out its downstream signaling, which causes the IR. Fructose addition to the high cholesterol diet seems to exacerbate up-regulation of PKC- $\delta$ . Vitamin D seems to protect against the IRS-1 degradation in high cholesterol diet but fails to protects from IR and fibrosis caused by fructose addition to the diet. Fructose-rich diet might also be source inflammation that underlies the progression from NAFLD to NASH.



**Figure 17: Protein kinase C- $\delta$  in Insulin resistance and NAFLD; effect of diet and vitamin D:** Reaction cascade resulting in IR pass first by up-regulation and translocation of PKC- $\delta$  by DAG. PKC- $\delta$ , in turn, inhibits IRS-1 (probably by degradation) to hinder the downstream reaction of the insulin signaling. High cholesterol and high fructose diet contribute to the phenomenon while Vit D protects against it. High fructose diet also may induce IR through inflammation or oxidative stress.

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