AMERICAN UNIVERSITY OF BEIRUT

POSSIBLE IMPLICATIONS OF TOLL-LIKE RECEPTOR 4 (TLR4) MUTATIONS IN PATIENTS WITH NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

by
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ABSTRACT
OF THE THESIS OF

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Title: Possible Implications of Toll-like receptor 4 mutations in patients with Non-Alcoholic Fatty Liver Disease

Non-Alcoholic Fatty Liver Disease (NAFLD) is defined as the accumulation of fats, mainly triglycerides, in the liver exceeding 5% of its weight in the absence of any significant alcohol consumption. It comprises a wide spectrum of diseases that include non-alcoholic fatty liver (NAFL) characterized by simple steatosis and non-alcoholic steatohepatitis (NASH) characterized by steatosis with inflammation and fibrosis. The prevalence of NAFLD has increased in the recent years reaching 37.3% in 2019 due to the rise in metabolic disorder. NAFLD is a multifactorial disease that is associated with several factors of the metabolic syndrome including obesity, type-2 diabetes, and insulin resistance. Several genes also affect the development and pathogenesis of NAFLD such as Patatin-like phospholipase domain-containing protein 3 (PNPLA3), monocyte differentiation antigen CD14 (CD14), tumor necrosis factor (TNF), and toll-like receptor 4 (TLR4).

In the current study, we examined the role of TLR4 polymorphisms in the progression of NAFLD in a sample of Lebanese individuals, and we attempted to identify a genotype-phenotype correlation between TLR4 gene mutations and the fibrosis stage of the patients. NAFLD patients (30) and healthy controls (19) underwent a number of clinical tests and examinations including liver function tests, hematological tests and lipid profiling. Moreover, blood samples were collected form all individuals for nucleotide base changes in the TLR4 gene. DNA extraction was performed followed by PCR amplification of the 3 exons, followed by agarose gel electrophoresis and sequencing of the amplicons to identify mutations and polymorphisms in patients and healthy individuals.

The sequencing of TLR4 gene revealed the presence of many heterozygous missense mutations and frameshifts in exon-1 and 3. No frame shifts were detected in exon-2. The majority of mutations were located in exon-3 encoding the extracellular domain involved in ligand recognition and binding. Our findings were not conclusive when attempts to correlate NAFLD with obesity, diabetes or age were examined. Furthermore, we have detected in our sample: a) the two mutations Thr399Ile and Asp299Gly reported to exhibit a protective role in line with literature; b) A new homozygous mutation Gly249Val, in exon -3 present in patients with F0 fibrosis stage, may have a protective role in NAFLD progression; and c) frameshift mutations localized

between positions 241-251 identified in most patients with F0 stage and controls suggesting a possible protective role, that may be attributed to the disruption of ligand binding or recognition. To sum up, TLR4 gene is highly polymorphic making it difficult to correlate the genotype with the NAFLD progression and fibrosis stage. The role of other genes may not be excluded and should be investigated to delineate the role of these genes in disease progression and fibrosis.

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ABBREVIATIONS

AA: amino acid

ABCC2: ATP-binding cassette, sub-family C, member 2

ALT: alanine transaminase

AP-1: activator protein 1

AST: aspartate transaminase

BMI: body mass index

BAMBI: bone morphogenic protein and active membrane bound inhibitor

BP: base pair

BUN: blood urea nitrogen

CD14: monocyte differentiation antigen CD14

CT: computed tomography

CXCL10: Interferon gamma-induced protein 10

CAD: coronary artery disease

CHF: congestive heart failure

DAMPs: damage-associated molecular patterns

DD: death domain

DL: dyslipidemia

DM: diabetes mellitus

DM2T: Type 2 diabetes mellitus

EV: extracellular vesicles

ER: endoplasmic reticulum

E-1: exon 1

E-2: exon 2

E-3: exon 3

FGF21: fibroblast growth factor 21

FFA: free fatty acids

GGT: gamma-glutamyltransferase

GCKR: Glucokinase regulatory protein

GSH: glutathione

HSCs: hepatic stellate cells

HTN: hypertension

HDL: high-density lipoprotein

HMG: high mobility group

HIV: human immunodeficiency virus

iNKT: invariant natural killer T cells

ICD: International Classification of Diseases

IL-6: interleukin-6

IRS: insulin receptor substrate

IRAK-4: IL-1 receptor-associated kinase-4

IκB: Inhibitor of NF-κB

IKK: IκB kinase

IRF3: interferon regulatory factor 3

PNPLA3: Patatin-like phospholipase domain-containing protein 3

IR: insulin resistance

IRS1: Insulin receptor substrate 1

TLR4: toll-like receptor 4

IRB: Institutional Review Board

JAK: Janus kinase

KCs: Kupffer cells

LFTs: liver function tests

LPC: lysophoshoatidylcholine

LPS: lipopolysaccharides

LRRs: leucine-rich repeats

LBP: LPS-binding protein

LDL: low-density lipoprotein

MyD88: myeloid differentiation factor 88

MAL: MyD88-adaptor-like

MD2: myeloid differentiation protein 2

MAPK: mitogen-activated protein kinase

mg: milligram

MRI: magnetic resonance imaging

NAFL: alcoholic fatty liver

NASH: non-alcoholic steatohepatitis

NF-κB: nuclear factor kappa B

Ob-Rb: leptin receptor

PAMPs: pathogen-associated molecular patterns

PT: prothrombin time

PL: platelet count

PPRE: PPAR response elements

ROS: reactive oxygen species

RAS: renin-angiotensin system

TNF: tumor necrosis factor

TIR: Toll-interleukin-1 receptor

TRAIL: TNF-related apoptosis inducing ligand

TRIF: TIR domain-containing adaptor inducing interferon-β

TRAM: TRIF-related adaptor molecule

TNFR: tumor necrosis factor receptor

TRAF6: TNFR-associated factor 6

TAK1: TGF-β-activated kinase 1

TG: triglycerides

TE: transient elastography

TFR: transferrin receptor

TM6SF2: transmembrane 6 superfamily member 2

VLDL: very low-density lipoprotein

VLCS: very long-chain acyl-CoA synthetase

WBC: white blood cell count

CHAPTER 1

INTRODUCTION

1.1.The Liver: An Essential Organ of the Body

The liver is the second largest organ in the body following the skin. It is located in the upper right-hand segment of the abdominal cavity, shaped like a cone, and weighs 3 pounds (Johns Hopkins, n.d). It is divided into lobes that are separated by a thin connective tissue layer (McCuskey, 2012). The liver is made up of several cell types including hepatocytes that are responsible for secreting aspartate transaminase (AST) and alanine transaminase (ALT). The synthesis of cholesterol and triglycerides occurs in the hepatocytes where the end product is released into the blood stream as very low-density lipoprotein (VLDL) (Phang-Lyn & Llerena, 2022; Rui, 2014). Moreover, the liver executes specific metabolic processes including the biotransformation and the generation of the majority of intermediate metabolites such as triglycerides and cholesterol.

The liver is a crucial frontline immune tissue that detects, traps, and removes bacteria, viruses, and macromolecules that enter the body through the gut (Kubes & Jenne, 2018). It contains the highest number of phagocytes in the body, thus serving as a crucial barrier between the human body and the outside world. The liver's default immune status is immunotolerant due to the significant number of foreign but innocuous molecules (e.g., food antigens) that enter the liver by the portal blood; however, the liver generates a strong and rapid immune response following injuries and infections. The immune response is mediated mainly by invariant natural killer T cells (iNKT). This delicate balance of immunity and tolerance is critical to the liver’s

function, where any imbalance may cause chronic infections, malignancies, and tissue damage (Kubes & Jenne, 2018).

1.2. Non-Alcoholic Fatty Liver Disease (NAFLD): Overview

Non-Alcoholic Fatty Liver Disease (NAFLD) is the most common cause of chronic liver disease (Li et al., 2018). It is defined as fat accumulation in the liver to a level exceeding 5% of its weight with no significant chronic alcohol consumption (>30 g per day for men and >20 g for women, equivalent to 1-2 glasses of alcohol) (Papatheodoridi & Cholongitas, 2018). According to the International Classification of Diseases (ICD), the prevalence of NAFLD increased from 21.9% to 37.3% from 1991 to 2019. This increase has been attributed to the surge in obesity and type 2 diabetes mellitus in the recent years (Le et al., 2022; Wang & Malhi, 2018; Younossi et al., 2018). NAFLD is strongly linked to obesity, type 2 diabetes mellitus, insulin resistance, hypertension, dyslipidemia, and other cardiovascular risk factors (Huang, 2009).

NAFLD encompasses a wide spectrum of diseases that vary between two subtypes: nonalcoholic fatty liver (NAFL) characterized by simple steatosis with the absence of hepatocellular injury, and non-alcoholic steatohepatitis (NASH) characterized by steatosis with inflammation and hepatocellular injury (figure 1) (Wang & Malhi, 2018). Hepatic Steatosis is the accumulation of fats in the liver, mainly triacylglycerols in liver cells (Briseño-Bass et al., 2019). Most patients with NAFLD present to the hospital with NAFL whereas the minority are diagnosed with NASH (Mann et al., 2020).

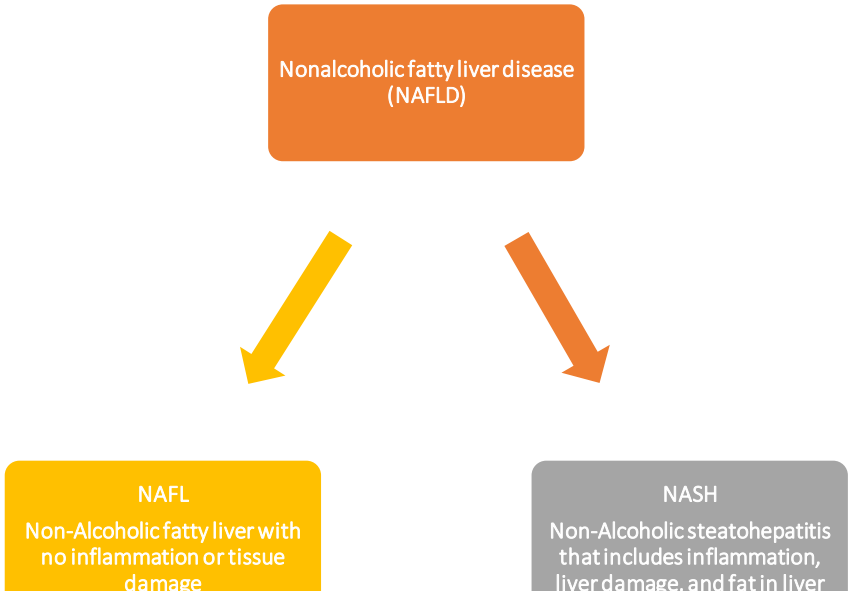

Figure 1- NAFLD and its subtypes, their characteristics and differences.

NAFLD is characterized by hepatic fibrosis, which is the thickening and scarring of liver tissues. A study conducted by Briseño-Bass et al showed that around 20% of NAFLD patients have increased liver fibrosis that may lead to liver cirrhosis (figure 2) (Briseño-Bass et al., 2019). The fibrotic state of the liver is divided into 5 stages ranging from F0-F4 (Table 1). F0 is the normal stage with no fibrosis. Stage F1 indicates early fibrosis with no septation, described as peri-portal, as it spares the portal system. Stage F2 represents moderate fibrosis with slightly more septa being evident compared to stage F1 but shares the same characteristics. Stage F3 represents severe fibrosis characterized by numerous septa, and stage F4 is the state of cirrhosis (Poynard et al., 2012).

Table 1- The 5 stages of liver fibrosis in NAFLD patients.

Score	Histologic stage of liver fibrosis
F0	No fibrosis
F1	Early fibrosis: periportal fibrosis without septa
F2	Moderate fibrosis: periportal fibrosis with rare septa
F3	Severe fibrosis: numerous septa without cirrhosis
F4	Cirrhosis: lobular regeneration between septa

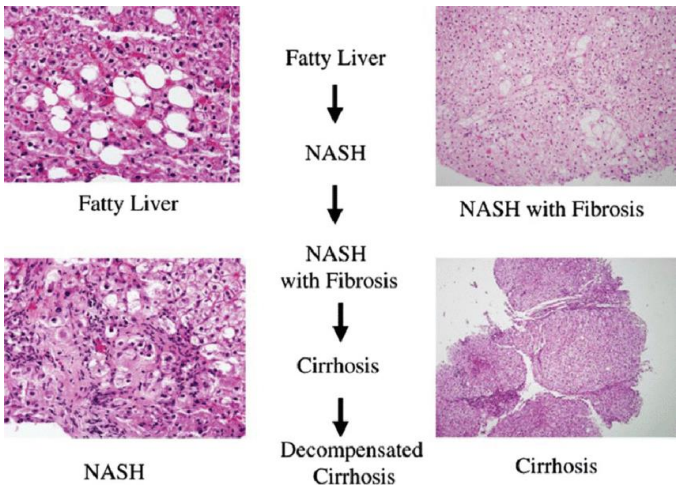

Figure 2- Progression of NAFLD from fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH), followed by cryptogenic cirrhosis (Sharp et al., 2018)

The degree of fibrosis is identified quantitatively by the 'Fibro Sure Score', a value that estimates the degree of liver damage (fibrosis and inflammation) in the different liver diseases including NAFLD (Zarghamravanbakhsh et al., 2021; Zeremski et al., 2014). It is estimated depending on age, gender, and a group of 6 biomarkers (a2-macroglobulin, g-glutamyl transpeptidase, total bilirubin, haptoglobin, apolipoprotein A1, and alanine aminotransferase) (Sweet et al., 2017). Another potential diagnostic biomarker of fibrosis is the serum levels of ALT, AST and gamma-glutamyltransferase

(GGT). Although Xie et al. showed that abnormally high levels of ALT, AST and GGT indicate liver damage due to fibrosis, recent studies demonstrate that ALT is not a reliable marker of liver disease (Xie et al., 2017).

NAFLD, including NAFL and NASH, is mostly asymptomatic. However, in some cases, however, individuals may develop a few symptoms including fatigue or discomfort in the upper right quadrant. Other symptoms such as weight loss and easy bruising manifest after years of disease progression (Kopec & Burns, 2011). Therefore, NAFLD represents a major challenge for the healthcare sector due to its difficulty in diagnosis, complex pathogenesis, and absence of approved therapy (Neuschwander-Tetri, 2017).

1.3. NAFLD Diagnosis

The initial diagnosis of NAFLD depends on liver function tests (LFTs) which assess the levels of liver enzymes released in blood. LFTs are followed by diagnostic imaging to confirm the presence of steatosis. For more accurate diagnosis and prognosis, liver biopsy is further required for some patients (Adams et al., 2005).

1.3.1. Liver Function Tests

NAFLD is initially diagnosed by performing liver function tests to determine the serum level of aminotransferases (AST and ALT). Serum AST and ALT levels are elevated (>25-50 IU/L) in a significant number of NAFLD patients (50-90%) (Sanyal, 2002; Yin & Tong, 2009). AST and ALT are normally present in hepatocytes. Liver necrosis and hepatocyte damage causes AST and ALT levels to increase (Yin & Tong, 2009). Hence, AST and ALT are surrogate markers of liver injury. A potential marker of

NAFLD is the high triglyceride serum levels. Although the mechanism of fat buildup in NAFLD is complex and poorly understood, triglycerides account for the majority of intrahepatic lipids (Galiero et al., 2021). Triglyceride accumulation may result either from the excessive fatty acid uptake from the blood which causes lipid deposition or from the disruption of mitochondrial β-oxidation which affects lipid removal (Kawano & Cohen, 2013).

Serum GGT is a potential NAFLD marker as it is present in elevated amounts in NAFLD patients. GGT activity is affected by the environmental factors that are associated with NAFLD such as BMI, smoking, alcohol consumption, serum glucose levels, and lipid metabolism (Fujii et al., 2020). GGT is an indicator of alcohol intake due to its role in the homeostasis of glutathione and the detoxification of xenobiotics (Lala et al., 2021; Whitfield, 2001).

A serological marker for NAFLD detection is fibroblast growth factor 21 (FGF21), one of the main regulators of hepatic glucose and lipid metabolism. Multiple studies have shown that FGF21 levels are high in obese individuals, and are associated with markers of insulin resistance. A study by Li et al. showed that FGF21 serum level was higher in NAFLD patients compared to controls (402.38 pg/ml and 198.62 pg/ml respectively) suggesting that FGF21 is a potential biomarker for NAFLD (Li et al., 2010). The increase in FGF21 levels is attributed to lipotoxicity, oxidative stress, and endoplasmic reticulum stress that are induced by NAFLD (Rusli et al., 2016). However, one of the limitations of FGF21 is the inability to distinguish between NASH and NAFL. Its levels are not yet correlated with liver histological assessment of liver biopsy, the most important detection method of NAFLD (Morris-Stiff & Feldstein, 2010).

1.3.2. Imaging

For the precise diagnosis of NAFLD in individuals having normal or elevated LFTs, imaging techniques are required. Typical examples are ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI), or transient elastography (TE) (Saadeh et al., 2002; Stern & Castera, 2017). These imaging techniques, however, may not distinguish between NAFL and NASH (Farrell & Larter, 2006).

1.3.2.1. Ultrasound

Ultrasound is considered the first line of imaging due to its safety, accuracy, and reliability in fatty liver diagnosis. NAFLD patients show an increased echogenicity represented by a light-colored liver (Farrell & Larter, 2006). Ultrasound has a low cost, high sensitivity, and high specificity (84% and 93% respectively) (Hernaez et al., 2011); however, it is not accurate in determining the liver steatosis stage (Farrell & Larter, 2006; Hernaez et al., 2011).

1.3.2.2. Computed Tomography (CT) Scan

CT scan is a method that consists of taking X-ray images from different angles and combining them using a computer processor to get a cross-sectional, slice like image of different parts of the body organs. The liver CT scans of NAFLD patients show increased radiolucency where the liver is hypodense and darker than the spleen (Farrell & Larter, 2006). While CT scan has high specificity (100%) in detecting moderate hepatic steatosis, it cannot detect hepatic inflammation (Dinani et al., 2020).

CT scan is not used as the first line of detection due to it being expensive compared to the ultrasound imaging, and due to the risk of exposing patients to radiation (Dinani et al., 2020).

1.3.2.3.Magnetic Resonance Imaging (MRI)

MRI is the most accurate imaging technique. It utilizes a magnetic field in the presence of radio waves that penetrate body organs from different angles to generate a detailed image. It is used to detect and quantify fatty liver change. However, MRI is unable to differentiate between the stages of steatosis and fibrosis unless advanced fibrosis and cirrhosis are present (Abd El-Kader & El-Den Ashmawy, 2015). Moreover, when the fat distribution appears heterogeneous on a CT scan (cannot be differentiated from hepatic malignancies), MRI is used in NAFLD diagnosis. The restrictions of MRI include the high cost, prolonged time of examination, and difficulty to use in patients with implantable devices (Dinani et al., 2020)

1.3.2.4. Transient Elastography (TE)

TE is a simple, non-invasive method that accurately measures liver stiffness and fibrosis using vibration or sheer wave. It has a sensitivity and specificity similar to that of an ultrasound (77% and 91% respectively), with the advantage of quantifying liver fibrosis (Dinani et al., 2020).

1.3.3. Liver Biopsy

Liver biopsy is the gold standard for the diagnosis of NAFLD (Heyens et al., 2021). It distinguishes between NAFL and NASH and determines the stage of fibrosis

(Farrell & Larter, 2006). A liver biopsy helps to detect NAFLD in patients with uncertain diagnosis (Adams et al., 2005). While a biopsy may determine the prognosis of NAFLD, (Abd El-Kader & El-Den Ashmawy, 2015) it is an invasive technique that should be performed based on the medical condition of each individual.

1.4. Pathogenesis and Progression of NAFLD

One of the widely supported key mechanisms that lead to hepatic steatosis followed by steatohepatitis is the "multiple-hit" hypothesis (Zarghamravanbakhsh et al., 2021). The multiple-hit hypothesis is initiated by a "first hit" characterized by the accumulation of lipids in the hepatocytes in a macrovesicular pattern. The first hit increases the liver's susceptibility to the injury mediated by the secondary hits that include lipotoxicity, innate immune activation, diet (saturated fat and fructose), and sedentary lifestyle (Zarghamravanbakhsh et al., 2021). The secondary hits were found responsible for the consequent liver inflammation and fibrosis that develop as an outcome (Zarghamravanbakhsh et al., 2021) (Figure 3). However, the metabolic processes that cause intrahepatic triglycerides accumulation should be further understood to facilitate NAFLD treatment development. We will discuss the major mechanisms and signaling pathways for the pathogenesis of NAFLD.

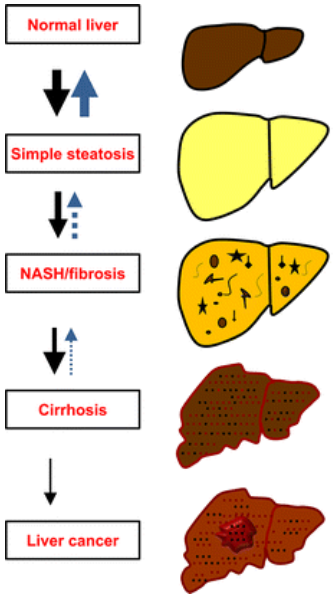

Figure 3- NAFLD progression (Alwahsh & Gebhardt, 2017)

1.4.1. Lipotoxicity

Lipotoxicity is the damaging effect caused by accumulation of lipids in non-adipose tissues resulting in organelle dysfunction, cellular injury, and death (Dowman et al., 2010; Engin, 2017). Lipotoxicity has a critical role in the pathogenesis of NAFLD depending on the quantity and the type of lipid molecules involved (Wasilewska & Lebensztejn, 2021). It occurs during increased lipolysis which results in the release of excess free lipids into the blood that are consequently absorbed by the liver (Engin, 2017; Schweiger et al., 2017). The fatty acids are transformed into triglycerides, ceramides, diacylglycerols, or prostaglandins which cause fatty acid-induced lipotoxicity leading to the development of NAFLD (Schweiger et al., 2017).

1.4.2. Lipogenesis and Extracellular Vesicles formation

Lipogenesis refers to the metabolic process yielding fatty acids. Lipogenesis is elevated in NAFLD patients compared to healthy individuals (Lambert et al., 2014) and

contributes to the pathogenesis of NASH through extracellular vesicles (EV) signaling (Koyama & Brenner, 2017). Hepatocytes become steatotic due to the decrease in vVLDL and β-oxidation and the increase in de novo lipogenesis. Lipogenesis causes the amounts of free fatty acids such as stearate and palmitate to increase to a cytotoxic level. Palmitate is a key element in ceramide and lysophoshoatidylcholine (LPC) synthesis. Both products can cause EV release from the cells in the form of exosomes, microvesicles, and apoptotic bodies which enclose a plethora of molecules. EV carry signaling proteins such as TNF-related apoptosis inducing ligand (TRAIL), sphingosine-1-phosphate, and Interferon gamma-induced protein 10 (CXCL10) which is a mediator of inflammation in NAFLD (Koyama & Brenner, 2017; Zhang et al., 2014). The signaling molecules released through EV activate macrophages enhancing the pathogenesis of NASH (Koyama & Brenner, 2017).

1.4.3. Endoplasmic Reticulum Stress and Reactive Oxygen Species

Studies reported a mutual bidirectional interaction between endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) production that drives the progression from NAFLD to NASH (Win et al., 2015). The accumulation of misfolded proteins in the ER causes ER stress which may result in the accumulation of ROS and causes oxidative stress (Cao & Kaufman, 2014; Oakes & Papa, 2015). The incomplete oxidation of the accumulated liver fats leads to inflammation which produces toxic metabolites and causes hepatocyte apoptosis (Jiménez-Cortegana et al., 2021). When the hepatic cells are injured in a pattern known as fatty balloon degeneration, inflammatory cells such as neutrophils are recruited to the parenchyma of the organ to help repair the damaged tissue by depositing proteins on the extracellular matrix. Chronic liver

inflammation leads to the development of fibrosis which advances to a perisinusoidal fibrotic state that spreads widely causing hepatocellular loss and physiologic dysfunction of the organ. Fibrosis progresses with a decline in inflammation due to the fewer available hepatocytes to be injured, ultimately leading to cirrhosis (Sweet et al., 2017).

1.4.4. NAFLD in Lean Individuals

NAFLD can occur in lean individuals who do not possess excess adipose tissues. The most common cause for the development and progression of NAFLD in lean individuals is an underlying insulin resistance and an increase in visceral adiposity. Studies suggest that NAFLD in lean individuals is associated with a number of genetic disorders such as lipodystrophies, cholesterol ester storage disease, and single nucleotide polymorphisms (SNPs) in Patatin-like phospholipase domain-containing protein 3 (PNPLA3) (Honda et al., 2016). Infectious-inflammatory disorders (hepatitis C and human immunodeficiency virus (HIV) infections), the administration of certain drugs (tamoxifen and amiodarone), and a slight change in body weight in lean individuals may also increase the incidence of NAFLD (Chang et al., 2009; Honda et al., 2016).

1.5. NAFLD Risk Factors

Several risk factors accompany NAFLD development and enhance its progression. Obesity, hyperlipidemia, diabetes mellitus type 2 (DM2T), insulin resistance, and sleep deprivation are some of the major risk factors for NAFLD development.

1.5.1. Obesity

Obesity is defined as having a body mass index (BMI) > 30 kg/m^2, and morbid obesity, a subgroup of obesity, represents individuals with a BMI > 40 kg/m^2 (Milić et al., 2014). NAFLD is strongly correlated to obesity with 80% prevalence in obese patients due to the high amount of visceral adipose tissues in individuals with morbid obesity. The progression from steatosis to NASH occurs in 18.5% of the obese patients and in 3.5% of normal weight patients, suggesting that obesity represents a major risk factor for NAFLD progression (Andronescu et al., 2018). Obese patients with NAFLD have excess free fatty acids from visceral adipose tissue and de novo lipogenesis which may promote further liver injury (Milić et al., 2014). A recent cohort study, held over approximately a 4-year follow-up period, showed a significant association between NAFLD incidence and visceral, but not subcutaneous, adipose tissue suggesting that the incidence of NAFLD depends on the location and type of fats (Kim et al., 2016).

In obese patients, M1 macrophages proliferate leading to increased production of proinflammatory cytokines (tumor necrosis factor (TNF-α) - alpha and interleukin-6 (IL-6)). Proinflammatory cytokines activate downstream signaling cascades and cause inflammation, lipolysis, and insulin resistance (Khan et al., 2019). Elevated cytokine levels are also correlated with liver fibrosis and NASH progression (Giby & Ajith, 2014).

Leptin is a molecule released from adipocytes and regulates metabolism and energy homeostasis (Perakakis et al., 2021). It is also responsible for decreasing the storage of triglycerides in adipocytes and non-adipose tissues such as the liver. In obese individuals, leptin levels are elevated and correlated to NAFLD pathogenesis and liver steatosis. The steatotic effect of leptin in obese patients is partially mediated by the phosphorylation of leptin receptor (Ob-Rb) which causes leptin resistance and reduces

leptin signaling (Jiménez-Cortegana et al., 2021). Decreased leptin signaling may promote triglyceride accumulation which drives NAFLD pathogenesis.

1.5.2. Diabetes

Approximately 70% of diabetic individuals develop NAFLD (Leite et al., 2009). A prospective cohort study, carried out on approximately 41,000 patients, showed a significant correlation between the severity of liver steatosis and the increased risk of diabetes (Shen et al., 2018). Moreover, type 2 diabetes mellitus (T2DM) patients with elevated BMI, lower HDL, and poor dietary control are at higher risk of developing NAFLD (Alsabaani et al., 2018). In T2DM patients, the lipolysis of insulin-resistant adipose tissues leads to the release of free fatty acids and their deposition in the liver causing steatosis (Cusi, 2009).

1.5.3. Insulin Resistance

Insulin is a hormone that contributes to glucose metabolism by suppressing the production of hepatic glucose, enhancing glucose uptake by the liver and adipose tissues, and inhibiting lipolysis (Tanase et al., 2020). Insulin resistance is referred to when the normal insulin levels cannot induce a proper biological response, and the pancreas produces more insulin to facilitate cellular glucose consumption (Wilcox, 2005). Several studies revealed that the development of NASH is mediated by IL6, a key effector of insulin resistance. IL6 is secreted due to prolonged inflammation. It binds to its receptor and activates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway. JAK/STAT signaling activates the transcription of downstream genes including the suppressor of cytokine signaling (SOCS). SOCS

phosphorylates insulin receptor substrate (IRS), a key factor for insulin activity, causing IRS inhibition and leading to insulin resistance (Asrih & Jornayvaz, 2013). Insulin resistance causes adipose tissues to resist lipolysis, which results in the breakdown of triglycerides and produces free fatty acids and glycerol (Schweiger et al., 2017). The accumulation of free fatty acids in the liver increases the susceptibility to lipotoxicity promoting the development of NASH (Zarghamravanbakhsh et al., 2021).

1.5.4. Sleep Deprivation

According to current epidemiological studies, sleep disturbances are common medical problems. Sleep deprivation is associated with obesity which plays a major role in NAFLD pathogenesis (Perumpail et al., 2017). A recent population cohort study stated that lack of sleep may be independently related to NAFLD. Furthermore, a positive correlation was revealed between NAFLD and poor sleep quality in men and women (Kim et al., 2013).

1.6. Toll-like Receptor 4

The variability between individuals in the occurrence and progression of NAFLD, along with the increased prevalence among twins indicate that the genetic background contributes to the development of NAFLD (Loomba et al., 2015). Several genes play a role in the pathogenesis of NAFLD such as PNPLA3, IRS1, TNF, and toll-like receptor 4 (TLR4) (Severson et al., 2016). In this study, we will focus on TLR4 gene polymorphisms and their effects on NAFLD progression.

Toll-like receptors (TLRs) are pattern recognition receptors that were first described as a type I transmembrane receptors that control the embryonic dorsal-ventral

development pattern in drosophila. TLR proteins contain an extracellular amino terminus, an intracellular carboxy terminal, and a transmembrane domain. In humans, the TLR family comprises 10 members (TLR1-10) that function in mediating innate and adaptive immunity by recognizing microbial components such as pathogen-associated molecular patterns (PAMPs) (Guo & Friedman, 2010; Li & Sun, 2007).

TLR4, a member of the TLR protein family, is normally expressed in the liver in parenchymal and non-parenchymal cells including hepatocytes, Kupffer cells, liver sinusoidal endothelial cells, hepatic stellate cells, and intrahepatic lymphocytes (Broering et al., 2011). TLR4 expressing cells are activated by binding to exogenous microbes or endogenous ligands such as damage-associated molecular patterns (DAMPs) released from injured cells and degraded matrices (Guo & Friedman, 2010). TLR4 ligands such as lipopolysaccharides (LPS) and free fatty acids (FFA) activate TLR4 signaling and enhance inflammation through the production of pro-inflammatory, antiviral, and antibacterial cytokines, (Sharifnia et al., 2015). TLR4 is associated with several diseases including insulin resistance, gastrointestinal diseases such as Crohn's diseases, airway inflammatory diseases such as asthma, and sepsis by inducing chronic low-grade inflammation (Jialal et al., 2014; Lin et al., 2012). TLR4 is also correlated to atherosclerosis since TLRs and their ligands are detected in human atherosclerotic plaque and adventitia (de Kleijn & Pasterkamp, 2003). In the liver, TLR4 responds to hepatic injury resulting from various diseases such as viral hepatitis and NAFLD. The inflammatory phenotype in the injured hepatic tissues is caused by TLR4 signaling in hepatic stellate cells (HSCs) which are the major fibrogenic cells (Guo & Friedman, 2010).

1.6.1. Signaling Pathway

TLR4 gene is located on chromosome 9, q arm, section 33.1, and has 3 exons separated by intronic sequences (Figure 4) (Smirnova et al., 2000; Wang et al., 2016). The extracellular domain is encoded by the 3 exons where exon 1 encodes the first amino acids (aa), exon 2 encodes the initial leucine-rich repeats (LRRs), and exon 3 encodes the remaining LRRs and the hypervariable region. Exon 3 also encodes the cytoplasmic and the transmembrane domains, and exon 1 encodes the signal peptide (Vaure & Liu, 2014). The extracellular domain of TLR4 contains 624 aa spanning the residues 1–624, and is highly polymorphic compared to the transmembrane and intracellular domain of the receptor (Li & Sun, 2007). It contains LRRs spanning aa residues 55–569, and it allows precise binding of the ligand to the receptor with the help of co-receptors (Guo & Friedman, 2010). The transmembrane domain connects the extra- and intra-cellular domains by a single 33-aa helix that spans the residues 625–658 (Matsushima et al., 2007). The intracellular domain spans the aa residues 659–838. It is a Toll-interleukin-1 receptor (TIR) domain which mediates the interaction between TLR4 and its signal transduction adaptor proteins including myeloid differentiation factor 88 (MyD88), TIR domain-containing adaptor inducing interferon-β (TRIF), MyD88-adaptor-like/TIR domain-containing adaptor protein (MAL/TIRAP) (Fitzgerald et al., 2001), and TRIF-related adaptor molecule (TRAM) (Hoebe et al., 2003; Lu et al., 2008; Ronni et al., 2003; Yamamoto et al., 2003).

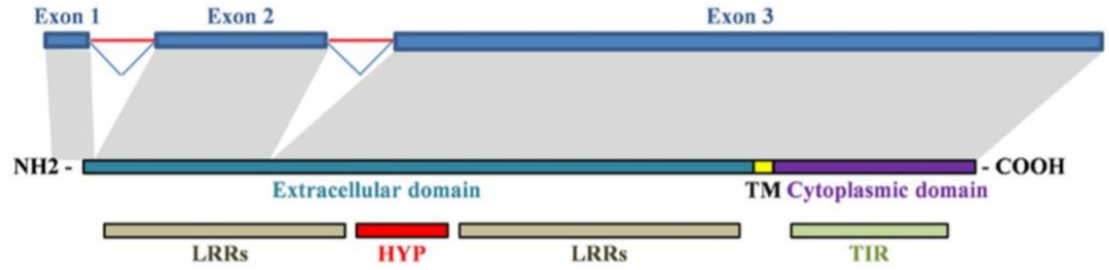

Figure 4- Alignment of human TLR4 gene and protein.

LPS is the main ligand of TLR4. Activation of TLR4 by LPS requires the binding of several proteins and co-receptors. LPS binds to the circulating LPS-binding protein (LBP), three LRR domain-containing proteins, TLR4, cluster of differentiation 14 (CD14), and myeloid differentiation protein 2 (MD2). CD14 is a TLR4 co-receptor that does not have a transmembrane domain, thus cannot initiate an intracellular signal. Upon LPS activation, CD14 transfers LPS to MD2, another co-receptor that lacks a transmembrane domain but adheres to the cell by interacting with TLR4. MD2 is crucial for the cell-surface expression of TLR4 and the activation of signaling (Guo & Friedman, 2010).

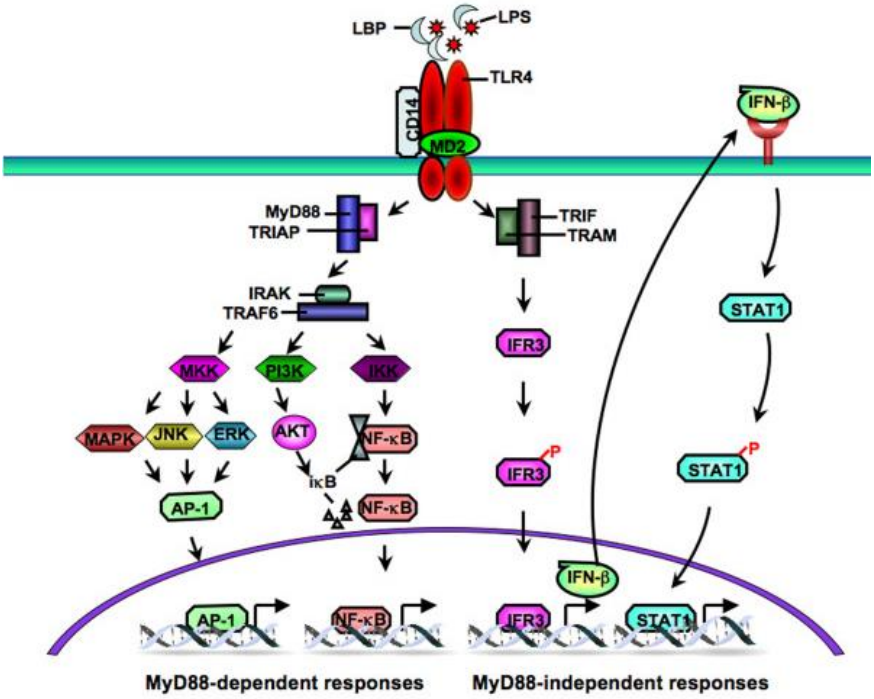

Figure 5- TLR4 activation through LPS binding

Abbreviations: AKT: protein kinase B; AP-1: activator protein 1; ERK: extracellular signal-regulated kinase; iκB: Inhibitor of NF-κB; IKK: iκB kinase; IRAK: IL-1 receptor-associated kinase; IRF3: interferon regulatory 3; IFN- β: interferon beta; JNK: c-Jun N-terminal kinase; LPS: lipopolysaccharide; LBP: LPS-binding protein; MAPK: mitogen activated protein kinase; MKK: MAPK kinase; MD2: myeloid differentiation protein; MyD88: myeloid differentiation factor 88; NF-κB: nuclear factor kappa B; STAT1: signal transducers and activators of transcription 1; TRAF6: TNFR-associated factor 6; TRIF: TIR domain-containing adaptor inducing interferon-β; TRAM: TRIF-related adaptor molecule; PI3K: phosphatidylinositol 3-kinase; P: phosphorylation.

TLR4 signaling activates a cascade of downstream targets such as receptors, co-receptors and adaptor proteins to regulate the transcription of genes that control cell survival, fibrogenesis, and immunity. TLR4 signaling is controlled by two major pathways (Figure 5): MyD88-dependent pathway which is responsible for the expression of proinflammatory cytokines; and MyD88-independent (TRIF-dependent) pathway responsible for the expression of type 1 interferons (Lu et al., 2008).

MyD88 is a downstream adaptor of TLR4. It is composed of a TIR domain and a death domain (DD). Following LPS stimulation, TLR4 oligomerizes and recruits MyD88

through binding to the TIR domain. MyD88 recruits IL-1 receptor-associated kinase-4 (IRAK-4) which activates tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) (Walsh et al., 2015). TRAF6 is an adaptor protein that stimulates the TGF-β-activated kinase 1 (TAK1), a prosurvival protein that induces 2 downstream signaling pathways: the IκB kinase (IKK) pathway and the mitogen-activated protein kinase (MAPK) pathway. IKK and MAPK signaling regulate the expression of pro-inflammatory cytokines through the induction of the nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) transcription factors respectively (Lu et al., 2008).

The MyD88-independent pathway is mediated by TRIF which is a TLR4 adaptor protein. Studies using TRIF-deficient macrophages demonstrate that TRIF plays a key role in the activation of the transcription factor interferon regulatory factor 3 (IRF3), and the late-phase activation of NF-κB and MAPK which cause the upregulation of Type I interferons such as IFN-β. IFN-β interacts with (STAT1) inducing the phosphorylation and dimerization of STAT1 which, in turn, activates janus protein tyrosine kinases (JAKs). Genes regulated by STAT1/JAK signaling include genes that mediate antibacterial and antiviral responses (Lu et al., 2008).

1.6.2. TLR4 Signaling in Healthy and Damaged Liver

TLR4 and some of its co-receptors (such as MD2) and adaptors (MyD88) are downregulated in healthy liver cells possibly due to the constant exposure of liver cells to the intestinal microbiota which renders the liver highly tolerant to TLR4 ligands. However, in an injured liver, the expression of TLR4 and its co-receptors is elevated to stimulate the inflammatory response mediated by TLR4 signaling (Kitazawa et al., 2008).

Each liver cell has a specific function mediated by TLR4 signaling. Hepatocytes are responsible for the uptake of LPS from the circulation through TLR4-dependent manner and the release of LPS into bile. Kupffer cells (KCs) play an important role in the liver immunity (Guo & Friedman, 2010). KCs may release proinflammatory cytokines and reactive oxygen species through LPS-mediated TLR4 activation. HSCs have a major role in hepatic fibrogenesis. TLR4 signaling induces fibrogenesis in HSCs by downregulating the inhibitory TGF-β1 pseudoreceptor (bone morphogenic protein and active membrane bound inhibitor (BAMBI)) (Seki et al., 2007). Moreover, CD14-TLR4-MD2 signaling has an anti-apoptotic effect on HSCs mediated by the resistance of HSCs to pro-apoptotic stimuli. Therefore, inducing the apoptosis of HSCs represents a possible therapeutic strategy for hepatic fibrosis (Guo & Friedman, 2010).

1.6.3. Implications of TLR4 in NAFLD

Obesity, diabetes, and insulin resistance are major risk factors for NAFLD. Particularly, insulin resistance causes an elevated level of FFA to circulate which disrupts glucose uptake and leads to increased glucose production by the liver. Macrophages and adipocytes respond to the increased amounts of FFAs by inducing an inflammatory response via TLR4 activation. TLR4 signaling activates NF-κB and enhances the expression of proinflammatory cytokines which leads to the development of NAFLD (Zeyda & Stulnig, 2009). NAFLD is induced by dietary fructose intake. A high fructose intake causes plasma triglyceride levels to increase. This increase causes triglyceride accumulation in the liver and increased bacterial growth in the intestine, which results in the activation of TLR4 signaling due to the elevated endotoxin levels. Therefore, TLR4

represents an association between NAFLD, lipids, and dietary intake (Spruss et al., 2009; Thuy et al., 2008).

KCs are activated by several factors including TLR4 activation by LPS. The activation of KCs promotes the upregulation of inflammatory cytokines (e.g. TNF-α and IL-6) and affects the expression of genes that regulate fibrogenesis and oxidative stress (Chen et al., 2020). KCs play an important role in the pathogenesis of NAFLD by inducing reactive oxygen species and activating X-box binding protein 1 transcription factor which is responsible for regulating the expression of cell survival genes. The disruption of TLR4 receptor signaling due to gene polymorphisms affects the binding of TLR4 to its ligands and causes an imbalance between anti-inflammatory and proinflammatory cytokines potentially causing NAFLD development and progression.

Dysregulaion in TLR4 signaling is a major factor in the pathogenesis NAFLD. Sharifnia et al identified a correlation between TLR4 activity and NAFLD pathogenesis. The expression of TLR4 and its downstream adaptors (MYD88 and TRIF) are upregulated in NASH compared to NAFL patients. The enhanced TLR4 signaling in NASH patients is attributed to the inflammatory phenotype of liver.

TLR4 gene is polymorphic, thus single-nucleotide polymorphisms (SNPs) can occur at specific positions in the genome. SNPs are the most common type of genetic variations. SNPs cause polymorphisms among the same population, affecting the susceptibility of an individual to develop a certain disease, the severity of an illness, and the response to treatment. TLR4 gene polymorphisms are linked with several liver diseases. Sghaier et al. showed that TLR4 rs3775290 minor T genotype and rs4986790 minor G genotype are associated with increased risk for the chronic infection with hepatitis B and C viruses, suggesting that TLR4 gene polymorphisms are potential

biomarkers for hepatitis (B and C)-associated cirrhosis (Sghaier et al., 2019). Kiziltas et al investigated TLR4 gene polymorphism in NAFLD. A heterozygous substitution mutation detected in TLR4 gene in healthy individuals caused aspartic acid (Asp) to glycine (Gly) shift at position 299 (Asp299Gly) suggesting a protective role against NAFLD development (Kiziltas et al., 2014). Furthermore, the specific nonsynonymous gene variants (Asp299Gly and Thr399Ile) were reported to decrease TLR4 mediated inflammation and fibrosis in the liver, protecting against NAFLD through the disruption of the extracellular domain of TLR4, and the decreased ligand recognition (Guo et al., 2009).

The few studies that assessed the correlation between TLR4 gene polymorphism and progression to NAFL in humans are summarized in Table 2.

Table 2- Summary of literature findings for TLR4 gene SNPs and NAFLD correlation

Publication	**Mutation (SNP)**	**Amino acid difference**	**Effect**
Guo, et. al, (2009)	1196C>T	T399I	Protective effect
	896A>G	D299G	Protective effect
Kiziltas, et. al, (2014)		D299G	Protective effect
		T399I	No effect

1.7. Aim of the study:

NAFLD is the most common etiology of chronic liver disease (Li et al., 2018). It affects 30% of the general population and 75% of obese patients in the Western world (Tiniakos et al., 2010). NAFLD is caused by the accumulation of fats in the liver that exceeds 5% of its weight with no significant chronic alcohol consumption (Papatheodoridi & Cholongitas, 2018). Several factors are implicated in the onset and

development of NAFLD including diabetes, obesity, and insulin resistance. Moreover, various genetic polymorophisms have been identified in several genes that are implicated in the pathogenesis of NAFLD such as PNPLA3, Insulin receptor substrate 1 (IRS1), Glucokinase regulatory protein (GCKR), monocyte differentiation antigen CD14 (CD14), and toll-like receptor 4 (TLR4). TLR4 is a polymorphic gene that has a major role in the pathogenesis of NAFLD. Endotoxins and endogeneous ligands such as LPS activate TLR4 signaling which enhances inflammation through the expression of proinflammatory cytokines and affects the fibrosis stage of the patients. Studies suggest that the presence of TLR4 polymorphisms may have a protective role against NAFLD development and progression. Therefore, the aim of the current study is to:

1. Identify the mutations and polymorphisms in TLR4 gene of Lebanese NAFLD patients and healthy controls.
2. Compare the identified mutations to check if a genotype-phenotype correlation exists.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Equipment

The equipment used in this study were supplied by:

- **Fisher Scientific:** Vortex Genie2TM
- **Eppendorf:** Mini spin Centrifuge
- **BioRad:** MyCycler Thermal Cycler for Polymerase Chain Reactions (PCR); Agarose Gel Electrophoresis setup type (III); Wide Mini-Sub Cell GT
- **Thermo Scientific:** Centrifuge MicroCL 21R
- Filtered tips (0.5-10µL CAT#: 5030030; 100µL CAT#: 5030066: 1000µL CAT#: 5130150)
- Fibroscan

2.1.2. Kits:

The following kits were supplied and purchased from:

- **QIAGEN:** DNA Blood Mini Kit (Cat#: 51106); QIA quick gel extraction kit (CAT#: 28704)
- **Machery-Nagel, Germany:** Nucleospin extract-2 kit
- **Applied biosystems, USA:** ABI PRISM BigDye terminator v3.1 ready reaction cycle sequencing kit

2.1.3. Chemicals and Reagents:

The following reagents were supplied and purchased from:

- **BioRad, USA:** iTaq Universal SYBER Green Supermix (Cat#: 172-5121); 500g Molecular Biology Agarose powder (Cat#: 161-3102); 10ml ethidium bromide (10mg/ml) (Cat#: 161-0433); DNA molecular weight ladder EZ 100bp; Loading Dye 5x, 1ml (CAT#: 970344; Loading buffer 5x (Cat#: 161-0767)
- **Solis BioDyne:** DNA high molecular weight ladder 1 kb (Cat#: 07-12-00050); DNA low molecular weight ladder (100bp) size range 100-300bp (Cat#: 07-11-00000)
- **QIAGEN:** DNA protease (proteinase K)
- **Lonza:** 1L AccuGENE Molecular Biology Grade Water (Cat#: 51200)
- **Acros Organic:** 1L TBE buffer (CAT#: A0257333)
- **VWR:** 2.5L ethanol absolute (CAT#: 18G034016)
- **Sigma Aldrich:** 2.5L Isopropanol (2-propanol) (CAT#:24137)

2.2. Methods

2.2.1. Human Subjects:

All subjects in this study presented for follow up or check in to the gastroenterology private clinics or the endoscopy unit at AUBMC. Recruited subjects were asked to sign a consent form (IM.FD.08, supplement 1) approved by the Institutional Review Board (IRB) and the Research Committee at AUBMC. The total number of individuals recruited in this study was 49 composed of 19 healthy controls and 30 NAFLD patients.

The following criteria were used in recruiting Healthy subjects and NAFLD patients. Regardless of age and sex, Healthy subjects presenting for medical reason other than NAFLD, and planning to undergo endoscopy were eligible for the study with normal LFTs. However, healthy subjects who refused to sign the informed consent were excluded.

Eligible NAFLD patients included in the study, are patients with or without elevated LFTs, with ultrasound findings suggestive of fatty liver, in addition to patients having idiopathic compensated cirrhosis labeled as possibly secondary to NASH. Subjects were excluded if they have BMI>35, cancer and/or liver metastasis, consumers of more than 2 alcoholic drinks per day, had viral hepatitis before, have ingested hepatotoxic drugs (chlorpromazine, halothane, isoniazid and amoxicillin-clavulanate), or any known liver diseases were excluded from the study.

2.2.2. Data collection:

Human subjects consisted of a full history including data on gender, age, smoking habits, and alcohol consumption, in addition to anthropometric data (height, and weight). Moreover, clinical history of subjects was collected to elucidate if the patients had a stroke, peripheral artery disease, coronary artery disease (CAD), congestive heart failure (CHF), hypertension (HTN), dyslipidemia (DL), and diabetes mellitus (DM).

Peripheral blood samples were collected from normal and patient subjects. The blood was used for performing standard biochemical tests including liver function tests and for genotyping purposes. In addition, standard clinical biochemical tests that assesses liver performance were determined including the measuring of the production

of several proteins, the clearance of bilirubin, and the release of enzymes in response to damage or disease.

Liver function tests conducted in this study included tests for alanine transaminase (ALT/SGPT), aspartate transaminase (AST/SGOT), alkaline phosphatase (ALP), albumin and total protein, bilirubin, GGT, and prothrombin time (PT).

Lipid profile is also performed in this study include tests for serum triglycerides (TG), total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL).

In addition, hematological test is performed including white blood cell count (WBC), blood urea nitrogen (BUN), platelet count (PLT), glucose, hemoglobin, ferritin, iron, and creatinine. Moreover, all subjects normal and patients underwent liver Ultrasonographic and fibroscan that were recorded for each patient at the endoscopy unit at AUBMC to identify the presence of a fatty liver and to determine the stage of steatosis or fibrosis if present.

2.2.3. Genotype:

In this study we screened normal and NAFLD subjects for mutation in TLR4 gene and we examined if a possible correlation exists between NAFLD patients and mutation in TLR4 gene.

The genotypic screening for SNPs and mutations was carried on all subjects. Blood samples were collected from all subjects, followed by DNA isolation, amplification, observed through agarose gel electrophoresis, then sequenced and screened for exons bearing mutations or SNPs and followed by comparison between controls and patients.

2.2.4. DNA Extraction from Blood Samples

DNA was isolated from Peripheral blood samples collected from all subjects in EDTA tubes (figure 6). DNA was extracted using a DNA Blood Mini Kit supplied by QIAGEN (Cat#: 51106). Blood sample (200 µL) was added to a microcentrifuge tube containing 20 µL of QIAGEN protease (Prokinase K) and 200 µL of Buffer Al. The contents were vortexed for 15 seconds, incubated for 10 minutes (56ºC) and centrifuged. Ethanol (200 µL, 96%) was then added to the supernatant and the tubes were vortexed for 15 seconds, centrifuged at 6000xg (8000 rpm) for one minute in the QIAamp Mini Spin column. The contents were washed with 500 µL of Buffer AW1 and centrifuged again at 6000xg (8000 rpm) for one minute. Buffer AW2 (500 µL) were added to the QIAamp Mini Spin column and centrifuged at full speed 20000xg (14000 rpm) for three minutes. Buffer AE (100 µL) was finally added, and the contents were incubated at room temperature for one minute, then centrifuged at 6000xg (8000 rpm) for one minute. The DNA eluant were quantified for DNA level using Nanodrop stored at -20ºC.

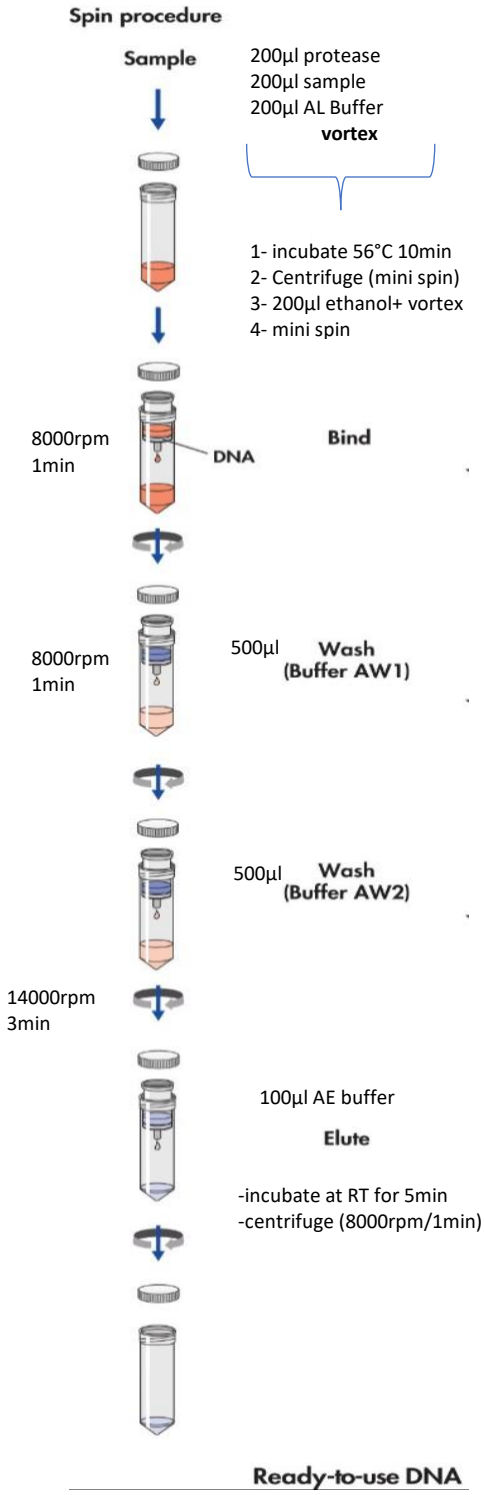

Figure 6-DNA extraction protocol

2.2.5. PCR Protocol

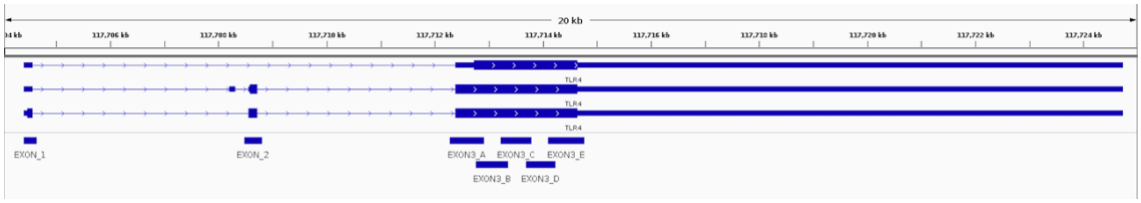

Figure 7- schematic diagram showing the exons of TLR4 gene

TLR4 gene (is made up of 3 exons of 242 (E-1), 338 (E-2) AND 3023 (E-3) Bp size. Exons 1 and 2 were amplified using primers, whereas exon 3 was amplified as 5 different segments using the primers for each segment (figure 7). Amplification of exons 1-3 of TLR4 gene was carried on all samples using My Cycler Thermal Cycler PCR machine (BIORAD). The PCR reaction mixture of final volume 25 µL contained of: water RNase (8.5 ml); forward and the reverse primers diluted (each 1 µL) , DNA (2 µL each)and iTaq universal SYBR (12.5 µL) from BioRad (cat#: 172-5121). Primers flanking exons' boundaries (exons 1 and 2) were designed. As for exon 3, which was divided to 5 amplicons have primers spanning the exon boundaries and internal sequences. Forward and reverse primers are tabulated with amplicon size indicated.

Two PCR programs were used in amplifying the various amplicons:

Program 1 was used to amplify exons 2 and 3b, 3c, and 3d. It involved the activation of Taq polymerase (94ºC for 5 minutes), and 30 cycles of denaturation (at 94ºC for 1 minute,), annealing (at 58ºC for 1 minute) and extension (at 72ºC for 2 minutes), followed by a hold step. A final phase of extension was also conducted at 72ºC for 7 minutes.

Program 2 was similar to program 1 except for the annealing temperature which was 56ºC. This program was used to amplify exons 1, 3a, and 3e.

2.2.5.1. List of Primers used:

Primers were purchased from Microgen (30nmol) and diluted to a final concentration of 0.6 nmol. The primer sequences and their corresponding exons are displayed in table 3.

Table 3- Exons used and their primer sequences

Exons	Name	Sequence	Size (Bp)
Exon 1	Exon 1 Left	5'ACGGTGATAGCGAGCCAC 3'	5533.6
	Exon 1 Right	5' AATAAACAAACCAGGGCACAC 3'	6723.4
Exon 2	Exon 2 Left	5' CCTCTCCACCATCTCTGGTC 3'	5940
	Exon 2 Right	5' CTCCACAAACCAAGCTTTCC 3'	5966
Exon 3	Exon 3 (a) Left	5'TCACATCTGTATGAAGAGCTGG 3'	6774.4
	Exon 3 (a) Right	5'TCTCCCAGAACCAAACGATG 3'	6055
	Exon 3 (b) Left	5' CCCTATGAACTTTATCCAACCAG 3'	6927.6
	Exon 3 (b) Right	5' GGTAATAACACCATTGAAGCTCAG 3'	7369.8
	Exon 3 (c) Left	5' TCAGGTACTAAATATGAGCCACA3'	7007.6
	Exon 3 (c) Right	5' CAGAGCTGAAATGGAGGCAC 3'	8337.4
	Exon 3 (d) Left	5' CATCTGTATGAAGAGCTGGATG 3'	7643
	Exon 3 (d) Right	5' TCTCCAGAACCAAACGATG 3'	6200
	Exon 3 (e) Left	5'TTCAGAAGTTGATCTACCAAGCC 3'	6050
	TLR4 Exon 3 (e) Right	5'CATTATGTGATTGAGACTGTAATCAAG3'	6350.2

2.2.6. Agarose Gel electrophoresis

Amplified PCR amplicon were separated on 2% agarose gel compared to molecular weight standards to verify the size. PCR products were mixed with a loading

dye at a 6:1 ratio, introduced into gel wells, and run at 80 volts. The amplicon size was verified against a genomic molecular size ladder ranging between 100 to 1000 bp, and the amplified exons were separated using 2% agarose gel containing 10 µL of 1mg/ml ethidium bromide. The gel was prepared by adding 1g of agarose into an Erlenmeyer flask containing 50ml of buffer. The agarose was dissolved using a microwave, and 3µl of ethidium bromide was added. The ladder was then placed in the tray and the gel was poured. When the gel solidified (after 30min), PCR products were mixed with a loading dye at 6:1 ratio, introduced into agarose gel wells, and run at 80 volts. The separated DNA fragments were visualized under UV light. Following electrophoresis, the purified PCR products were sequenced.

2.2.7. DNA Sequencing

The purified amplicons were sequenced at the AUBMC. The sequencing reaction contained 1µL of either the forward or reverse primers (1.6 pmol/ µL); purified amplicons of volume 1-3 µL depending on the intensity of the PCR fragment on the gel after purification; and DNAse/RNAse free water up to a final volume of 5.5 µL. BigDye terminator v3.1 ready reaction cycle sequencing kit (2 µL) was then added to the sequencing reaction, followed by amplification of the specific exon for 25 cycles: denaturation at 96 °C x10 sec, hybridization at 50 °C x5 sec, and elongation at 60 °C x4 min. For the removal of excess ddNTPs, purification of the sequencing reaction was achieved by gel exclusion chromatography: Sephadex resin, deposited in a 96-well-ELISA plate and hydrated by 300 µL of water for 3 hours. The obtained sephadex plate was positioned above a genetic analyzer recuperation plate (ABI 3130A machine) where sequencing reaction mixtures were added to the wells of the sedaphex plate and

centrifuged (5 mins at 2300 rpm). Purified DNA is eluted in the recuperation plate, lyophilized and resuspended in 25 µL formamide. The obtained sequences were analyzed using the sequence analyzer software and compared to the published normal sequences in the different databanks through either Blast at NCBI or Blat at UCSC.

2.2.8. Sequence Alignment and Analysis

Alignment and variant calling of the Sanger sequences in AB1 file format were performed using "Tracy" (Rausch et al., 2020) command-line tool, by applying the "decompose" module. Hg38 chromosome 9 sequence was used as a reference for the alignment. The output of the "decompose" module is a BCF file for each sequenced exon, containing all the called variants. Seven BCF files were obtained for each patient (either normal or case) and they were concatenated and were sorted using "bcftools concat" (Danecek et al., 2021) and "bcftools sort" modules respectively to obtain one VCF file for each patient. Normal samples were merged together into one VCF file to obtain a union set of all variants found in normal samples using "bcftools merge" module. The same was applied for the case samples (figure 8). The two obtained VCF files were then used to extract the variants found only in at least one case sample using "bcftools isec" module. The same procedure was also applied for the normal samples: variants that are found exclusively in at least one normal sample were extracted for further analysis. The obtained variants were annotated using "ensembl-vep" – Variant Effect Predictor (McLaren et al., 2016) command-line tool with homo-sapiens annotation reference version 104. Tables were generated for the annotated variants using "bcftools +split-vep" module, including a table of the union set of all mutations in all samples (cases and normal samples). Additional filters were applied by excluding 3

prime UTR variants, 5 prime UTR variants, intronic variants, and upstream gene variants. The count of variants in patients and normal samples was done using a customized bash script.

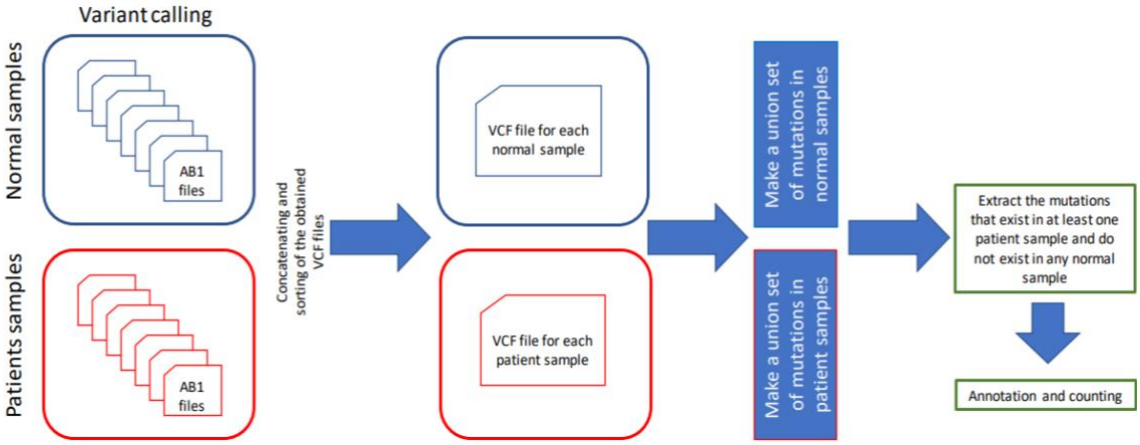

Figure 8- Sequence alignment and analysis

CHAPTER 3

RESULTS

3.1. Clinical Evaluation of Patients and Healthy Individuals

This study aims to determine the mutations and polymorphisms in the TLR4 gene of Lebanese NAFLD patients and healthy controls. Findings will be then compared to check if a genotype - phenotype correlation exists. Recruited individuals (19 healthy controls were labelled as C1-C17 AND C35-C36 and 30 NAFLD patients referred to as P18-P34 and P37-P49) presenting to AUBMC, underwent clinical assessment and were also screened for nucleotide base changes in the 3 exons of TLR4 gene, (Access #: ENST00000355622.8) using PCR followed by sequencing.

3.1.1. BMI, Smoking and Alcohol Habits Of Recruited Subjects

A total of 49 subjects were recruited, composed of 19 healthy controls (52% females, 48% males) and 30 NAFLD patients (33% females, 67% males). Their ages ranged between 24-66 years and 23-70 years for controls and patients respectively. Information on their smoking and alcohol drinking habits were collected (Table-4). Around 47% and 52% of all healthy individuals, versus 24% and 75% of NAFLD patient are smokers and not alcohol consumers respectively.

Body-mass index (BMI) is one criterion that is used in identifying overweight and obese subjects. By definition, BMI it is the ratio of bodyweight in kilograms (or pounds) to the square of height in meters (or feet). In our recruited subjects, 50% of normal were obese and overweight while 55% of the NAFLD patients, with higher percentage of subjects being overweight in males than females (Table 4).

Table 4- Comparison of smoking habits, alcohol consumption, and BMI in NAFLD patients and healthy controls

Data		Healthy controls			NAFLD patients		
		%Males	%Females	%Total	%Males	%Females	%Total
Smoking	Smokers	26	21	47	10	13	23
	Occasional	0	0	0	7	0	7
	Former Smokers	5	11	16	13	0	13
	Non-Smokers	16	21	37	37	20	57
Alcohol Consumption	None	19	33	52	50	25	75
	Occasional	19	14	33	17	8	25
	Daily	5	5	10	0	0	0
	2-3 glasses daily	5	0	5	0	0	0
BMI (Weight)	Normal (BMI<25)	20	30	50	9	6	15
	Overweight (25≤BMI<30)	20	15	35	31	13	44
	Obese (BMI>30)	10	5	15	22	19	41

3.1.2. Liver Function Tests and Lipid Profile:

Liver function tests were assessed in healthy control subjects included: SGOT, SGPT, and GGT. All were in the normal range (100%). Similarly, the lipid profile of healthy control individuals showed normal values in 63% for TC, 79% for HDL while 74% had high LDL levels (Table 5).

Table 5- Lipid profile and liver function tests in control healthy patients.

		Healthy controls		
Clinical data	**Normal Range**	**%normal**		
SGPT(U/l)	7-56	100		
SGOT(U/l)	8-40	100		
GGT(U/l)	9-48	100		
Lipid Profile				
Clinical data	**Normal range**	**%low**	**%Normal**	**%High**
TC (mg/dl)	125 – 200	-	63	37
HDL (mg/dl)	M: 35 – 65 W: 35 – 80	21	79	-
LDL (mg/dl)	<100	-	26	74

Abbreviations: SGPT, Alanine transaminase; SGOT, aspartate transaminase; GGT, gamma-glutamyl transferase; TG, serum triglycerides; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein

Liver function tests were also performed on NAFLD patients. Majority of patients (>88%) had normal level of SGPT, SGOT, and ALP while 55% had normal levels of GGT.

As for the lipid profile, more than 60% of NAFLD patients had normal TG, TC, HDL, and LDL. Surprisingly, LDL level was elevated only in 39% of the patients compared to 74% LDL elevation in healthy controls. The low percentage of patients having high LDL levels is attributed to statin treatment in NAFLD patients.

Hematological findings of the NAFLD patients revealed levels within normal ranges of: WBC, Platelet counts, Hemoglobin, Ferritin, and Creatinine. Total Bilirubin was normal in 93% of patients with high level of direct bilirubin in 24% of patients. Furthermore around 33% of NAFLD patients had HBA1C level >5.6% (Table 6).

Table 6- Liver function tests, lipid profile, and hematological tests in NAFLD patients

		NAFLD patients		
Clinical data	**Normal Range**	**%low**	**%normal**	**%high**
Liver Function Tests				
SGPT **(U/l)**	7-56	0	88	12
SGOT **(U/l)**	8-40	0	89	11
ALP **(U/l)**	20 – 140	0	97	3
Total bilirubin **(mg/dl)**	0.2 – 1.2	0	93	7
Direct bilirubin **(mg/dl)**	<0.3	0	76	24
Prot **(g/l)**	60 – 83	0	100	0
GGT **(U/l)**	9 – 48	0	55	45
Lipid Profile				
TG **(mg/dl)**	<150	0	66	34
TC **(mg/dl)**	125 – 200	0	82	18
HDL **(mg/dl)**	M: 35 – 65 W: 35 – 80	35	65	0
LDL **(mg/dl)**	<100	0	61	39
Hematological Tests				
WBC **(µl)**	4,000 – 11,000	6	89	6
BUN **(mg/dl)**	7 – 22	0	96	4
Plt(/**mcl)**	150,000 – 450,000	3	97	0
Glucose **(mg/dl)**	70 – 100	0	88	13
Hb **(g/dl)**	M: 14.0 – 17.5 W: 12.3 – 15.3	6	94	0
Ferritin **(µg/l)**	M: 18 – 270 W: 18 – 160	10	90	0
Iron **(µg/dl)**	M: 70 – 180 W: 60 - 180	24	76	0
Creatinine **(mg/dl)**	0.6 – 1.3	0	100	0
Diabetes Test				
HBA1C	4 – 5.6 %	0	67	33

Abbreviations: SGPT, Alanine transaminase; SGOT, aspartate transaminase; ALP, alkaline phosphatase; Protein, total protein; GGT, gamma-glutamyltransferase; TG, serum triglycerides; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; WBC, white blood cell count; BUN, blood urea nitrogen; PLT, platelet count; Hb hemoglobin; HBA1C, hemoglobin A1C.

3.2. Overall Genotypes

A total of 49 individuals were recruited in this study (30 NAFLD patients and 19 healthy controls). TLR4 gene exons were amplified and sequenced. Exons 1 and 2 were amplified as one segment, whereas exon 3 was amplified as 5 segments using the primers spanning each segment.

The primers used for the amplification of each exon segment were optimized (Figure 9). Nucleotide base changes were identified in healthy controls (SNPs) and NAFLD patients (mutations) and compared.

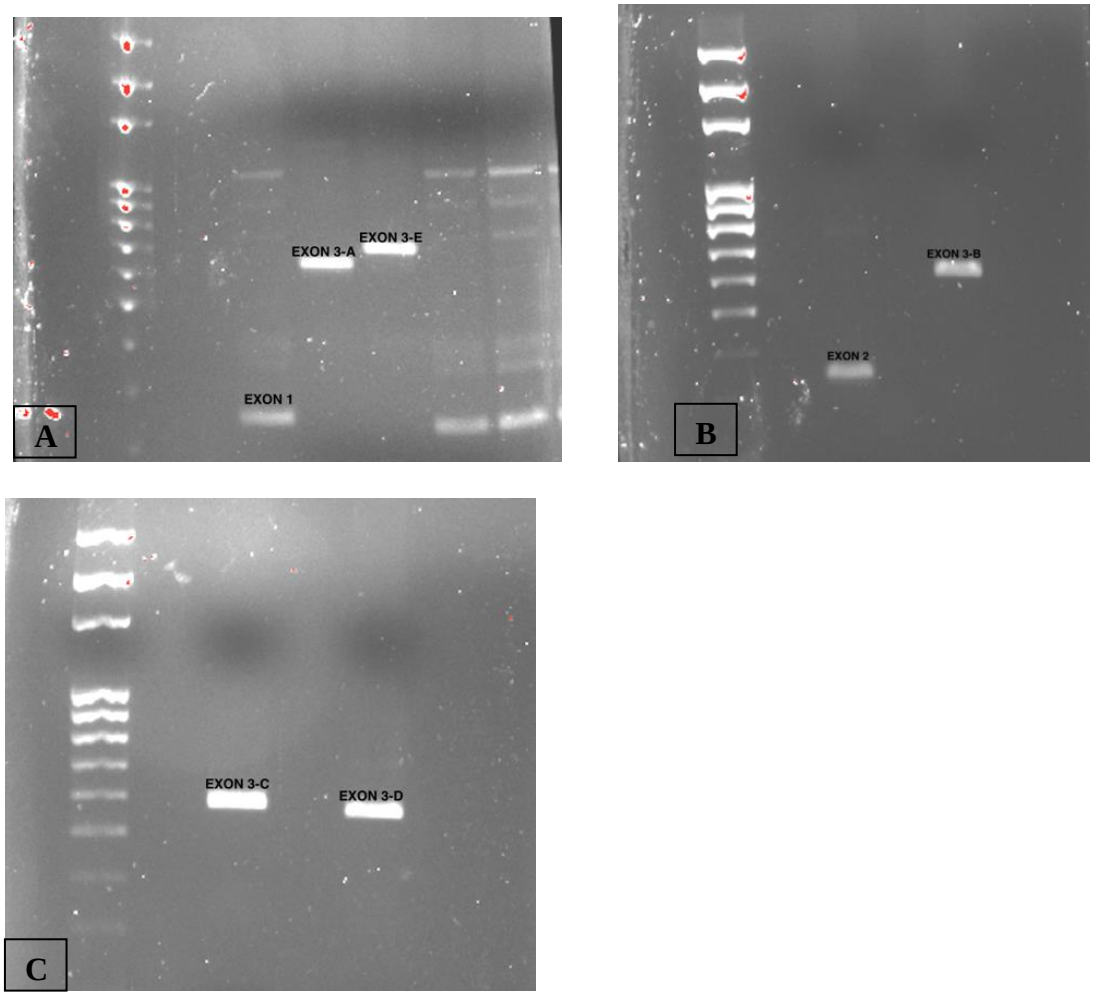

Figure 9- Optimization of the primers for TLR4 PCR.

Comparing sequences of the different amplicons, using different software identified a total of 1765 mutations in our control and NAFLD. The intronic sequences, 5'3' UTR, and synonymous sequences were disregarded, leaving us with 1098 TLR4 mutations (Figure 10). Most of the mutations (1040) were present in exon 3; 31 mutations were present in exon 1, and 27 mutations were present in exon 2. The most common mutations were missense mutations which result in codon changes (498 missense), and 367 mutations were frameshift mutations including deletions and insertions. Other mutations were detected such as Stop-gain mutations accounted for 69 mutations resulting a shortened protein, stop-loss mutation, with deletions and

insertions accounting for 25 and 36 mutations respectively. Protein altering variants and coding sequence variants comprised 24 and 76 mutations respectively out of the total mutations present (1098) (Table 7).

Table 7- The different types of mutations in the 3 exons of TLR4 gene.

Mutation Type	**Exon 1**	**Exon 2**	**Exon 3**	**Total**
Missense	13	19	465	498
Frameshift	11	6	349	367
Stop-gain	3	1	64	69
Stop-loss	0	0	1	1
In frame deletions	2	1	36	39
In frame insertions	0	0	22	22
Protein altering variants	0	0	23	23
Coding sequence variants	2	0	74	76
Total	**31**	**27**	**1040**	**1098**

Out of the 31 mutations present in exon 1, 13 were missense mutations, 11 were frameshift, 3 were stop-gain, 2 were in frame deletions, and 2 were coding sequence variants. In frame insertions, stop-loss, and protein altering variants were absent in exon 1. In exon 2, 19 out of the 27 mutations were missense; one mutation corresponded to as in frame deletion; and one corresponded to a stop-gain mutation. Six frameshift mutations were identified in exon 2 that were present only in healthy individuals. In frame insertions, stop-loss mutations, protein altering variants, and coding sequence variants were absent in exon 2. Exon 3 contained 1040 total mutations; 465 mutations were missense and 349 were frameshift. All TLR4 in frame deletions identified in our

sample (36) were present in exon 3. Also, 64 stop-gain mutations, 74 coding sequence variants, 23 protein altering variants, 22 in frame insertions, and one stop-loss mutation were found in exon 3 (Table 7).

Due to the effect of frameshift mutations on the whole amino acid sequence of TLR4, we considered mutations that were only present before the frameshift in each healthy control and NAFLD patient. A total of 24 missense mutations were identified in healthy individuals, and 40 missense mutations were found in NAFLD patients. All the mutations were heterozygous except for 3 homozygous mutations (1 in healthy individuals and 2 in patients).

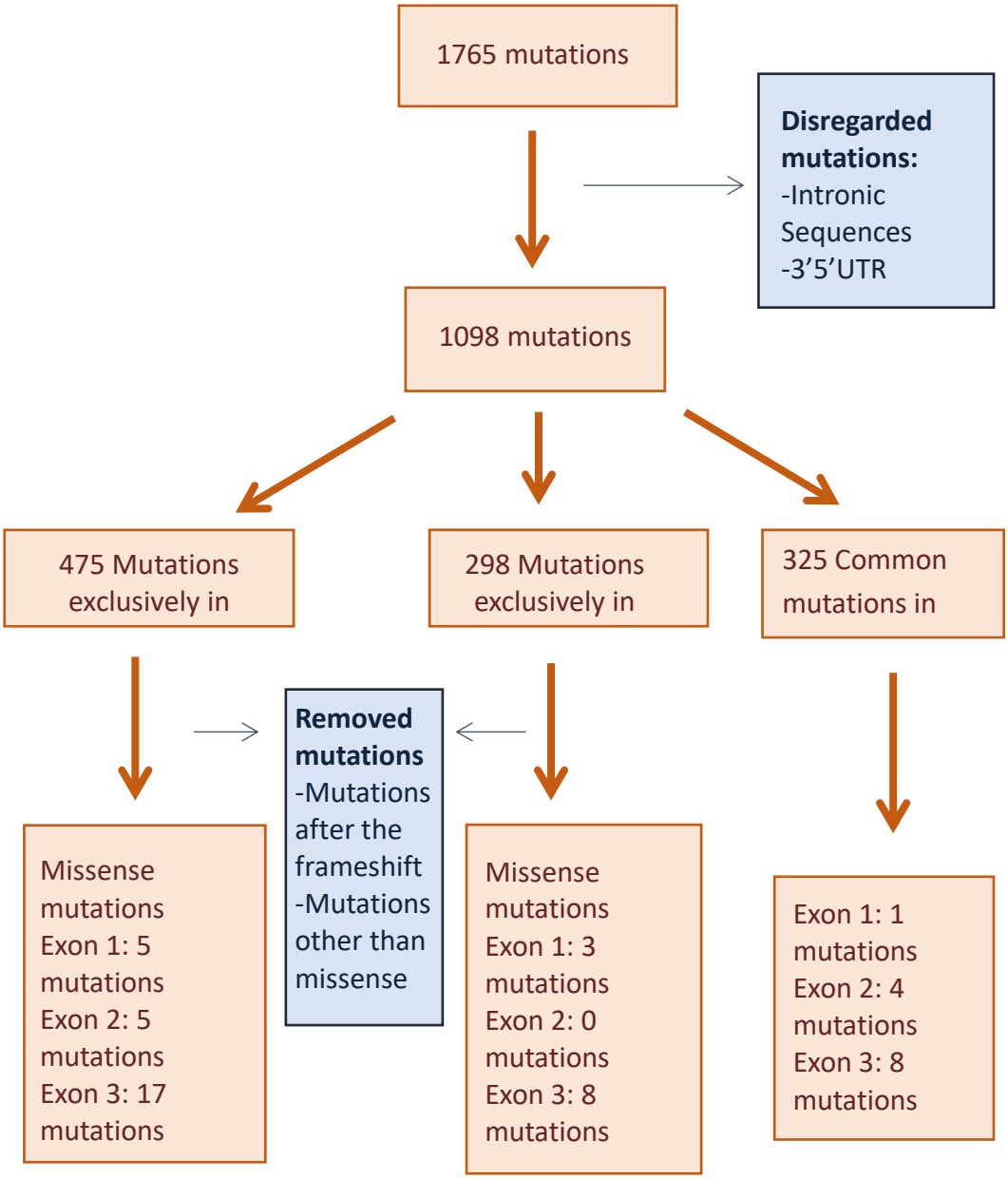

Figure 10- Mutations identified in our study

Mutations in exon 1 affect the signal peptide and the first amino acids of the extracellular domain. Exon 2 mutations also affect the extracellular domain encoding the initial LRRs. Exon 3 is responsible for encoding the remaining LLRs and the hypervariable region of the extracellular domain, the transmembrane domain and the cytoplasmic domain (Vaure & Liu, 2014).

3.3. Mutations Present in Healthy Controls

3.3.1. Mutations in Exon 1

Two frameshift mutations were identified in exon 1 at locations 18 (in C8 and C11) and 22 (in C12). Exon 1 harbors a total of 4 mutations Leu7Gln (non-polar neutral hydrophobic to polar neutral hydrophilic) present in C11, Ala8Asp (non-polar neutral hydrophobic to polar ionizable acidic hydrophilic) present in C9 and C11, Leu11Gln (non-polar hydrophobic to polar neutral hydrophilic) present in C8 and C11, and Ser25Cys (polar neutral to polar neutral) present in C1, C2, C6, C9, C10, C13, and C36 (Table 8). All 4 mutations were heterozygous, resulted in a major change in aa nature. Moreover, Ser25Cys was common among 7 healthy individuals.

Table 8- Exon 1 mutations, aa position, aa nature, and the healthy controls possessing the mutations

AA CHANGE	**AA POSITION**	**AA Nature**	**CONTROLS POSSESSING THE MUTATION**
Mutations in Exon 1			
Leu->Gln	7	Non-polar neutral hydrophobic -> Polar neutral hydrophilic	C11
Ala->Asp	8	Non-polar hydrophobic -> Polar Ionizable acidic hydrophilic	C9, C11
Leu->Gln	11	Non-polar hydrophobic -> Polar neutral hydrophilic	C8, C11

AA CHANGE	AA POSITION	AA Nature	CONTROLS POSSESSING THE MUTATION
Ser-> Cys	25	Polar Neutral-> Polar neutral	C1, C2, C6, C9, C10, C13, C36

Abbreviation: AA, amino acid; Ala: Alanine; Asp: Aspartic acid; Cys: Cysteine; Gln: Glutamine; Leu: Leucine; Ser: Serine

3.3.2. Mutations in Exon 2

Frameshift mutations were absent from exon 2. We identified 4 heterozygous missense mutations in exon 2 Val82Glu (non-polar hydrophobic to polar neutral hydrophilic) present in C7 and C17, Leu83Arg (non-polar hydrophobic to polar ionizable, basic hydrophilic) present in C6, Leu83Val (Conserved nature of aa non-polar hydrophobic) present in C13 and C17, Ser86Tyr (polar neutral aliphatic to polar neutral aromatic) present in C6 and C7 (table 9).

Table 9-Exon 2 mutations, aa position, aa nature, and the healthy controls possessing the mutation

AA CHANGE	AA POSITION	AA Nature	CONTROLS POSSESSING THE MUTATION
Mutations in Exon 2			
Val->Gln	82	Non-polar hydrophobic -> polar neutral hydrophilic	C7, C17
Leu-> Arg	83	Non-polar hydrophobic -> polar ionizable, basic hydrophilic	C6
Leu-> Val	83	No change (Non-polar hydrophobic)	C13, C17
Ser-> Tyr	86	Polar Neutral aliphatic -> polar neutral aromatic	C6, C7

Abbreviation: AA, amino acid; Arg: Arginine; Gln: Glutamine; Leu: Leucine; Ser: Serine; Tyr: Tyrosine; Val: Valine

3.3.3. Mutations in Exon 3

Frameshift mutations were present in 63% of all healthy individuals (12 out of 19) between the positions 246-249. A total of 16 missense mutations were identified in exon 3 (table 10). All 16 mutations were heterozygous except for one homozygous mutation (Ile247Thr), accounting for an isoleucine to threonine shift at position 247. A common heterozygous mutation, Cys246Arg, was identified in 4 out of 19 healthy controls.

Table 10-Exon 3 mutations, aa position, aa nature, and the healthy controls possessing the mutation

AA CHANGE	**AA POSITION**	**AA Nature**	**CONTROLS POSSESSING THE MUTATION**
Mutations in Exon 3			
Leu->Pro	155	Neutral No change	C13
His-> Pro	179	Basic ionizable hydrophilic -> neutral	C13
Leu-> Ser	180	Neutral hydrophobic -> neutral Polar	C13
Leu-> Pro	210	neutral No change	C9
Ser-> Phe	211	Polar Neutral ->neutral aromatic hydrophobic	C9
Leu-> Met	212	No change(non-polar hydrophobic)	C9
Ile-> Thr	226	Neutral hydrophobic -> neutral polar	C13
Arg->Tyr	227	Basic hydrophilic -> polar aromatic neutral	C9, C13
Leu-> Ile	228	No change (non-polar hydrophobic)	C16
Leu-> Val	228	No change (non-polar hydrophobic)	C9, C36
Leu->His	228	Neutral hydrophobic -> basic ionizable hydrophilic	C3, C6
Lys->Thr	244	Basic ionizable hydrophilic-> neutral polar	C36
Lys-> Asn	244	Basic ionizable hydrophilic-> polar neutral hydrophilic	C14
Cys-> Tyr	246	Polar neutral->polar aromatic neutral	C5
Cys->Arg	246	Polar neutral -> polar basic ionizable hydrophilic	C5, C7, C9, C15
Ile-> Thr	247	Neutral hydrophobic -> polar neutral	C14

Abbreviation: AA, amino acid; Asn: Asparagine; Arg: Arginine; Cys: Cysteine; His: Histidine; Ile: Isoleucine; Leu: Leucine; Met: Methionine; Phe: Phenylalanine; Pro: Proline; Ser: Serine; Val: Valine; Thr: Threonine; Tyr: Tyrosine; Lys: Lysine

3.4. Mutations in NAFLD Patients

3.4.1. Mutations in Exon 1

Out of the 30 NAFLD patients recruited in this study, only 3 showed frameshift mutations in exon 1: P33 had a frameshift mutation at position 3, and P29 and P48 had a frameshift at position 28. A total of 6 mutations were identified in exon 1; however, Ser25Cys conversion was excluded since it is common between patients and controls, thus 5 mutations were exclusively present in the patients (table 11). The mutations were Leu11Arg (neutral hydrophobic to basic ionizable hydrophilic) present in P26; Ala14Pro (no change -neutral hydrophobic) present in P30 and P48; Leu18Pro (no change- neutral hydrophobic) present in P19; Pro28His (neutral hydrophobic to basic ionizable hydrophilic), present in P30; and Cys29Phe (polar neutral to non-polar aromatic hydrophobic) present in P22, P25, and P27. All 5 mutations in exon 1 were heterozygous.

Table 11-Exon 1 mutations, aa position, aa nature, and the patients possessing the mutation. The highlighted mutations represent the common mutations between patients and controls

AA CHANGE	AA NATURE	AA POSITION	PATIENTS POSSESSING THE MUTATION
leu-> arg	Neutral hydrophobic -> basic ionizable hydrophilic	11	P26
Ala->pro	No change (neutral hydrophobic)	14	P30, P48
Leu->pro	No change (neutral hydrophobic)	18	P19
Ser->cys	No change (Polar Neutral)	25	P22, P25, P26, P27,P28,P30, P38,P40,P41,P43,P44,P46, P47,P49
Pro->his	neutral hydrophobic -> Polar, basic ionizable hydrophilic	28	P30
Cys-> phe	Polar neutral -> non-polar aromatic hydrophobic	29	P22, P25, P27

Abbreviation: AA, amino acid; Ala: Alanine; Arg: Arginine; Cys: Cysteine; His: Histidine; Leu: Leucine; Phe: Phenylalanine; Pro: Proline; Ser: Serine

3.4.2. Mutations in Exon 2

The sequencing of exon 2 in NAFLD patients shows that frameshift mutations were absent from exon 2 and a total of 9 missense mutations were identified. Five mutations were exclusive to the patients while the remaining 4 were excluded being present in both healthy controls and patients (Val82Glu, Leu83Val, Leu83Gln, and Leu83Arg) (table 12). The 5 mutations were Met41Arg (non-polar neutral hydrophobic to polar basic ionizable hydrophilic) present in P49, Pro53Ser (hydrophobic to polar hydrophilic neutral) present in P32, Leu61Val (no change in nature- non-polar hydrophobic) present in P32 and P49, Leu66Met (no change in nature- non-polar hydrophobic) present in P32 and P49, and Phe77Ser (non-polar neutral aromatic hydrophobic to polar neutral hydrophilic) present in P32. All mutations present in exon 2 were heterozygous mutations.

Table 12-Exon 2 mutations, aa position, aa nature, and the patients possessing the mutation. The highlighted mutations represent the common mutations between patients and controls

AA CHANGE	AA NATURE	AA POSITION	PATIENTS POSSESSING THE MUTATION
Met-> arg	Non-polar neutral hydrophobic-> Polar basic ionizable hydrophilic	41	P49
Pro ->ser	hydrophobic to polar hydrophilic neutral	53	P32
Leu->Val	No change (non-polar hydrophobic)	61	P32, P49
Leu-> met	No change (non-polar hydrophobic)	66	P32, P49
Phe-> ser	Non-polar neutral aromatic hydrophobic-> Polar hydrophilic neutral	77	P32
Val-> Glu	Neutral hydrophobic-> polar acidic ionizable hydrophilic	82	P32, P25

AA CHANGE	AA NATURE	AA POSITION	PATIENTS POSSESSING THE MUTATION
Leu->Val	No change (non-polar hydrophobic)	83	P18, P21, P24, P25
Leu-> Gln	Neutral hydrophobic -> Polar neutral hydrophilic	83	P32
Leu-> arg	Non-polar Neutral hydrophobic -> polar basic ionizable hydrophilic	83	P25, P30

Abbreviation: AA, amino acid; Arg: Arginine; Cys: Cysteine; Glu: Glutamic acid; Gln: Glutamine; Leu: Leucine; Met: Methionine; Phe: Phenylalanine; Pro: Proline; Ser: Serine; Val: Valine

3.4.3. Mutations in Exon 3

The genotyping of exon 3 indicated the presence frameshift mutations localized at various positions in: 20 out of 30 patients (67%) of all the patients between positions 241-251; 4 out of 30 patients (13%) between positions 367-401; and 3 out of 30 patients (10%) at position 215. A total of 25 mutations in exon 3 were identified, however 8 mutations were excluded since they were present in healthy controls; Therefore 17 missense mutations were exclusive to NAFLD patients (table 13). All exon 3 mutations are heterozygous except for 2 homozygous mutations, GLy249Val present in P20, P41, and P42 and Ile207Met identified in P34.

Table 13- Exon 3 mutations, aa position, aa nature, and the patients possessing the mutation. The highlighted mutations represent the common mutations between patients and controls.

AA CHANGE	AA NATURE	AA POSITION	PATIENTS POSSESSING THE MUTATION
Leu-> ser	Neutral hydrophobic -> polar neutral hydrophilic	167	P18
Tyr-> his	Polar aromatic neutral -> Basic polar ionizable	170	P33
Phe-> ser	neutral hydrophobic -> Polar neutral hydrophilic	171	P33
Ser>phe	Polar Neutral Hydrophilic -> Non-polar neutral aromatic hydrophobic	172	P46

AA CHANGE	AA NATURE	AA POSITION	PATIENTS POSSESSING THE MUTATION
Leu-> ser	neutral hydrophobic -> Polar neutral hydrophilic	180	P18
Asn-> ser	Polar neutral hydrophilic -> polar neutral hydrophilic	185	P18
Asp-> ala	Polar ionizable acidic -> non-polar Neutral hydrophobic	194	P46
Leu-> pro	no change (non-polar hydrophobic)	195	P46
Ile-> met	No change (non-polar hydrophobic)	207	P34
Leu-> ser	Neutral hydrophobic -> Polar neutral Hydrophilic	208	P18, P20
Asp-> his	Polar ionizable acidic -> Polar ionizable basic	209	P46
Leu-> ser	Neutral hydrophobic -> Polar neutral Hydrophilic	210	P46
Asn-> serine	Polar neutral hydrophilic -> Polar neutral Hydrophilic	213	P18
Ile-> phe	No change (non-polar hydrophobic)	218	P19
Leu-> Ile	No change (non-polar hydrophobic)	228	P34, P32
Leu-> arg	Neutral hydrophobic -> Polar ionizable basic hydrophilic	228	P24, P32
Lys-> thre	Polar ionizable basic hydrophilic-> Polar neutral hydrophilic	244	P47
Cys->Tyr	Polar neutral -> aromatic Polar neutral	246	P34
Ile-> Leu	No change(non-polar hydrophobic)	247	P34, P42
Ile-> thr	Neutral hydrophobic-> Polar neutral Hydrophilic	247	P18
Lys-> asn	Polar ionizable Basic hydrophilic-> Polar neutral hydrophilic	248	P47
Gln-> pro	Polar neutral hydrophilic -> neutral hydrophobic	248	P34
Gly->val	Non-Polar neutral-> Neutral hydrophobic	249	P20, P41, P42
Gly-> arg	Non-Polar Neutral -> Polar ionizable basic hydrophilic	249	P34
Leu-> glu	Neutral hydrophobic -> Polar Ionizable acidic hydrophilic	250	P34
Mutations in Exon 3: Stop gained and protein altering			
Stop gained		219	P24
Protein Altering		247	P34
Stop gained		248	P24, P34

Abbreviation: AA, amino acid; Ala: Alanine; Asn: Asparagine; Arg: Arginine; Asp: Aspartic acid; Cys: Cysteine; Gly: Glycine; Glu: Glutamic acid; Gln: Glutamine; His: Histidine; Ile: Isoleucine; Leu: Leucine; Met: Methionine; Phe: Phenylalanine; Pro: Proline; Ser: Serine; Val: Valine; Lys: Lysine; Thr: Threonine; Tyr: Tyrosine;

3.5. Fibro scan Assessment of Controls and Patients

All the recruited individuals (controls and patients) undertook a fibroscan to assess the level of steatosis and fibrosis level. Patients were categorized into 4 groups according to the fibrosis stage (f0, f1, f3, and f4). Stage f0 is characterized by the absence of fibrosis, f1 is characterized by early fibrosis, f3 is characterized by severe fibrosis, and f4 is characterized by cirrhosis (Table-14). The fibroscan results indicated that all healthy individuals have a steatosis level of S0 (68%) or S1 (32%) and a fibrosis level of f0 (100%). On the other hand, steatosis level in NAFLD patients showed S2 in (27%) and S3 in (73%) with fibrosis stage of f0 (66%), f1 (10%), f3 (17%), and f4 (7%) (Table-15). No f2 stage was detected in any of the patients.

Table 14-The histologic characteristics of the 5 fibrosis stages (f0-f4)

Score	Histologic stage of liver fibrosis
F0	No fibrosis
F1	Early fibrosis: periportal fibrosis without septa
F2	Moderate fibrosis: periportal fibrosis with rare septa
F3	Severe fibrosis: numerous septa without cirrhosis
F4	Cirrhosis: lobular regeneration between septa

To investigate the effects of the genotype on the progression of NAFLD and the stage of fibrosis, we examined whether a genotype-phenotype correlation exists in NAFLD patients. The genotypes of the patients and the identified mutations were

assessed to test if a correlation exited with the fibrosis stage of the patients in our sample.

3.5.1. Genotype with F0 Stage Patients

The fibroscan results showed that 20 out of 30 NAFLD patients had f0 fibrosis stage (Table 15). Two patients with f0 stage had a frameshift mutation in exon 1 at position 28, and 19 patients had a frameshift in exon 3 at 3 locations. The frameshift in exon 3 occurred at position 218 in one patient, positions 241-251 in 15 patients, and positions 367-396 in 2 patients. Moreover, we identified a total of 5 mutations in exon 1, 4 mutations in exon 2 all identified in one patient (p32), and 13 mutations in exon 3. All the mutations were heterozygous except for one homozygous mutation present in p41 and p42 (Gly249Val) (tables 16, 17, 18).

3.5.2. Genotype with F1 Stage Patients

Three patients out of 30 showed f1 fibrosis stage upon clinical examination (Table 15). We identified 3 frameshift mutations in the patients at positions 215, 250, and 396. Moreover, a total of 2 mutations were found: one heterozygous mutation in exon 1 and one homozygous mutation in exon 3. The homozygous mutation was present only in one patient (p20) and resulted in glycine to valine conversion at position 249 (Gly249Val) (table 19).

3.5.3. Genotype with F3 Stage Patients

The fibro scan of NAFLD patients showed 5 patients with f3 fibrosis stage (Table 15). Frameshift mutations were present at positions 3, 215, 246, 249, and 251.We also

identified one exon 1 mutation, 3 exon 2 mutations, all present in one patient (p49), in addition to 5 exon 3 mutations. All the mutations were heterozygous, and patient 34 possessed 4 mutations in exon 3 (table 20).

3.5.4. Genotype with F4 Stage Patients

Two out of 30 NAFLD patients were diagnosed with f4 fibrosis stage (Table 15). TLR4 sequencing indicated that they exhibited frameshift mutations at positions 246 and 401. They also harbored one common heterozygous mutation in exon 1 and no mutations in exons 2 and 3 (table 21).

Table 15. The fibrosis stage of NAFLD patients and the percentage of patients in each stage

Fibrosis stage	**Percentage of Patients**	**Patients**
F0	66	P19, P18,P21,P23,P27,P28,P29,P30 P31, P32,P37,P39,P40,P41,P42,P43, P44, P45,P46,P47,P48
F1	10	P20, P22
F3	17	P24, P26,P33,P34,P49
F4	7	P25, P38

Table 16- Exon 1 mutations of all patients diagnosed with F0 fibrosis.
HT (yellow): heterozygous mutation in exon 1; HT (blue): heterozygous mutation in exon 1; arrows (red): frameshift mutation

EXON	GENDER	EXON1	EXON1	EXON1	EXON1	EXON1	EXON1	PHENO
MUT		ALA-PRO	LEU-PRO	PRO-HIS	CYS-PHE	PRO-SER	LEU-VAL	
P.P		14	18	28	29	53	61	
P18	M							S2 F0
P19	F		HT					S2 F0
P21	F							S3 F0
P23	M							S2 F0
P27	M				HT			S3 F0
P28	M							S2 F0
P29	M			>>>>>>				S3 F0
P30	F	HT		HT				S3 F0
P32	M					HT	HT	S2 F0
P37	M							S3 F0
P39	F							S2 F0
P40	M							S3 F0
P41	M							S3 F0
P42	F							S3 F0
P43	F							S3 F0
P44	M							S2 F0
P45	M							S3 F0
P46	M							S3 F0
P47	M							S3 F0
P48	M	HT		>>>>>>				S3 F0

Abbreviations: Ala: Alanine; Cys: Cysteine; His: Histidine; Leu: Leucine; Phe: Phenylalanine; Pro: Proline; Ser: Serine; Val: Valine; S: Steatosis stage; F; fibrosis stage; MUT: mutation; P.P: patient; Pheno: phenotype; M: male; F: female.

Table 17- Exon 2 mutations of all patients diagnosed with F0 fibrosis.

HT (blue): heterozygous mutation in exon 2; HT (green): heterozygous mutation in exon 2

EXON	GENDER	EXON2	EXON2	EXON2	EXON2	EXON2	PHENO
MUT		LEU-MET	PHE-SER	LEU-SER	SER-PHE	ASN-SER	
P.P		66	77	167	172	185	
P18	M			HT		HT	S2 F0
P19	F						S2 F0
P21	F						S3 F0
P23	M						S2 F0
P27	M						S3 F0
P28	M						S2 F0
P29	M						S3 F0
P30	F						S3 F0
P32	M	HT	HT				S2 F0
P37	M						S3 F0
P39	F						S2 F0
P40	M						S3 F0
P41	M						S3 F0
P42	F						S3 F0
P43	F						S3 F0
P44	M						S2 F0
P45	M						S3 F0
P46	M				HT		S3 F0
P47	M						S3 F0
P48	M						S3 F0

Abbreviations: Asn: Asparagine; Leu: Leucine; Met: Methionine; Phe: Phenylalanine; Ser: Serine; S: Steatosis stage; F; fibrosis stage; MUT: mutation; P.P: patient; Pheno: phenotype; M: male; F: female.

Table 18- The mutations in exon 3 of each patient having a fibrosis stage of f0. HT (green): heterozygous mutation in exon 3; HM (green): homozygous mutation in exon 3; arrows (red): frameshift mutation

MUT	G	ASP-ALA	LEU-PRO	LEU-SER	ASP-HIS	LEU-SER	ASN-SER		ILE-PHE	LEU-ARG			ILE-MET	GLN-PRO	GLY-VAL	ALA-PRO			
P.P		194	195	208	209	210	213	215	218	228	241	246	247	248	249	251	367	396	
P18	M			HT			HT							>>> >>>					S2F0
P19	F								HT				>> >> >>						S2F0
P21	F												>> >> >>						S3F0
P23	M												>> >> >>						S2F0
P27	M											>> >> >>							S3F0
P28	M															>>> >>>			S2F0
P29	M																		S3F0
P30	F											>> >> >>							S3F0
P32	M									HT			>> >> >>						S2F0
P37	M											>> >> >>							S3F0
P39	F											>> >> >>							S2F0
P40	M											>> >> >>							S3F0
P41	M														HM	HT	>> >> >>		S3F0

P42	F														HM			>>>>>>	S3F0
P43	F											>>>>>>							S3F0
P44	M										>>>>>>								S2F0
P45	M											>>>>>>							S3F0
P46	M	HT	HT		HT	HT		>>>>>>											S3F0
P47	M														>>>>>>				S3F0
P48	M																		S3F0

Abbreviations: Ala: Alanine; Arg: Arginine; Asp: Aspartic acid; Gly: Glycine;Gln: Glutamine; His: Histidine; Ile: Isoleucine; Leu: Leucine; Met: Methionine; Phe: Phenylalanine; Pro: Proline; Ser: Serine; Val: Valine; S: Steatosis stage; F; fibrosis stage; MUT: mutation; P.P: patient; Pheno: phenotype; M: male; F: female.

Table 19-The mutations in exon 1, 2, and 3 of each patietns having a fibrosis stage of f1. HT (yellow): heterozygous mutation in exon 1; HM (green): homozygous mutation in exon 3; arrows (red): frameshift mutation.

EXON	GENDER	EXON1	EXON3	EXON3	EXON3		PHENO
MUT		CYS-PHE		GLY-VAL	LEU-GLU		
P.P		29	215	249	250	396	
P20	M			HM	>>>>>>		S3 F1
P22	F	HT	>>>>>>				S3 F1
P31	F					>>>>>>	S2 F1

Abbreviations: Cys: Cysteine; Gly: Glycine; Glu: Glutamic acid; Leu: Leucine; Phe: Phenylalanine; Val: Valine; S: Steatosis stage; F; fibrosis stage; MUT: mutation; P.P: patient; Pheno: phenotype; M: male; F: female.

Table 20-The mutations in exon 1, 2, and 3 of each patietns having a fibrosis stage of f3. HT (yellow): heterozygous mutation in exon 1; HT (blue): heterozygous mutation in exon 2; HT (green): heterozygous mutation in exon 3; arrows (red): frameshift mutation.

EXON	GENDER	EXON1	EXON1	EXON2	EXON2	EXON2	EXON3	EXON3		EXON3	EXON3	EXON3	EXON3	EXON3	EXON3	PHENO
MUT			LEU-ARG	MET-ARG	LEU-VAL	LEU-MET		LEU-ARG		ILE-MET	GLN-PRO	GLY-VAL	GLY-ARG	LEU-GLU	ALA-PRO	
P.P		3	11	41	61	66	215	228	246	247	248	249	249	250	251	
P24	F							HT				>>>>>>				S3 F3
P26	F		HT				>>>>>>									S3 F3
P33	M	>>>>>>														S3 F3
P34	M									HT	HT		HT	HT	>>>>>>	S3 F3
P49	M			HT	HT	HT			>>>>>>							S3 F2-3

Abbreviations: Ala: Alanine; Arg: Arginine; Gly: Glycine; Glu: Glutamic acid; Gln: Glutamine; Ile: Isoleucine; Leu: Leucine; Met: Methionine; Pro: Proline; Ser: Serine; Val: Valine; S: Steatosis stage; F; fibrosis stage; MUT: mutation; P.P: patient; Pheno: phenotype; M: male; F: female.

Table 21.The mutations in exon 1, 2, and 3 of each patient having a fibrosis stage of f4. HT (yellow): heterozygous mutation in exon 1; arrows (red): frameshift mutation.

EXON	GENDER	EXON1	EXON3	EXON3	PHENO
MUT		CYS-PHE			
P.P		29	246	401	
P25	M	HT	>>>>>>		S3 F4
P38	M			>>>>>>	S3 F4

Abbreviations: Cys: Cysteine; Phe: Phenylalanine; S: Steatosis stage; F; fibrosis stage; MUT: mutation; P.P: patient; Pheno: phenotype; M: male; F: female.

CHAPTER 4

DISCUSSION

NAFLD refers to a spectrum of liver dysfunctions that include steatosis (fat infiltration of the liver parenchyma), NASH (fat accumulation and inflammation), and cirrhosis. NAFLD is considered to be the hepatic manifestation of the metabolic syndrome associated with obesity, insulin resistance, type 2 diabetes, and dyslipidemia. It is a complex disease where the environmental and genetic factors determine the disease phenotype and progression. Mutations in many genes have been suggested to play a role in the development of NAFLD including (Table 22), but not limited to: Patatin-like phospholipase domain-containing protein 3 (PNPLA3), monocyte differentiation antigen CD14 (CD14), TNF, and toll-like receptor 4 (TLR4).

The current study is the first in Lebanon to examine the potential role of the TLR4 gene in NAFLD development and progression. Compared to healthy subjects, genetic mutations in the TLR4 gene were determined in a sample of Lebanese NAFLD patients. Genotypic variations were then examined to check if they have any bearing on the clinical phenotype and progression of the disease. More specifically we checked if possible correlations exist between genotypic variations and Fibrotic stages (F0-F4) in NAFLD patients.

Recruited NAFLD patients were mostly males (67%), their clinical assessment showed no significant changes in liver function tests or serum enzymes. While we expected the lipid profile, specifically LDL (cholesterol carrying lipoprotein), to be significantly higher than normal levels, only 39% of patients had high LDL. This however may be attributed to the fact that most patients were being treated with LDL lowering agents such as statins.

Obesity and Diabetes type 2 are among the many risk factors underlying NAFLD development and progression. Prevalence of NAFLD in obese subjects (BMI>30) have been attributed to enhanced liver lipogenesis that promote liver injury (Milić et al., 2014) . Other studies correlated obesity with NAFLD progression and Fibrosis (Sweet et al., 2017). In our study, while 83% of obese individuals had NAFLD in line with literature; our findings with fibrosis were discordant. Most of the patients (10 out of 13 obese NAFLD patients) showed a stage of F0 indicating no fibrosis, one had F1 stage, and two had F3 stage. This mild phenotype may be attributed to presence of protective TLR4 mutations in NAFLD patients resisting thus the impact of obesity on NAFLD progression. Out of 13 patients, 3 had homozygous possibly protective mutation in exon 3 (Gly249Val) that has not been reported before. In addition, 6 patients had frameshift between amino acids 246-248 that interfere with TLR4 ligand/co-receptor complex interaction, reducing thus the level of pro-inflammatory cytokines and decreasing inflammation and fibrosis.

Insulin resistance (IR) that leads to Type 2 diabetes mellitus (DM2T) has been also associated with NAFLD with 70% prevalence rate (Leite et al., 2009). IR enhances hepatic de novo lipogenesis and gluconeogenesis resulting in increased serum glucose levels hence DM2T development (Dabravolski et al., 2021). Among the NAFLD patients in this study, 33% were diabetic. Most of the diabetic patients (6 out of 10 patients) had no to low fibrosis (F0 and F1 stages), which may be attributed to the presence of protective mutations in TLR4 gene controlling inflammation and progression of the disease.

NAFLD is known to increase with age, being highest in patients aged between 45-49 years (Wu et al., 2022) who possess factors favoring the metabolic syndrome

such as hypertension, hyperlipidemia, obesity, and diabetes. In addition, other complications that increase visceral fat accumulation and decrease hepatic blood flow are more predominant in elder individuals, enhancing thus NAFLD development (Wu et al., 2022). In our study, our NAFLD patients aged between 23-70 years, with those aged between 45-49 years (20%) possessing fibrosis stages of F0-F1. Although all the patients who possessed F3-F4 stages were older than 50 years, one patient harboring F4 stage was 43. Moreover, most patients aged older than 50 had F0-F1 stages. Hence, no link between age and fibrosis stage of NAFLD patients in our sample could be established.

These findings indicate that other factors may be contributing in controlling the fibrotic progression of the disease into F4 stage. In our sample, the majority of NAFLD patients possessed F0 stage (67%), 17% showed F3 fibrosis, and only two (7%) were diagnosed with F4 stage.

Several genes are implicated in the pathogenesis of NAFLD including genes Involved in insulin signaling, Lipogenesis, Phospholipid transfer, Activation of long chain fatty acids Glucose transporters, regulator of energy homeostasis, monocyte differentiation antigen CD14 (CD14), tumor necrosis factor (TNF), and toll-like receptor 4 (TLR4) and others (Table 22). In this study, we examined the role of TLR4 in NAFLD progression and development of fibrosis stages. This is the first study performed on a Lebanese sample of NAFLD patients, that attempts to check if genotypic variations in the TLR4 gene has any significant implications on the phenotypic presentation of patients, more specifically their fibrosis stage.

Table 22- The genes that are implicated in the pathogenesis of NAFLD

Gene	Protein	Chromosome	Exons	Function
IRS-1	Insulin receptor substrate 1	2	2	no intrinsic enzyme activity, serves as a docking protein involved in binding and activating other signal transduction molecules after being phosphorylated on tyrosine by insulin receptor kinase
ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase family member 1	6	25	Enzyme that regulates pyrophosphate levels, and functions in bone mineralization and soft tissue calcification
GCKR	Glucokinase regulatory protein	2	19	Inhibits glucokinase (GCK) by forming an inactive complex with this enzyme
PPARG	Peroxisome proliferator-activated receptor gamma	3	9	Nuclear receptor that binds peroxisome proliferators such as hypolipidemic drugs and fatty acids. Once activated by a ligand, the nuclear receptor binds to DNA specific PPAR response elements (PPRE) and modulates the transcription of its target genes, such as acyl-CoA oxidase. It therefore controls the peroxisomal beta-oxidation pathway of fatty acids. Key regulator of adipocyte differentiation and glucose homeostasis
TCF7L2	Transcription factor 7- like 2	10	17	high mobility group (HMG) box-containing transcription factor implicated in blood glucose homeostasis
SLC2A1	Solute carrier family 2, facilitated glucose transporter member 1	1	10	major glucose transporter in the mammalian blood-brain barrier
SLC27A5	Solute carrier family 27 (fatty acid transporter), member 5	19	10	an isozyme of very long-chain acyl-CoA synthetase (VLCS). It is capable of activating very long-chain fatty-acids containing 24- and 26-carbons
LIPN1 LIPN	lipase, family member N	10	9	lipase that is highly expressed in granular keratinocytes, plays a role in the differentiation of keratinocytes
MTTP	microsomal triglyceride transfer protein *(large subunit)*	4	19	catalyzes the transport of triglyceride, cholesteryl ester, and phospholipid between phospholipid surfaces
PEMT	phosphatidylethanol amine N-methyltransferase	17	9	an enzyme which converts phosphatidylethanolamine to phosphatidylcholine by sequential methylation in the liver
ADIPOQ	adiponectin, C1Q and collagen domain containing	3	3	Adiponectin is a hormone secreted by adipocytes that regulates energy homeostasis and glucose and lipid metabolism, expressed in adipose tissue exclusively,
ADIPOR2	adiponectin receptor 2	12	7	receptor for globular and full-length adiponectin, activates a signaling cascade that leads to increased PPARA activity, and ultimately to increased fatty acid oxidation and glucose uptake
ApoC3	apolipoprotein C-III	11	4	Component of triglyceride-rich very low density lipoproteins (VLDL) and HDL in plasma, Intracellularly, promotes hepatic very low density lipoprotein 1 (VLDL1) assembly and secretion; extracellularly, attenuates hydrolysis and clearance of triglyceride-rich lipoproteins (TRLs). Impairs the lipolysis of TRLs by inhibiting lipoprotein lipase and the hepatic uptake of TRLs by remnant receptors
ApoE	Apolipoprotein E	19	4	main apoprotein of the chylomicron, binds to a specific receptor on liver cells and peripheral cells, essential for the normal catabolism of triglyceride-rich lipoprotein constituents
NR1I2/PXR	nuclear receptor subfamily 1 group I member 2 (pregnane X receptor)	3	9	Activates cytochrome P450-3A expression in response to a wide variety of xenobiotics and plays a critical role in mediating dangerous drug-drug interactions
PPARA	Peroxisome proliferator-activated receptor alpha	22	8	transcription factor and a major regulator of lipid metabolism in the liver: promotes uptake, utilization, and catabolism of fatty acids by upregulation of genes involved in fatty acid transport, fatty binding and activation, and peroxisomal and mitochondrial fatty acid β-oxidation
FADS1	Fatty acid desaturase 1	11	8	component of a lipid metabolic pathway that catalyzes biosynthesis of highly unsaturated fatty acids from

Gene	Protein	Chromosome	Exons	Function
				linoleic acid and alpha-linolenic acid. Catalyzes the desaturation of dihomo-gamma-linoleic acid and eicosatetraenoic acid to generate arachidonic acid and eicosapentaenoic acid, respectively
PNPLA3	Patatin-like phospholipase domain-containing protein 3	22	9	Multifunctional enzyme which has both triacylglycerol lipase and acylglycerol Oacyltransferase activities.
TM6SF2	Transmembrane 6 superfamily member 2	19	10 12	Regulator of liver fat metabolism influencing triglyceride secretion and hepatic lipid droplet content. May function as sterol isomerase
HFE	Hereditary hemochromatosis protein	6	7	Binds to transferrin receptor (TFR) and reduces its affinity for iron-loaded transferrin
SOD2	Superoxide dismutase 2	6	5	mitochondrial matrix enzyme that scavenges oxygen radicals produced by the extensive oxidation-reduction and electron transport reactions occurring in mitochondria
GCLC	Glutamate-cysteine ligase catalytic subunit	6	16	first rate-limiting enzyme in glutathione (GSH) biosynthesis.
MRP2 (ABCC2)	ATP-binding cassette, sub-family C, member 2	10	32	Mediates hepatobiliary excretion of numerous organic anions
MTHFR	5,10-Methylenetetrahydrofolate reductase	1	11	Catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine
TLR4	**Toll-like receptor 4**	**9**	**3**	**recognizes bacterial lipopolysaccharide, along with several other components of pathogens and endogenous molecules produced during abnormal situations, such as tissue damage which leads to the synthesis of pro-inflammatory cytokines and chemokines and the expression of co-stimulatory molecules**
CD14	Monocyte differentiation antigen CD14	5	2	Coreceptor for bacterial lipopolysaccharide, leads to NF-kappa-B activation, cytokine secretion and the inflammatory response
TNF	Tumor necrosis factor	6	4	Cytokine that binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. It is mainly secreted by macrophages and can induce cell death of certain tumor cell lines
sTNFr-2	Tumor necrosis factor receptor 2	1	10	main TNF receptor found on circulating T cells and is the major mediator of autoregulatory apoptosis in CD8+ cells. TNFR2 may act with TNFR1 to kill nonlymphoid cells.
FDFT1	Farnesyldiphosphate farnesyl transferase 1 (Squalene synthase)	8	8	This gene encodes a membrane-associated enzyme located at a branch point in the mevalonate pathway. The encoded protein is the first specific enzyme in cholesterol biosynthesis, catalyzing the dimerization of two molecules of farnesyl diphosphate in a two-step reaction to form squalene
IL6	Interleukin-6	7	5	Cytokine with a wide variety of biological functions. It is a potent inducer of the acute phase response. Plays an essential role in the final differentiation of Bcells into Ig-secreting cells Involved in lymphocyte and monocyte differentiation
AGT	Angiotensinogen	1	5	Essential component of the renin-angiotensin system (RAS), a potent regulator of blood pressure, body fluid and electrolyte homeostasis.
AGTR1	Angiotensinogen II Receptor 1	3	5	type 1 receptor, mediates the major cardiovascular effects of angiotensin II
KLF6	Krueppel-like factor 6	10	4	Transcriptional activator (By similarity). Binds a GC box motif. functions as a tumor suppressor. Multiple transcript variants encoding different isoforms have been found for this gene, some of which are implicated in carcinogenesis
TGFb1	Transforming growth factor beta-1	19	7	high levels localized in developing cartilage, endochondral and membrane bone, and skin, suggesting a role in the growth and differentiation of these tissues

Gene	Protein	Chromosome	Exons	Function
COL13A1	Collagen, Type XIII, Alpha-1	10	42	nonfibrillar transmembrane collagen that plays an autocrine role in the development and maturation of the neuromuscular junction
CDKN1A	Cyclin-dependent kinase inhibitor 1	6	3	critical role in the cellular response to DNA damage, and its overexpression results in cell cycle arrest

TLR4 plays a key role in the activation of inflammatory pathways in various liver diseases including NAFLD. Being highly polymorphic gene; the occurrence of single-nucleotide polymorphisms (SNPs) in TLR4 gene among certain individuals alters the capacity of TLR4 receptor to bind or respond to its ligands. Screening normal subjects and NAFLD patients for mutations in their TLR4 gene, we have identified a large number of mutations indicating it is a highly polymorphic gene. Mutations in exon-1would influence the signal peptide and the first few amino acids of the extracellular domain, while mutations in exon-2 affect the initial LRRs of the extracellular domain. All nucleotide base changes were missense mutations with no frameshift identified in either the control or the patients' sequences in exon-2. Exon-3 harbored most of the identified mutations including missense mutations (93%) and many frameshifts (95%) spanning amino acid residues 241-251. Changes in nucleotides of exon-3 will modify the translated protein encoding the extracellular domain (aa residues 1–624) including the hypervariable region, the cytoplasmic (aa residues 659–838), and the transmembrane (aa residues 625–658) domains.

In previous studies, 2 mutations in TLR4 gene were identified in homozygous and heterozygous states (Thr399Ile and Asp299Gly) that exhibited a protective effect against NAFLD progression (Guo et al., 2009; Kiziltas et al., 2014). Both mutations are located in exon-3 affecting the extracellular domain of TLR4 receptor, disrupting the binding of TLR4 to its co-receptors and reducing ligand recognition (Rallabhandi et al., 2006). In hepatic stellate cells (HSCs), TLR4 signaling causes fibrogenesis via the

downregulation of BAMBI, an inhibitory TGF-β1 pseudoreceptor, in addition to enhancing the resistance of HSCs to pro-apoptotic stimuli, prolonging cell survival (Guo & Friedman, 2010; Seki et al., 2007). The presence of protective TLR4 mutations (Thr399Ile and Asp299Gly) enhances apoptosis through reducing the expression of anti-apoptotic protein, Bcl-2 (Guo et al., 2009), and may decrease TLR4-mediated fibrogenesis by disrupting receptor-ligand recognition. Thr399Ile was detected in 3 patients and 1 control whereas the Asp299Gly was detected in one patient and 1 control. All patients possessing either mutation have steatosis stage of S2-S3 with F0 fibrotic stage indicating no progression of fibrotic stage confirming their protective effect. Being located in exon 3 these mutations will affect the extracellular domain of TLR4 and disrupt the binding of TLR4 to its co-receptors and ligands (Rallabhandi et al., 2006). Hence, the dysregulation in TLR4 signaling is expected to reduce the immune and fibrogenic response of liver cells, decreasing thus the expression of downstream pro-inflammatory cytokines, and inhibiting TLR4-mediated fibrogenesis. A study (Guo et al., 2009) has confirmed the role of Thr399Ile and Asp299Gly mutations in enhancing HSCs apoptosis by reducing the activity of NF-κB and Bcl-2, an anti-apoptotic protein.

A new mutation was identified in 3 NAFLD patients in the homozygous state Gly249Val. While the patients had S3 steatosis, their fibrosis stage was F0-F1 suggesting that Gly249Val might possess a protective effect against the progression of fibrosis in NAFLD patients. Exon-3 Gly249Val mutation, the hydrogen side chain is replaced by branched chain hydrophobic residue in the extracellular domain of TLR4 receptor. This causes disruption in the hypervariable region of the ectodomain affecting in turn the binding and the recognition of TLR4 to its co-receptors and ligands (Vaure

& Liu, 2014). A decrease in ligand recognition reduces the TLR4 signaling via MyD88-dependent and independent pathways, resulting in a decline in the expression of pro-inflammatory cytokines and IFN-β respectively, consequently attenuating the inflammatory response and reducing fibrosis.

Attempts to correlate genotype to phenotype were not conclusive due to small sample size, age difference, and clinical history of patients. Other contributing genes may not be excluded and need be investigated. However, the following pattern may be suggested when mutations and fibrosis stages are being compared or correlated.

1. A striking finding in our study is the high number of frame shifts mutations identified in exon -3 of TLR4 gene. Frameshifts in 67% of NAFLD patients were identified between amino acid position 241-251 of exon-3 where most patients have S2 -S3 steatosis stages but F0 fibrosis stage. Interestingly healthy controls harbored frameshift mutations in the same positions (246-249). In a previous study Ferguson et al. reported an attenuated response to LPS, in rat models with homozygous frameshift mutation in TLR4 at position 25 (Ferguson et al., 2013). It is plausible to suggest that identified frameshift mutations, in our study, may influence TLR4 expression, transport to cellular membrane, or ligand recognition (Rallabhandi et al., 2006). Hence, in patients with no to early fibrosis, frameshift mutations may be present on the active TLR4 allele causing a major change in TLR4 expression, attenuating receptor-ligand response, disrupting the signaling pathway, and leading to decreased inflammation and fibrogenesis.

Two frameshift mutations in exon-3 were identified at positions 367 and 396 in 2 patients, (P41 and P42 respectively) diagnosed with S3F0 stage. The absence of fibrosis may be attributed to the deleterious effect of the frameshift mutation on the

extracellular domain, which may affect the hypervariable region leading to reduced binding to ligands (e.g. LPS). Moreover, patients with frameshift mutations at positions 367 and 396 also possess the homozygous mutation Gly249Val which has a potential protective role in NAFLD.

A frameshift mutation in exon 1, present at position 28, was identified in 2 patients diagnosed with stage S3 steatosis and F0 fibrosis. The frameshift mutation occurred at an early position in the aa sequence, thus causing a severe alteration in the TLR4 signal peptide or the extracellular domain, potentially leading to a disruption in the vesicular transport of the protein to the ER and the cellular surface, or causing a dysregulation in the ligand-receptor-co-receptor complex. Reduced TLR4 signaling results in decreased inflammation and fibrogenesis. A study on TLR4/LDL receptor knockout mice revealed that the lack of TLR4 in diet-induced NAFLD induces the oxidation of fatty acids and prevents triglycerides formation in the liver (Ferreira et al., 2015).

Most NAFLD diabetic patients in our study (6 out of the 10) had F0-F1 fibrosis. The mild phenotype is attributed to the presence of protective missense mutations, homozygous Gly249Val mutation, and frameshifts in the diabetic patients. The presence of frameshift mutations in all NAFLD diabetic patients could potentially lead to disrupted TLR4 signaling in F0 patients where the active TLR4 allele harbored deleterious frameshift mutations.

2. Three NAFLD patients had F1 fibrosis stage. The presence of frameshift mutations in exon 3 in all 3 patients may have resulted in blocking TLR4-co-receptor-ligand complex. The dysregulation of TLR4 signaling may have caused a decrease in inflammation and fibrogensis through reducing the pro-inflammatory cytokines.

3. Five patients had F3 fibrosis stage with identified missense mutations in exons 1, 2, and 3 were identified. Most of the identified missense mutations in the extracellular region had a drastic change in aa nature from hydrophobic to hydrophilic basic or acidic amino acids (Leu11Arg, Met41Arg, Leu228Arg, Gly249Arg, and Leu250Glu) contributing to new negative or positive charge, thus altering protein structure consequently impaired signaling and function.

Mediated by TLR4 signaling, kupffer cells (KCs) are known to downregulate pro-inflammatory cytokines by increasing the level of anti-inflammatory cytokine IL-10 (Guo & Friedman, 2010). Thus, it is possible to suggest that deleterious mutations will disrupt TLR4 signaling and enhance liver inflammation through the reduction of anti-inflammatory cytokines.

Although the frameshift mutations localized between positions 241-251 were mostly present in patients with F0 fibrosis stage, several NAFLD patients who had F3 stage also harbored the same frameshift mutations. The variation in the phenotype between patients possessing the same mutation can be attributed to heterozygous frameshifts mutations where one of the alleles is inactive leading to increased expression of pro-inflammatory cytokines and increased immune response promoting inflammation and fibrosis.

Other possible explanation for the variation in the fibrosis stage among patients possessing the same frameshift relates to difference in inheritance patterns of TLR4 alleles among patients. Patients with no to early fibrosis may have homozygous TLR4 frameshift mutation which impedes TLR4 signaling, decreasing the expression of pro-inflammatory cytokines, and reducing thus the inflammation and fibrogenesis; on the other hand, patients showing late fibrotic stages may possess a heterozygous frameshift

with one active copy of TLR4 responsible for normal signaling, immune system activation, and fibrogenesis.

4. Only two NAFLD patients were diagnosed with F4 fibrosis stage. They harbored frameshift mutations localized at positions 246 and 401. Both frameshifts belong to exon 3 and affect the extracellular region of TLR4. The F4 phenotype may result due to the deleterious effect of the frameshift mutations.

It is worth noting that polymorphisms in different genes were associated with the progression of NAFLD to cirrhosis. Ile148Met mutation in PNPLA3 gene (Valenti et al., 2010) and Glu167Lys mutation in the transmembrane 6 superfamily member 2 (TM6SF2) were associated with an increased risk of cirrhosis and advanced fibrsosis. TM6SF2 contributed to the progression of NAFLD through increased oxidative stress, cell damage, and serum transaminase levels (Liu et al., 2014). Similarly, deleterious TLR4 mutations may promote TLR4 signaling leading to enhanced inflammation, fibrogenesis, and cirrhosis. A study (Fels Elliott et al., 2017) discovered that TLR4 mutations may contribute to the pathogenesis of esophageal tumorigenesis. The study suggested that impaired TLR4 signaling may affect the ability of epithelial cells to repair which decreases the functionality of the epithelial barrier against microbes in a tumor environment. In TLR4 mutant liver cells, a similar mechanism may alter the capacity of the cells to respond to microbes, thereby enhancing liver inflammation and cirrhosis.

To sum up, TLR4 is a polymorphic gene with lots of mutations spanning the 3 exons. The majority of the identified mutations are hetrozygous mostly identified in exon-3 which encodes for the extracellular domain involved in ligand recognition and binding. The homozygous mutation Gly249Val was identified in NAFLD patients who

have F0-F1 fibrosis stages which may reflect its protective role. Frame shifts were frequent in exon 3, to lesser extent in exon 1 and not present in exon 2. Frameshifts in exon 3 located between aa residue 241 and 251 may exhibit a protective role in the majority of patients presenting with F0 although their steatosis stage varied between S2-S3. Due to the multi-factorial nature of NAFLD, other genes may be contributing to the phenotypic variability among patients and need to be investigated as well.

Limitation of study

- Small size sample size (number of patients and healthy controls)
- Clinical corelation with genotype was not conclusive because some patients were on treatment.
- Functional assay to prove the protective effect of TLR4 needs to be performed. We could generate cell lines with specific TLR4 mutations to test TLR4 responsiveness to its ligands (e.g. LPS).

BIBLIOGRAPHY

Abd El-Kader, S. M., & El-Den Ashmawy, E. M. (2015). Non-alcoholic fatty liver disease: The diagnosis and management. *World J Hepatol*, *7*(6), 846-858. https://doi.org/10.4254/wjh.v7.i6.846

Adams, L. A., Angulo, P., & Lindor, K. D. (2005). Nonalcoholic fatty liver disease. *Cmaj*, *172*(7), 899-905. https://doi.org/10.1503/cmaj.045232

Alsabaani, A. A., Mahfouz, A. A., Awadalla, N. J., Musa, M. J., & Al Humayed, S. M. (2018). Non-Alcoholic Fatty Liver Disease among Type-2 Diabetes Mellitus Patients in Abha City, South Western Saudi Arabia. *Int J Environ Res Public Health*, *15*(11). https://doi.org/10.3390/ijerph15112521

Andronescu, C. I., Purcarea, M. R., & Babes, P. A. (2018). Nonalcoholic fatty liver disease: epidemiology, pathogenesis and therapeutic implications. *J Med Life*, *11*(1), 20-23.

Asrih, M., & Jornayvaz, F. R. (2013). Inflammation as a potential link between nonalcoholic fatty liver disease and insulin resistance. *J Endocrinol*, *218*(3), R25-36. https://doi.org/10.1530/joe-13-0201

Briseño-Bass, P., Chávez-Pérez, R., & López-Zendejas, M. (2019). Prevalence of hepatic steatosis and its relation to liver function tests and lipid profile in patients at medical check-up. *Rev Gastroenterol Mex (Engl Ed)*, *84*(3), 290-295. https://doi.org/10.1016/j.rgmx.2018.05.007 (Prevalencia y relación de esteatosis hepática con perfil lipídico y hepático en pacientes de chequeo médico.)

Broering, R., Lu, M., & Schlaak, J. F. (2011). Role of Toll-like receptors in liver health and disease. *Clin Sci (Lond)*, *121*(10), 415-426. https://doi.org/10.1042/cs20110065

Cao, S. S., & Kaufman, R. J. (2014). Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. *Antioxid Redox Signal*, *21*(3), 396-413. https://doi.org/10.1089/ars.2014.5851

Chang, Y., Ryu, S., Sung, E., Woo, H. Y., Cho, S. I., Yoo, S. H., Ahn, H. Y., & Choi, N. K. (2009). Weight gain within the normal weight range predicts ultrasonographically detected fatty liver in healthy Korean men. *Gut*, *58*(10), 1419-1425. https://doi.org/10.1136/gut.2008.161885

Chen, J., Deng, X., Liu, Y., Tan, Q., Huang, G., Che, Q., Guo, J., & Su, Z. (2020). Kupffer Cells in Non-alcoholic Fatty Liver Disease: Friend or Foe? *Int J Biol Sci*, *16*(13), 2367-2378. https://doi.org/10.7150/ijbs.47143

Cusi, K. (2009). Nonalcoholic fatty liver disease in type 2 diabetes mellitus. *Curr Opin Endocrinol Diabetes Obes*, *16*(2), 141-149. https://doi.org/10.1097/MED.0b013e3283293015

Dabravolski, S. A., Bezsonov, E. E., Baig, M. S., Popkova, T. V., Nedosugova, L. V., Starodubova, A. V., & Orekhov, A. N. (2021). Mitochondrial Mutations and Genetic Factors Determining NAFLD Risk. *Int J Mol Sci*, *22*(9). https://doi.org/10.3390/ijms22094459

Danecek, P., Bonfield, J. K., Liddle, J., Marshall, J., Ohan, V., Pollard, M. O., Whitwham, A., Keane, T., McCarthy, S. A., Davies, R. M., & Li, H. (2021). Twelve years of SAMtools and BCFtools. *Gigascience*, *10*(2). https://doi.org/10.1093/gigascience/giab008

de Kleijn, D., & Pasterkamp, G. (2003). Toll-like receptors in cardiovascular diseases. *Cardiovasc Res*, *60*(1), 58-67. https://doi.org/10.1016/s0008-6363(03)00348-1
Dinani, A. M., Lewis, S., Branch, A. D., & Perumalswami, P. (2020). Working Up an Incidental Finding of Hepatic Steatosis on Imaging. *Clin Liver Dis (Hoboken)*, *16*(2), 58-62. https://doi.org/10.1002/cld.926
Dowman, J. K., Tomlinson, J. W., & Newsome, P. N. (2010). Pathogenesis of non-alcoholic fatty liver disease. *Qjm*, *103*(2), 71-83. https://doi.org/10.1093/qjmed/hcp158
Engin, A. B. (2017). What Is Lipotoxicity? *Adv Exp Med Biol*, *960*, 197-220. https://doi.org/10.1007/978-3-319-48382-5_8
Farrell, G. C., & Larter, C. Z. (2006). Nonalcoholic fatty liver disease: from steatosis to cirrhosis. *Hepatology*, *43*(2 Suppl 1), S99-s112. https://doi.org/10.1002/hep.20973
Fels Elliott, D. R., Perner, J., Li, X., Symmons, M. F., Verstak, B., Eldridge, M., Bower, L., O'Donovan, M., Gay, N. J., & Fitzgerald, R. C. (2017). Impact of mutations in Toll-like receptor pathway genes on esophageal carcinogenesis. *PLoS Genet*, *13*(5), e1006808. https://doi.org/10.1371/journal.pgen.1006808
Ferguson, C., McKay, M., Harris, R. A., & Homanics, G. E. (2013). Toll-like receptor 4 (Tlr4) knockout rats produced by transcriptional activator-like effector nuclease (TALEN)-mediated gene inactivation. *Alcohol*, *47*(8), 595-599. https://doi.org/10.1016/j.alcohol.2013.09.043
Ferreira, D. F., Fiamoncini, J., Prist, I. H., Ariga, S. K., de Souza, H. P., & de Lima, T. M. (2015). Novel role of TLR4 in NAFLD development: Modulation of metabolic enzymes expression. *Biochim Biophys Acta*, *1851*(10), 1353-1359. https://doi.org/10.1016/j.bbalip.2015.07.002
Fitzgerald, K. A., Palsson-McDermott, E. M., Bowie, A. G., Jefferies, C. A., Mansell, A. S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M. T., McMurray, D., Smith, D. E., Sims, J. E., Bird, T. A., & O'Neill, L. A. (2001). Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature*, *413*(6851), 78-83. https://doi.org/10.1038/35092578
Fujii, H., Doi, H., Ko, T., Fukuma, T., Kadono, T., Asaeda, K., Kobayashi, R., Nakano, T., Doi, T., Nakatsugawa, Y., Yamada, S., Nishimura, T., Tomatsuri, N., Sato, H., Okuyama, Y., Kimura, H., Kishimoto, E., Nakabe, N., & Shima, T. (2020). Frequently abnormal serum gamma-glutamyl transferase activity is associated with future development of fatty liver: a retrospective cohort study. *BMC Gastroenterol*, *20*(1), 217. https://doi.org/10.1186/s12876-020-01369-x
Galiero, R., Caturano, A., Vetrano, E., Cesaro, A., Rinaldi, L., Salvatore, T., Marfella, R., Sardu, C., Moscarella, E., Gragnano, F., Calabrò, P., & Sasso, F. C. (2021). Pathophysiological mechanisms and clinical evidence of relationship between Nonalcoholic fatty liver disease (NAFLD) and cardiovascular disease. *Rev Cardiovasc Med*, *22*(3), 755-768. https://doi.org/10.31083/j.rcm2203082
Giby, V. G., & Ajith, T. A. (2014). Role of adipokines and peroxisome proliferator-activated receptors in nonalcoholic fatty liver disease. *World J Hepatol*, *6*(8), 570-579. https://doi.org/10.4254/wjh.v6.i8.570
Guo, J., & Friedman, S. L. (2010). Toll-like receptor 4 signaling in liver injury and hepatic fibrogenesis. *Fibrogenesis Tissue Repair*, *3*, 21. https://doi.org/10.1186/1755-1536-3-21

Guo, J., Loke, J., Zheng, F., Hong, F., Yea, S., Fukata, M., Tarocchi, M., Abar, O. T., Huang, H., Sninsky, J. J., & Friedman, S. L. (2009). Functional linkage of cirrhosis-predictive single nucleotide polymorphisms of Toll-like receptor 4 to hepatic stellate cell responses. *Hepatology*, *49*(3), 960-968. https://doi.org/10.1002/hep.22697

Hernaez, R., Lazo, M., Bonekamp, S., Kamel, I., Brancati, F. L., Guallar, E., & Clark, J. M. (2011). Diagnostic accuracy and reliability of ultrasonography for the detection of fatty liver: a meta-analysis. *Hepatology*, *54*(3), 1082-1090. https://doi.org/10.1002/hep.24452

Heyens, L. J. M., Busschots, D., Koek, G. H., Robaeys, G., & Francque, S. (2021). Liver Fibrosis in Non-alcoholic Fatty Liver Disease: From Liver Biopsy to Non-invasive Biomarkers in Diagnosis and Treatment. *Front Med (Lausanne)*, *8*, 615978. https://doi.org/10.3389/fmed.2021.615978

Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S. O., Goode, J., Lin, P., Mann, N., Mudd, S., Crozat, K., Sovath, S., Han, J., & Beutler, B. (2003). Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature*, *424*(6950), 743-748. https://doi.org/10.1038/nature01889

Honda, Y., Yoneda, M., Kessoku, T., Ogawa, Y., Tomeno, W., Imajo, K., Mawatari, H., Fujita, K., Hyogo, H., Ueno, T., Chayama, K., Saito, S., Nakajima, A., & Hotta, K. (2016). Characteristics of non-obese non-alcoholic fatty liver disease: Effect of genetic and environmental factors. *Hepatol Res*, *46*(10), 1011-1018. https://doi.org/10.1111/hepr.12648

Hopkins, J. (n.d). *Liver: Anatomy and Functions*. Johns Hopkins Medicine. https://www.hopkinsmedicine.org/health/conditions-and-diseases/liver-anatomy-and-functions

Huang, P. L. (2009). A comprehensive definition for metabolic syndrome. *Dis Model Mech*, *2*(5-6), 231-237. https://doi.org/10.1242/dmm.001180

Jialal, I., Kaur, H., & Devaraj, S. (2014). Toll-like receptor status in obesity and metabolic syndrome: a translational perspective. *J Clin Endocrinol Metab*, *99*(1), 39-48. https://doi.org/10.1210/jc.2013-3092

Jiménez-Cortegana, C., García-Galey, A., Tami, M., Del Pino, P., Carmona, I., López, S., Alba, G., & Sánchez-Margalet, V. (2021). Role of Leptin in Non-Alcoholic Fatty Liver Disease. *Biomedicines*, *9*(7). https://doi.org/10.3390/biomedicines9070762

Kawano, Y., & Cohen, D. E. (2013). Mechanisms of hepatic triglyceride accumulation in non-alcoholic fatty liver disease. *J Gastroenterol*, *48*(4), 434-441. https://doi.org/10.1007/s00535-013-0758-5

Khan, R. S., Bril, F., Cusi, K., & Newsome, P. N. (2019). Modulation of Insulin Resistance in Nonalcoholic Fatty Liver Disease. *Hepatology*, *70*(2), 711-724. https://doi.org/10.1002/hep.30429

Kim, C. W., Yun, K. E., Jung, H. S., Chang, Y., Choi, E. S., Kwon, M. J., Lee, E. H., Woo, E. J., Kim, N. H., Shin, H., & Ryu, S. (2013). Sleep duration and quality in relation to non-alcoholic fatty liver disease in middle-aged workers and their spouses. *J Hepatol*, *59*(2), 351-357. https://doi.org/10.1016/j.jhep.2013.03.035

Kim, D., Chung, G. E., Kwak, M. S., Seo, H. B., Kang, J. H., Kim, W., Kim, Y. J., Yoon, J. H., Lee, H. S., & Kim, C. Y. (2016). Body Fat Distribution and Risk of Incident and Regressed Nonalcoholic Fatty Liver Disease. *Clin Gastroenterol Hepatol*, *14*(1), 132-138.e134. https://doi.org/10.1016/j.cgh.2015.07.024

Kitazawa, T., Tsujimoto, T., Kawaratani, H., Fujimoto, M., & Fukui, H. (2008). Expression of Toll-like receptor 4 in various organs in rats with D-galactosamine-induced acute hepatic failure. *J Gastroenterol Hepatol*, *23*(8 Pt 2), e494-498. https://doi.org/10.1111/j.1440-1746.2007.05246.x

Kiziltas, S., Ata, P., Colak, Y., Mesçi, B., Senates, E., Enc, F., Ulasoglu, C., Tuncer, I., & Oguz, A. (2014). TLR4 gene polymorphism in patients with nonalcoholic fatty liver disease in comparison to healthy controls. *Metab Syndr Relat Disord*, *12*(3), 165-170. https://doi.org/10.1089/met.2013.0120

Kopec, K. L., & Burns, D. (2011). Nonalcoholic fatty liver disease: a review of the spectrum of disease, diagnosis, and therapy. *Nutr Clin Pract*, *26*(5), 565-576. https://doi.org/10.1177/0884533611419668

Koyama, Y., & Brenner, D. A. (2017). Liver inflammation and fibrosis. *J Clin Invest*, *127*(1), 55-64. https://doi.org/10.1172/jci88881

Kubes, P., & Jenne, C. (2018). Immune Responses in the Liver. *Annu Rev Immunol*, *36*, 247-277. https://doi.org/10.1146/annurev-immunol-051116-052415

Lala, V., Goyal, A., & Minter, D. A. (2021). Liver function tests. In *StatPearls [Internet]*. StatPearls Publishing.

Lambert, J. E., Ramos-Roman, M. A., Browning, J. D., & Parks, E. J. (2014). Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. *Gastroenterology*, *146*(3), 726-735. https://doi.org/10.1053/j.gastro.2013.11.049

Le, M. H., Yeo, Y. H., Li, X., Li, J., Zou, B., Wu, Y., Ye, Q., Huang, D. Q., Zhao, C., Zhang, J., Liu, C., Chang, N., Xing, F., Yan, S., Wan, Z. H., Tang, N. S. Y., Mayumi, M., Liu, X., Liu, C., Rui, F., Yang, H., Yang, Y., Jin, R., Le, R. H. X., Xu, Y., Le, D. M., Barnett, S., Stave, C. D., Cheung, R., Zhu, Q., & Nguyen, M. H. (2022). 2019 Global NAFLD Prevalence: A Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol*, *20*(12), 2809-2817.e2828. https://doi.org/10.1016/j.cgh.2021.12.002

Leite, N. C., Salles, G. F., Araujo, A. L., Villela-Nogueira, C. A., & Cardoso, C. R. (2009). Prevalence and associated factors of non-alcoholic fatty liver disease in patients with type-2 diabetes mellitus. *Liver Int*, *29*(1), 113-119. https://doi.org/10.1111/j.1478-3231.2008.01718.x

Li, B., Zhang, C., & Zhan, Y. T. (2018). Nonalcoholic Fatty Liver Disease Cirrhosis: A Review of Its Epidemiology, Risk Factors, Clinical Presentation, Diagnosis, Management, and Prognosis. *Can J Gastroenterol Hepatol*, *2018*, 2784537. https://doi.org/10.1155/2018/2784537

Li, H., Fang, Q., Gao, F., Fan, J., Zhou, J., Wang, X., Zhang, H., Pan, X., Bao, Y., Xiang, K., Xu, A., & Jia, W. (2010). Fibroblast growth factor 21 levels are increased in nonalcoholic fatty liver disease patients and are correlated with hepatic triglyceride. *J Hepatol*, *53*(5), 934-940. https://doi.org/10.1016/j.jhep.2010.05.018

Li, H., & Sun, B. (2007). Toll-like receptor 4 in atherosclerosis. *J Cell Mol Med*, *11*(1), 88-95. https://doi.org/10.1111/j.1582-4934.2007.00011.x

Lin, Y. T., Verma, A., & Hodgkinson, C. P. (2012). Toll-like receptors and human disease: lessons from single nucleotide polymorphisms. *Curr Genomics*, *13*(8), 633-645. https://doi.org/10.2174/138920212803759712

Liu, Y. L., Reeves, H. L., Burt, A. D., Tiniakos, D., McPherson, S., Leathart, J. B., Allison, M. E., Alexander, G. J., Piguet, A. C., Anty, R., Donaldson, P., Aithal,

G. P., Francque, S., Van Gaal, L., Clement, K., Ratziu, V., Dufour, J. F., Day, C. P., Daly, A. K., & Anstee, Q. M. (2014). TM6SF2 rs58542926 influences hepatic fibrosis progression in patients with non-alcoholic fatty liver disease. *Nat Commun*, *5*, 4309. https://doi.org/10.1038/ncomms5309

Loomba, R., Schork, N., Chen, C. H., Bettencourt, R., Bhatt, A., Ang, B., Nguyen, P., Hernandez, C., Richards, L., Salotti, J., Lin, S., Seki, E., Nelson, K. E., Sirlin, C. B., & Brenner, D. (2015). Heritability of Hepatic Fibrosis and Steatosis Based on a Prospective Twin Study. *Gastroenterology*, *149*(7), 1784-1793. https://doi.org/10.1053/j.gastro.2015.08.011

Lu, Y. C., Yeh, W. C., & Ohashi, P. S. (2008). LPS/TLR4 signal transduction pathway. *Cytokine*, *42*(2), 145-151. https://doi.org/10.1016/j.cyto.2008.01.006

Mann, J. P., Carter, P., Armstrong, M. J., Abdelaziz, H. K., Uppal, H., Patel, B., Chandran, S., More, R., Newsome, P. N., & Potluri, R. (2020). Hospital admission with non-alcoholic fatty liver disease is associated with increased all-cause mortality independent of cardiovascular risk factors. *PLoS One*, *15*(10), e0241357. https://doi.org/10.1371/journal.pone.0241357

Matsushima, N., Tanaka, T., Enkhbayar, P., Mikami, T., Taga, M., Yamada, K., & Kuroki, Y. (2007). Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors. *BMC Genomics*, *8*, 124. https://doi.org/10.1186/1471-2164-8-124

McCuskey, R. (2012). *Anatomy of the liver. Zakim and Boyer's hepatology. A textbook of liver disease. 2012; 3–21* (6 ed.). Saunders.

McLaren, W., Gil, L., Hunt, S. E., Riat, H. S., Ritchie, G. R., Thormann, A., Flicek, P., & Cunningham, F. (2016). The Ensembl Variant Effect Predictor. *Genome Biol*, *17*(1), 122. https://doi.org/10.1186/s13059-016-0974-4

Milić, S., Lulić, D., & Štimac, D. (2014). Non-alcoholic fatty liver disease and obesity: biochemical, metabolic and clinical presentations. *World J Gastroenterol*, *20*(28), 9330-9337. https://doi.org/10.3748/wjg.v20.i28.9330

Morris-Stiff, G., & Feldstein, A. E. (2010). Fibroblast growth factor 21 as a biomarker for NAFLD: integrating pathobiology into clinical practice. *J Hepatol*, *53*(5), 795-796. https://doi.org/10.1016/j.jhep.2010.07.003

Neuschwander-Tetri, B. A. (2017). Non-alcoholic fatty liver disease. *BMC Med*, *15*(1), 45. https://doi.org/10.1186/s12916-017-0806-8

Oakes, S. A., & Papa, F. R. (2015). The role of endoplasmic reticulum stress in human pathology. *Annu Rev Pathol*, *10*, 173-194. https://doi.org/10.1146/annurev-pathol-012513-104649

Papatheodoridi, M., & Cholongitas, E. (2018). Diagnosis of Non-alcoholic Fatty Liver Disease (NAFLD): Current Concepts. *Curr Pharm Des*, *24*(38), 4574-4586. https://doi.org/10.2174/1381612825666190117102111

Perakakis, N., Farr, O. M., & Mantzoros, C. S. (2021). Leptin in Leanness and Obesity: JACC State-of-the-Art Review. *J Am Coll Cardiol*, *77*(6), 745-760. https://doi.org/10.1016/j.jacc.2020.11.069

Perumpail, B. J., Khan, M. A., Yoo, E. R., Cholankeril, G., Kim, D., & Ahmed, A. (2017). Clinical epidemiology and disease burden of nonalcoholic fatty liver disease. *World J Gastroenterol*, *23*(47), 8263-8276. https://doi.org/10.3748/wjg.v23.i47.8263

Phang-Lyn, S., & Llerena, V. A. (2022). Biochemistry, Biotransformation. In *StatPearls*. StatPearls Publishing

Poynard, T., Lenaour, G., Vaillant, J. C., Capron, F., Munteanu, M., Eyraud, D., Ngo, Y., M'Kada, H., Ratziu, V., Hannoun, L., & Charlotte, F. (2012). Liver biopsy analysis has a low level of performance for diagnosis of intermediate stages of fibrosis. *Clin Gastroenterol Hepatol*, *10*(6), 657-663.e657. https://doi.org/10.1016/j.cgh.2012.01.023

Rallabhandi, P., Bell, J., Boukhvalova, M. S., Medvedev, A., Lorenz, E., Arditi, M., Hemming, V. G., Blanco, J. C., Segal, D. M., & Vogel, S. N. (2006). Analysis of TLR4 polymorphic variants: new insights into TLR4/MD-2/CD14 stoichiometry, structure, and signaling. *J Immunol*, *177*(1), 322-332. https://doi.org/10.4049/jimmunol.177.1.322

Rausch, T., Fritz, M. H., Untergasser, A., & Benes, V. (2020). Tracy: basecalling, alignment, assembly and deconvolution of sanger chromatogram trace files. *BMC Genomics*, *21*(1), 230. https://doi.org/10.1186/s12864-020-6635-8

Ronni, T., Agarwal, V., Haykinson, M., Haberland, M. E., Cheng, G., & Smale, S. T. (2003). Common interaction surfaces of the toll-like receptor 4 cytoplasmic domain stimulate multiple nuclear targets. *Mol Cell Biol*, *23*(7), 2543-2555. https://doi.org/10.1128/mcb.23.7.2543-2555.2003

Rui, L. (2014). Energy metabolism in the liver. *Compr Physiol*, *4*(1), 177-197. https://doi.org/10.1002/cphy.c130024

Rusli, F., Deelen, J., Andriyani, E., Boekschoten, M. V., Lute, C., van den Akker, E. B., Müller, M., Beekman, M., & Steegenga, W. T. (2016). Fibroblast growth factor 21 reflects liver fat accumulation and dysregulation of signalling pathways in the liver of C57BL/6J mice. *Sci Rep*, *6*, 30484. https://doi.org/10.1038/srep30484

Saadeh, S., Younossi, Z. M., Remer, E. M., Gramlich, T., Ong, J. P., Hurley, M., Mullen, K. D., Cooper, J. N., & Sheridan, M. J. (2002). The utility of radiological imaging in nonalcoholic fatty liver disease. *Gastroenterology*, *123*(3), 745-750. https://doi.org/10.1053/gast.2002.35354

Sanyal, A. J. (2002). AGA technical review on nonalcoholic fatty liver disease. *Gastroenterology*, *123*(5), 1705-1725. https://doi.org/10.1053/gast.2002.36572

Schweiger, M., Romauch, M., Schreiber, R., Grabner, G. F., Hütter, S., Kotzbeck, P., Benedikt, P., Eichmann, T. O., Yamada, S., Knittelfelder, O., Diwoky, C., Doler, C., Mayer, N., De Cecco, W., Breinbauer, R., Zimmermann, R., & Zechner, R. (2017). Pharmacological inhibition of adipose triglyceride lipase corrects high-fat diet-induced insulin resistance and hepatosteatosis in mice. *Nat Commun*, *8*, 14859. https://doi.org/10.1038/ncomms14859

Seki, E., De Minicis, S., Osterreicher, C. H., Kluwe, J., Osawa, Y., Brenner, D. A., & Schwabe, R. F. (2007). TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med*, *13*(11), 1324-1332. https://doi.org/10.1038/nm1663

Severson, T. J., Besur, S., & Bonkovsky, H. L. (2016). Genetic factors that affect nonalcoholic fatty liver disease: A systematic clinical review. *World J Gastroenterol*, *22*(29), 6742-6756. https://doi.org/10.3748/wjg.v22.i29.6742

Sghaier, I., Zidi, S., Mouelhi, L., Ghazoueni, E., Brochot, E., Almawi, W. Y., & Loueslati, B. Y. (2019). TLR3 and TLR4 SNP variants in the liver disease resulting from hepatitis B virus and hepatitis C virus infection. *Br J Biomed Sci*, *76*(1), 35-41. https://doi.org/10.1080/09674845.2018.1547179

Sharifnia, T., Antoun, J., Verriere, T. G., Suarez, G., Wattacheril, J., Wilson, K. T., Peek, R. M., Jr., Abumrad, N. N., & Flynn, C. R. (2015). Hepatic TLR4 signaling in obese NAFLD. *Am J Physiol Gastrointest Liver Physiol*, *309*(4), G270-278. https://doi.org/10.1152/ajpgi.00304.2014

Shen, X., Cai, J., Gao, J., Vaidya, A., Liu, X., Li, W., Chen, S., Zhou, Y., Li, Y., Zhang, Y., Zhao, J., Hu, F. B., Wu, S., & Gao, X. (2018). Nonalcoholic Fatty Liver Disease and Risk of Diabetes: A Prospective Study in China. *Endocr Pract*, *24*(9), 823-832. https://doi.org/10.4158/ep-2018-0098

Smirnova, I., Poltorak, A., Chan, E. K., McBride, C., & Beutler, B. (2000). Phylogenetic variation and polymorphism at the toll-like receptor 4 locus (TLR4). *Genome Biol*, *1*(1), Research002. https://doi.org/10.1186/gb-2000-1-1-research002

Spruss, A., Kanuri, G., Wagnerberger, S., Haub, S., Bischoff, S. C., & Bergheim, I. (2009). Toll-like receptor 4 is involved in the development of fructose-induced hepatic steatosis in mice. *Hepatology*, *50*(4), 1094-1104. https://doi.org/10.1002/hep.23122

Stern, C., & Castera, L. (2017). Non-invasive diagnosis of hepatic steatosis. *Hepatol Int*, *11*(1), 70-78. https://doi.org/10.1007/s12072-016-9772-z

Sweet, P. H., Khoo, T., & Nguyen, S. (2017). Nonalcoholic Fatty Liver Disease. *Prim Care*, *44*(4), 599-607. https://doi.org/10.1016/j.pop.2017.07.003

Tanase, D. M., Gosav, E. M., Costea, C. F., Ciocoiu, M., Lacatusu, C. M., Maranduca, M. A., Ouatu, A., & Floria, M. (2020). The Intricate Relationship between Type 2 Diabetes Mellitus (T2DM), Insulin Resistance (IR), and Nonalcoholic Fatty Liver Disease (NAFLD). *J Diabetes Res*, *2020*, 3920196. https://doi.org/10.1155/2020/3920196

Thuy, S., Ladurner, R., Volynets, V., Wagner, S., Strahl, S., Königsrainer, A., Maier, K. P., Bischoff, S. C., & Bergheim, I. (2008). Nonalcoholic fatty liver disease in humans is associated with increased plasma endotoxin and plasminogen activator inhibitor 1 concentrations and with fructose intake. *J Nutr*, *138*(8), 1452-1455. https://doi.org/10.1093/jn/138.8.1452

Tiniakos, D. G., Vos, M. B., & Brunt, E. M. (2010). Nonalcoholic fatty liver disease: pathology and pathogenesis. *Annu Rev Pathol*, *5*, 145-171. https://doi.org/10.1146/annurev-pathol-121808-102132

Valenti, L., Al-Serri, A., Daly, A. K., Galmozzi, E., Rametta, R., Dongiovanni, P., Nobili, V., Mozzi, E., Roviaro, G., Vanni, E., Bugianesi, E., Maggioni, M., Fracanzani, A. L., Fargion, S., & Day, C. P. (2010). Homozygosity for the patatin-like phospholipase-3/adiponutrin I148M polymorphism influences liver fibrosis in patients with nonalcoholic fatty liver disease. *Hepatology*, *51*(4), 1209-1217. https://doi.org/10.1002/hep.23622

Vaure, C., & Liu, Y. (2014). A comparative review of toll-like receptor 4 expression and functionality in different animal species. *Front Immunol*, *5*, 316. https://doi.org/10.3389/fimmu.2014.00316

Walsh, M. C., Lee, J., & Choi, Y. (2015). Tumor necrosis factor receptor- associated factor 6 (TRAF6) regulation of development, function, and homeostasis of the immune system. *Immunol Rev*, *266*(1), 72-92. https://doi.org/10.1111/imr.12302

Wang, M., Xu, G., Lü, L., Xu, K., Chen, Y., Pan, H., Burstrom, B., Burstrom, K., & Wang, J. (2016). Genetic polymorphisms of IL-17A, IL-17F, TLR4 and miR-

146a in association with the risk of pulmonary tuberculosis. *Sci Rep*, *6*, 28586. https://doi.org/10.1038/srep28586
Wang, X. J., & Malhi, H. (2018). Nonalcoholic Fatty Liver Disease. *Ann Intern Med*, *169*(9), Itc65-itc80. https://doi.org/10.7326/aitc201811060
Wasilewska, N., & Lebensztejn, D. M. (2021). Non-alcoholic fatty liver disease and lipotoxicity. *Clin Exp Hepatol*, *7*(1), 1-6. https://doi.org/10.5114/ceh.2021.104441
Whitfield, J. B. (2001). Gamma glutamyl transferase. *Crit Rev Clin Lab Sci*, *38*(4), 263-355. https://doi.org/10.1080/20014091084227
Wilcox, G. (2005). Insulin and insulin resistance. *Clin Biochem Rev*, *26*(2), 19-39.
Win, S., Than, T. A., Le, B. H., García-Ruiz, C., Fernandez-Checa, J. C., & Kaplowitz, N. (2015). Sab (Sh3bp5) dependence of JNK mediated inhibition of mitochondrial respiration in palmitic acid induced hepatocyte lipotoxicity. *J Hepatol*, *62*(6), 1367-1374. https://doi.org/10.1016/j.jhep.2015.01.032
Wu, W., Feng, A., Ma, W., Li, D., Zheng, S., Xu, F., Han, D., & Lyu, J. (2022). Worldwide long-term trends in the incidence of nonalcoholic fatty liver disease during 1990-2019: A joinpoint and age-period-cohort analysis. *Front Cardiovasc Med*, *9*, 891963. https://doi.org/10.3389/fcvm.2022.891963
Xie, W., Cao, Y., Xu, M., Wang, J., Zhou, C., Yang, X., Geng, X., Zhang, W., Li, N., & Cheng, J. (2017). Prognostic Significance of Elevated Cholestatic Enzymes for Fibrosis and Hepatocellular Carcinoma in Hospital Discharged Chronic Viral Hepatitis Patients. *Sci Rep*, *7*(1), 10289. https://doi.org/10.1038/s41598-017-11111-5
Yamamoto, M., Sato, S., Hemmi, H., Uematsu, S., Hoshino, K., Kaisho, T., Takeuchi, O., Takeda, K., & Akira, S. (2003). TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat Immunol*, *4*(11), 1144-1150. https://doi.org/10.1038/ni986
Yin, L. K., & Tong, K. S. (2009). Elevated Alt and Ast in an Asymptomatic Person: What the primary care doctor should do? *Malays Fam Physician*, *4*(2-3), 98-99.
Younossi, Z., Anstee, Q. M., Marietti, M., Hardy, T., Henry, L., Eslam, M., George, J., & Bugianesi, E. (2018). Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol*, *15*(1), 11-20. https://doi.org/10.1038/nrgastro.2017.109
Zarghamravanbakhsh, P., Frenkel, M., & Poretsky, L. (2021). Metabolic causes and consequences of nonalcoholic fatty liver disease (NAFLD). *Metabol Open*, *12*, 100149. https://doi.org/10.1016/j.metop.2021.100149
Zeremski, M., Dimova, R. B., Benjamin, S., Makeyeva, J., Yantiss, R. K., Gambarin-Gelwan, M., & Talal, A. H. (2014). FibroSURE as a noninvasive marker of liver fibrosis and inflammation in chronic hepatitis B. *BMC Gastroenterol*, *14*, 118. https://doi.org/10.1186/1471-230x-14-118
Zeyda, M., & Stulnig, T. M. (2009). Obesity, inflammation, and insulin resistance--a mini-review. *Gerontology*, *55*(4), 379-386. https://doi.org/10.1159/000212758
Zhang, X., Shen, J., Man, K., Chu, E. S., Yau, T. O., Sung, J. C., Go, M. Y., Deng, J., Lu, L., Wong, V. W., Sung, J. J., Farrell, G., & Yu, J. (2014). CXCL10 plays a key role as an inflammatory mediator and a non-invasive biomarker of non-alcoholic steatohepatitis. *J Hepatol*, *61*(6), 1365-1375. https://doi.org/10.1016/j.jhep.2014.07.006