HAMAD BIN KHALIFA UNIVERSITY

COLLEGE OF HEALTH AND LIFE SCIENCES

IDENTIFICATION OF THE LONG NON-CODING RNAs AND THE SIGNALING PATHWAYS INVOLVED IN THE PROTECTIVE EFFECT OF THE GLUCAGON-LIKE PEPTIDE-1 RECEPTOR AGONIST EXENDIN-4 ON HEPATIC STEATOSIS

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ABSTRACT

Nonalcoholic Fatty Liver Disease (NAFLD) is characterized by lipid accumulation in the hepatocytes without a coexisting etiology of chronic liver diseases, such as medications, excessive alcohol drinking, or viral hepatitis. NAFLD encompasses steatosis, a benign and reversible condition marked by excessive hepatic triglycerides above 5% of the liver's weight, and the more aggressive non-alcoholic steatohepatitis (NASH), marked by steatosis and the presence of lobular and portal inflammation, and hepatocyte ballooning. NASH can progress to fibrosis, leading to cirrhosis and eventually to hepatocellular carcinoma. NASH is the fastest growing indication for liver transplantation in Western countries. The exact etiology of NAFLD remains elusive. However, obesity, type 2 diabetes (T2D), dyslipidemia, and insulin resistance are its primary drivers. The pathophysiology of NAFLD is complex and implicates alterations in the metabolism of carbohydrates, lipids, proteins, and insulin signaling. There is also evidence for an element of heritability of NAFLD.

There is currently no approved pharmacotherapy for NAFLD. Recent research suggests that glucagon-like peptide-1 receptor agonists (GLP1-RAs) are novel NAFLD therapies because they have a positive effect on liver fat accumulation in NAFLD patients. Despite this beneficial effect, however, the underlying molecular mechanisms remain elusive due to the pleiotropic functions of these drugs. Indeed, GLP-1R agonists could reduce the liver fat content by inducing overall weight loss, mainly visceral fat. These medications, however, could possibly improve NAFLD by directly activating the hepatic GLP-1Rs and their downstream signaling pathways, which would modify the pathways for lipid metabolism and reduce the amount of fat in the liver. Given the difficulty of losing weight and mainly maintaining it, attempting to discover novel liver fat-reducing medications independently of weight loss is of critical therapeutic value. Understanding the GLP-

1Rs' protective effect on NAFLD necessitates a deep comprehension of the underlying molecular mechanisms. LncRNAs are a broad family of non-encoding transcribed RNA molecules with a length of more than 200 nucleotides. LncRNAs play a position in a variety of liver processes, including lipid metabolism, inflammation, cell death, and development. . In previous investigations, the expression of LncRNAs was examined for dysregulation and modification in NAFLD, and many LncRNAs, including MALAT1, NEAT1, H19, and CCAT1, were found to be associated with NAFLD. The present thesis looked at the signaling pathways and LncRNAs that are involved in the GLP1RA Exendin-4's anti-steatosis effects.

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DEDICATION

To my late father, Mohamed Errafii, who passed away while I was pursuing my PhD. I hope you are proud of how far I have come and what I have accomplished so far as you look down from heaven. It is in your honor that I did it. Rest in peace for all eternity.

CHAPTER1: Introduction

NATURAL HISTORY OF NAFLD

Non Alcoholic fatty liver disease (NAFLD), first defined by Ludwig and colleagues (Ludwig, Viggiano, Mcgill, & Oh, 1980) is a disease whose prevalence continues to rise concomitantly with the global obesity epidemic (Benedict & Zhang, 2017). Ludwig's team observed that hepatic steatosis, defined as lipid accumulation in the liver, in about twenty patients is associated with inflammatory histological lesions like those encountered in alcoholic hepatitis. However, most of those patients were abstinent from alcohol but were obese and had type 2 diabetes (T2D). Nearly 30 years later, this condition, which Ludwig et al. referred to as "Non-Alcoholic Steatohepatitis" or NASH, emerged as one of the most prevalent liver disorders in the world. NASH is a component of NAFLD, which includes a variety of liver conditions such as simple steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma in addition to NASH (HCC). The diagnosis of NAFLD requires the exclusion of secondary causes such as medications, Wilson's disease, overgrowth of gut microbial, parenteral nutrition, severe undernutrition or hypothyroidism, viral hepatitis, and alcoholism (Ratziu, Bellentani, Cortez-Pinto, Day, & Marchesini, 2010).

Steatosis refers to the accumulation of triglycerides in more than 5% of hepatocytes, while the combination of steatosis and inflammation defines NASH. Fibrosis is not part of the histological definition of NASH and is characterized by scarring of liver tissue. Cirrhosis, liver failure, and portal hypertension are side effects of advanced liver fibrosis, which frequently necessitates liver transplantation. Liver cirrhosis can, in some cases, progress to hepatocellular carcinoma. **Figure 1** depicts the different stages of NAFLD.

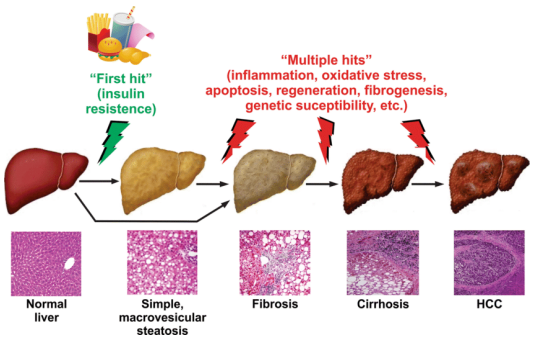

Figure 1. Different stages of NAFLD. ***Taken and adapted from (Bessone, Razori, & Roma, 2019)***

NAFLD Histopathology

The early stage of NAFLD, also called NAFL or steatosis, is defined by an accumulation of more than 5% of lipids in the liver. Lipids, predominantly triglycerides (TGs), are stored in hepatocytes as droplets. Steatosis can be of two types:

a. Macrovesicular: characterised by a large drop of lipids, structurally well-defined, and occupies the cytoplasm of hepatocytes by repulsing their nuclei at the periphery.
b. Micro vesicular: in this case, the hepatocyte cytoplasm is filled with a myriad of lipid microdroplets and maintains a central core.

While NAFLD is primarily a form of macrovesicular steatosis, 10% of patients will have a microvesicular shape (Burt, Mutton, & Day, 1998). NASH is characterized by the presence of various inflammatory infiltrates such as kupffer cells (liver resident macrophages), eosinophils, lymphocytes, and neutrophils (Nati et al., 2016). Most often, intra-lobular

hepatic inflammation is observed (Takahashi & Fukusato, 2014). In the most severe forms, a ballooning of the hepatocytes can develop. One of NASH's progression characteristics is the development of liver fibrosis, predominantly pericellular. The liver thickens and its function is impaired (Lackner & Tiniakos, 2019). Fibrosis can, in some cases, lead to the advent of cirrhosis, which may progress towards HCC and may require liver transplantation when possible (Estes, Razavi, Loomba, Younossi, & Sanyal, 2018).

EPIDEMIOLOGY OF NAFLD

- **Geographical data**

Analysis of geographical data on NAFLD easily correlates with the explosion of obesity and T2D worldwide and reflects changing population habits of food and lifestyle. The true incidence of NAFLD across the world remains difficult to assess precisely because of diagnostic difficulties. The various international epidemiological studies (HEPAHEALTH, NHANES) estimate that about 25% of the global population has NAFLD and that 3–5% of the world's population has NASH (Z. Younossi et al., 2019). These general epidemiological trends are modulated according to geographic and diagnostic criteria. On the American continent, it seems that South American countries are more affected by NAFLD than the United States. Thus, a diagnosis of NAFLD is made for 24% of the population in the US versus 30.45% of the South American population (Younossi et al., 2016). Within the same region, there are inter-country disparities: for example, NAFLD affects 23% of adults in Chile but only 13% in Peru (Pegah Golabi et al., 2021). In Asia, there is a drastic increase in cases of NAFLD related to the urbanization of populations. This

change in lifestyle is accompanied by sedentary lifestyles and overeating. Thus, 25% of the Asian population is suspected of having NAFLD (Wong et al., 2018). In China, data reveal that the incidence of metabolic fatty liver disease has doubled in the last 20 years and affects 15% of the total population (Fan & Farrell, 2009). A similar development is noted in Japan, where 29.7% of the population is estimated to have NAFLD (T. Ito et al., 2021). South Asian countries are following the same trend, with a clear disparity between rural and urbanized populations. In India, for example, NAFLD affects is about 9% of rural populations compared to almost 33% of urban populations, thus reaffirming the impact of lifestyle on the advent of this disease (Younossi, 2019). The epidemiological trends in Europe are similar; approximately 23.71% of the total European population has NAFLD (Pimpin et al., 2018). A North-South gradient appears with a greater prevalence of the disease in the countries of South Europe. In Australia, NAFLD is the number one liver disease in the country and affects 20–30% of the population, while it affects only 13% of the population in New Zealand (Adams et al., 2020). Finally, the NAFLD prevalence in the countries of the Middle East and North Africa ranges between 20 and 30% (Pegah Golabi et al., 2021). Little data is available on the prevalence of NAFLD in Central African countries.

- **Age- gender, and ethnicity-related data**

Age plays an important role in the progression of NAFLD. A study of over 300 patients of various ages found an increased incidence of NAFLD in people over 60, as well as an increase in fibrosis and the occurrence of NASH (Younossi, 2019). This can be explained particularly by the increase in the prevalence of metabolic diseases such as T2D in older populations.

One of the major concerns of health professionals is the increase of NAFLD in the pediatric population. Childhood obesity is soaring among children. This is extremely concerning because obesity and childhood liver injury is a major risk factor for developing adult complications such as cirrhosis or HCC (Nobili et al., 2019).

Gender-related discrepancies in NAFLD patient populations were shown by analyzing NAFLD and NASH epidemiology data. However, these observations remain controversial. Based on data from USA, China, and Europe, it appears that the prevalence of NAFLD is similar for both sexes up to 20 years old, but it is more prevalent in men as they get older (Younossi, 2019). On the contrary, some Asian population studies show the opposite trend, with an increase in NAFLD cases among females (Ascha et al., 2010).

Available data show the existence of ethnic differences between populations, just as there is a disparity in the prevalence of NAFLD among countries within the same continent. In USA for example, the patients most affected by NAFLD are Hispanics, followed by non-Hispanic Caucasian patients. African Americans are the population least likely to develop NAFLD. These ethnic disparities are thought to stem from the intricate interactions between specific genetic polymorphisms and environmental factors, but the exact mechanisms are still to be elucidated (Younossi, 2019).

NAFLD DIAGNOSIS

- **Clinical Diagnosis**

NAFLD is most typically asymptomatic, and its diagnosis is frequently discovered through routine screening, suggesting an underestimation of its actual impact. The diagnosis of NAFLD remains an exclusion diagnosis for any other liver disease that could respond to abnormalities detected by imaging or after the discovery of impaired hepatic biological function (viral, autoimmune, alcoholic, or toxic-induced hepatitis). In the early stages of the disease, most patients do not show any alteration in liver parameters, and transaminases and markers of hepatic cytolysis may be normal. Some non-specific symptoms may be present.

The diagnosis of NAFLD is often primarily established by imaging techniques (ultrasound, MRI or CT-scan) that show steatosis. Current imaging systems do not allow for a precise assessment of the severity of the fibrosis and cannot determine the state of the disease. In addition, for ultrasound, steatosis is detectable only when it is greater than 30% (Q. Li, Dhyani, Grajo, Sirlin, & Samir, 2018). Diagnosis of NASH may require invasive methods to assess the degree of liver fibrosis. Hepatic biopsy thus provides indications of the liver's condition, hepatocyte morphology, steatosis, necrosis, and the general organization of the liver to prevent fibrosis (Hashimoto, Taniai, & Tokushige, 2013; Pappachan, Babu, Krishnan, & Ravindran, 2017). Although it is the most appropriate and finest way to assess the disease, liver biopsy is only indicated in NAFLD patients labeled as high risk of developing NASH or who are suspected of having chronic liver disease concurrently (Hashimoto et al., 2013).

Despite the evolution of imaging techniques, liver biopsy is still the gold standard reference method for diagnosing NASH and determining the stage of disease. Due to the risks associated with this diagnostic procedure and the evolution of cases of NAFLD, one of the major challenges is the development of specific and sensitive biomarkers that enable a reliable and non-invasive

diagnosis of the disease, usable on a large-scale in a reproducible manner. Some algorithms such as Fibrotest allow one to have an idea of the stage of fibrosis and have constituted a major advance in the diagnosis of patients with NASH (Cheah, McCullough, & Goh, 2017). Several avenues are being explored to assess the activities of the NAFLD at all stages. These include markers of cell death and apoptosis such as cytokeratin 18 (CK18) fragments, structural components of hepatocytes that are found in NASH patients. In addition, the determination of adipocytokines involved in the development of NASH (adiponectin, ghrelin, interleukin-6...) could sharpen the assessment of the NASH stage, as their circulating rate is closely correlated with the severity of the disease (Vilar-Gomez & Chalasani, 2018). Finally, hopes are placed in the OMICS approaches where the use of genomics, transcriptomics, epigenomics, metabolomics, and proteomics analyses could define a signature for the disease. Different models are being studied based on the determination of polyunsaturated fatty acids (Loomba et al., 2019), on circulating levels of glutamate, taurine, and lactate or lipid metabolite assays (Zhou et al., 2016).

- **NAFLD grading**

The different stages of NAFLD are evaluated and graded according to the histological findings in the liver. The evaluation of the stage of the pathology considers steatosis, the presence of fibrosis and the inflammatory signals present in situ. In terms of hepatic inflammation, three stages emerge depending on the degree of hepatocellular steatosis, ballooning and disorganization of hepatocytes, and the type of inflammation observed (lobular or periportal). Stage 1 corresponds to a mild necro-inflammatory state while stage 3 is considered severe. The degree of liver fibrosis is an important

factor in determining the severity of liver disease. There are four stages (F1 to F4), with stage 4 corresponding to cirrhosis.

Several scores that consider physiological, histological, and pathological factors are used to grade NAFLD (Hashimoto et al., 2013; Pappachan et al., 2017; Takahashi & Fukusato, 2014)

- The NAFLD activity score (NAS) introduced in 2005 by Kleiner and Brunt, is based on the amount of steatosis, the presence of hepatocyte ballooning and the degree of hepatic inflammation (Kleiner et al., 2005). The sum of the three entities determines a score of 0 to 8 where 8 is the most advanced stage of the pathology. Fibrosis is assessed separately in 7 stages (0,1A,1B,1C,2,3,4).
- The Steatosis Activity Fibrosis (SAF) set up in 2012 by Bedossa evaluates independently the factors involved in the disease, namely steatosis (0 to 3), activity, i.e., hepatic inflammation (0 to 2) + ballooning of hepatocytes (0 to 2) and fibrosis (0 to 4) (Bedossa et al., 2012).

New algorithms that are based either on clinical data such as the Fibrotest or Hepascore, or on plasma data such as the ELF test or the Fibrometer are also used. The Fibrotest is used to diagnose advanced fibrosis in NAFLD patients, but its diagnostic performance is reduced in the presence of certain interfering elements such as acute inflammation, sepsis or extra-hepatic cholestasis. Hepascore is an algorithm widely used in the detection of fibrosis in chronic liver disease and combines clinical variables such as age, gender and biological data including bilirubin and GGT levels. The enhanced liver fibrosis test (ELF test) (uses data related to type III procollagen levels (PIIINP) and metalloproteinase 1 inhibitor (TIMP-1). Its performance is, however, affected by the gender and age and its effectiveness in NAFLD remains to be proven (Vilar-Gomez & Chalasani, 2018). Finally, the Fibrometer is a blood test whose diagnostic accuracy is recognized in patients

with advanced fibrosis. The interest of the scores based on the measurement of serum biomarkers lies in the fact that they are non-invasive, quantitative, and reproducible tests where we are freed from the operator-induced bias. However, they have shortcomings, including a lack of hepatic specificity and interferences not allowing an optimal precision of diagnostic. Thus, their interest appears to be in the orientation of the hypothetic diagnosis and the follow-up, rather than in the diagnosis itself (Vilar-Gomez & Chalasani, 2018).

- **Etiological classification of NAFLD**

NAFLD/NASH is proposed to be categorized according to its etiological origin in the guidelines published by the organizations EASL"European Association for the Study of the Liver", EASD "European Association for the Study of Diabetes", EASO "European Association for the Study of Obesity", and AASLD "American Association for the Study of Liver Diseases". Primary NAFLD/NASH refers to steatosis caused by metabolic diseases such as obesity, T2D, dyslipidemia, and, in this case, is often associated with insulin resistance (Flisiak-Jackiewicz, Bobrus-Chociej, Wasilewska, & Lebensztejn, 2021). Among the primary NAFLD/NASH cases, some do not present impairment of glucose tolerance and are the consequence of either a genetic polymorphism or cryptogenic, i.e., of an undetermined origin (Maheshwari & Thuluvath, 2006). Secondary NAFLD/NASH includes fatty liver disease associated with endocrine disorders such as, polycystic ovary syndrome or hypothyroidism, consumption of drugs such as glucocorticoids, amiodarone, methotrexate or tamoxifen, or hospital procedures such as parenteral nutrition (Lonardo, Mantovani, Lugari, & Targher, 2019) . They also involve fatty liver disease of viral or

autoimmune origin (Araújo, Rosso, Bedogni, Tiribelli, & Bellentani, 2018; Hashimoto et al., 2013).

If NAFLD is a predicted side effect of obesity and the metabolic syndrome, we also refer to the condition as "lean NAFLD" when it first manifests in people with a normal body mass index. Lean NAFLD is thought to affect 7% of patients in the USA, but in rural Asia, where it affects 25% to 30% of the population, the prevalence is still very high (Paik, 2020 #36; Sayiner, 2016 #38; Sayiner, 2019 #37). Despite having a healthier metabolic profile than patients with classic NAFLD, recent reports claim that "lean NAFLD," which primarily affects young patients, would result in a greater morbidity and mortality with a reduced survival time, particularly in the event of hepatic transplantation. (DiStefano, 2022 #81)

ECONOMIC IMPACT OF NAFLD

Due to its associated comorbidities, the explosion of NAFLD cases is a major public health issue in the light of the economic constraints associated with it. The economic impact of NAFLD and NASH is mainly related to the health cost induced by the treatment management associated with the disease itself and its complications.

In the USA, it has been estimated that the total annual cost of caring for a NAFLD patient without private insurance was on average $8,000 for primary diagnosis and about $3,500 for long-term care, while for patients with the same comorbidities but without NAFLD the cost of care was on average $2300. The management of a NAFLD patient involves imaging procedures, biopsies, and extremely costly periods of hospitalization (Younossi, 2019).

In the USA, an annual expenditure of 62 billion dollars is predicted in the future years taking into account the increase in NAFLD cases, i.e., approximately 39 million people (Center, 2018). This annual expenditure would amount to 35 billion dollars in Europe to care for approximately 52