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Topic

Action of β2-Adrenergic receptor agonist on hepatic steatosis in Wistar male rats

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Dedications

*I dedicate this work to my parents, my mother **Nora** and my father **Essaid**, who never had the chance to learn to read or write, but who passed on to me, with greatness, the noblest values: dignity, courage, respect, and unconditional love.*

This thesis is much more than just an academic work. It is a reflection of my gratitude, the continuation of your sacrifices. I am, and will always be, your voice. My success is above all, yours.

Thank you for instilling in me, every single day, the meaning of healthy competition, excellence, and perseverance. Thank you for teaching me to dream, to believe, and to never give up.

*This thesis is also a love letter to the **curious-eyed little girl** I once was. It is the result of her dreams, her ambitions, and her determination.*

"We must have perseverance and above all confidence in ourselves.

We must believe that we are gifted for something and that this thing must be attained."

Marie Curie

Thinhinane Medjkane

Dedications

*To those whose love carved the path beneath my feet, to my cherished family, both **MADJI** and **ABDELHADI**, to my loving parents and wise grandparents, and to my loyal friends, this is for you.*

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List of abbreviations

4-HNE: 4-Hydroxynonenal.

ALAT: Alanine Aminotransferase.

ALP: Alkaline Phosphatase.

ApoB100: Apolipoprotein B100.

ApoB: Apolipoprotein B.

ASAT: Aspartate Aminotransferase.

ATP: Adenosine Triphosphate.

ATP-ase: Adenosine Triphosphatase.

ATP7B: ATPase Copper-transporting Beta.

CAMP: Cyclic Adenosine Monophosphate.

CCl4: Carbon Tetrachloride.

CD36: Cluster of Differentiation 36.

CE: Cholesterol Ester.

CGI-58: Comparative Gene Identification-58 (also known as ABHD5).

CH: Cholesterol.

ChREBP: Carbohydrate Response Element-Binding Protein.

CK18: Cytokeratin 18.

CO: Carbon Monoxide.

CREB: cAMP Response Element-Binding Protein.

CRP: C - reactive protein.

CTL: Control.

DAG: Diacylglycerol.

DNL: De Novo Lipogenesis.

DNA: Deoxyribonucleic Acid.

ER: Endoplasmic Reticulum.

FA: Fatty Acid.

FATP: Fatty Acid Transport Protein.

FFA: Free Fatty Acid.

FOR-HD: Formoterol High Dose.

FOR-LD: Formoterol Low Dose.

GCKR: Glucokinase Regulator.

GGT: Gamma-Glutamyl Transferase.

Gi: Inhibitory G protein alpha subunit.

GK: Glycerol Kinase.

GKRP: Glucokinase Regulatory Protein.

GOD: Glucose Oxidase.

GOT: Glutamate Oxaloacetate Transaminase (AST).

GPCRs: G Protein-Coupled Receptors.

GPO: Glycerol-3-Phosphate Oxidase.

GPT: Glutamate Pyruvate Transaminase (ALT / TGP).

GQ: G protein alpha q subunit.

GRK: G Protein-Coupled Receptor Kinase.

Gs: Stimulatory G protein alpha subunit.

HCC: Hepatocellular Carcinoma.

HDL: High-Density Lipoprotein.

HFD: High-Fat Diet.

HSD17B13: Hydroxysteroid 17-beta-dehydrogenase 13.

IKK-NF-κB: IκB Kinase / Nuclear Factor Kb.

IKK2: IκB Kinase beta2.

IL-1 : Interleukin 1.

IL-10 : Interleukin 10.

IL-6 : Interleukin 6.

IR : Insulin Receptor.

JNK : c-Jun N-terminal Kinase.

JNK-AP-1 : c-Jun N-terminal Kinase / Activator Protein 1.

LDH: Lactate Dehydrogenase.

LDL: Low-Density Lipoprotein.

LPL: Lipoprotein Lipase.

MBOAT7: Membrane-bound O-acyltransferase domain-containing 7.

MDA: Malondialdehyde.

MDH: Malate Dehydrogenase.

MRI: Magnetic Resonance Imaging.

NAFLD: Non Alcoholic Fatty Liver disease.

NASH: Non-Alcoholic Steatohepatitis.

NH_4^+: Ammonium ion.

NF-kB: Nuclear Factor kappa B.

PGC-1α: Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha.

PH: Potential of Hydrogen.

PKA: Protein Kinase A.

PKC: Protein Kinase C.

PNPLA3: Patatin-like phospholipase domain-containing protein 3.

POD: Peroxidase.

PPARα: Peroxisome Proliferator-Activated Receptor Alpha.

RNA: Ribonucleic Acid.

ROS: Reactive Oxygen Species.

SAL-HD: Salbutamol High Dose.

SAL-LD: Salbutamol low Dose.

Ser659: Serine 659.

Ser660: Serine 660.

SNP: Single Nucleotide Polymorphism.

SREBP1c: Sterol Regulatory Element-Binding Protein 1c.

T2D: Type 2 Diabetes.

TG: Triglycerides.

TGP: Transaminase Glutamic Pyruvic (ALT).

Thr1160: Threonine at position 1160.

TIMPs: Tissue Inhibitors of Metalloproteinases.

TM6SF2: Transmembrane6 superfamily 2.

TNF-α: Tumor Necrosis Factor alpha.

VLDL: Very Low Density Lipoprotein.

Introduction

Introduction

The liver is the largest organ, accounting for approximately 2% to 3% of average body weight **(Abdel-Misih and Bloomston, 2010).** It is dynamic, heterogeneous organs that participate in numerous physiological processes, but unfortunately hepatic pathologies continue to have significant global morbidity and mortality burdens and the causes of liver disorders are diverse **(Trefts et *al*., 2017)**. In recent decades, non-alcoholic fatty liver disease (NAFLD) has become the most common liver disease worldwide, affecting approximately 25% of the global population **(Younossi *et al*., 2019;Tagkou and Goossens, 2023).** NAFLD is considered the hepatic manifestation of a multisystem metabolic syndrome and encompasses a spectrum of diseases ranging from isolated steatosis (excessive accumulation of triglycerides) to nonalcoholic steatohepatitis (NASH), which can potentially lead to liver fibrosis, cirrhosis, or hepatocellular carcinoma (HCC) **(Donnelly *et al*., 2005).**

Several risk factors promote the appearance of NAFLD, such as metabolic factors (insulin resistance, obesity, type 2 diabetes, dyslipidemia, and hypertension), Lifestyle changes and genetic factors (**Younossi *et al*., 2017; Stols *et al*., 2019).** In fact, fat may accumulate in the liver as a consequence of multiple abnormalities of hepatic lipid metabolism, including fatty acid uptake, increased de novo lipogenesis, decreased β-oxidation of fatty acids, and/or decreased synthesis or secretion of very low-density lipoproteins (VLDL) **(Donnelly *et al*., 2005, Stefan *et al*., 2008).** Several Signaling pathways are involved in the progression of NAFLD like oxidative stress, mitochondrial dysfunction, inflammatory pathways and MicroRNAs **(Buzzetti *et al*., 2016; Fang *et al*., 2021).**

Sympathetic nervous system plays a key role in the overall physiology of the human body, its activation are mediated through the action of the endogenous catecholamines on adrenergic receptors **(Ferguson and Feldman, 2014).** β2-adrenoceptors largely expressed in the body, belong to the superfamily of G protein-coupled receptors, and they are involved in different function, such as dilatation of blood vessels and bronchioles, relaxation of the muscles of the uterus, bladder and gastrointestinal duct, and also decreased platelet aggregation and glycogenolysis in the liver (resulting in glucose release) **(Rasmussen *et al*., 2007; Motiejunaite *et al*., 2020).** Adrenergic pathways are the subject of increasing scientific interest due to their role in the regulation of lipid metabolism, confirmed by the study of **Kim *et al*. (2010)** which examined the direct effect of clenbuterol, a β2-agonist, on apoptosis, adipogenesis and lipolysis *in vitro*, using the 3T3-L1 cell line and primary rat adipocytes. This study showed that direct activation of the β2-adrenoceptor resulted in stimulation of lipolysis and inhibition of adipogenesis (**Kim *et al*., 2010)**

Based on these observations, we hypothesized the potential of this pathway in the context of hepatic steatosis. The ability of these agonists to mobilize lipids in adipose tissue suggests a possible beneficial effect on hepatic accumulation. Thus, we conducted this experimental study in a model of hepatic steatosis induced by a high-fat diet in albino Wistar rats. The main objective of this work is to evaluate, *in vivo*, the action of β2-adrenergic agonists (Salbutamol or Formoterol) against non-alcoholic fatty liver disease. This evaluation is based first on the effective induction of hepatic steatosis in the animal model to simulate human pathological conditions; this will allow evaluating the impact of treatments on lipid accumulation in the liver and adipose tissue. Next, we examined the effect of 14 days of β2 –adrenoceptors stimulation by salbutamol or formoterol on tissue damage through biochemical markers of liver damage and liver histology. We also examined the hypothesis the canonical activation pathway of lipolysis in response to salbutamol or formoterol administration is associated by alterations in the lipid profile and glycemia. Thus, all of these aim to examine the effects of β2-agonists on the repair of liver damage and the improvement of the metabolic profile.

Chapter 01
Bibliographic Synthesis

I. Liver; physiology and pathology

I.1. Anatomical Structure

The liver is a large organ weighing between 1.6 and 2 kg. The liver is located on the right, below the diaphragm, and is reddish-brown in color. It is surrounded and protected by the fibrous Glisson's capsule. It consists of two distinct lobes, separated by the falciform ligament, which also connects it to the anterior abdominal wall. The liver is one of the most vascularized organs, receiving 25 to 30% of the cardiac output **(Bessaguet and Desmouliére, 2021).**

The liver is composed of several cell types of different embryological origin, including hepatocytes, biliary epithelial cells (cholangiocytes), stellate cells, kupffer cells, and hepatic sinusoidal endothelial cells. Hepatocytes constitute the majority of the liver's volume and perform many functions **(Trefts et *al*., 2017)** (Figure 01).

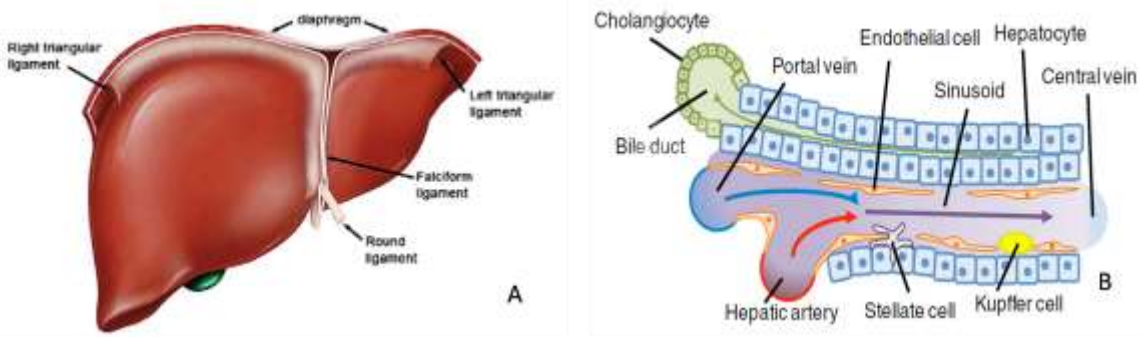

Figure 01: Anatomical Structure of the liver; A: Macroanatomy / B: Microanatomy **(Abdel-Misih and Bloomston, 2010; Trefts *et al.*, 2017).**

I.2.Main roles and functions

The liver participates in hematopoiesis during the fetus before the bone marrow can take over this job in the seventh month of development **(Sumadewi, 2023).** The liver is a center for many physiological processes, it is essential for the synthesis of many molecules, including lipoproteins, albumin, most plasma globulins, and some steroid hormones **(Baudin, 2017).** It has the ability to store and metabolize glucose in the form of glycogen, as well as vitamins A, B12, D, E, K, and iron **(Bessaguet and Desmoulière, 2021).**

The exocrine function of the liver is represented by bile secretion, which facilitates the digestion of lipids and participates in the elimination of unnecessary or toxic substances from

the body. It is composed of acids, bile salts, and pigments such as bilirubin; the heme degradation product **(Baudin, 2017).** The liver is a detoxification organ responsible for transforming toxic substances into eliminable compounds, whether endogenous products, such as ammonium (NH4+), or exogenous substances, such as xenobiotics **(Baudin, 2017).** This is achieved through hepatic enzymes that increase the hydrophilicity of molecules, thus facilitating their urinary elimination **(Almazroo *et al*., 2017).** Moreover, the liver is often exposed to intestinal antigens and low concentrations of bacterial endotoxins. Several regulatory mechanisms are involved in maintaining immune tolerance and forming an anti-inflammatory microenvironment. These include Kupffer cells, resident liver macrophages secreting interleukin (IL)-10 **(Ju and Tacke, 2016)**.

I.3.Liver disorders

Due to the diversity and importance of liver functions, the pathologies that can affect the liver are numerous and varied. The causes of liver disease are diverse and include excessive alcohol consumption (greater than 20 g/day for both men and women), viral infections, prolonged medication use, autoimmune disorders, genetic conditions such as hemochromatosis and Wilson's disease, and extended exposure to potentially hepatotoxic substances **(Mantovani *et al*., 2019).** The following sections outline some of the major liver diseases.

I.3.1.Hepatitis : Hepatitis is classified into viral hepatitis (mainly viruses A, B, C, and E) and non-viral hepatitis with various causes, including toxic hepatitis (CCl4, methanol, Amanita phalloides...) or iatrogenic hepatitis (paracetamol poisoning) **(Baudin,2017).**

I.3.2.Steatosis: Steatosis, known as nonalcoholic fatty liver disease (NAFLD) is characterized by hepatic fat accumulation (≥ 5% of the liver) **(Younossi *et al*., 2019).**

I.3.3.Cholestasis: It is characterized by an obstruction of the bile ducts which leads to an accumulation of bilirubin in the blood, skin and mucous membranes **(Baudin, 2017).** In cases of cholestasis, conjugated bilirubin, ALP, and GGT levels increase simultaneously. The rise in GGT occurs earlier than that of ALP and is more specific to the liver and bile ducts. **(Whitfield, 2001)**.

I.3.4.Hemochromatosis: Is a genetic disorder characterized by excessive iron accumulation in the body, primarily affecting the heart, liver, and skin. It results from mutations leading to

reduced or absent hepcidin expression or a loss of ferroportin function **(Bessaguet and Desmouliére, 2021).**

I.3.5.Wilson's disease: Is an inherited metabolic disorder caused by biallelic mutations in the ATP7B gene .The loss of function of copper transporter ATP-ase results in an impaired excretion of copper into the bile and subsequent accumulation of copper in the liver, and later in the brain and other organs. Excessive free copper leads to cytotoxic effects in hepatic and central nervous tissues **(Hedera, 2019).**

I.4. Liver diseases progression

All of these pathologies can lead to liver fibrosis, which results in deposits of fibrous components resulting from excessive synthesis and/or impaired degradation, leading to remodeling of the extracellular matrix **(Ju and Tacke, 2016)**. Following the progression of fibrosis, it can progress to cirrhosis, an irreversible stage characterized by structural disorganization of the liver, which can become the site of hepatocellular carcinoma, also known as hepatoma, with hepatocellular insufficiency and predominant inflammation **(Drescher *et al*., 2019)** Its diagnosis is generally based on liver imaging, liver biopsy, and the measurement of specific tumor markers, notably alpha-fetoprotein **(Baudin, 2017).** Thus, liver diseases follow a progressive course, from fibrosis to hepatocellular carcinoma, with increasing functional and structural alterations **(Bessaguet and Desmoulière, 2021)**.

II. Non-Alcoholic Fatty Liver Disease (NAFLD)

II.1. Definition and characteristics of NAFLD

Non-alcoholic fatty liver disease is defined by an accumulation of lipid vacuoles in the cytoplasm of at least 5% of hepatocytes **(Younossi *et al*., 2019)**. It is the most common chronic liver disease in individuals without alcohol abuse, encompasses a spectrum of pathological changes ranging from steatosis to progressive inflammatory injury (termed nonalcoholic steatohepatitis, or NASH), ultimately with potential development of cirrhosis and/or hepatocellular carcinoma **(Stols *et al*., 2019).**

NAFLD is asymptomatic and often discovered incidentally. Although this disease is silent, some patients may present with asthenia, abdominal discomfort or hepatomegaly.
Biologically, NAFLD is characterized by an increase in transaminases (ALAT>ASAT) **(Anty,**

2023). Dyslipidemia is also observed, characterized by a decrease in HDL cholesterol and an increase in triglycerides **(Zhang and Lu, 2015).**

II.2. Prevalence of NAFLD

Non-alcoholic fatty liver disease (NAFLD) is a major public health problem due to its high prevalence worldwide, estimated at 25%, the highest prevalence rates have been reported in the Middle East **(Younossi *et al*., 2019)** (Figure 02).

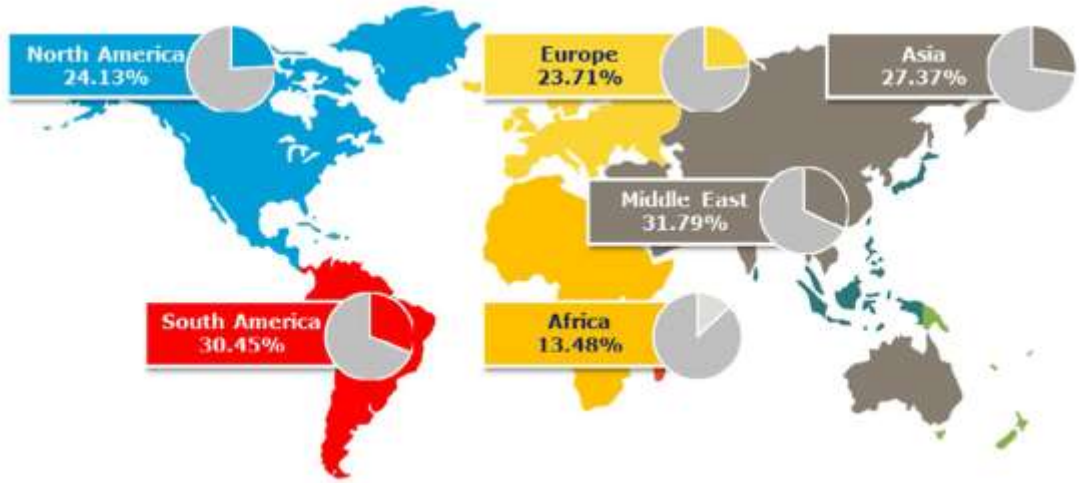

Figure 02: The Global Prevalence of Nonalcoholic Fatty Liver Disease (NAFLD) **(Younossi *et al*., 2019).**

The prevalence of NAFLD varies in parallel with that of obesity, but this does not prevent the presence of a significant proportion of lean patients with abdominal obesity. It is also important to note that NAFLD does not only affect adults; there is also a high prevalence in children and adolescents **(Younossi *et al*., 2019).**

II.3. Diagnosis of NAFLD

Diagnosis of NAFLD is generally based on non-invasive strategies that rely either on serum scores and biomarkers or on measurements of liver elasticity by imaging, using ultrasound or MRI-based techniques **(Caussy *et al*., 2018)** On the other hand, Biopsy Although generally well tolerated, it is an invasive procedure that carries a risk of complications such as bleeding, infection, bile leakage, damage to other organs and a rare risk of mortality **(Tagkou and Goossens, 2023).**

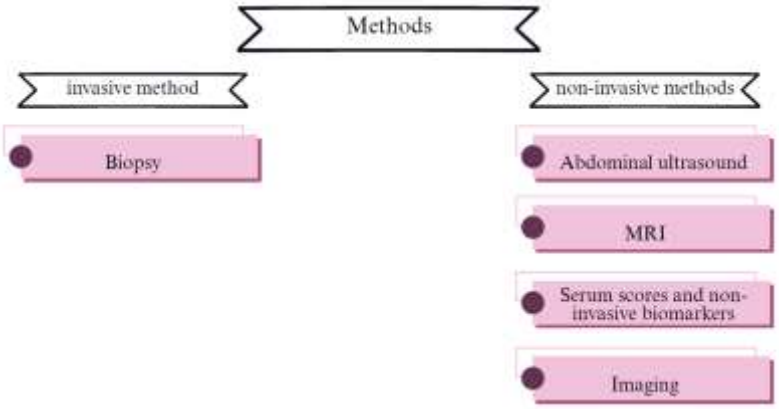

Figure 03: Diagnosis methods (**inspired by Tagkou and Goossens, 2023).**

II.4.Risk factors and etiology

II.4.1.Metabolic factors

NAFLD is considered the hepatic manifestation of insulin resistance, obesity, type 2 diabetes, dyslipidemia, and hypertension **Stols *et al*., 2019).**

-Type 2 diabetes: NAFLD affects up to 70–80% of patients with type 2 diabetes (T2D). These patients are at risk of developing severe histological forms of NAFLD, including hepatocellular carcinoma, by inducing inflammation, oxidative stress, and genomic instability, which causes hepatocyte hyper proliferation and reduced apoptosis **(Mantovani *et al*., 2019; Yang *et al*., 2020).**

-Obesity: is the main cause of fatty liver disease, especially when it begins in childhood and adolescence, which increases the rate of liver mortality **(Younossi *et al*., 2017).** The first treatment to consider for NAFLD is weight loss through calorie restriction and exercise. Weight loss of ≥ 10% has been shown to resolve NASH and reduce fibrosis **(Stols *et al*., 2019).**

-Insulin resistance: causes persistent hyperglycemia, impairs nitric oxide production, increases the expression of vascular adhesion molecules, and enhances the uptake of oxidized LDL **(Pasterkamp, 2013; Arab, 2018).**

-Hypertension: Contributes to NAFLD through the activation of renin–angiotensin–aldosterone system **(Kumar *et al*., 2017),** which worsens insulin resistance and stimulates liver fibrosis **(Fonseca *et al*., 2006; Toblli *et al*., 2008).**

II.4.2.Lifesyle

Lifestyle changes are the main cause of several metabolic diseases such as NAFLD, which has become a global health problem **(Younossi *et al*., 2017).** Numerous studies have demonstrated the beneficial role of lifestyle changes in NAFLD **(Semmler *et al*., 2021)**

Dietary composition has been well documented to influence hepatic fat accumulation. Physical activity also reduces lipid accumulation and markers of hepatocellular injury, independent of weight loss **(Leslie *et al*., 2014; Sung *et al*., 2016).**

II.4.3.Genetics and epigenetic factors

Several studies have demonstrated a hereditary component of NAFLD (Please see Table 1), including family and twin studies **(Zeybel *et al*., 2012; Zarrinpar *et al*., 2016).** Furthermore, in the United States, NAFLD prevalence rates vary by ethnicity, with the highest prevalence observed among Hispanic Americans, followed by European Americans, and then African Americans. The main explanations are related to genetic differences in lipid metabolism **(Younossi *et al*., 2017).**

A recent study by Brouwers et al. (2019) identified 12 genes linked to NAFLD susceptibility. The following table summarizes the key genetic variants that promote NAFLD progression: Table 01

Table 01: key genetic variants that promote NAFLD

Gene	function	Mutation	References
PNPLA3 Patatin-like phospholipase domain-containing protein 3	Codes for adiponutrin, a hydrolase involved in TG metabolism by transferring essential FA from TG to phospholipids in hepatic lipid droplets	The I148M variant (SNP rs738409 C>G) reduces TG hydrolase activity, thus implying deregulation of lipid droplets in hepatocytes	(Mitsche *et al*., 2018) (Pingitore and Romeo, 2019)
TM6SF2 Transmembrane6 superfamily 2	promotes the secretion of VLDL and nascent ApoB-containing lipoproteins in hepatocytes	The E167K mutation disrupts lipid export in lipoproteins, thus promoting hepatic fat accumulation. However, this mutation	(Li *et al*., 2018)

		reduces the risk of cardiovascular disease by decreasing circulating levels of VLDL and LDL	
MBOAT7 Membrane-bound O-acyltransferase domain-containing 7	MBOAT7 is an enzyme with acyltransferase activity. It plays a role in the remodeling of the acyl chain of phospholipids via the Lands cycle. It regulates inflammation by modulating arachidonic acid levels in neutrophils.	MBOAT7 variants may contribute to inflammation-induced hepatic fibrogenesis	(Buch *et al*., 2015) (Caddeo *et al*., 2019)
GCKR **glucokinase regulator**	Encodes glucokinase regulatory protein (GKRP). It regulates de novo lipogenesis by controlling glucose influx into hepatocytes	Genetic variants of GCKR influence TG levels, fasting blood glucose, and insulin regulation. Therefore, they are always linked to metabolic disorders such as NAFLD.	(Drescher *et al*., 2019). (Jonas and Schürmann, 2021).
HSD17B13 Hydroxysteroid 17-beta-dehydrogenase 13	Encodes a hepatic enzyme associated with lipid droplets, upregulated in patients with NAFLD.	HSD17B13 variants confer protection against steatosis progression	(Liu *et al*., 2023).

II.5. Associated conditions

Several associated conditions are observed in patients with NAFLD, including extrahepatic diseases, such as chronic renal failure and cardiovascular complications such as atherosclerosis **(Targher *et al*., 2016; Mantovani *et al*., 2019).**

In these patients, there is a bidirectional relationship between risk factors and associated conditions, particularly metabolic ones, which contribute to the onset of NAFLD. Once established, NAFLD aggravates the progression of these conditions. This creates a vicious cycle and complicates the management of these patients **(Younossi *et al*., 2017).**

III.1. Global Pathogenesis

The pathophysiological mechanisms leading from steatosis to NASH are still debated and are based on the concept of the two hit hypothesis proposed by Day and James. The first hit would be the consequence of insulin resistance and would induce steatosis, i.e. the

accumulation of hepatic lipids; the second hit would correspond to the development of fibro-inflammatory lesions whose causes are multifactorial **(Day and James, 1998).** However, it became rapidly evident that the pathogenesis of NAFLD and its progression is a complex process, which cannot be completely explained by the 'two hit' hypotheses. A number of diverse parallel processes acting synergistically and contribute to the development and progression of steatosis and liver inflammation, which is explained by the new "multiple hits" hypothesis **(Buzzetti *et al*., 2016).**

Dietary and environmental factors, together with obesity, lead to raised serum levels of fatty acids (FFAs) and cholesterol (CH), development of insulin resistance, adipocyte proliferation and dysfunction and changes in the intestinal microbiome **(Bugianesi *et al* ., 2010).**

Insulin resistance acts on adipose tissue, exacerbating adipocyte dysfunction, inducing lipolysis and the release of adipokines and pro-inflammatory cytokines such as TNF-α and IL-6, which also contribute to the maintenance of insulin resistance **(Guilherme *et al* ., 2008 ; Kirpich *et al* ., 2015)**. At the hepatic level, insulin resistance amplifies de novo lipogenesis **(Bugianesi *et al*., 2010).**

As shown in Figure 4, lipid accumulation causes mitochondrial dysfunction with oxidative stress and endoplasmic reticulum (ER) stress, leading to hepatic inflammation and fibrosis (NASH) or persistence of the disease at a stable stage (NAFLD) **(Buzzetti *et al*., 2016).**

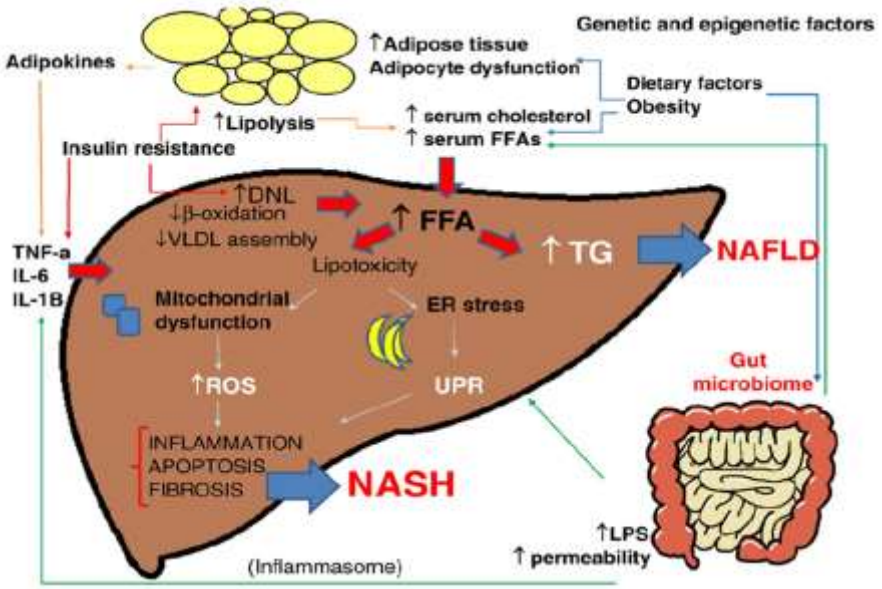

Figure 04: Multiple hit hypothesis for the development of NAFLD **(Buzzetti *et al*., 2016).**

III.2. Liver Lipid accumulation

Hepatic fat accumulation results from an imbalance between lipid acquisition and elimination, meaning that fatty acid uptake and de novo lipogenesis exceed fatty acid oxidation and export, constituting the four main pathways of hepatic lipid homeostasis (**Ipsen *et al.,* 2018).** Indeed**,** a study using tracers in patients with metabolic steatosis showed that the origin of fatty acids was 59% via lipolysis, 26% via de novo lipogenesis and only 15% via diet **(Donnelly *et al* ., 2005).** The uptake of circulating fatty acids by the liver is largely dependent on fatty acid transporters, while passive diffusion contributes less. The transport is predominately mediated by fatty acid transport proteins (FATP), cluster of differentiation 36 (CD36), and caveolins located in the hepatocyte plasma membrane **(Koo, 2013).**

Recent work has shown that adipocyte lipolysis also represents a major source of lipids; insulin resistance increases the release of FFAs from white adipose tissue, due to the decrease in the anti-lipolytic action of insulin and therefore a significant influx of free fatty acids into the portal blood and the liver **(Parlati *et al*., 2023)**. Lipid accumulation in NAFLD is promoted by increased lipogenesis, defined as the de novo synthesis of FA from non-lipid precursors, e.g., acetyl-CoA **(Benhamed *et al*., 2012)**. Transcriptional regulation of DNL is mainly orchestrated by two key transcription factors: sterol regulatory element-binding protein 1c (SREBP1c), activated by insulin and liver X receptor α, and carbohydrate regulatory element-binding protein (ChREBP), activated by carbohydrates **(Sanders and Griffin, 2016)**.

In this case a compensatory increase in fatty acid oxidation is observed; controlled by PPARα and occurs primarily in the mitochondria. However, very long-chain fatty acids are preferentially metabolized via peroxisomal β-oxidation. In cases of lipid overload, such as NAFLD, ω-oxidation also contributes to this process in cytochromes. However, this oxidation is insufficient to normalize lipid levels and may even promote cell damage and disease progression by inducing oxidative stress **(Ipsen *et al*., 2018).** In addition to fatty acid oxidation, export of triglycerides as VLDL is the only way to reduce hepatic lipid content **(Perry *et al*., 2014).** Although moderate exposure to fatty acids increases apoB100 secretion, prolonged exposure leads to endoplasmic reticulum stress and post-translational degradation of apoB100, and consequently to a decrease in its secretion **(Zhang *et al* .,2014).**

III.3. Signaling pathways involved in the progression of NAFLD

Lipids that accumulate in the early stages of NAFLD generate cytotoxic effects that contribute to the initiation of inflammatory lesions. However, hepatocyte lipotoxicity alone is insufficient to trigger the full range of tissue damage that corresponds to all stages of NAFLD.

The molecular mechanisms governing hepatic lipid homeostasis and the counterregulatory mechanisms related to chronic lipid overload are very complex and closely interconnected **(Parlati *et al*., 2023).**

III.3.1. Oxidative stress

Oxidative stress results from an imbalance between the production of pro- and antioxidant molecules, leading to cellular damage. In steatosis liver, efforts to reduce lipid overload and lipotoxicity may even promote disease progression because fatty acid oxidation is a major source of reactive oxygen species (ROS) (O-, OH-, H2O2, O2-) and pro-oxidant molecules **(Parlati *et al*., 2023)**. These molecules induce DNA damage, alteration of cell membranes, a decrease in ATP stores, and production of pro-inflammatory cytokines **(Lemoine and Serfaty, 2012)**. Also, *in vitro* and *in vivo* studies have clearly shown that reactive aldehydes resulting from lipid peroxidation (malondialdehyde; MDA and 4-hydroxynonenal; 4-HNE) are important pro-inflammatory mediators, capable of activating hepatic stellate cells and increasing the synthesis of type 1 collagen, found mainly in hepatic fibrous tissue **(Bedossa *et al*., 1994).**

III.3.2. Mitochondrial dysfunction

Structural and functional alterations in mitochondria contribute to the pathogenesis of NAFLD. Structural alterations encompass depletion of mitochondrial DNA, morphological and ultra structural changes, while functional alterations include the respiratory chain and mitochondrial β-oxidation **(Pessayre and Fromenty, 2005)** , inducing impairment of fat homeostasis, generation of lipid derived toxic metabolites and overproduction of ROS **(Begriche *et al*., 2006)** All these molecules activate inflammatory pathways contributing to hepatocytes necroinflammation and worsening of mitochondrial damage **(Buzzetti *et al*., 2016).**

In NASH (one of the consequences of these alterations is the activation of caspases 3 which will cleave intracellular substrates such as cytokeratin 18 (CK18) and induce apoptosis of hepatocytes **(Wieckowska *et al*., 2006).**

Thus, lipid oxidation and oxidative damage to mitochondrial DNA further decrease mitochondrial function, creating a vicious cycle that exacerbates mitochondrial dysfunction and oxidative stress **(Ipsen *et al*., 2018).**

III.3.3. Inflammatory pathways

Non-alcoholic steatosis is associated with an inflammatory state characterized by the production of numerous cytokines involved in the inflammatory response. Several studies have shown that NASH results from hepatocyte necroptosis and macrophage infiltration, leading to higher serum levels of TNF-α, IL-1, IL-6, NF-βB and CRP **(Diehl *et al.*, 2005).** Indeed, two main inflammatory pathways, JNK-AP-1 and IKK-NF-κB, are involved in the development of the chronic inflammatory state in NAFLD. JNK is a member of mitogen activated protein kinases, associated with activation of apoptosis and development of NASH. Nuclear factor-jB kinase-b (NF-kB) is a transcription factor and a primary regulator of inflammatory activation, and its IKK2 subunit is the major component required for its activation during the acute inflammatory response , her over-expression and persistent activation of NF-kB in hepatocytes lead to a chronic inflammatory state and insulin resistance **(Buzzetti *et al.*, 2016).**

Adipose tissue, in addition to its role as energy storage, also participates in the secretion of numerous proteins involved in inflammation, such as adipocytokines **(Targher *et al.*, 2006)**. All of these molecules trigger inflammatory cascades and also contribute to liver repair. The chronic inflammatory state of NASH promotes the differentiation of stellate cells into myofibroblasts, leading to the deposition of type 1 collagen and the production of tissue inhibitors of metalloproteinases (TIMPs), ultimately leading to fibrosis (**Stols *et al.*, 2019).**

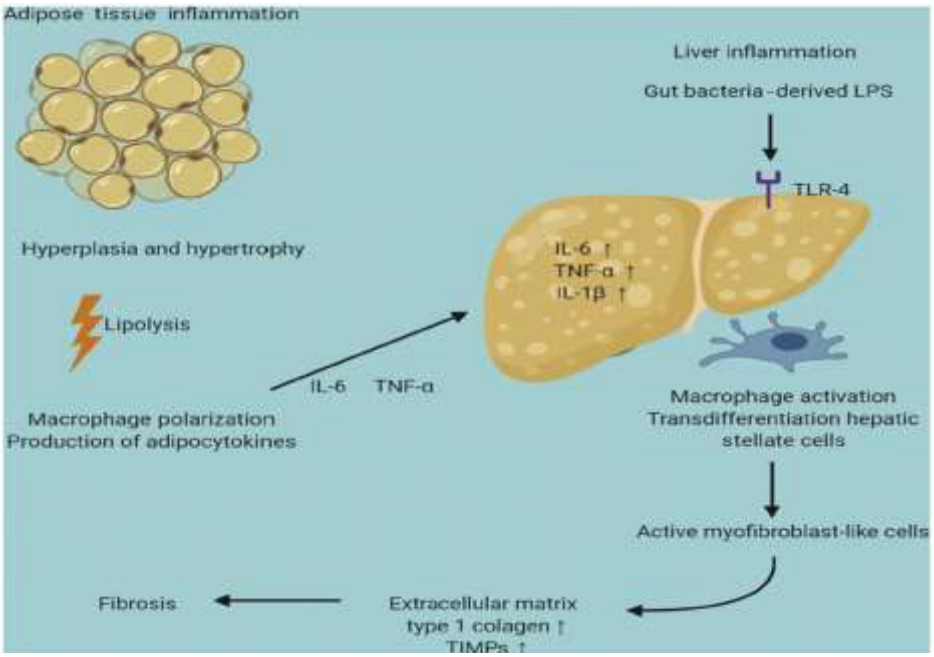

Figure 05: Inflammation role in NAFLD at different levels (**Stols *et al.*, 2019).**

III.3.4.MicroRNAs

MiRNAs are small (19–23 nucleotides) non-coding RNA molecules that participate in the post-transcriptional fine tuning of gene regulation and, consequently, their protein synthesis. They are indispensable for numerous biological processes, such as proliferation, apoptosis, development, differentiation, metabolism, and carcinogenesis **(Fang *et al*., 2021).** The pathogenesis of NAFLD is very complex, MicroRNAs (miRNAs) dysrégulation are suggested to play a key role in the occurrence and development of NAFLD (Figure 06) **(Baffy *et al*., 2015)**.

Circulating miRNAs have been proposed as attractive diagnostic tools for distinguishing, non-invasively, NAFLD diseased individuals from healthy ones. miRNAs concentrations are significantly upregulated in patients with NAFLD. However, miR-122, miR-192, and miR-375 have the potential to distinguish NASH from simple steatosis (Figure 06), but only miR-122 distinguishes liver fibrosis **(Pirola *et al*., 2015).** NAFLD exhibits a distinct circulating miRNAs signature, which is a reliable fingerprint of morphological changes occurring in liver tissue (Figure 06). In addition, by characterizing the dysregulated miRNAs in the circulation, we might be able to identify key signaling pathways involved in the pathogenesis of the disease and identifying individuals at risk. **(Pirola *et al*., 2014)** (Figure 05).

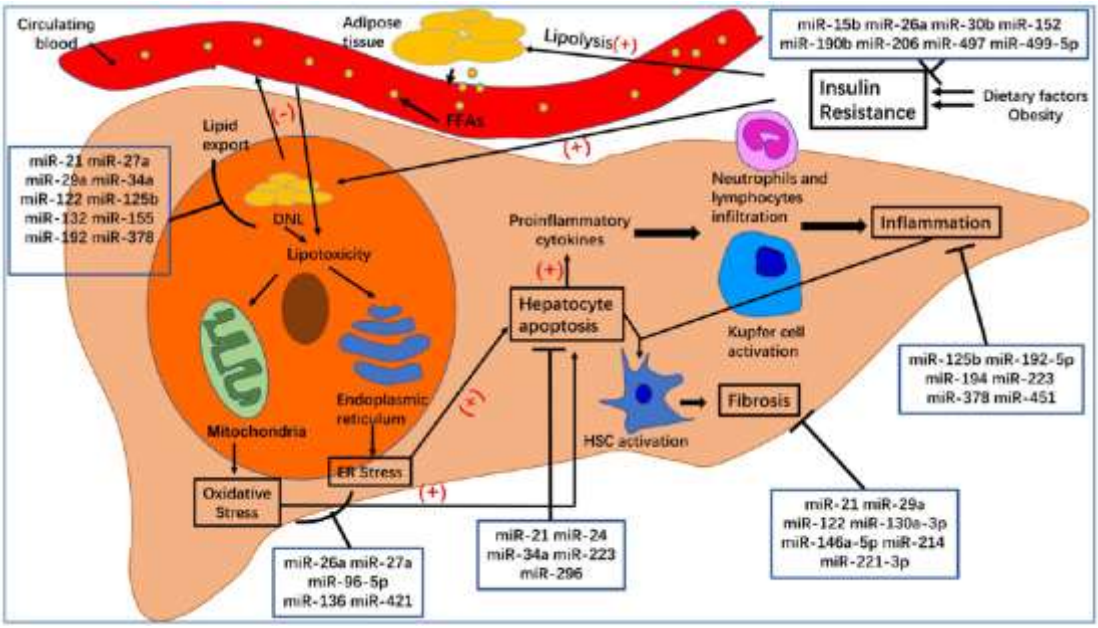

Figure 06: The roles of specific miRNAs in the pathogenesis of NAFLD (**Fang *et al*., 2021**).

IV .The adrenergic system and its receptors

Activation of the sympathetic nervous system is responsible for the body's "fight or flight" reaction and is essential to maintain homeostasis in a constantly changing environment. The

physiological and metabolic responses to sympathetic activation are mediated through the action of the endogenous catecholamines: norepinephrine (or noradrenaline) and epinephrine (or adrenaline) on adrenergic receptors **(Motiejunaite *et al*., 2020).** This is why the adrenergic system plays a key role in the overall physiology of the human body.

IV.1.Overview of the adrenergic receptors

IV.1.1.Structure of adrenergic receptors

Adrenergic receptors, also called adrenoceptors belong to the superfamily of G protein-coupled receptors. All GPCRs have in common architecture with 7 transmembrane domains, organized in helices, connected to each other by extracellular and intracellular loops. Structurally, GPCRs have an amino-terminal end in contact with the extracellular medium and a cytoplasmic carboxy-terminal end **(Rasmussen *et al*., 2007).** They are membrane receptors that activate G proteins following the binding of a ligand, Gs and Gi proteins activate and inhibit the enzyme adenylyl-cyclase respectively, while Gq proteins activate phospholipase C **(Pfleger *et al*., 2019).** Adrenergic receptors are typically divided into alpha1, alpha2, beta1, beta2 and beta3 receptors (**Motiejunaite *et al*., 2020).**

IV.1.2.Distribution of β2 adrenergic receptors in the body

β2-adrenergic receptors are expressed predominantly in bronchial smooth muscle cells, but are also largely expressed by epithelial and endothelial cells of the lung, by hepatocytes, and by genito-urinary smooth muscle cells (**Motiejunaite *et al*., 2020).**

IV.2. Mechanism of action of β2 agonists

β2- agonists are traditionally used for the treatment of bronchospasm associated with asthma and the treatment of symptomatic patient with chronic obstructive pulmonary disease (COPD) to induce a bronchodilatation by relaxing airway smooth muscle **(Cazzola et al., 1997)**.

β2- adrenergic receptors are coupled to the stimulatory G protein, which in turn activates adenylate cyclase, triggering the production of cAMP from ATP, cAMP then catalyzes the activation of protein kinase A, which in turn phosphorylates proteins involved in controlling muscle tone **(Rasmussen *et al*., 2011).** These activated protein kinases then induce three types of responses, resulting in smooth muscle relaxation: reduction of cytosolic calcium, inhibition of actin-myosin interaction, and opening of high-conductance calcium-gated potassium channels **(Devillier *et al*., 1996).**

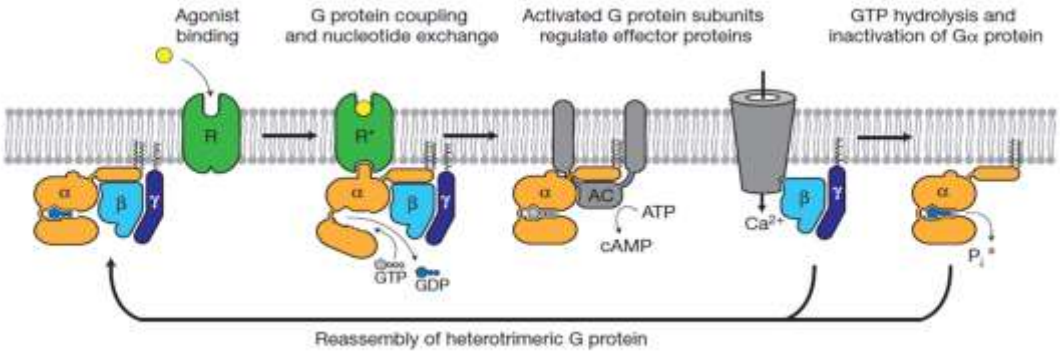

Figure 07: β2-adrenoceptors -Gs signaling pathway **(Rasmussen *et al.*, 2011).**

Prolonged β2-adrenergic stimulation leads to desensitization and rapid attenuation of the biological response. This process involves receptor phosphorylation at serine residues 355 and 356 located at the COOH terminus of the receptor by protein kinase A (PKA) and certain members of the G protein-coupled receptor kinase (GRK) family **(Seibold *et al.*, 2000).**

After receptor phosphorylation by GRKs, β-arrestin is recruited and then binds weakly to the COOH terminus of the receptor. β-arrestin binds to β2-adaptin and clathrin, structural components of vesicles formed at the plasma membrane and allowing internalization of the protein assembly **(Kim & Benovic, 2002).** They will then be dephosphorylated and directed towards membrane recycling or degradation **(Moore *et al.*, 2007).**

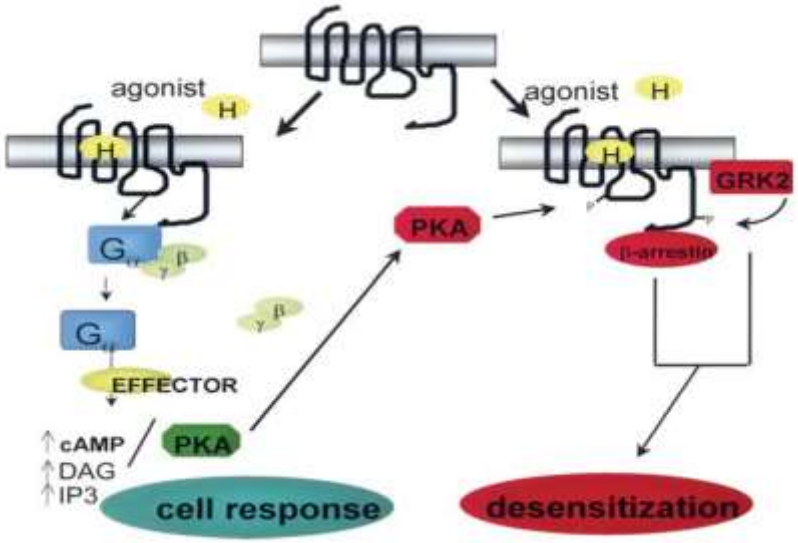

Figure 08: role of *β*-arrestin: desensitization **(Shenoy and Lefkowitz, 2003).**

IV.3.Examples of β2-agonists

β2-agonists are classified into three categories based on their duration of action: short-acting (3 to 6 h) agonists (e.g., salbutamol), long-acting (12 h) agonists (e.g., salmeterol or formoterol), and very long-acting (24 h) agonists (e.g., indacaterol) **(Motiejunaite *et al*., 2020).** The onset of action, as well as the duration of action of β2-agonists, depends mainly on the physicochemical properties responsible for the binding of β2-agonists to β-adrenergic receptors **(Anderson *et al*., 1994).**

IV.3.1.Salbutamol

Salbutamol is a hydrophilic molecule that directly accesses the receptor's active site from the aqueous extracellular compartment . This results in a rapid action on respiratory tissue relaxation and bronchodilation in patients. However, due to its hydrophilic nature, salbutamol's persistence at the receptor's active site is short-lived, resulting in duration of action of 4 to 6 hours **(Price and Clissold, 1989).**

IV.3.2.Formoterol

Formoterol is slightly lipophilic in nature. It interacts directly with the receptor's active site, explaining the rapid onset of action observed clinically. In addition, a small amount of formoterol is captured in the cell membrane, where it gradually interacts with the receptor's active site. This explains the long duration of action of formoterol (12 hours) **(Anderson *et al*., 1994; Palmqvist *et al*., 1997).**

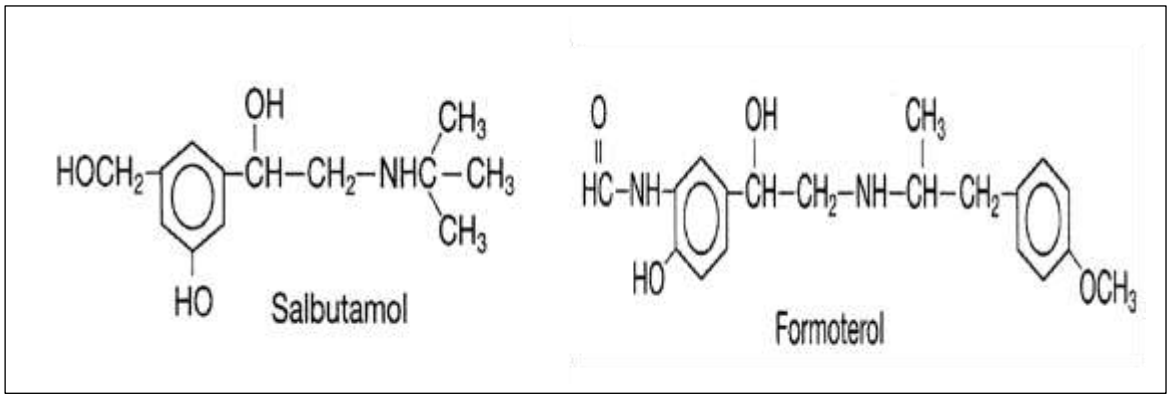

Figure 09: Chemical structure of Salbutamol and Formoterol **(Sears and Lottvall, 2005).**

IV.4.Side effects of β2 agonists

Prolonged exposure to β2-agonists results in numerous side effects such as migraines, heart palpitations and tremors, thus limiting their use **(Palmqvist *et al*., 1997; Guhan *et al*., 2000).** That's why long-acting β2-adrenergic agonists were developed to reduce the number of administrations required and the risk of side effects associated with frequent dosing **(Anderson *et al*., 1994).** On the other hand, the administration of β2-agonists at doses higher than the therapeutic dose shows anabolic effects on skeletal–muscle, as well as significant lipolysis **(Joassard et al., 2013).** For example, the administration of clenbuterol reduces the content of lipid droplets, decreases the size of fat cells **(Zhang *et al*., 2007)** and increases apoptosis of adipose tissue **(Page *et al*., 2004).**

Chapter 02
Materials and Methods

I. Animal model

To evaluate *in vivo* the anti-steatotic effects of beta-2 adrenergic receptor agonists, 50 middle-aged Wistar albino rats (males) weighing approximately 100 g, were imported from the Pasteur Institute (Algiers). Wistar albino rats are a popular animal model in many fields of research. Their high reproducibility, ease of handling, genetic uniformity, and adaptability to laboratory conditions make them an ideal model for analyzing complex biological functions and generating new models of human disease **(Krubaa and Yogitha, 2024).**

Figure 10: Wistar albino rats **(original photo).**

II.Experimental protocol

II.1. Housing conditions

The experiment was carried out at the animal house of faculty of nature and life sciences, university of Bejaia, for 14 weeks; 12 weeks of disease induction of non-alcoholic fatty liver disease is done through a high-fat diet and 2 weeks of treatment.

The rats were kept in a 12/12h cycle (light/dark), an ambient temperature, with free access to food and water. They were identified using a color system at the level of their tail by indelible markers and they are weighed every 2 days using an electronic scale.

II.2. Experimental groups

After the adaptation period, the rats were divided into six groups to receive different diets and treatments; this is explained in the following table;

Table 02: experimental groups

Groups	**Diet and treatment**
Control (CTL) group (negative control)	Standard diet and water for 14 weeks
HFD group (positive control)	High-fat diet for 14 weeks
HFD-Formoterol low dose group	high-fat diet for 14 weeks + treatment with Formoterol for the last two weeks: intraperitoneal injection
HFD-Formoterol high dose group	high-fat diet for 14 weeks + treatment with Formoterol for the last two weeks: intraperitoneal injection
HFD-Salbutamol low dose group	high-fat diet for 14 weeks + treatment with Salbutamol for the last two weeks: intraperitoneal injection
HFD-Salbutamol high dose group	high-fat diet for 14 weeks + treatment with Salbutamol for the last two weeks: intraperitoneal injection

II.3. Sample preparation

The animals were anesthetized by intraperitoneal injection of a mixture of Ketamine 50 mg/kg and Xylazine 10 mg/kg, in order to induce stable anesthesia, and then they were sacrificed. Blood samples were collected from the jugular vein of rats using a micropipette and then deposited into commercial HEPARIN blood collection tubes. The samples were centrifuged at 2000 g for 12 min and the obtained serum was stored at -20°C until use, particularly in biochemical analyses.

After opening the abdominal cavity, the organs of the rats were removed, rinsed with physiological water to remove residual blood on their surfaces and then the organs were weighed. Then parts of the livers, muscles and testes were placed at 4 °C in formalin (10% formaldehyde), which is the standard histological fixative to preserve the cellular structure for later histological studies. For other tissues, notably adipose tissues, the rest of the livers, hearts and muscles were preserved by freezing at -80°C directly after dissection to keep broader analysis options for the rest of the project such as molecular biology analyses, tissue biochemistry and histology by cryosection.

III. Biochemical analyses

The dosage of biochemical parameters was carried according to the protocols mentioned in the technical sheets of the kits used. This is explained in the following table.

Table 03: Biochemical analysis parameters and their principles

Parameters	**Principle**
AST (GOT) BIOLABO REF :80025	Method developed by Karmen and al, and optimized by Henry and al. Aspartate aminotransferase, also known as glutamate-oxaloacetate transaminase (GOT), catalyzes the following reaction : L-Aspartate + 2-Oxoglutarate $\overset{AST}{\longleftrightarrow}$ Oxaloacetate + L-Glutamate Oxaloacetate + NADH+H^+ $\overset{MDH}{\longleftrightarrow}$ L-Malate +NAD^+ The decrease in absorbance proportional to AST activity in the specimen is measured at 340 nm.
ALT (TGP) BIOLABO REF : 80027B	Method developed by Wrobleski and Due, and optimized by Henry and al. Alanine aminotransferase, also known as Glutamate-pyruvate transferase (TGP), catalyzes the following reaction: L-Alanine + 2-Oxoglutarate $\overset{AST}{\longleftrightarrow}$ Pyruvate + L-Glutamate Pyruvate + NADH+H^+ $\overset{LDH}{\longleftrightarrow}$ L-Lactate +NAD^+ The decrease in absorbance proportional to ALT activity in the specimen, is measured at 340 nm
GammaGT (GGT) BIOLABO REF : 81310	Szasz ,Rosalki and Tarlow method Gamma Glutamyltransferase , catalyzes the following reaction : L-G-Glutamyl-3-carboxy-4-nitroanilide $\overset{GGT}{\longleftrightarrow}$ L-G-Glutamyl-glycylglycine +Glycyglycine +p-nitroaniline The rate of formation of p-nitroaniline, directly proportional to GGT activity in the specimen, is measured at 405 nm.
Albumin BIOLABO	In buffered solution at PH 4.2, bromocresol green binds albumin to form a colored compound which absorbance, measured at 630 nm (620-640) is proportional to the albumin concentration in the specimen.

REF : 80002	
Cholesterol BIOLABO REF: LP80106	Enzymatic method described by Allain and al., which reaction scheme is as follows : Cholesterol esters $\xrightarrow{CE}$ Cholesterol + free fatty acids Cholesterol + O_2 $\xrightarrow{CO}$ Cholesten 4 one 3 + H_2O_2 2 H_2O_2+ Phenol +PAP $\xrightarrow{POD}$ Quinoneimine (pink) + 4 H_2O_2 The rate of appearance of Quinoneimine is measured at 500 nm (480-520) whose absorbance intensity is proportional to the cholesterol concentration.
Triglycerides CYPRESS DIAGNOSTICS REF: HBL060	Enzymatic method which reaction scheme is as follows : Triglyceride + 3 H_2O $\xrightarrow{LPL}$ Glycerol + 3 fatty acids Glycerol + ATP $\xrightarrow{GK}$ Glycerol 3-Phosphate + ADP Glycerol 3-phosphate + O_2 $\xrightarrow{GPO}$ DHAP + H_2O_2 H_2O_2 + 4-AAP + DHBS $\xrightarrow{POD}$ Quinoneimine + 2 H_2O The intensity of the color measured at 510 nm corresponds to the Quinoneimine compound and is proportional to the triglyceride concentration in the sample.
Glucose CYPRESS DIAGNOSTICS REF: HBL04	Enzymatic method which reaction scheme is as follows : Glucose +O_2 +H_2O $\xrightarrow{GOD}$ gluconic acid + H_2O_2 H_2O_2 + 4-CP + 4-PAP $\xrightarrow{POD}$ Quinoneimine + HCL +2 H_2O The intensity of the color measured at 510nm is proportional to the glucose concentration in the sample.
Lipase CYPRESS DIAGNOSTICS	The sequence of reactions involved in the enzymatic direct lipase determination is the following :

REF: HBE09	1-2-O-dilauryl-rac-glycero-3-glutaric-(6'-methylresorufin)-ester $\xrightarrow{lipase}$ 1-2-O-dilauryl-rac-glycerol + glutaric-6'-methylresorufin-ester (not stable) $\xrightarrow{OH-}$ Glutaric acid + Methylresorufin The rate of Methylresorufin formation measured at 580nm is proportional to the catalytic concentration of lipase.
ALP BIOLABO REF :92214	Optimized method based on German Society of Clinical Chemistry, 1972 and Scandinavian Society of Clinical Chemistry recommendations. In alkaline solution, Alkaline phosphatase catalyzes the hydrolysis of p-nitrophenyl phosphate in p-nitrophenol and phosphate. The rate of formation of p-nitrophenol, proportional to the ALP activity, is measured at 405nm.

IV. Liver histology

The Liver histology was carried out at the histology laboratory in the faculty of medicine of the A-MIRA University, Bejaia according to the protocols optimized by the laboratory.

IV.1. Macroscopic observation

The samples were examined with naked eye to detect any abnormalities. Then, coarse sections (Figure 11) were taken and placed in histology cassettes, and immersed in 10% formalin for better fixation for 24 hours.

Figure 11: Macroscopic observation **(original photo).**

IV.2. Dehydration

It is based on the phenomenon of osmosis. The process is carried out by a dehydration machine, also called a circulator (Figure 12), and involves three main steps:

- Dehydration: the samples are passed through eight ethanol baths of increasing concentrations.

- Clarification or brightening with xylene (transition medium), to completely eliminate the ethanol and ensure absorption of the paraffin by the tissues.

- Paraffin impregnation and preparation for embedding: the samples remain in the paraffin for three hours.

IV.3. Paraffin embedding

Samples are infiltrated into hot paraffin (melting at 70°C) in an embedding station, allowing them to be embedded in paraffin blocks.
The entire sample is then placed on a cold plate to allow for rapid cooling of the blocks. (Figure12).

Figure 12: Paraffin blocks preparation **(original photo).**

IV.4. Microtomy

After mounting the blocks on the Manual Rotary Microtome (Leica RM 2125 RTS), two main steps are performed:

- Trimming (20 µm) to remove the first layers of paraffin to reach the most preserved tissue area.
- Thin ribbon sections (2 µm) are cut. The goal is to have a single layer of cells, which allows good light transmission during observation under an optical microscope (Figure 13).

Figure 13: Microtomy process **(original photo).**

IV.5. Preparation of the slides

The main steps of slide preparation of slide preparation are presented in the following table

Table 04: Steps of slide preparation

Steps	Details
Spreading	- Stretching the sections in a hot water bath to remove any wrinkles or deformations; - Collecting the sections on adhesive slides.
Deparaffinization	- Incubate in an incubator at 90°C for at least 2 hours; - Immerse the slides in xylene for 30 minutes; - Hydrate for 10 minutes in ethanol + 10 minutes in tap water.
Staining	- Use of Haris hematoxylin ; 3 to 5 min for nuclear staining; -Use of Eosin; 1-3 min for cytoplasmic staining. After a thorough rinse, the slides are successively transferred into: -Ethanol: removal of impurities; -Ethanol/xylene: transition medium; -Xylene: ensures better diffusion of the biological glue.
Montage	-Mounting the cover glass using biological glue (eukkit) -The slides are left to air dry.

IV.6. Microscopic Observation

The prepared slides were observed under a light microscope using the 20x objective, for detailed visualization of tissue structures and cellular organization (Figure 14).

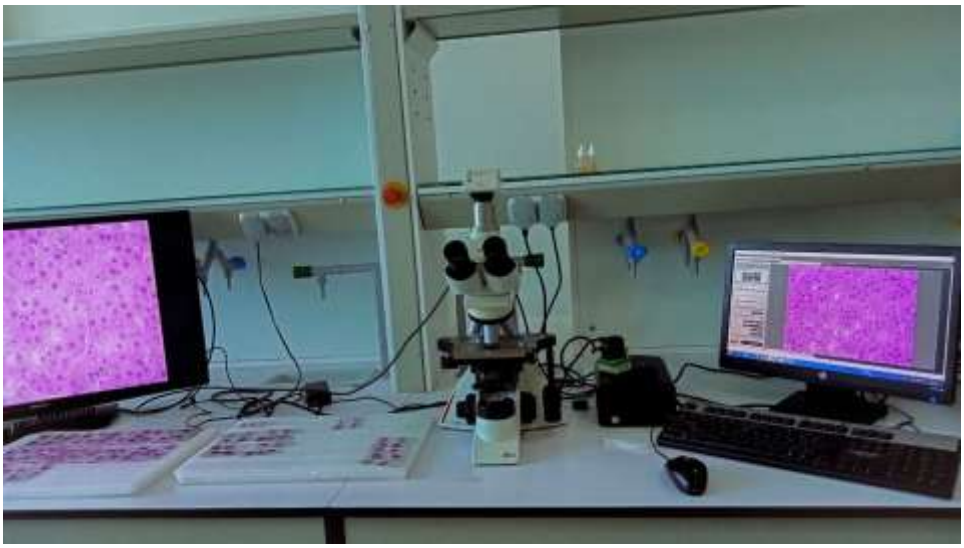

Figure 14: Microscopic observation **(original photo)**.

V. Statistics

Various statistical tests including unpaired t-tests as well as one-way analysis of variance followed by Tukey test were used to determine whether specific group mean differences were significant. Each test performed is specified in the figure legends. The minimum a-level of significance was set at 0.05. Data are presented as means ± SEM throughout.

Chapter 03

Results

The aim of our work is to evaluate the action of b2-adrenoceptor agonists in hepatic steatosis. We have therefore carried out a series of tests as described above to evaluate and demonstrate the therapeutic effect of these molecules. The results obtained are presented in this section.

I.β2-agonist administration does not alter weight of different organs

After regular monitoring of changes in the rats weight, we found that neither the diet nor the administration of formoterol and salbutamol significantly altered the animal's growth. The evaluation of the effect of the administration of formoterol or salbutamol on different organs such as the liver, spleen, kidneys, lungs and heart revealed that there was no significant difference between the administration of β2-agonists and the negative control with regard to tissue weight in the different groups. The statistical analysis clearly shows that the differences between the diet group, the diet with treatment groups and the control groups are negligible. Consequently, these results highlight the lack of impact of the diet and treatments on tissue weight in the groups studied. The results are presented in the following table (Table 05).

Table 05: Comparative organ weights between experimental groups

	Liver	**Heart**	**Kidney**	**Rate**	**Lungs**
CTRL	11,48 ± 1,18	1,35 ± 0,13	1,49 ± 0,04	1,04 ± 0,11	2,17 ± 0,46
HFD	11,32 ± 1,20	1,37 ± 0,18	1,39 ± 0,06	0,918 ± 0,10	1,86 ± 0,47
FOR-LD	11,78 ± 1,30	1,39 ± 0,10	1,49 ± 0,07	0,95 ± 0,12	2,33 ± 0,36
FOR-HD	12,04 ± 1,87	1,50 ± 0,19	1,54 ± 0,05	1,06 ± 0,40	2,08 ± 0,20
SAL-LD	12,31 ± 2,77	1,44 ± 0,20	1,49 ± 0,06	0,96 ± 0,18	2,33 ± 0,17
SAL-HD	12,20 ± 0,79	1,38 ± 0,18	1,43 ± 0,06	1,02 ± 0,21	2,64 ± 1,27

II.β2-agonist administration leads to decrease tissue adipose in animals model HFD

We also aimed to assess the effects of administering formoterol and salbutamol on different tissues (Figure 15). Adipose tissue weight was significantly (p <0, 05) increased in HFD group compared the control. Noted that the adipose tissue was drastically (p <0, 01) decreased with both low and high dose of formoterol. However, we don't found difference of adipose tissue and other groups treated with salbutamol.

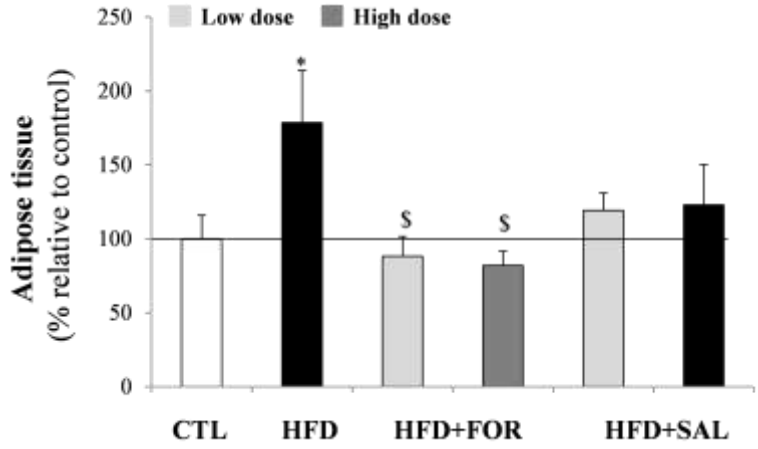

Figure 15: Histogram representing the percentage of adipose tissue weight in different groups (CTL: Control, HFD: High Fat Diet, FOR: High Fat Diet Formoterol, SAL: High Fat Diet Salbutamol) compared to the control group (CTL: Control). Values are means ± SE (n=6-8). *P<0.05; **P<0.01 relative to control group, $P<0.05; $$P<0.01 relative to HFD group: One-way ANOVA and Tukey as post hoc test.

III.β2-agonist administration leads to decrease liver enzymes in animals HFD

Changes in liver enzyme levels are one of the common biological manifestations in non-alcoholic fatty liver disease, including alanine aminotransferase (ALAT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) **(Anty, 2023)**. As shown in figure 16, AST levels increased in the HFD group by 81% compared to the control group. However, formoterol or salbutamol treatment decrease levels of AST activity, with varying proportions similar to those in the CTL group (Figure16). Surprisingly, ASAT level was highly reduced (43%) in the group treated with high dose of salbutamol compared to the control group (Figure16). In addition, we observed that ALT levels was (p <0,05) significantly increased in the HFD group by 18% compared to the CTL group. On the other hand, all treatments

decreased ALT levels, with greater efficacy in the case of salbutamol high-dose by 48% compared to the HFD group (Figure16). For alkaline phosphatase levels is significantly ($p < 0,05$) increased by about 77% in the HFD group compared to the CTL group. We have also observed an increase ALP in response to salbutamol or formoterol treatments compared to CTL (Figure 16).

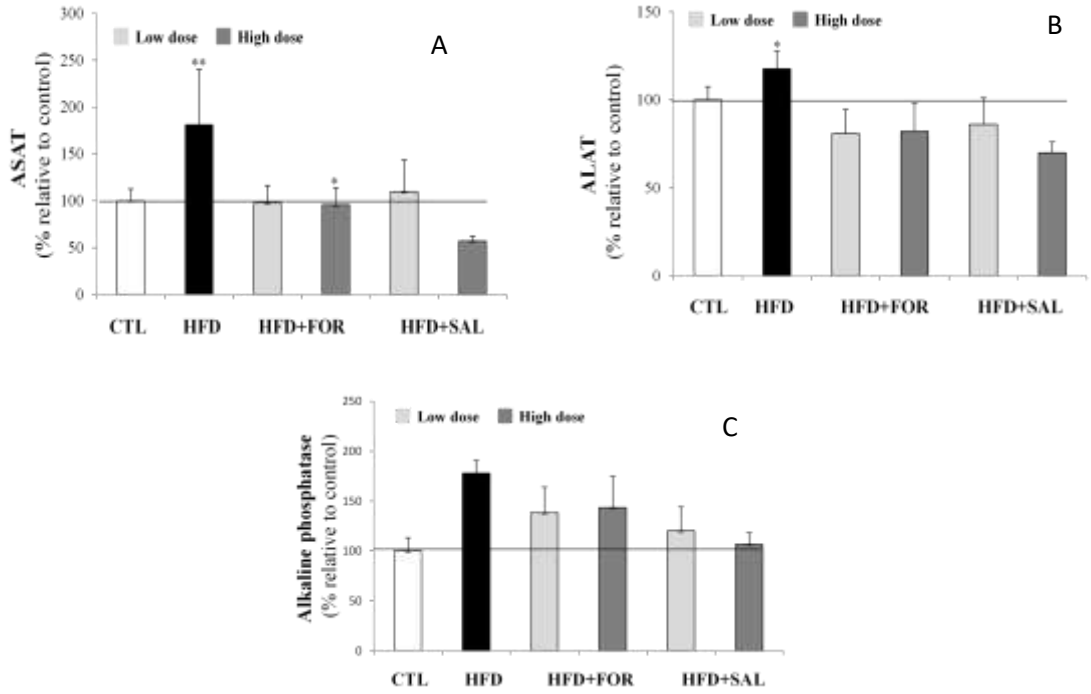

Figure16: Plasma levels of different liver enzymes (A: ALAT, B: ASAT, C: ALP) in different groups (CTL: Control, HFD: High Fat Diet, FOR: High Fat Diet Formoterol, SAL: High Fat Diet Salbutamol) compared to the control group (CTL: Control). Values are means ± SE (n=6-8). *P<0.05; **P<0.01 relative to control group: One-way ANOVA and Tukey as post hoc test.

IV.β2-agonist administration leads to decrease Triglyceride level

Hepatic steatosis is characterized by an accumulation of triglycerides in hepatocytes (**Ipsen *et al.*, 2018).** We examined whether β2 –adrenoceptors stimulation by salbutamol or formoterol affected the triglyceride level. As expected, triglyceride levels was highly ($p < 0,05$) increased in the HFD group compared to the CTL group. However, we observed a decrease of triglycerides levels in response of low dose of β2 –adrenoceptors stimulation by salbutamol or formoterol (Figure 17). This finding leads us to conclude that salbutamol or formoterol treatment enhances lipid metabolism.

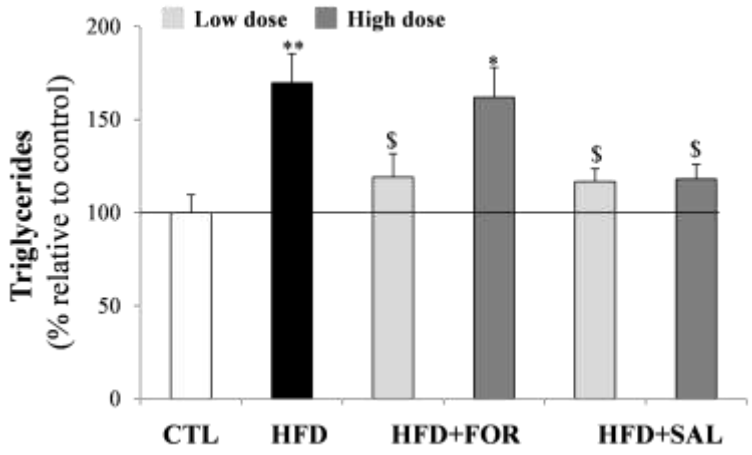

Figure 17: Histogram representation % of triglyceride levels in different groups (CTL: Control, HFD: High Fat Diet, FOR: High Fat Diet Formoterol, SAL: High Fat Diet Salbutamol) compared to the control group (CTL: Control). Values are means ± SE (n=6-8). *P<0.05; **P<0.01 relative to control group, $P<0.05; $$P<0.01 relative to HFD group: One-way ANOVA and Tukey as post hoc test.

V.β2-agonist administration does not alter cholesterol level

Dyslipidemia is frequently associated with non-alcoholic fatty liver disease, including decreased HDL cholesterol **(Zhang and Lu, 2015).** Administration of β2 agonists with formoterol or salbutamol lead to increase (p <0,05) total cholesterol levels in treated groups compared to the control group or HFD group. The graph below shows unchanged total cholesterol levels between the CTL and HFD group (Figure 18).

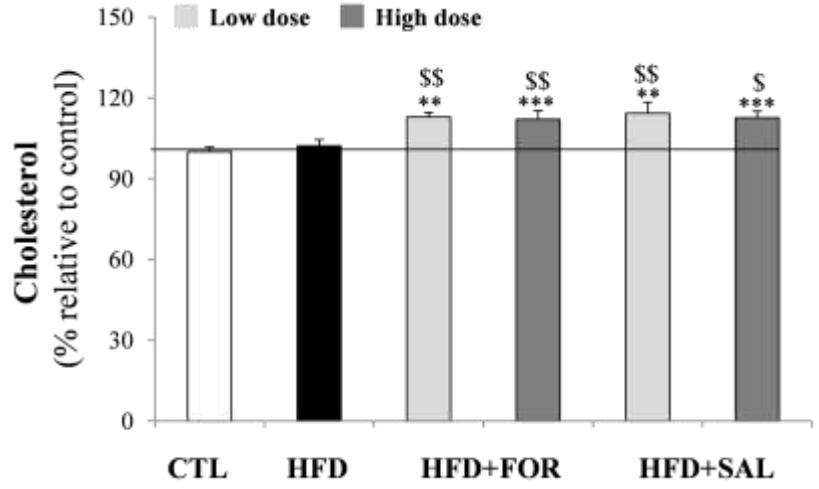

Figure18: Histogram representation % of cholesterol levels in different groups (CTL: Control, HFD: High Fat Diet, FOR: High Fat Diet Formoterol, SAL: High Fat Diet Salbutamol) compared to the

control group (CTL: Control). Values are means ± SE (n=6-8).*P<0.05; **P<0.01 relative to control group, $P<0.05; $$P<0.01 relative to HFD group: One-way ANOVA and Tukey as post hoc test.

VI.β2-agonist administration leads to decrease glucose level

Steatosis is characterized by the fat accumulation in the liver, which increases insulin resistance, thus causing persistent hyperglycemia **(Stols *et al.*, 2019).** Surprisingly, ours data demonstrates that glucose levels increased significantly (p <0,05) in the HFD group by 57% compared to the CTL group. In contrast, Formoterol or Salbutamol treatment induced a decrease (p <0,05) of glucose levels by 28%, 20%, 30% in formoterol LD, formoterol HD and salbutamol LC respectively compared to control group (Figure19).

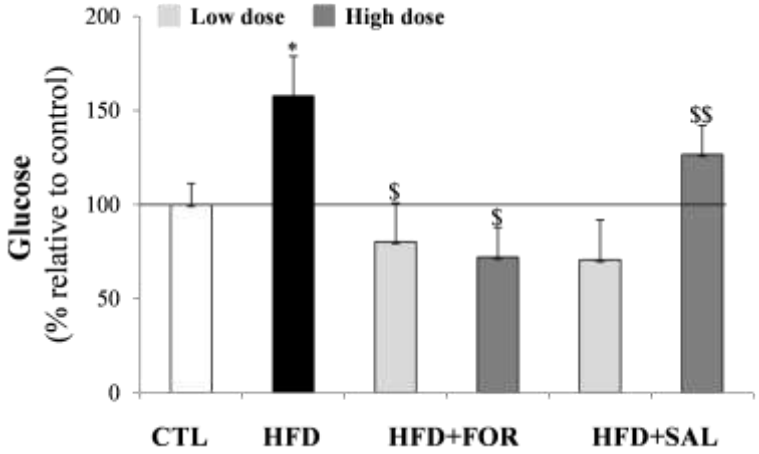

Figure 19: Histogram representation % of glucose levels in different groups (CTL: Control, HFD: High Fat Diet, FOR: High Fat Diet Formoterol, SAL: High Fat Diet Salbutamol) compared to the control group (CTL: Control). Values are means ± SE (n=6-8). *P<0.05; **P<0.01 relative to control group, $P<0.05; $$P<0.01 relative to HFD group: One-way ANOVA and Tukey as post hoc test.

VII.β2-agonist administration leads to decrease lipase

Lipases (EC 3.1.1.3, triacylglycerol hydrolases) are mainly active against water-insoluble molecules, such as triglycerides composed of long-chain fatty acids **(Lopes *et al.*, 2011)**, therefore they play an important role in the pathophysiology of hepatic steatosis characterized by the accumulation of triglycerides in the liver **(Younossi *et al.*, 2019)**. Confirmed by the obtained graph, lipase levels increase significantly (p <0,05) increased only in treated groups compared untreated or control groups (Figure 20). However, we don't observe any difference of lipase activity between control and untreated group.

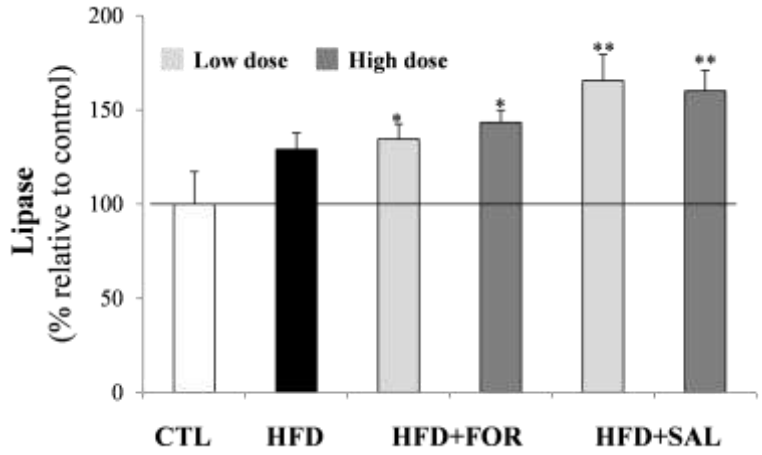

Figure 20: Histogram representation % of lipase levels in different groups (CTL: Control, HFD: High Fat Diet, FOR: High Fat Diet Formoterol, SAL: High Fat Diet Salbutamol) compared to the control group (CTL: Control). Values are means ± SE (n=6-8). *P<0.05; **P<0.01 relative to control group: One-way ANOVA and Tukey as post hoc test.

VIII.β2-agonist administration does not alter albumin level

The liver participates in the synthesis of several molecules, notably albumin **(Baudin, 2017), w**hich is the most abundant circulating protein in the plasma of healthy individuals (3.5-5 g/dL). It plays a crucial role in maintaining oncotic pressure, which regulates fluid distribution in the body, and acts as a transporter of variety of hydrophobic molecules **(Caraceni *et al*., 2013).** This is why the albumin level is a key parameter in the diagnosis of liver diseases **(Baudin, 2017).** As shown in (figure 21), our results demonstrate that, there is no significant difference in albumin level in different groups. So β2-agonist administration does not alter albumin level.

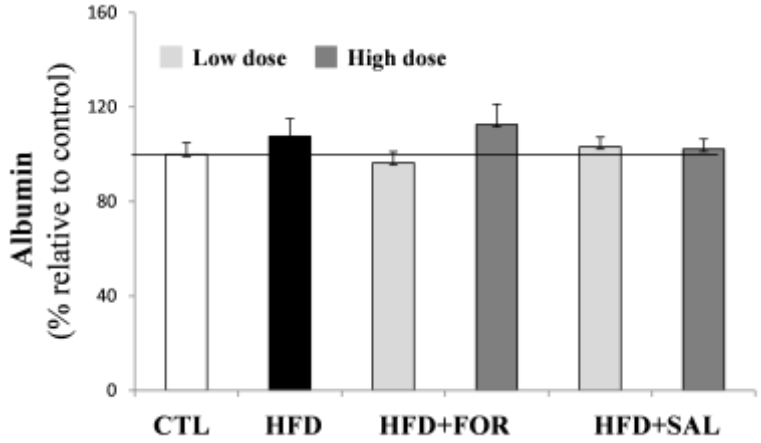

Figure 21: Histogram representation % of albumin levels in the different groups (CTL: Control, HFD: High Fat Diet, HFD+FOR: High Fat Diet Formoterol, HFD+SAL: High Fat Diet Salbutamol) relative to the control group (CTL: Control). Values are means ± SE (n=6-8) relative to CTL group: One-way ANOVA and Bonferroni as post hoc test.

IX.β2-agonist administration leads to decrease percentage of steatosis in animals HFD

Non-alcoholic fatty liver disease (NAFLD) is defined as an excess of fat in the liver (≥ 5% of hepatocytes laden with lipid droplets upon histological analysis. After a histological study, the lipid droplets in the liver tissue were quantified and the percentage of hepatic steatosis was estimated.

After 12 weeks of high fat diet, we determined the accumulation of fat in the liver in different groups. As illustrated in (Figure 22), the steatosis is highly significant increase in HFD group by about 5.89% compared to negative control. The steatosis in HFD- treated with formoterol or salbutamol at low concentration is respectively significant 1.28 % and 1.75 % compared to untreated group (HFD). Moreover, there is no difference in percentage of steatosis between HFD-treated with formoterol (3.03%) or salbutamol (4.11%) at high concentration compared to untreated group (HFD).

Finally, the effect of formoterol or salbutamol administration at low concentration is highly marked than formoterol or salbutamol at high concentration treatment compared to HFD group.

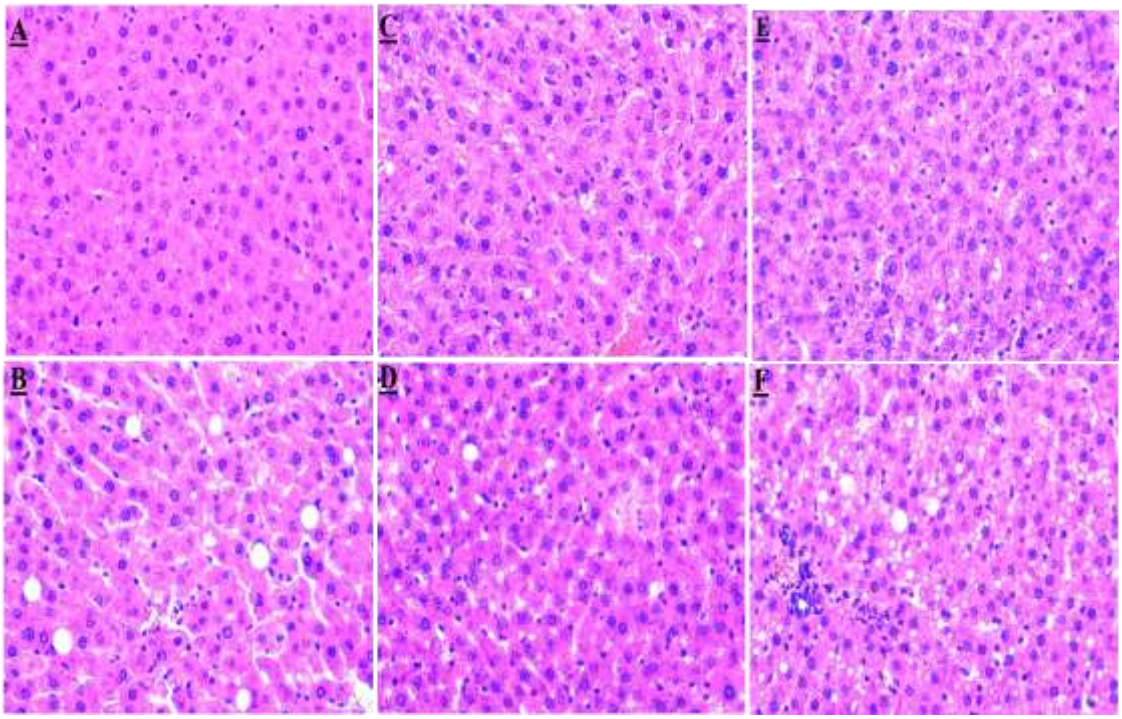

Figure 22: Hemalun-eosin-safran staining of liver cross-sectional area. A : Control group, healthy hepatocytes (CTL), B : High Fat Diet (HFD) group, steatosis characterized by lipid droplets (indicated by arrows), C : High Fat Diet Formoterol-treated group (FOR), uniform appearance of hepatocytes without obvious signs of swelling or degeneration, D : High Fat Diet Formoterol-treated group (FOR-

HD), uniform appearance of hepatocytes with steatosis characterized by lipid droplets, E :High Fat Diet Salbutamol-treated group (SAL-LD), hepatocytes appear normal, with no obvious signs of cellular stress or degeneration. There are few lipid droplets. F: High Fat Diet Salbutamol-treated group (SAL-HD), steatosis characterized by lipid droplets.

Chapter 04

Discussion

Discussion

The results obtained show that the induction of hepatic steatosis is successful, which was confirmed by the tissue damage caused and the increased liver injury markers (ASAT, ALAT, …), the success of the model also appears in the disruption of metabolism, in particular the lipid profile (TG, cholesterol, lipase) and blood sugar. However, the administration of treatments, either formoterol or salbutamol, has allowed overall improvement of metabolism and repair of liver tissue damage, which has been confirmed by histology.

Liver damage, including steatosis, is characterized by hepatic cytolysis resulting from the rupture of the plasma membrane of hepatocytes with the release of their cytosolic contents into the extracellular environment and then into the blood plasma. The two transaminases, ASAT or GOT, and ALAT or GPT, are the two most frequently requested biochemical tests, demonstrating their great utility in exploring liver damage **(Baudin *et al*., 2017).** They are both cytosolic, but ASAT is also mitochondrial. Their activities increase in hepatic, cardiac, muscular and renal cytolysis; their return to normal indicates healing; a further increase, a relapse, and a high level of maintenance indicate the transition to chronic hepatitis **(Bismuth *et al*., 2007).** The administration of a high-fat diet (HFD) in 24 Swiss albino mice for 18 weeks led to an increase in liver enzymes, particularly ASAT and ALAT. This elevation reflects early liver changes associated with fat accumulation and moderate hepatic stress **(Vu *et al*., 2025).** The alkaline phosphatase level, which must be assessed when exploring any liver disease, is very rarely elevated during NAFLD **(Anty, 2023).** The results obtained in our study confirm previous studies; there was an increase in liver enzyme levels in the group HFD indicating liver stress and damage. And after fourteen days of stimulation of β2-adrenergic receptors by salbutamol or formoterol, the levels of liver enzymes compared to those of the control group are normalized. Moreover, we noted that the alkaline phosphatase levels were increased in HFD group as well as in other groups after 14 days of salbutamol or formoterol administration. Noted that, the impact on liver enzymes have not been documented in response to β2 -agonist administration. Therefore, our results suggest a potential effect of these beta-2 adrenergic agonists on the regulation of liver enzymes.

In recent decades, high-fat diets (HFDs) have been widely used in rodent research to study the mechanism of insulin resistance and metabolic syndrome **(Wali *et al*., 2020).** Several studies have reported that a high-fat diet induces profound metabolic alterations, including increased adipose tissue mass, hypertriglyceridemia, hypercholesterolemia, (**Akagiri *et al* .,2008 ; Xie *et al* .,2008)**,insulin resistance, and elevated fasting glucose levels **(Buettner *et al*., 2007)** These disturbances contribute to the development of metabolic syndrome and NASH **(Picchi *et al.,* 2011).** Fructose added in the diet of overweight and obese humans who

consumed fructose-sweetened drinks for 10 weeks. Strongly stimulates de novo lipogenesis (DNL) in the liver and promotes visceral obesity **(Stanhope *et al.*, 2009)**, which involved hyperplasia and hypertrophy of adipocytes **(Reuter, 2007).** Communally, hepatic steatosis is mainly characterized by dyslipidemia including a decrease in HDL cholesterol and an increase in triglycerides **(Zhang and Lu, 2015).** In agreement with our observations in response to β2 -agonist administration. In fact, triglyceride levels and adipose tissue mass significantly decreased.

Zhang *et al*, have demonstrated that β2-agonists administration shows significant lipolysis-stimulating effects such as clenbuterol administration which reduces lipid droplet content and decreases fat cell size **(Zhang *et al*., 2007).** From a molecular point of view, in another study isoproterenol, β1–agonist induces lipolysis by allowing the phosphorylation and activation of hormone-sensitive lipase dependent on protein kinase A, primarily at residues Ser-659 and Ser-660 **(Anthonsen *et al*., 1998 ;Clifford *et al*., 2000).** In addition, isoproterenol phosphorylates perilipin A, which dissociates from the protein CGI-58, a coactivator of adipose triglyceride lipase and thus activates triglyceride hydrolysis **(Zechner *et al*., 2009).** Concomitant with the activation of adipose triglyceride lipase, the hormone-sensitive lipase migrates from the cytosol into the lipid droplets and hydrolyzes the diglyceride, produced by adipose triglyceride lipase **(Zechner *et al*., 2009) .**In our study, lipase activity was remarkably high in the groups treated with Salbutamol and Formoterol, as in the HFD group.

It is well-established that high-fat diets (HFD) contribute to the development of hyperglycemia through mechanisms involving insulin resistance and altered glucose uptake. In the study elaborated by **Picchi *et al*. (2011)**, animals subjected to a high-fat diet exhibited significantly elevated fasting blood glucose levels, confirming the hyperglycemic effect of the diet. Increased hepatic triglycerides impair the ability of insulin to inhibit glucose production in humans **(Buettner *et al.*, 2007).** Insulin resistance is characterized by impaired insulin action in target tissues and includes reduced glucose uptake in adipose and muscle tissues, decreased suppression of endogenous glucose production in the liver, decreased suppression of lipolysis in adipose tissue, and reduced insulin-induced glycogen synthesis **(Petersen and Shulman, 2018; Roden and Shulman, 2019).** In rodents, a high-fat diet increases diacylglycerol (DAG) levels in liver and muscle. DAG accumulation in tissues activates novel isoforms of the calcium-independent protein kinase C (PKC) family (**Roden and Shulman, 2019).** DAG-mediated PKC-activation has been shown to inhibit hepatic insulin signaling by phosphorylation of Thr1160 in the IR activation loop, thereby blocking its kinase activity **(Petersen *et al*., 2016)**. In line with our study, the HFD diet increased blood glucose levels,

suggesting insulin resistance and induced glucose intolerance. In this study, blood sugar levels were highly decreased in response of salbutamol or formoterol treatments. This unexpected improvement suggests a potential hypoglycemic effect of β2-adrenergic agonists, which could go beyond simple normalization. The improvement in the glycemic profile is linked to the down regulation of PGC-1α expression (a key regulator of gluconeogenesis) in the liver and muscle after salbutamol treatment **(Saleh *et al*., 2018).** Although the literature regarding the effect of salbutamol and formoterol on blood glucose remains limited, our results are consistent with these observations and highlight the potential of β2-agonists to modulate glucose homeostasis in the context of diet-induced metabolic disorders. Indeed, although significant lipolysis was observed under treatment (with increased release of fatty acids), no significant increase in blood glucose levels were observed. This could indicate an improvement in insulin sensitivity. One hypothesis would be that the released fatty acids are mainly directed towards β-oxidation, thus limiting their deleterious effect on glycemic regulation. However, this interpretation remains to be confirmed. Additional studies, particularly at the molecular level, would be necessary to better understand the interactions between lipolysis, β-oxidation and insulin sensitivity in this context.

It is generally known that during non-alcoholic liver injury, TG accumulation in hepatocytes is the first step leading to inflammation and lipotoxicity **(Younossi *et al*., 2019)**. The histological analysis in our study focused on the observation and quantification of lipid droplets developed in hepatocytes in all experimental groups. As expected, liver sections from the control group showed almost no lipid droplets, reflecting normal liver architecture and the absence of steatosis. In contrast, the HFD group exhibited an accumulation of lipid droplets, confirming the successful induction of hepatic steatosis by a high-fat diet. Interestingly, treatment with low doses of β_2-adrenergic agonists (salbutamol or formoterol) resulted in a notable reduction in lipid droplet accumulation compared to the untreated HFD group. This suggests a partial protective effect against hepatic fat accumulation. However, treatment with high doses of the same β-agonists showed a paradoxical effect, with lipid accumulation levels almost comparable to the untreated HFD group. These results are confirmed with a previous study which demonstrates that acute activation of β2-adrenoceptors in vivo increases hepatic lipid accumulation. They also showed that overexpression of β2-adrenoceptors in rodent hepatocytes increases lipid accumulation **(Ghosh *et al.,* 2012).** A decrease in the transcription of β2-adrenoceptors is observed and would be linked to a decrease in the phosphorylation of CREB, a key factor in the regulation of gene expression. Prolonged stimulation of a

membrane receptor by its ligand often induces a state of desensitization that interrupts signal transmission **(Devillier *et al*., 1996).**

Our results suggest a potentially beneficial and protective effect of β2-agonists on hepatic steatosis, with enhance of liver lesions and improvement of metabolism. However, the therapeutic effect is dose-dependent and requires further study to standardize it.

Conclusion And Perspectives

Lifestyle changes over the past decades have largely contributed to the emergence and progression of metabolic diseases such as non-alcoholic fatty liver disease, so understanding their triggering mechanisms is essential for implementing prevention strategies. Our work devoted to the study of the involvement of adrenergic pathways in NAFLD is part of this dynamic.

In our case the results found indicate that the new treatment strategy explored in this study by β2-agonists (Salbutamol or Formoterol) against hepatic steatosis induced by a high-fat diet is a promising therapeutic approach that has allowed to reduce the percentage of hepatic steatosis but also to improve hepatic and lipid profiles. Our results suggest a dose-dependent effect. We observed after liver histology a better reduction of steatosis in the case of low doses of both treatments. On the other hand, in the biochemical parameters we did not observe a significant difference between the doses administered. The mechanisms behind this effect, although not yet demonstrated, but the involvement of the Gs-cAMP pathway is strongly implicated. However, to fully validate this approach, molecular studies are necessary to fully understand the mechanisms involved, such as tissue studies of the lipolysis and β-oxidation cycles, as well as a dosage of β2-adrenergic receptors.

The validation of this strategy by additional studies remains the ultimate objective. This will allow the off-target effects of approved drugs to be valued, no longer considering them as adverse effects, but exploiting them as opportunities for the development of new drugs. Such a strategy could contribute to reducing the prevalence of hepatic steatosis, which is increasingly becoming a major public health problem and an economic burden for many countries.

Bibliographic References

Bibliographic References

1. Abdel-Misih SRZ, Bloomston M. Liver Anatomy. Surg Clin North Am. 2010; 90: 643-653.

2. Akagiri S, Naito Y, Ichikawa H, Mizushima K, Takagi T, Handa O, et al. A Mouse Model of Metabolic Syndrome; Increase in Visceral Adipose Tissue Precedes the Development of Fatty Liver and Insulin Resistance in High-Fat Diet-Fed Male KK/Ta Mice. J Clin Biochem Nutr. 2008; 42: 150-157.

3. Almazroo OA, Miah MK, Venkataramanan R. Drug Metabolism in the Liver. Clin Liver Dis. 2017; 21:1–20.

4. Anderson G, Linden A, Rabe K. Why are long-acting beta-adrenoceptor agonists long-acting? Eur Respir J. 1994; 7:569–578.

5. Anthonsen MW, Rönnstrand L, Wernstedt C, Degerman E, Holm C. Identification of novel phosphorylation sites in hormone-sensitive lipase that are phosphorylated in response to isoproterenol and govern activation properties in vitro. J Biol Chem. 1998; 273:215–221.

6. Anty R. Clinical signs and diagnosis of NASH and NAFLD. Correspond Metab Horm Diab Nutr. 2022; 26:93–7.

7. Arab JP, Arrese M, Trauner M. Recent Insights into the Pathogenesis of Nonalcoholic Fatty Liver Disease. Annu Rev Pathol. 2018; 13: 321-350.

8. Baffy G. MicroRNAs in Nonalcoholic Fatty Liver Disease. J Clin Med. 2015; 4:1977–1988.

9. Baudin B. Exploration biochimique du foie en 2017. Rev Fr Lab. 2017; 490:25–33.

10. Bedossa P, Houglum K, Trautwein C, Holstege A, Chojkier M. Stimulation of collagen alpha 1(I) gene expression is associated with lipid peroxidation in hepatocellular injury: a link to tissue fibrosis? Hepatology. 1994; 19:1262–1271.

11. Begriche K, Igoudjil A, Pessayre D, Fromenty B. Mitochondrial dysfunction in NASH: causes, consequences and possible means to prevent it. Mitochondrion. 2006; 6:1–28.

12. Benhamed F, Denechaud PD, Lemoine M, Robichon C, Moldes M, Bertrand-Michel J, et al. The lipogenic transcription factor ChREBP dissociates hepatic steatosis from insulin resistance in mice and humans. J Clin Invest. 2012; 122:2176–2194.

13. Bessaguet F, Desmoulière A. Le foie. Act Pharm. 2021; 60:57–61.

14. Imbert-Bismuth F, Myara A, Gerhard MF, Voitot H, Bonyhay L, Thibault V. Biological assessment in the diagnosis and monitoring of liver disease: decision support diagrams. Rev Fr Lab 2007; 394:51–62.

15. Buch S, Stickel F, Trépo E, Way M, Herrmann A, Nischalke HD, et al. A genome-wide association study confirms PNPLA3 and identifies TM6SF2 and MBOAT7 as risk loci for alcohol-related cirrhosis. Nat Genet. 2015; 47:1443–1448.

16. Buettner R, Schölmerich J, Bollheimer LC. High-fat diets: modeling the metabolic disorders of human obesity in rodents. Obesity (Silver Spring). 2007; 15:798–808.

17. Bugianesi E, Moscatiello S, Ciaravella MF, Marchesini G. Insulin resistance in nonalcoholic fatty liver disease. Curr Pharm Des. 2010; 16:1941–1951.

18. Buzzetti E, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). Metabolism. 2016; 65:1038–1048.

19. Caddeo A, Jamialahmadi O, Solinas G, Pujia A, Mancina RM, Pingitore P, et al. MBOAT7 is anchored to endomembranes by six transmembrane domains. J Struct Biol. 2019; 206: 349–360.

20. Caraceni P, Tufoni M, Bonavita ME. Clinical use of albumin. Blood Transfus. 2013; 11: 18–25.

21. Caussy C, Reeder SB, Sirlin CB, Loomba R. Noninvasive, Quantitative Assessment of Liver Fat by MRI-PDFF as an Endpoint in NASH Trials. Hepatology. 2018; 68:763–772.

22. Cazzola M, Spina D, Matera MG. The use of bronchodilators in stable chronic obstructive pulmonary disease. Pulm Pharmacol Ther. 1997; 10:129–144.

23. Clifford GM, Londos C, Kraemer FB, Vernon RG, Yeaman SJ. Translocation of Hormone-sensitive Lipase and Perilipin upon Lipolytic Stimulation of Rat Adipocytes. J Biol Chem. 2000; 275: 5011-5015.

24. Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology 1998; 114:842–845.

25. Devillier P. Aspects actuels des recepteurs 132-adr6nergiques et des 132-stimulants dans I'asthme. Rev fr Allergol. 1996; 36:269-281.

26. Diehl AM, Li ZP, Lin HZ, Yang SQ. Cytokines and the pathogenesis of non-alcoholic steatohepatitis. Gut. 2005; 54:303–306.

27. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. J Clin Invest. 2005; 115:1343–1351.

28. Drescher H, Weiskirchen S, Weiskirchen R. Current Status in Testing for Nonalcoholic Fatty Liver Disease (NAFLD) and Nonalcoholic Steatohepatitis (NASH). Cells. 2019; 8:845.

29. Fang Z, Dou G, Wang L. MicroRNAs in the Pathogenesis of Nonalcoholic Fatty Liver Disease. Int J Biol Sci. 2021; 17:1851–1863.

30. Ferguson SSG, Feldman RD. β-Adrenoceptors as Molecular Targets in the Treatment of Hypertension. Can J Cardiol. 2014; 30:S3–S8.

31. Fonseca VA. Insulin resistance, diabetes, hypertension, and renin-angiotensin system inhibition: reducing risk for cardiovascular disease. J Clin Hypertens (Greenwich). 2006; 8: 713-720.

32. Ghosh PM, Shu ZJ, Zhu B, Lu Z, Ikeno Y, Barnes JL, et al. Role of β-adrenergic receptors in regulation of hepatic fat accumulation during aging. J Endocrinol. 2012; 213:251–261.

33. Guhan A, Cooper S, Oborne J, Lewis S, Bennett J, Tattersfield A. Systemic effects of formoterol and salmeterol: a dose-response comparison in healthy subjects. Thorax. 2000; 55: 650-656.

34. Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat Rev Mol Cell Biol. 2008; 9:367–377.

35. Hedera P. Wilson's disease: A master of disguise. Parkinsonism Relat Disord. 2019; 59:140–145.

36. Ipsen DH, Lykkesfeldt J, Tveden-Nyborg P. Molecular mechanisms of hepatic lipid accumulation in non-alcoholic fatty liver disease. Cell Mol Life Sci. 2018; 75:3313–3327.

37. Joassard OR, Amirouche A, Gallot YS, Desgeorges MM, Castells J, Durieux AC, et al. Regulation of Akt-mTOR, ubiquitin-proteasome and autophagy-lysosome pathways in response to formoterol administration in rat skeletal muscle. Int J Biochem Cell Biol. 2013; 45:2444–2455.

38. Jonas W, Schürmann A. Genetic and epigenetic factors determining NAFLD risk. Mol Metab. 2021; 50: 101–111.

39. Ju C, Tacke F. Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. Cell Mol Immunol. 2016; 13:316–327.

40. Kim HK, Della-Fera MA, Hausman DB, Baile CA. Effect of clenbuterol on apoptosis, adipogenesis, and lipolysis in adipocytes. J Physiol Biochem. 2010; 66:197–203.

41. Kim YM, Benovic JL. Differential roles of arrestin-2 interaction with clathrin and adaptor protein 2 in G protein-coupled receptor trafficking. J Biol Chem. 2002; 277: 30760-30768.

42. Kirpich IA, Marsano LS, McClain CJ. Gut-liver axis, nutrition, and non-alcoholic fatty liver disease. Clin Biochem. 2015; 48:923–930.

43. Koo SH. Nonalcoholic fatty liver disease: molecular mechanisms for the hepatic steatosis. Clin Mol Hepatol. 2013; 19:210–215.

44. Krubaa P, Yogitha PS. Albino Wistar Rats: Advantages and Limitations in Biomedical Research. SBV J Basic Clin Appl Health Sci. 2024; 7:61–65.

45. Kumar A, Blackshear C, Subauste JS, Esfandiari NH, Oral EA, Subauste AR. Fatty Liver Disease, Women, and Aldosterone: Finding a Link in the Jackson Heart Study. J Endocr Soc. 2017; 1:460–469.

46. Lemoine M, Serfaty L. Stéatopathies métaboliques. Presse Med. 2012; 41:169–189.

47. Leslie T, Pawloski L, Kallman-Price J, Escheik C, Hossain N, Fang Y, et al. Survey of health status, nutrition and geography of food selection of chronic liver disease patients. Ann Hepatol. 2014; 13:533–540.

48. Li TT, Li TH, Peng J, He B, Liu LS, Wei DH, et al. TM6SF2: A novel target for plasma lipid regulation. Atherosclerosis. 2018; 268:170–176.

49. Liu S, Sommese RF, Nedoma NL, Stevens LM, Dutra JK, Zhang L, et al. Structural basis of lipid-droplet localization of 17-beta-hydroxysteroid dehydrogenase 13. Nat Commun. 2023; 14:51–58.

Lopes DB, Fraga LP, Fleuri LF, Macedo GA. Lipase and esterase: to what extent can this classification be applied accurately? Ciênc Tecnol Aliment. 2011;31:603–613.

50. Mantovani A, Turino T, Lando MG, Gjini K, Byrne CD, Zusi C, et al. Screening for non-alcoholic fatty liver disease using liver stiffness measurement and its association with chronic kidney disease and cardiovascular complications in patients with type 2 diabetes. Diabetes Metab. 2020; 46:296–303.

51. Mitsche MA, Hobbs HH, Cohen JC. Patatin-like phospholipase domain–containing protein 3 promotes transfer of essential fatty acids from triglycerides to phospholipids in hepatic lipid droplets. J Biol Chem. 2018; 293:695–868.

52. Moore RH, Millman EE, Godines V, Hanania NA, Tran TM, Peng H, et al. Salmeterol stimulation dissociates beta2-adrenergic receptor phosphorylation and internalization. Am J Respir Cell Mol Biol. 2007; 36:254–261.

53. Motiejunaite J, Amar L, Vidal-Petiot E. Adrenergic receptors and cardiovascular effects of catecholamines. Ann Endocrinol. 2021; 82:193–197.

54. Page KA, Hartzell DL, Li C, Westby AL, Della-Fera MA, Azain MJ, et al. beta-Adrenergic receptor agonists increase apoptosis of adipose tissue in mice. Domest Anim Endocrinol. 2004; 26:23–31.

55. Palmqvist M, Persson G, Lazer L, Rosenborg J, Larsson P, Lötvall J. Inhaled dry-powder formoterol and salmeterol in asthmatic patients: onset of action, duration of effect and potency. Eur Respir J. 1997; 10:2484–2489.

56. Parlati L, Régnier M, Postic C, Guillou H. Physiopathologie de la stéatose hépatique non-

alcoolique (NAFLD). Médecine Mal Métab. 2023; 17:222–227.

57. Pasterkamp G. Methods of accelerated atherosclerosis in diabetic patients. Heart. 2013;99:743-749

58. Perry RJ, Samuel VT, Petersen KF, Shulman GI. The role of hepatic lipids in hepatic insulin resistance and type 2 diabetes. Nature. 2014; 510:84–91.

59. Pessayre D, Fromenty B. NASH: a mitochondrial disease. J Hepatol. 2005; 42: 928-940.

60. Petersen MC, Madiraju AK, Gassaway BM, Marcel M, Nasiri AR, Butrico G, et al. Insulin receptor Thr1160 phosphorylation mediates lipid-induced hepatic insulin resistance. J Clin Invest. 2016; 126: 4361-4371.

61. Petersen MC, Shulman GI. Mechanisms of Insulin Action and Insulin Resistance. Physiol Rev. 2018; 98: 2133-2223.

62. Pfleger J, Gresham K, Koch WJ. G protein-coupled receptor kinases as therapeutic targets in the heart. Nat Rev Cardiol. 2019; 16: 612-622.

63. Picchi MG, Mattos AMD, Barbosa MR, Duarte CP, Gandini MDA, Portari GV, et al. A high-fat diet as a model of fatty liver disease in rats. Acta Cir Bras. 2011; 26:25–30.

64. Pingitore P, Romeo S. The role of PNPLA3 in health and disease. Biochim Biophys Acta Mol Cell Biol Lipids. 2019; 1864: 900-906.

65. Pirola CJ, Fernández Gianotti T, Castaño GO, Mallardi P, San Martino J, Mora Gonzalez Lopez Ledesma M, et al. Circulating microRNA signature in non-alcoholic fatty liver disease: from serum non-coding RNAs to liver histology and disease pathogenesis. Gut. 2015; 64: 800-812.

66. Price AH, Clissold SP. Salbutamol in the 1980s. A reappraisal of its clinical efficacy. Drugs. 1989; 38: 77-122.

67. Rasmussen SGF, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, et al. Crystal structure of the human β2 adrenergic G-protein-coupled receptor. Nature. 2007; 450: 383-387.

68. Rasmussen SGF, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, et al. Crystal structure of the β2 adrenergic receptor–Gs protein complex. Nature. 2011 Sep 29; 477: 549-555.

69. Reuter TY. Diet-induced models for obesity and type 2 diabetes. Drug Discovery Today: Dis Model Mech. 2007; 4:3–8.

70. Roden M, Shulman GI. The integrative biology of type 2 diabetes. Nature. 2019; 576:51–60.

71. Saleh N. The Effect of Salbutamol on Pgc-1 α and Glut4 Mrna Expression in the Liver

and Muscle of Elderly Diabetic Mice. Acta Endo (Buc). 2018; 14:184–191.

72. Sanders FWB, Griffin JL. De novo lipogenesis in the liver in health and disease: more than just a shunting yard for glucose. Biol Rev Camb Philos Soc. 2016; 91:452–468.

73. Sears MR, Lötvall J. Past, present and future— β 2 -adrenoceptor agonists in asthma management. Respir Med. 2005; 99:152–170.

74. Seibold A, Williams B, Huang ZF, Friedman J, Moore RH, Knoll BJ, et al. Localization of the sites mediating desensitization of the beta(2)-adrenergic receptor by the GRK pathway. Mol Pharmacol. 2000; 58: 1162-1173.

75. Semmler G, Datz C, Reiberger T, Trauner M. Diet and exercise in NAFLD/NASH: Beyond the obvious. Liver Int. 2021; 41: 2249-2268.

76. Shenoy SK, Lefkowitz RJ. Multifaceted roles of β-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling. Biochem J. 2003; 375:503–515.

77. Stanhope KL, Schwarz JM, Keim NL, Griffen SC, Bremer AA, Graham JL, et al. Consuming fructose-sweetened, not glucose-sweetened, beverages increases visceral adiposity and lipids and decreases insulin sensitivity in overweight/obese humans. J Clin Invest. 2009; 119: 1322-1334.

78. Stols-Gonçalves D, Hovingh GK, Nieuwdorp M, Holleboom AG. NAFLD and Atherosclerosis: Two Sides of the Same Dysmetabolic Coin? Trends Endocrinol Metab. 2019; 30:891–902.

79. Sumadewi KT. Embryology, anatomy and physiology of the liver: Review. IJCAP. 2023; 10:138–144.

80. Sung KC, Ryu S, Lee JY, Kim JY, Wild SH, Byrne CD. Effect of exercise on the development of new fatty liver and the resolution of existing fatty liver. J Hepatol. 2016; 65: 791-797.

81. Tagkou NM, Goossens N. Stéatose hépatique non alcoolique : diagnostic et traitement en 2022. Schweiz Gastroenterol. 2023; 4:27–37.

82. Targher G, Byrne CD, Lonardo A, Zoppini G, Barbui C. Non-alcoholic fatty liver disease and risk of incident cardiovascular disease: A meta-analysis. J Hepatol. 2016; 65:589–600.

83. Toblli JE, Cao G, Olivieri L, Angerosa M. Comparative study of gastrointestinal tract and liver toxicity of ferrous sulfate, iron amino chelate and iron polymaltose complex in normal rats. Pharmacology. 2008; 82:127–137.

84. Trefts E, Gannon M, Wasserman DH. The liver. Current Biology. 2017; 27:1147–1151.

85. Vu TD, Ngo AD, Nguyen ST, Khuong YN, Phan VB, Pham TD, et al. Effects of high fat diet on blood lipids and liver enzymes in murine model: the systemic and experimental

study. Obesity Medicine. 2025; 55:100614.

86. Wali JA, Jarzebska N, Raubenheimer D, Simpson SJ, Rodionov RN, O'Sullivan JF. Cardio-Metabolic Effects of High-Fat Diets and Their Underlying Mechanisms—a Narrative Review. Nutrients. 2020; 12:1505.

87. Whitfield JB. Gamma glutamyl transferase. Crit Rev Clin Lab Sci. 2001; 38:263–355.

88. Wieckowska A, Zein NN, Yerian LM, Lopez AR, McCullough AJ, Feldstein AE. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. Hepatology. 2006; 44:27–33.

89. Xie W, Zhang Y, Wang N, Zhou H, Du L, Ma X, et al. Novel effects of macrostemonoside A, a compound from Allium macrostemon Bung, on hyperglycemia, hyperlipidemia, and visceral obesity in high-fat diet-fed C57BL/6 mice. Eur J Pharmacol. 2008; 599:159–165.

90. Yang JD, Ahmed F, Mara KC, Addissie BD, Allen AM, Gores GJ, et al. Diabetes Is Associated With Increased Risk of Hepatocellular Carcinoma in Patients With Cirrhosis From Nonalcoholic Fatty Liver Disease. Hepatology. 2020; 71:907–916.

91. Younossi Z, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, et al. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. Nat Rev Gastroenterol Hepatol. 2018; 15:11–20.

92. Younossi Z, Tacke F, Arrese M, Chander Sharma B, Mostafa I, Bugianesi E, et al. Global Perspectives on Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis. Hepatology. 2019; 69: 2672-2682.

93. Zarrinpar A, Gupta S, Maurya MR, Subramaniam S, Loomba R. Serum microRNAs explain discordance of non-alcoholic fatty liver disease in monozygotic and dizygotic twins: a prospective study. Gut. 2016; 65: 1546-1554.

94. Zechner R, Kienesberger PC, Haemmerle G, Zimmermann R, Lass A. Adipose triglyceride lipase and the lipolytic catabolism of cellular fat stores. J Lipid Res. 2009; 50:3–21.

95. Zeybel M, Hardy T, Wong YK, Mathers JC, Fox CR, Gackowska A, et al. Multigenerational epigenetic adaptation of the hepatic wound-healing response. Nat Med. 2012; 18: 1369-1377.

96. Zhang J, He Q, Liu QY, Guo W, Deng XM, Zhang WW, et al. Differential gene expression profile in pig adipose tissue treated with/without clenbuterol. BMC Genomics. 2007; 8:433.

97. Zhang J, He Q, Liu QY, Guo W, Deng XM, Zhang WW, et al. Differential gene expression profile in pig adipose tissue treated with/without clenbuterol. BMC Genomics. 2007; 8:433.

98. Zhang QQ, Lu LG. Nonalcoholic Fatty Liver Disease: Dyslipidemia, Risk for Cardiovascular Complications, and Treatment Strategy. J Clin Transl Hepatol. 2015; 3:78–84.

99. Zhang XQ, Xu CF, Yu CH, Chen WX, Li YM. Role of endoplasmic reticulum stress in the pathogenesis of nonalcoholic fatty liver disease. World J Gastroenterol. 2014; 20: 1768-1776.

Abstract

In view of the increasing prevalence of non-alcoholic fatty liver disease (NAFLD), new therapeutic approaches are explored. This study aimed to investigate the potential therapeutic role of β2-adrenergic receptor agonists salbutamol and formoterol on hepatic steatosis induced by a high-fat diet (HFD) in Wistar male rats. The experimental design involved six groups: a control group, an HFD group, and four treated groups receiving either salbutamol or formoterol at low and high doses following disease induction. Biochemical parameters, including liver enzymes, lipid profile, and glucose levels, were measured, and histological examination of hepatic tissue was performed. The results revealed a significant increase in hepatic damage markers and lipid accumulation in the HFD group. However, treatment with low doses of both salbutamol and formoterol led to noticeable improvements in biochemical and histological profiles, exerting a protective effect against the progression of steatosis. Interestingly, high doses of both formoterol and salbutamol did not demonstrate the same level of efficacy, highlighting a potential dose-dependent therapeutic effect. These findings support the hypothesis that β2-agonists, particularly at lower concentrations, may offer a protective effect against diet-induced hepatic steatosis. Further studies are required to confirm these results and to define optimal dosing strategies for clinical application.

Key words: Liver, HFD, lipolysis, Formoterol, Salbutamol.

Résumé

Face à la prévalence croissante de la stéatose hépatique non alcoolique (NAFLD), de nouvelles approches thérapeutiques sont explorées. Cette étude visait à évaluer le rôle thérapeutique potentiel des agonistes des récepteurs β2-adrénergiques, le salbutamol et le formotérol, sur la stéatose hépatique induite par un régime riche en graisses (HFD) chez des rats mâles Wistar. Le protocole expérimental comportait six groupes : un groupe témoin, un groupe HFD et quatre groupes traités recevant soit du salbutamol, soit du formotérol à faibles et fortes doses après l'induction de la maladie. Les paramètres biochimiques, notamment les enzymes hépatiques, le profil lipidique et la glycémie, ont été mesurés, et un examen histologique du tissu hépatique a été réalisé. Les résultats ont révélé une augmentation significative des marqueurs de lésions hépatiques et de l'accumulation lipidique dans le groupe HFD. Cependant, le traitement par de faibles doses de salbutamol et de formotérol a entraîné des améliorations notables des profils biochimiques et histologiques, exerçant un effet protecteur contre la progression de la stéatose. Il est intéressant de noter que les doses élevées n'ont pas démontré le même niveau d'efficacité, soulignant un potentiel effet thérapeutique dose-dépendant. Ces résultats étayent l'hypothèse selon laquelle les β2-agonistes, en particulier à faibles concentrations, pourraient offrir un effet protecteur contre la stéatose hépatique induite par l'alimentation. Des études complémentaires sont nécessaires pour confirmer ces résultats et définir des stratégies posologiques optimales pour une application clinique.

Mots clés : Foie, HFD, Lipolyse, Formotérol, Salbutamol

ملخص

في ضوء تزايد انتشار مرض الكبد الدهني غير الكحولي، يجري استكشاف مناهج علاجية جديدة. هدفت هذه الدراسة إلى تقييم الدور العلاجي المحتمل لمنبهات مستقبلات بيتا 2 الأدرينالية، سالبوتامول وفورموتيرول، في تدهن الكبد الناتج عن نظام غذائي غني بالدهون لدى ذكور فئران ويستار. تألف التصميم التجريبي من ست مجموعات: مجموعة ضابطة، ومجموعة نظام غذائي عالي الدهون، وأربع مجموعات معالجة تلقت إما جرعات منخفضة أو عالية من سالبوتامول أو فورموتيرول بعد تحريض المرض. تم قياس المعايير الكيميائية الحيوية، بما في ذلك إنزيمات الكبد، تراكم ومستوى الدهون، ومستوى السكر في الدم، وأُجري فحص نسيجي لأنسجة الكبد. كشفت النتائج عن زيادة ملحوظة في علامات إصابة الكبد و الدهون في مجموعة النظام الغذائي عالي الدهون. ومع ذلك، أدى العلاج بجرعات منخفضة من سالبوتامول وفورموتيرول إلى تحسينات ملحوظة من المثير للاهتمام أن الجرعات العالية لم تُظهر نفس في الملامح الكيميائية الحيوية والنسيجية، مما مارس تأثيرًا وقائيًا ضد تطور تدهن الكبد. مستوى الفعالية، مما يُبرز تأثيرًا علاجيًا محتملًا يعتمد على الجرعة. تدعم هذه النتائج الفرضية القائلة بأن مُنشِّطات بيتا 2، وخاصةً بتركيزات منخفضة، قد تُوفر تأثيرًا وقائيًا ضد تدهن الكبد الناتج عن النظام الغذائي. هناك حاجة إلى مزيد من الدراسات لتأكيد هذه النتائج وتحديد استراتيجيات الجرعات المثلى للتطبيق السريري.

الكلمات المفتاحية: الكبد، النظام الغذائي عالي الدهون، تحلل الدهون، فورموتيرول، سالبوتامول.