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MASTER

Topic

The action of β2-agonists formoterol and salbutamol on non-alcoholic fatty liver disease induced by a high-fat diet (HFD): Biochemical approaches.

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Summary

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Abbreviation list

NAFLD: Non-Alcoholic Fatty Liver Disease

NASH: Non-Alcoholic Steatohepatitis

HCC: Hepatocellular Carcinoma

NAFL: Non-Alcoholic Fatty Liver

Gβγ-Akt-eNOS-sGC: Gβγ subunits - Protein Kinase B (Akt) - Endothelial Nitric Oxide Synthase (eNOS) - Soluble Guanylate Cyclase (sGC)

DALY: Disability-Adjusted Life Year

T2DM: Type 2 Diabetes Mellitus

CVD: Cardiovascular Disease

CKD: Chronic Kidney Disease

RAS: Renin-Angiotensin System

FFAs: Free Fatty Acids

HSL: Hormone-Sensitive Lipase

CPT-1: Carnitine Palmitoyltransferase-1

HSC: Hepatic Stellar Cells

LGDN: Low-Grade Dysplastic Nodule

HGDN: High-Grade Dysplastic Nodule

GPCRs: G-Protein Coupled Receptors

cAMP: cyclic adenosine monophosphate

PLC: Phospholipase C

TM: Transmembrane helices

H8: Helix 8

ECL2: Extracellular Loop 2

CL: Cytoplasmic Loop

CT: Cytoplasmic Tail

ECL: Extracellular Loop

GPCR: G-Protein-Coupled Receptor

NT: N Terminus

DRY: Asp-Arg-Tyr

Asp: Aspartic acid

Arg: Arginine

Tyr: Tyrosine

GABA: Gamma-amino butyrique-acide

COPD: Chronic Obstructive Pulmonary Disease

mRNA : messenger RNA

PKA: Protein Kinase A

GRKs: G Protein-Coupled Receptor Kinases

USP33: Ubiquitin-Specific Protease 33

USP20: Ubiquitin-Specific Protease 20

EBP50/NHERF1: Ezrin-Radixin-Moesin-Binding Phosphoprotein 50 / Na+/H+ Exchanger Regulatory Factor 1

NSF: N-Ethylmaleimide-Sensitive Factor

SNX27: Sorting Nexin 27

3T3-L1: A cell line derived from mouse embryonic fibroblasts

COPD: Chronic Obstructive Pulmonary Disease

CTL: negative control with water and libitum

HFD: High-Fat Diet without treatment

FOR: HFD treated with Formoterol

SAL: HFD treated with Salbutamol

IFCC: International Federation of Clinical Chemistry and Laboratory Medicine

AST: Aspartate Aminotransferase

NAD: Nicotinamide Adenine Dinucleotide

NADH + H+: Reduced Nicotinamide Adenine Dinucleotide + Proton

MDH : Malate Dehydrogenase

ALT : Alanine aminotransferase

LDH : Lactate Dehydrogenase

GGT: Gamma-Glutamyl Transferase

H_2O_2: Hydrogen Peroxide

PAP: Para-Aminophenol

GOD: Glucose Oxidase

POD: Peroxidase

MDA: Malondialdehyde

LPO: Lipid Peroxidation

TCA: Tricarboxylic Acid Cycle

TBA: Total Bile Acids

SEM: Standard Error of the Mean

SE: Standard Error

n: Sample Size

PNPLA3: Patatin-Like Phospholipase Domain-Containing 3

TM6SF2: Transmembrane 6 Superfamily Member 2

GCKR: Glucokinase Regulatory Protein

CETP: Cholesteryl Ester Transfer Protein

SREBP-1c: Sterol Regulatory Element-Binding Protein 1c

ChREBP: Carbohydrate-Responsive Element-Binding Protein

MBOAT7: Membrane-Bound O-Acyltransferase Domain-Containing 7

MTTP: Microsomal Triglyceride Transfer Protein

SOD2: Superoxide Dismutase 2

GST: Glutathione S-Transferase

TNF-α: Tumor Necrosis Factor Alpha

PPARA: Peroxisome Proliferator-Activated Receptor Alpha

APOC3 : Apolipoprotein C-III

IL6 : Interleukin 6

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a condition characterized by the accumulation of fat in the liver, not related to alcohol consumption **(Tagkou and Goossens, 2023)**. The non-alcoholic fatty liver disease (NAFLD) has emerged as the most common chronic liver disease, affecting a quarter of the global population **(Younossi and al., 2016)**. NAFLD is the hepatic manifestation of metabolic syndrome and is highly prevalent in obese, diabetic and hyperlipidemic subjects **(Estes and al., 2018; Younossi and al., 2019)**. NAFLD is characterized by the development of steatosis in >5% of hepatocytes identified either histologically or radiologically **(Huang and al., 2019; Yki-Jarvinen, 2014)**. The pathological picture resembles that of alcohol-induced liver injury but is not attributable to consumption of alcohol **(Angulo, 2002; Chalasani and al., 2012).** NAFLD includes a histological spectrum of liver disease, covering from simple steatosis with or without inflammation to NASH and liver cirrhosis with risk of hepatocellular carcinoma (HCC) **(Angulo and al., 2007)**. Over the last several years, converging lines of evidence has led the hypothesis that histological phenotype such as steatosis and non-alcoholic steatohepatitis (NASH) can progress to fibrosis and hepatocellular carcinoma (HCC) **(McPherson and al., 2015; Wong and al., 2010).** It is the most prevalent form of chronic liver disease worldwide and they set to surrogate viral hepatitis as the primary cause of cirrhosis, hepatocellular cancer and liver transplantation over the next decade **(Huang and al., 2019)**.

NAFLD has become the most prevalent liver disease worldwide, affecting roughly 25% of the global population **(Younossi and al., 2019)**. This prevalence is due, in part, to the growing rates of obesity and related metabolic disorders. NAFLD can lead to serious liver complications, including cirrhosis, liver cancer, and the need for liver transplantation **(Tagkou and Goossens, 2023)**. Lifestyle changes, such as adopting a balanced diet, exercising regularly, and maintaining a healthy weight, are critical in managing NAFLD and its more severe form, non- alcoholic steatohepatitis (NASH). In more extreme cases, surgical interventions like sleeve gastrectomy or gastric bypass can support weight loss and improve liver function. There are also emerging treatments, such as fecal microbiota transplantation, aimed at altering the gut microbiota to benefit liver health **(Zhang and Yang, 2021)**.

In terms of pharmacological treatments, several drug classes are used to address the various aspects of NAFLD and NASH. For instance, antidiabetic and anti-obesity drugs can help reduce liver fat, while antioxidants may decrease inflammation. Anti-fibrosis drugs and anti-cell death agents are also under investigation to prevent the progression of fibrosis **(Zhang and Yang, 2021)**. However, these existing medications often target only specific aspects of

NAFLD. Our research explores the potential of β2-agonists, like formoterol, as a broader therapeutic approach, focusing on both lipid metabolism and inflammation. Formoterol has been shown to activate the Gβγ-Akt-eNOS-sGC signaling pathway, potentially leading to unique metabolic benefits. Its distinct molecular structure allows it to interact with more amino acids in the β2-adrenergic receptor, enhancing Gβγ-dependent signaling **(Cameron and al., 2017).** This feature might explain formoterol's significant effects on gene expression and cellular functions, suggesting it could be a promising candidate for a more comprehensive treatment strategy for NAFLD.

Given these properties, exploring β2-agonists like formoterol could expand treatment options for NAFLD. The aim of this study was therefore to provide a detailed analysis of the biochemical events involved in the high fat diet of rats treated by beta 2-adrenoceptor stimulation by formoterol. Formoterol was used because unlike to the first generation of beta 2-agonists like clenbuterol, the addition of a long carbon chain containing a second benzene ring confers a rapid onset and long duration of action **(van Noord and al., 1996)**. In this study, we also examined the hypothesis that activation of beta 2-adrenoceptor in response to formoterol administration promotes lipolysis contributing to the decrease in adipose mass and enhance NAFLD damage.

I. Bibligraphic sythisis

Chapter 1
General Information on NAFLD

I.1.1 Definition

Non-Alcoholic Fatty Liver Disease (NAFLD) is a chronic lesion **(Yang and al., 2020).** It encompasses all forms of hepatic steatosis in individuals who do not significantly consume alcohol **(Tagkou & Goossens, 2023).**To diagnose non-alcoholic fatty liver disease (NAFLD), two criteria must be met: The presence of signs of hepatic steatosis, confirmed by imaging or histology, and the absence of secondary causes of fat accumulation in the liver, such as excessive alcohol consumption (significant alcohol consumption defined as ≥30 g per day for men and ≥20 g per day for women) **(Tagkou & Goossens, 2023).** Noted that the prolonged use of medications promoting steatosis, or monogenic genetic disorders (such as lecithin-cholesterol acyltransferase deficiency, cholesterol ester storage disease, or Wolman disease) (**Chalasani and al. 2018).**

NAFLD can be histologically classified into non-alcoholic fatty liver (NAFL) or non-alcoholic steatohepatitis (NASH) (**Tagkou & Goossens 2023).** Non-Alcoholic Fatty Liver (NAFL): Presence of >5% hepatic steatosis without evidence of hepatocellular injury in the form of ballooning of the hepatocytes or evidence of fibrosis **(Chalasani and al. 2018).** The risk of progression to cirrhosis and liver failure is considered minimal **(Chalasani and al. 2018).** Non- Alcoholic Steatohepatitis (NASH): At the cellular level, besides hepatic steatosis, NASH is defined by lobular inflammation and signs of hepatocellular injury characterized by ballooning of hepatocytes with varying degrees of fibrosis (Figure 1). This can progress to cirrhosis, liver failure, and rarely hepatocellular carcinoma (HCC) **(Chalasani and al. 2018).** In most cases, NAFLD is associated with metabolic comorbidities such as obesity, diabetes, insulin resistance and dyslipidemia **(Chalasani and al. 2018).**

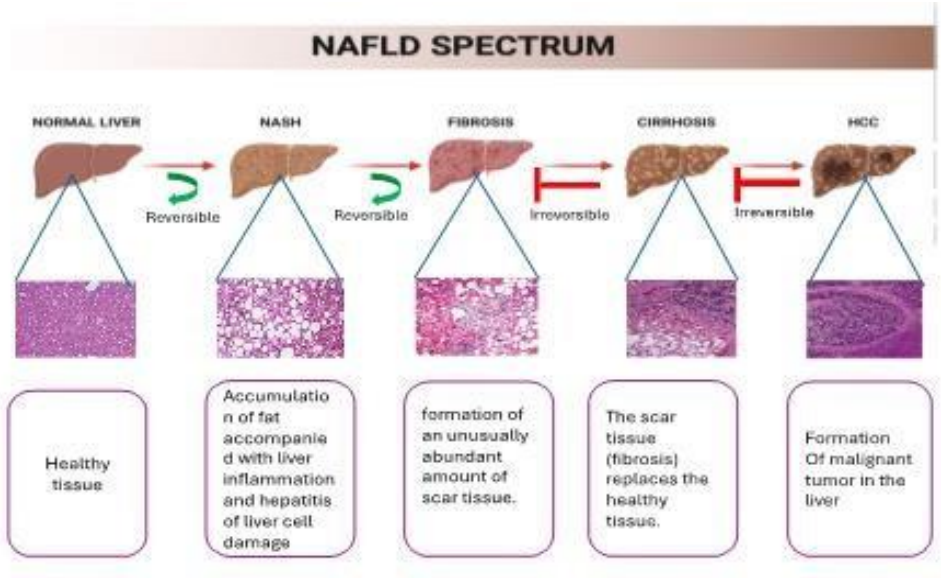


Figure 1: spectrum of liver damages modified from **(Razzaque and al., 2023)**

I.1.2 The Global Prevalence of NAFLD

There is a large discrepancy in estimation of NAFLD prevalence in general population. In fact, different techniques to diagnose NAFLD have been used such as imaging, liver biopsy and blood analysis. The prevalence of NAFLD by using blood tests (liver enzymes) consistently yielded lower estimates than those studies that used imaging **(Younossi and al., 2016).** A Meta analysis of studies between 2000 -2014 and conducted in adults age 18 or older, revealed that the global prevalence of NAFLD diagnosed by imaging is around 25% **(Estes and al., 2018; Younossi and al., 2016)**.

I.1.2.1 Prevalence in Africa

The prevalence of NAFLD (Non-Alcoholic Fatty Liver Disease) in Africa is challenging to determine due to limited data. In Algeria, Golabi et al. have reported that the hepatic complications due to non-alcoholic fatty liver disease was increased (Table 1) between 2009 to 2019 **(Golabi and al., 2021).** Unlike North America, Europe, and Asia, there have been fewer studies on NAFLD in Africa **(Z. Younossi and al., 2019)**. The lowest prevalence rate of NAFLD was reported from Africa with 14% (Figure 02) **(Younossi and al., 2016)**. This low rate may be explained by very few studies (Table 2) on the epidemiology of NAFLD from Africa **(Almobarak and al., 2014; Kruger and al., 2010; Onyekwere and al., 2011)**. Most challenge is to conducted research programs to explore NAFLD in different regions in Africa. NAFLD prevalence from Africa estimates were stratified by countries (Table 2).

Table 1: The key data for the years 2009 and 2019 concerning hepatic complications due to non- alcoholic fatty liver disease (NAFLD/NASH) in Algeria, Le DALY; Disability-Adjusted Life Year **(Golabi and al., 2021).**

Year	**Mortality Rate (per 100,000 population)**	**Incidence Rate (per 100,000 population)**	**DALY (per 100,000 population)**
2009	0.95 (0.62–1.38)	1.10 (0.70–1.63)	22.85 (15.23–32.82)
2019	1.32 (0.85–1.94)	1.79 (1.12–2.67)	30.22 (19.73–44.01)
Note	Increase in 2019 compared to 2009	Increase in 2019 compared to 2009	Increase in 2019 compared to 2009

Table 2: The prevalence of NAFLD (Non-Alcoholic Fatty Liver Disease) in Africa.

Region/ Country	**Prevalence NAFLD**	**Notes**
Africa	Estimated around 13.48%	Limited data available on NAFLD prevalence in Africa.
Sudan (2014)	reported a rate of 20%	The prevalence of NAFLD Africa is estimated based to these two studies
Nigeria (2011)	reported a rate of 8.67%	

I.1.2.2 Prevalence in Europe

The prevalence rates of NAFLD in Europe ranges from 24% to 28% (Table 3). Like other reports from industrialized societies, a third of population have NAFLD **(Younossi and al., 2019)**. NAFLD prevalence from Europe estimates were stratified by countries.

Table 3: Prevalence of NAFLD and NASH in Europe **(Younossi and al., 2019).**

Region/Country	Prevalence NAFLD	Prevalence NASH	Additional Information
Europe	20%-30%	Approximately 3%	NAFLD prevalence varies among European countries.
Germany (rural NE)	29.9%	-	A study found a prevalence of 29.9% for fatty liver by ultrasound, with 15.9% exhibiting elevated serum alanine aminotransferase levels.
Northern Italy	25%	-	Similar rates to rural Germany were observed.
Spain	25.8%	-	
United Kingdom	26.4%	-	
Hungary	22.6%	-	Prevalence reported by ultrasound.
Romania	20%	-	

I.1.2.3 Prevalence in Asia

In Asia, the rate of non-alcoholic fatty liver disease (NAFLD) can range widely (Table 4), from 15% to 40%, and non-alcoholic steatohepatitis (NASH) from 2% to 3% **(Younossi and al., 2019).** NAFLD prevalence from Asia estimates were stratified by countries.

Table 4: NAFLD prevalence in various regions of Asia, along with notable trends and estimates **(Younossi and al., 2019).**

Region	NAFLD Prevalence	Notes
India	28% to 31%	NAFLD prevalence is estimated at 30.7% in rural Haryana.
China	3.87% (1995) to 43.65% (2015)	Prevalence increased over time to 43.65% in 2015 among Shanghai adults. Additionally, 5.0% of schoolchildren in the Yangtze River delta region had NAFLD
Korea	24% to 40%	Range of NAFLD prevalence.
Taiwan	15% to 27%	
Japan	9% to 18%	Lower prevalence rats reported

I.1.2.4 Prevalence in the Americas

In the Americas, the prevalence of nonalcoholic fatty liver disease (NAFLD) varies significantly. In the North, it is estimated to be around 24% (Figure 02), while in the South, it reaches 32%. NAFLD prevalence from Americas estimates were stratified by countries (Table 5).

Table 5: Prevalence of NAFLD and NASH, Along with Obesity, in North and South America. NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis **(Younossi and al., 2019).**

Region/ Country	Prevalence NAFLD	Prevalence NASH	Prevalence of Obesity	Additional Information
North America	24%	-	-	
Dominican Republic	16%	-	-	Genetic factors, particularly those linked to Native American and Hispanic heritage, play a role in NAFLD prevalence. A polymorphism in the PNPLA3 gene has been associated with a higher prevalence of NAFLD among Hispanics (Mexicans).
Puerto Rico	18%	-	-	
Mexican	33%		-	
United States	24%	1.5% - 6.45%	-	Despite efforts to understand NAFLD and its severe form, NASH, precise data in the United States remain limited due to histological requirements.
Belize	29%	-	35%	NAFLD prevalence varies a ccording to obesity levels in each country.
South America	32%	-	-	
Peru	12.5%	-	15%	

I.1.2.5 Prevalence in the Middle East

The exact prevalence of non-alcoholic fatty liver disease (NAFLD) in Middle Eastern countries hasn't been thoroughly studied, but recent research sheds some light on the issue. A meta-analysis found that around 31.79% of people in the Middle East have NAFLD. In Iran, specifically, data suggests that 33.9% of the population has NAFLD, with rates going up to 39.3% in certain areas. These numbers are much higher than the reported 15.3% prevalence in rural parts of Iran **(Younossi and al., 2019).**

I.1.2.6 Prevalence in Australia and Pacific Countries

Like other reports from industrialized societies, the prevalence rates of NAFLD in Australia and Pacific Countries affecting a third of the population **(Younossi and al., 2019).** NAFLD prevalence from in Australia and New Zealand estimates were stratified by countries (Table 6).

Table 6: NAFLD prevalence in Australia and New Zealand, along with notable statistics and trends observed in the region. **(Younossi and al., 2019)**

Region	**NAFLD prevalence**	**Notes**
Australia	Affects a third of the population (5.5 million people); 40% of adults ≥50 years of age; 15% of schoolchildren.	NAFLD noted as the most common liver disease in Australia. High prevalence attributed to the country's high burden of overweight and obesity (63.4% of adults in 2014-2015).
New Zealand	13% of the population	NAFLD affects 13% of the population of New Zealand.
Australia (Population-based survey)	NAFLD reported as the most common cause of abnormal liver tests; 47% of the population had elevated alanine aminotransferase due to truncal obesity.	Recent nationwide survey of 9,447 individuals highlights NAFLD as a prevalent condition. Diagnosis and referral prompted by obesity.

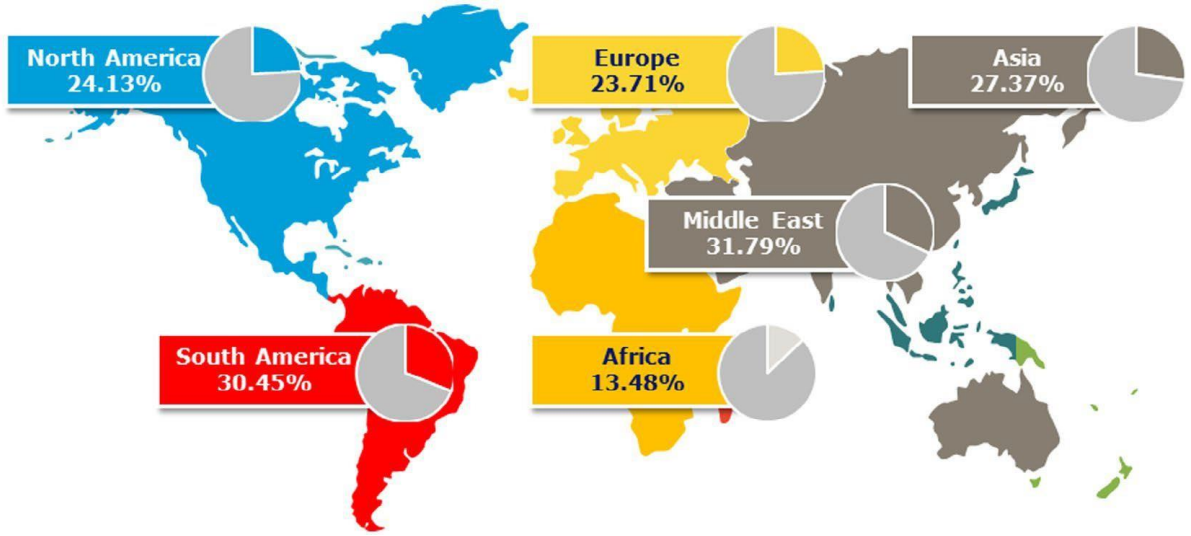


Figure 02: The Global Prevalence of Nonalcoholic Fatty Liver Disease (NAFLD) **(Younossi and al., 2019).**

I.3. Origin and factors

The pathogenesis of non-alcoholic fatty liver disease (NAFLD) is multifactorial, involving a combination of genetic factors with metabolic and environmental factors to promote the accumulation of fat in hepatocytes **(Marchisello and al., 2019)**. During the last decade of the 20th century, the most widely accepted theory was that of the "two-hit pathogenesis." According to this theory, insulin resistance leads to the deposition of triglycerides in the liver, causing steatosis and making it more susceptible to other triggering factors, such as oxidative stress, decreased ATP levels, and endotoxins **(Marchisello and al., 2019)**. These subsequent triggering factors ultimately lead to inflammation, fibrosis, and even cancer. Nowadays, this theory has been replaced by that of the "multiple hit pathogenesis". This new theory suggests that several factors act simultaneously or sequentially, and synergistically, on a genetically predisposed individual, thereby causing NAFLD and defining the spectrum of the disease phenotype **(Marchisello and al., 2019).**

I.1.3.1 Metabolic Comorbidity

The Metabolic Syndrome is a complex set of interconnected dysfunctions, involving disturbances in lipid and carbohydrate metabolism, as well as vascular issues, and a state promoting clotting and inflammation **(Marchisello and al., 2019)**.

It represents one of the main causes of non-alcoholic fatty liver disease (Figure 3). Cardiovascular risk factors associated with Metabolic Syndrome include abdominal obesity, dyslipidemia promoting atherosclerosis, high blood pressure, insulin resistance and/or glucose intolerance, as well as a predisposition to blood clot formation **(Marchisello and al., 2019).**

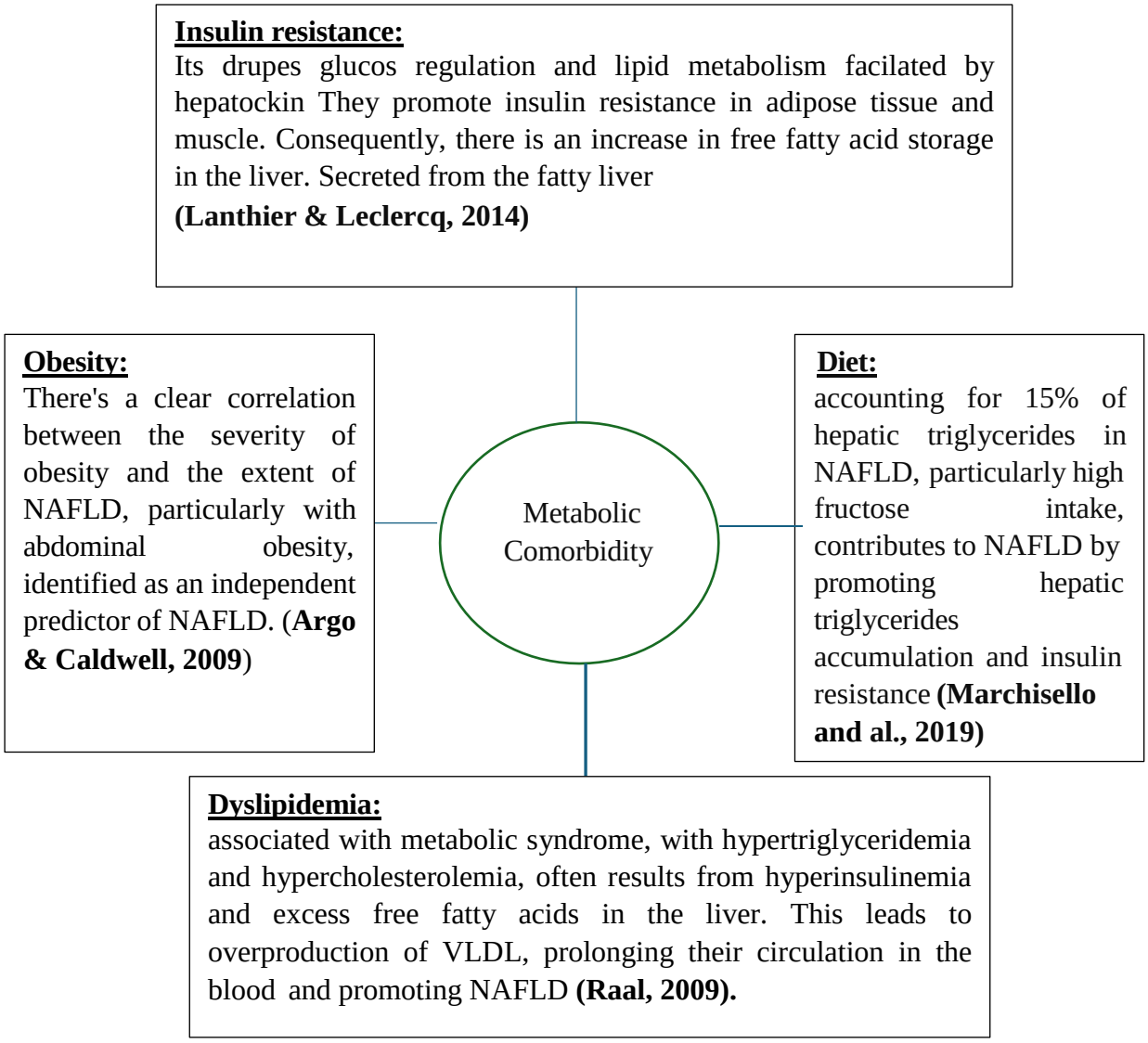


Figure 03: Diagram Representing Metabolic Comorbidities.

I.1.3.2 Genetic factors

Interethnic variances and familial clustering, coupled with the incomplete explanation of non-alcoholic fatty liver disease (NAFLD) by environmental and metabolic factors alone, have prompted the scientific community to consider genetic factors as potentially pivotal in its development **(Marchisello and al., 2019)**. Genome-wide association studies and gene expression analyses (Table 7) have pinpointed numerous genes involved in pathways like lipogenesis, fatty acid oxidation, lipoprotein transport, glucose regulation, detoxification, and inflammation, underscoring the multifaceted nature of NAFLD's pathogenesis and the ongoing significance of environmental interactions for its comprehension **(Marchisello and al., 2019).**

Table 7: Interethnic variances and familial clustering, associated with the NAFLD (**Marchisello and al., 2019).**

Gene	Function	Variant	Association with NAFLD
PNPLA3	Hepatic triglyceride degradation	rs738409	Increased risk of hepatic steatosis, particularly in individuals of Hispanic origin. Loss of function may promote liver fat accumulation and disease progression, independently of other metabolic factors.
TM6SF2	VLDL secretion	rs58542926	Loss of function linked to hepatic steatosis, inflammation, and fibrosis. This mutation also appears to offer some cardiovascular protection by reducing levels of apolipoprotein B-rich lipoproteins.
GCKR	Hepatic glucokinase Regulation	rs1260326-T	Variant associated with uncontrolled glucose uptake by hepatocytes, leading to hepatic lipid accumulation due to increased glycolysis and decreased beta-oxidation.
CETP	Reverse cholesterol Transport	rs1800777	Variant associated with hepatic steatosis and lobular inflammation in NAFLD patients.
SREBP-1c	Key regulator of hepatic gluconeogenesis	rs11868035	Variant associated with NAFLD development, insulin resistance, and atherogenic dyslipidemia.
MBOAT7	Arachidonic acid metabolism	rs641738 C>T	Variant associated with hepatic steatosis, while rs626283 variant may predispose obese Caucasian adolescents and children to NAFLD and insulin resistance.
MTTP	ApoB lipoprotein Assembly	493 G/T	Polymorphism associated with NAFLD and metabolic syndrome development.
Others	Various functions	Various variants	Several genes, including SOD2, GST, TNF-α, PPARA, APOC3, and IL6, have been studied for their potential involvement in NAFLD. These findings underscore the importance of understanding the interactions between genetic and environmental factors in NAFLD pathogenesis.

I.1.3.3 Age and sex

The pathogenesis of NAFLD is influenced by age, sex, and fertility **(Ullah and al., 2019).** It has been reported that the prevalence of NAFLD increases with age, ranging from 20% in individuals under 20 years old to over 40% in those over 60 years old **(Benedict and Zhang, 2017)**. Age is a significant factor in women, before the age of 50, women generally have less severe fibrosis than men due to the presumed protective effect of estrogen, which diminishes after menopause **(Fiz et al., 1987).**

I.1.4 The underlying diseases of NAFLD

Non-alcoholic fatty liver disease NAFLD, a prevalent chronic liver condition globally, represents a manifestation of metabolic syndrome within the liver. Growing evidence indicates a close association between NAFLD and several other conditions, such as diabetes, cardiovascular disease, kidney disease, and cancer **(Liu and al., 2020)**

I.1.4.1 Type 2 diabetes and NAFLD

Non-alcoholic fatty liver disease (NAFLD) is acknowledged as the most prevalent chronic liver disorder globally, with a higher prevalence observed among individuals with type 2 diabetes mellitus (T2DM), affecting up to 70–80% of such patients **(Mantovani and al., 2020)**. Moreover, those with T2DM are predisposed to developing severe histological forms of NAFLD, including non-alcoholic steatohepatitis (NASH), advanced fibrosis, and cirrhosis **(Mantovani and al., 2020)**. Recent meta-analytic findings reveal that biopsy-confirmed NASH affects approximately 38% of T2DM patients, while the prevalence of advanced fibrosis among individuals with both NAFLD and T2DM stands at around 17% **(Mantovani and al., 2020)**. While the precise mechanisms linking NAFLD and T2DM remain incompletely elucidated, the accumulation of liver lipid is known to correlate with hepatic insulin resistance and inflammation, hallmark features of NAFLD **(Targher and al., 2021)**. Consequently, interventions aimed at ameliorating hepatic lipid buildup in NAFLD could potentially mitigate the risk of T2DM by enhancing insulin sensitivity and curbing chronic inflammation **(Targher and al., 2021).** Given these insights, it is evident that targeting hepatic lipid accumulation is essential not just for managing NAFLD, but also for addressing T2DM, which often underlies NAFLD.

I.1.4.2 Cardiovascular disease

There could be either a bidirectional association or a shared origin between non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease (CVD), with NAFLD serving as a risk factor for premature coronary heart disease, cardiovascular events, and early changes in myocardial structure and function **(Tana et al., 2019)**. Additionally, NAFLD is linked to a higher risk of atrial fibrillation, regardless of concurrent valvular heart disease, hinting at its potential as a new risk factor for cardiac arrhythmias **(Ballestri, 2014)**. NAFLD complicates cardiovascular risk through a complex interplay of metabolic, cardiovascular, and hepatic factors, including systemic inflammation and oxidative stress **(Tana and al., 2019)**. Additionally, liver-specific abnormalities and shared genetic predispositions may further heighten cardiovascular risk **(Tana and al., 2019).**

I.1.4.3 Chronic kidney disease

Nonalcoholic fatty liver disease (NAFLD) and chronic kidney disease (CKD) are significant global health concerns, affecting approximately 25-30% of the population for NAFLD and 10-15% for CKD **(Byrne & Targher, 2020).** Furthermore, their association remains strong, unaffected by common conditions like obesity, hypertension, and type 2 diabetes (T2DM) **(Byrne & Targher, 2020).** The intricate connection between NAFLD and CKD involves complex mechanisms. NAFLD influences kidney function by modifying lipid metabolism and provoking inflammatory responses **(Marcuccilli & Chonchol, 2016).** Conversely, the kidneys react by further activating molecules like the renin-angiotensin system (RAS), perpetuating a harmful cycle leading to fibrosis **(Marcuccilli & Chonchol, 2016).**

I.1.4.4 Extra-hepatic cancers

NAFLD might significantly raise the likelihood of developing colorectal adenomas and cancer, as well as intrahepatic and extrahepatic cholangiocarcinoma, along with breast, gastric, pancreatic, prostate, and esophageal cancer **(Liu and al., 2020)**. Considering NAFLD as a notable influencer in clinical diagnosis and treatment approaches for extrahepatic cancers is warranted **(Liu and al., 2020).**

Chapter 2
Pathophysiological mechanism of NAFLD

I.2.1 Steatosis

Fatty liver results from an imbalance between the intake, storage and use of fats in the liver mainly triglycerides **(Grzych and al., 2023).**

I.2.1.1 The main mechanisms contributing to the development of fatty liver disease

Metabolic changes lead to the build-up of triglycerides in the liver during insulin resistance. Insulin resistance is shown by high insulin levels, increased glucose production in the liver, and reduced glucose uptake by cells **(Browning & Horton, 2004).** In fat cells, insulin resistance boosts the activity of hormone-sensitive lipase (HSL), increasing the breakdown of triglycerides and the flow of free fatty acids (FFAs) to the liver (Figure 4). These FFAs can be either burned in the mitochondria to produce energy (ATP) or converted into triglycerides for storage or incorporation into VLDL particles **(Browning & Horton, 2004).** In the liver, high insulin levels trigger the expression of SREBP-1c, which activates genes responsible for fat production **(Browning & Horton, 2004).** At the same time, high blood sugar levels activate ChREBP, which also turns on genes involved in fat production. Together, SREBP-1c and ChREBP enhance the activity of enzymes needed to convert excess glucose into fatty acids. This increased fat production leads to more malonyl-CoA (Figure 4), which blocks CPT-1, the protein that helps transport fatty acids into mitochondria **(Browning & Horton, 2004).** Therefore, in insulin resistance, FFAs coming from other parts of the body and those made in the liver are more likely to be turned into triglycerides **(Browning & Horton, 2004).**

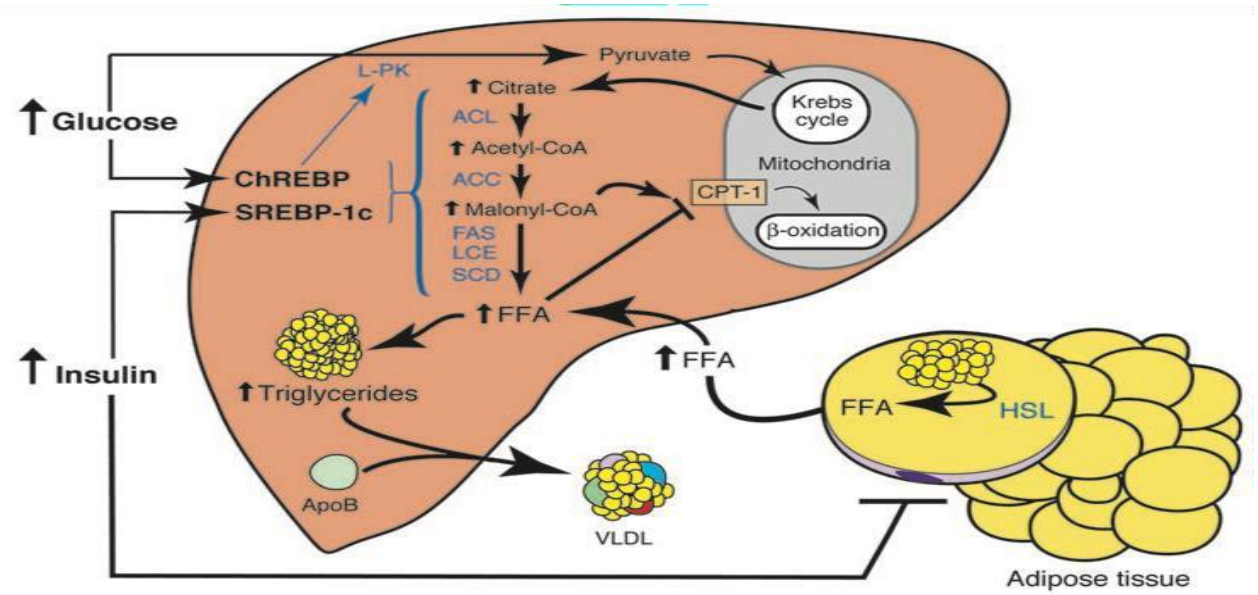


Figure 4: Metabolic changes leading to the accumulation of triglycerides in the liver during insulin resistance **(Browning & Horton, 2004)**

1.2.2 Fibrosis

Hepatic fibrosis is the result of excessive accumulation of scar tissue in the liver, resulting from inflammatory processes and activation of hepatic stellar cells (HSC) **(Koyama and al. 2016)**. Quiescent HSCs become activated in response to various stimuli (Figure 5), such as pro-inflammatory cytokines, oxidative stress, and certain lipids **(Chen and al., 2019)**. This activation transforms HSC into myofibroblasts, which lose their lipid content and acquire excessive extracellular matrix production properties **(Chen and al., 2019)**. Activated myofibroblasts produce and secrete extracellular matrix proteins such as collagen, fibronectin, and elastin. This overproduction of extracellular matrix leads to scarring and hepatic fibrosis (Figure 5). Activated HSCs recruit other types of inflammatory cells, such as macrophages, lymphocytes, and endothelial cells, into the liver **(Chen and al., 2019)**. These inflammatory cells participate in the inflammatory cascade and the progression of fibrosis **(Grzych and al. 2023).** Activated HSCs produce and release pro-inflammatory cytokines and chemokines that recruit and activate other inflammatory cells. These mediators play a key role in amplifying the inflammatory response and stimulating fibrogenesis **(Chen and al., 2019).**

Normally, the liver can degrade and resorb the extracellular matrix. However, in the case of hepatic fibrosis, this balance is disturbed, leading to an excessive accumulation of scar tissue. **(Grzych and al. 2023).**

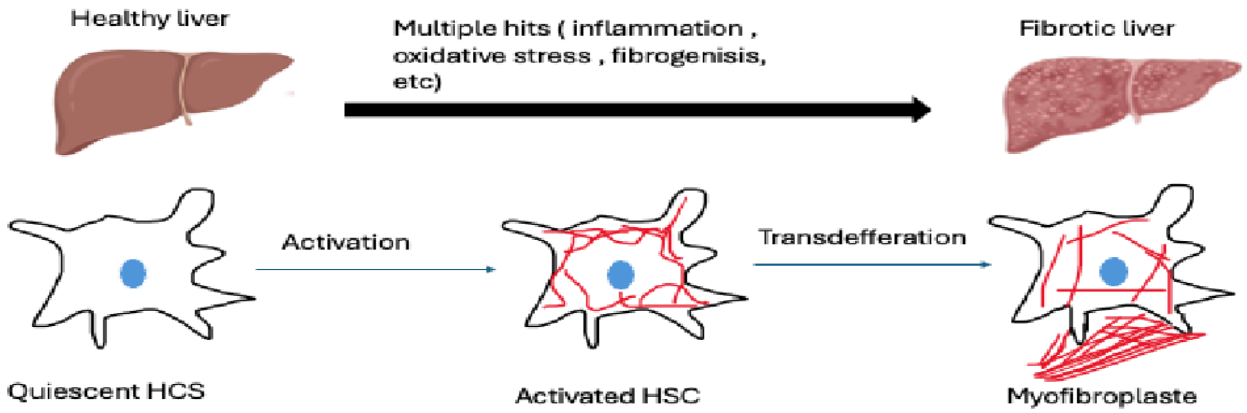


Figure 05: Activation process of quiescent HSCs into myofibroblasts during hepatic fibrosis. Modified from **(Chen and al., 2019).**

1.2.3 Cirrhosis

Cirrhosis is the advanced stage of liver fibrosis. It is characterized by an alteration of the hepatic architecture, with fibrosis surrounding nodules of hepatocyte regeneration **(Von Moos & Müllhaupt, 2015).**This condition results from chronic liver injury, leading to the destruction of hepatic cells and an accumulation of extracellular matrix components **(Von Moos & Müllhaupt, 2015).**Additionally, endothelial cells undergo modifications, resulting in the stiffening of sinusoids and collagen deposition in the space of Disse **(Von Moos & Müllhaupt, 2015).**Liver cirrhosis is characterized by a complex tissue transformation, like a scarring process. It involves three key elements: fibrosis (accumulation of scar tissue), angiogenesis (formation of new blood vessels), and regeneration of hepatic cells **(Von Moos & Müllhaupt, 2015).** These changes are triggered by chronic liver injury, often associated with chronic diseases such as cirrhosis. This process gradually alters the structure and function of the liver, contributing to disease progression **(Von Moos & Müllhaupt, 2015).**

I.2.4 Hepatocellular Carcinoma

Cirrhosis can progress to hepatocellular carcinoma and liver failure due to impaired liver regeneration resulting from unsuccessful attempts to restore a healthy hepatic architecture **(Amorim and al., 2023).** On cirrhotic liver, hepatocellular carcinogenesis is a multi-step process and involves the sequential appearance of the following tumor lesions: low-grade dysplastic nodule (NDBG), high-grade dysplastic nodule (NDHG), early hepatocellular carcinoma (HCC), and advanced hepatocellular carcinoma (HCC) **(Di Tommaso and al., 2013).** Dysplastic nodules are defined as precancerous lesions measuring between 1 mm and 2 cm and exhibiting regenerative characteristics **(Di Tommaso and al., 2013).** Arterial blood supply is commonly observed in NDHGs, followed by stromal invasion in early HCCs, and finally venous invasion as well as possible metastases in advanced HCCs **(Di Tommaso and al., 2013).**

The main histological features associated with the malignant transformation of liver cells are increased cell density and nucleus/cytoplasm ratio. HCCs are often described based on them architectural patterns (trabecular, pseudo-glandular, compact) and cytological appearance **(Calderaro and al., 2019).**

Chapter 3
Description of β2-agonists:
structure and classification

1.3.1 G Protein-Coupled Receptors

1.3.1.1 Definition

G-Protein Coupled Receptors (GPCRs) represent the largest family of membrane receptors, with approximately 750 different types identified in humans **(Assié and al., 2004)**. Their role extends far beyond endocrinology, as they can bind to a wide array of ligands as formoterol, including olfactory molecules, pheromones, light waves, nucleotides, and various mediators **(Assié and al., 2004)**. The signal transduction process involves G-proteins, which trigger specific signaling pathways, such as the cyclic adenosine monophosphate (cAMP) pathway and the phospholipase C (PLC) pathway, with responses that largely depend on the type of cell involved **(Assié and al., 2004)**.

1.3.1.2 Structure

G-Protein Coupled Receptors (GPCRs) share a common structural architecture, consisting of seven transmembrane helices (TM1 to TM7) (Figure 6), with an eighth helix (H8) running parallel to the inner surface of the plasma membrane **(Karnik and al., 2003).** These transmembrane helices are connected by three extracellular loops and three intracellular loops, whose varying sizes contribute to the structural diversity among GPCRs **(Karnik and al., 2003).** For example, the extracellular loops can form the ligand-binding site, as seen in the β1- adrenergic and β2-adrenergic receptors, where extracellular loop 2 (ECL2) is involved in this process (Figure 6). This structural arrangement not only provides a scaffold for ligand binding but also enables the conformational changes that are crucial for signal transduction **(Lebon & Tate, 2012).**

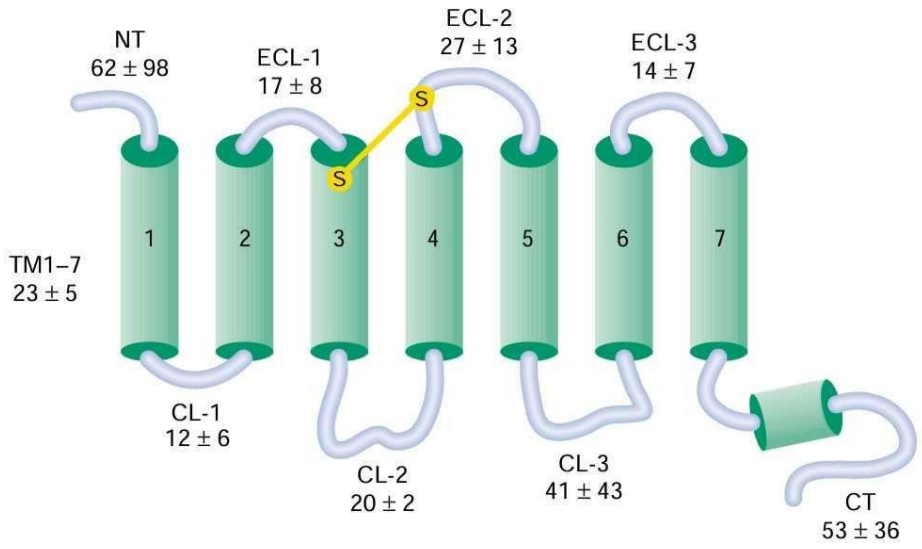


Figure 6: Secondary Structure of GPCRs: The Disulfide Bond Linking TM3 and ECL2, Conserved in 91.8% of GPCRs, with Variability Across Families. Abbreviations: CL

(cytoplasmic loop), (CT cytoplasmic tail), ECL (extracellular loop), GPCR (G-protein-coupled receptor), NT (N terminus), TM (transmembrane helix). Numbers indicate the numbers of residues in each region **(Karnik and al., 2003).**

I.3.1.3 The Classes of G Protein-Coupled Receptors

G protein-coupled receptors (GPCRs) represent a large family of transmembrane receptors involved in various biological processes. They are divided into multiple classes based on their sequences, structures, and functions **(Assié and al., 2004).** Below is an overview of the main GPCR families:

Class A (Family 1): Class A receptors, also known as rhodopsin-like receptors, form the largest group of GPCRs. Despite low overall sequence homology, they share a common structural feature: seven transmembrane alpha-helices connected by alternating intracellular and extracellular loops. These receptors are characterized by a conserved arginine in the Asp-Arg-Tyr (DRY) motif at the cytoplasmic end of the third transmembrane segment. This class includes key receptors such as the β2-adrenergic receptor and is extensively studied due to its relevance in numerous physiological processes and as drug targets **(Cavasotto and al., 2003)**.

Class B (Family 2): This family is defined by a large N-terminal domain containing cysteine residues that form disulfide bridges. The ligands for this class are typically large peptides such as glucagon, vasoactive intestinal peptide, or parathyroid hormone. The binding site is mainly located in the extracellular domain **(Assié and al., 2004).**

Class C (Family 3): Class C receptors share a common structure comprising two main domains. Like all GPCRs, they contain a heptahelical domain with seven transmembrane segments. What sets them apart is a large extracellular amino-terminal domain composed of 400 to 600 residues extending outside the membrane **(Galvez & Pin, 2003).** This class includes metabotropic glutamate receptors, GABA type B receptors, and calcium receptors **(Assié and al., 2004).**

There are other GPCR families, but their classification is less well-defined. The different classes of GPCRs vary in structure and function, which explains their wide range of roles in biological processes. Some families are more extensively studied than others, indicating the need for continued research to fully understand the diversity and complexity of GPCRs **(Assié and al., 2004).**

I.3.2 β-adrenergic receptor

Beta-adrenoceptors, also known as beta-adrenergic receptors, are a class of receptors distributed throughout the human body, notably in the respiratory system, the heart, and smooth

muscles **(Nials and al., 1993).** These receptors belong to the seven-transmembrane receptor family and mediate the effects of catecholamines like epinephrine and norepinephrine **(Nials and al., 1993).** Initially, beta-adrenoceptors were divided into beta-1 and beta-2 subtypes, with beta-1 primarily influencing cardiac function and beta-2 promoting bronchodilation. Despite this, beta-2 remains the dominant subtype along the trachea. In rats, the tracheal muscle responds to both beta-1 and beta-2 agonists, though the effects of beta-2 agonists are relatively weak, even with strong compounds **(Mustafa and al., 1999).**

I.3.3 Affinity of β2-agonists for β2-adrenergic receptors

The β2-adrenergic receptor is a type of G-protein-coupled receptor located deep within the cell membrane **(Johnson, 1998).** When an agonist like formoterol or salmeterol binds to this receptor, it triggers a cascade of events starting with the activation of a protein called Gs **(Johnson, 1998).** This then stimulates adenylate cyclase, an enzyme that converts ATP into cyclic AMP (cAMP). The increase in cAMP levels within the cell activates protein kinase A (PKA), which in turn phosphorylates proteins that play a role in relaxing the smooth muscles of the airways **(Johnson, 1998).**

Formoterol is a long-acting β2-adrenergic agonist used to manage asthma and chronic obstructive pulmonary disease (COPD) **(Patel et al., 2011).** Its extended duration of action is due to its ability to bind to both the active site and an additional "exo-site" on the β2-adrenergic receptor, enhancing its bronchodilator effect compared to other drugs like salbutamol. The moderate lipophilicity of formoterol allows it to form a deposit within the cell membrane, providing a gradual release that sustains its therapeutic effects over time **(Colman and al., 1996)**. Salbutamol is a fast-acting bronchodilator that typically has a duration of action lasting 4 to 6 hours. It demonstrates a quick onset of action because it binds directly to the β2-adrenoceptor, bypassing lipid membranes due to its high hydrophilicity. However, this low lipid affinity means it diffuses quickly from tissues, leading to its short duration of effect. This rapid dissipation contrasts with longer-acting bronchodilators like formoterol and salmeterol, whose effects persist due to stronger lipid interactions. Salbutamol's quick relief makes it ideal for immediate treatment of respiratory symptoms, but its shorter duration means it does not provide sustained bronchodilation **(Colman and al., 1996)**.

I.3.4 Regulation mechanisms of beta-2 agonists

I.3.4.1 Regulation of β2-adrenergic receptor density

The density of β2-adrenergic receptors is influenced by adrenaline administration and endurance training. An in vitro study demonstrated an increase in mRNA and membrane proteins of β2-adrenergic receptors following adrenaline administration **(Collins and al., 1989).** Endurance training for 10 to 18 weeks also leads to an augmentation in the density of β2- adrenergic receptors in certain muscles, while age does not significantly affect this density **(Buckenmeyer and al., 1990; Plourde and al., 1993; Ryall and al., 2004; Sillence and al., 1993).** However, pharmacological treatments such as clenbuterol and fenoterol can reduce the density of β2-adrenergic receptors, even at lower doses **(Ryall and al., 2002).** These findings suggest that the regulation of β2-adrenergic receptor density varies depending on the stimulus and may be specific to the muscle under study.

I.3.4.2 Phosphorylation and Desensitization of β2-Adrenergic Receptors

The desensitization of β2-adrenergic receptors is a crucial process involved in regulating the cellular response to adrenergic stimuli. This process is mediated by the phosphorylation of receptors by protein kinase A (PKA) and G protein-coupled receptor kinases (GRKs). GRKs, such as GRK2 and GRK5, specifically phosphorylate β2-adrenergic receptors on specific residues, thereby leading to their desensitization **(Benovic & Gomez, 1993; Jones and al., 2003; Seibold and al., s. d.).**

Phosphorylation of β2-adrenergic receptors can occur in two main ways: specifically (homologous) or non-specifically (heterologous) (Figure 7). In the first case, GRKs specifically phosphorylate receptors activated by β2-adrenergic agonists, while in the second case, PKA phosphorylates receptors independently of their specific activation by a β2-adrenergic agonist. This phosphorylation leads to a decrease in the receptors' ability to respond to agonists, which is crucial for maintaining cellular homeostasis and adaptive response to adrenergic stimuli. **(Nobles and al., 2011).**

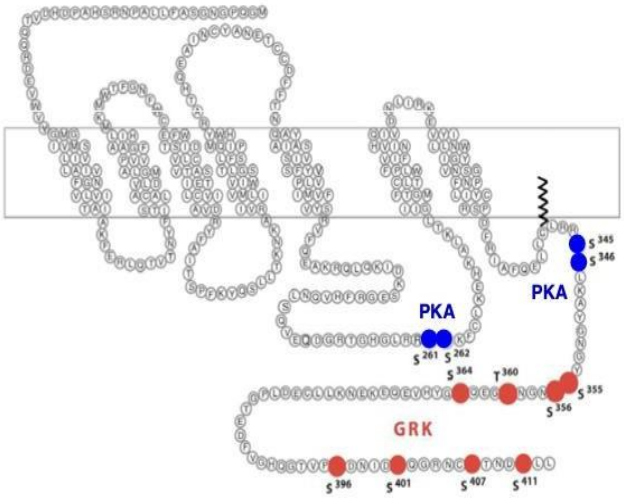


Figure 7: Phosphorylation site mapping of PKA (in blue) and GRK (in red) on the β2-adrenergic receptor. PKA, protein kinase A; GRK, G protein-coupled receptor kinase [modified from **(Nobles and al., 2011)**.

I.3.4.3 Internalization and degradation of β2-adrenergic receptors

After phosphorylation by GRKs, β-arrestin 2 recruits E3 ubiquitin ligases (Mdm2 and Nedd4), regulating the internalization and degradation of the β2-adrenergic receptor **(Kim & Benovic, 2002; Lin and al., 2002; Shenoy and al., 2001, 2008).** Internalized receptors are sorted in endosomes for recycling or degradation, a process influenced by proteins like ESCRT **(Hislop & Von Zastrow, 2011).** Deubiquitinating enzymes such as USP33, USP20, EBP50/NHERF1, NSF, and SNX27 facilitate receptor recycling (**Berthouze and al., 2009)**. Studies show that internalized receptors can return to the cell surface after cessation of agonist treatment **(Jensen and al., 2002).**

I.3.5 Effect of β2-adrenergic receptors

The agonists of beta-2 adrenergic receptors have various effects on different tissues of the body due to the distribution of these receptors throughout the body. Here are some of the main effects of beta-2 receptor agonists on different tissues **(Motiejunaite and al., 2021).**

I.3.5.1 Relaxation of smooth muscles

Activation of beta-2 adrenergic receptors triggers an intracellular signaling cascade that promotes relaxation of the smooth muscles in the respiratory airways, primarily through the activation of cAMP and PKA **(Motiejunaite and al., 2021).** This relaxation is crucial for treating

respiratory disorders such as asthma and bronchoconstriction, where excessive constriction of the airways leads to breathing difficulties. Agonists of beta-2 adrenergic receptors are widely used in these conditions to promote airway dilation and facilitate breathing **(Motiejunaite and al., 2021).**

I.3.5.2 strengthening skeletal Muscle

Increased Muscle Strength: Beta-2 receptor agonists can enhance the contraction strength of skeletal muscle, which can be beneficial in treating neuromuscular disorders and for enhancing athletic performance. **(Van Baak and al., 2004)**. Formoterol increases the number and surface area of mitochondria in the spinal cord and skeletal muscles. Increasing energy production in the cells of the spinal cord and skeletal muscles, which can promote better muscle function and enhance locomotor capacity **(Scholpa and al., 2019).**

I.3.5.3 Adipose tissue

First, they induce a significant decrease in adipose tissue mass. This decrease is attributed to several mechanisms. Initially, beta-2 agonists limit adipogenesis, i.e., the formation of new adipocytes, as shown by in vitro studies on 3T3-L1 adipocytes (**Kim and al., 2010).** This inhibition of adipogenesis contributes to the reduction of adipose mass mass **(Zhang and al., 2007).** Furthermore, these agonists promote lipolysis, the process of breaking down triglycerides stored in adipocytes into free fatty acids and glycerol. This increase in lipolysis results in an enhanced release of fatty acids into the bloodstream, thereby contributing to the decrease in adipose mass **(Zhang and al., 2007).** Additionally, beta-2 agonists reduce the size of adipocytes by decreasing the content of lipid droplets. This reduction in adipocyte size also contributes to the overall decrease in adipose mass **(Zhang and al., 2007).** Finally, these agonists promote apoptosis of adipocytes, which also contributes to the decrease in adipose mass **(Page and al., 2004).**

I.3.5.4 Muscle cardiac

The use of beta-2 agonists poses major cardiovascular risks **(Demoulin and al., 2018).** Studies have shown a correlation between the use of certain beta-2 agonists and an increased frequency of cardiac arrhythmias, myocardial infarctions, heart failure, and even sudden death, especially in patients with COPD and concomitant heart diseases **(Marquis and al., 2008).** Hypokalemia induced by beta-2 agonists could contribute to the exacerbation of these arrhythmias and other cardiovascular issues **(Marquis and al., 2008).**

II. Materials and methods

II.1. Experimental protocol

The protocol involves inducing steatosis through a specially prepared high fat diet to evaluate the progression of the disease among four groups: negative control with water ad libitum (CTL), HFD without treatment, treated with Formoterol (FOR), and treated with Salbutamol (SAL). The rats were identified using a color-coding system on their tails with indelible markers (red, green, black, and blue) and were weighed daily.

Thirty-two Fischer F344 rats (n=32) were divided into four groups (n=8 per group). A negative control group receiving water ad libitum, a group receiving a high-fat diet (HFD) for 10 weeks, a group receiving HFD for 10 weeks followed by treatment with Formoterol (15 µg/kg) for 2 weeks, and a group receiving HFD for 10 weeks followed by treatment with Salbutamol (15 µg/kg) for 2 weeks. At the end of the treatment period, tissue and serum samples were collected for biochemical assays to evaluate the effects of the treatments (Figure1).

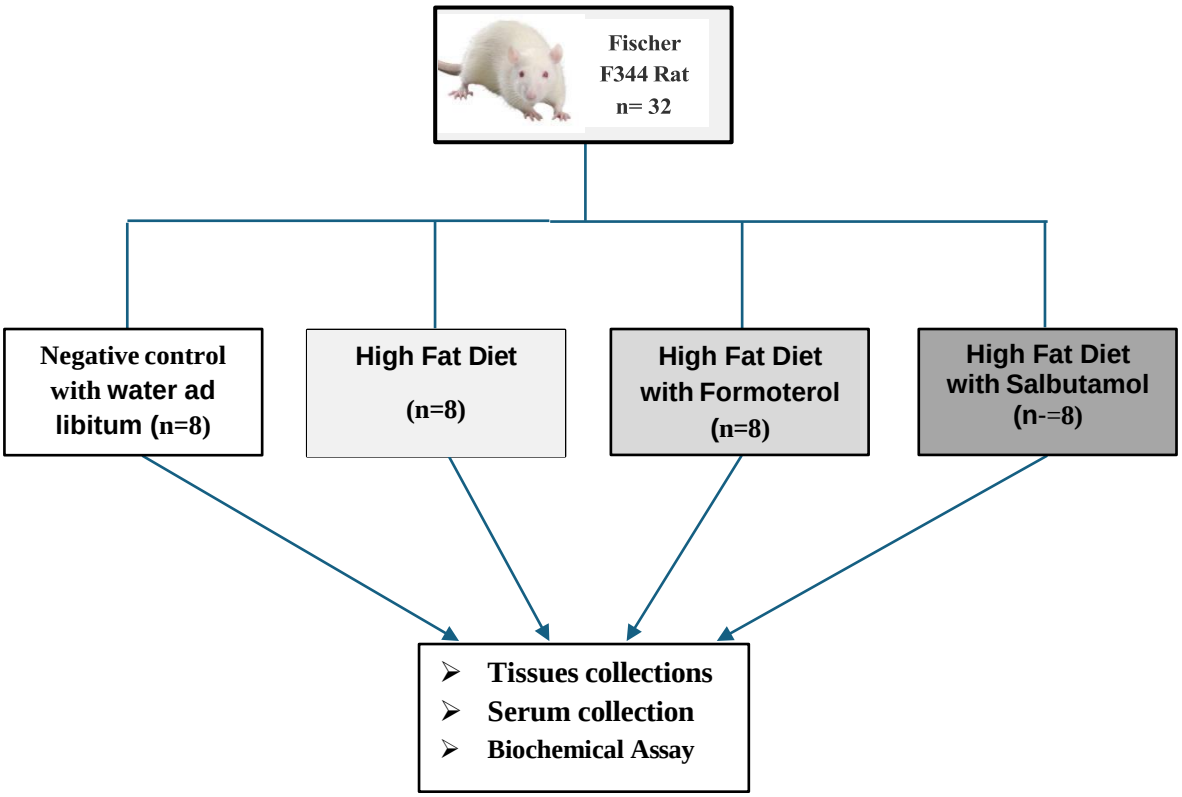


Figure 1: Experimental protocol.

II.2. Treatment and samples collections

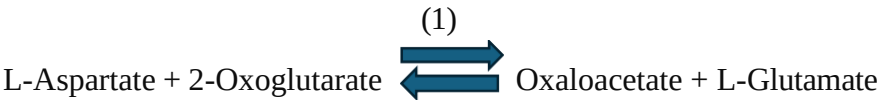
The treatment was prepared in the university's physico-chemical laboratory as an injectable solution. For in vivo experiments, 12-week-old rats received daily subcutaneously injections with formoterol at a concentration of 15µg/kg or salbutamol at a concentration 150 µg/kg. For control, age matched rats were injected with a sterile saline solution. After 2 weeks, the animals were anesthetized with intraperitoneally injections with ketamine (50 mg/kg) and xylazine (5 mg/kg).

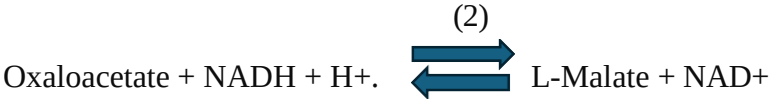
Organs (liver, heart, adipose tissue, kidney, lung, and spleen) were weighed and stored at -20°C. Blood samples were collected from the jugular vein in dry tubes, labeled, and centrifuged. The first centrifugation was at 4000 rpm for 10 minutes at 4°C to collect serum, which was then pipetted into Eppendorf tubes. The second centrifugation was at 2000 rpm for 10 minutes at 4°C to remove cellular debris, and the serum was stored at -20°C for subsequent biochemical tests. Vital organs (liver, heart, kidneys, lungs, spleen, pancreas, and adipose tissue) were dissected, weighed, and stored at -20°C.

II.3. Biochemical Assay

II.3.1. Aspartate aminotransferase (AST) Activity

Method developed by Karmen et al. and optimized by Henry et al (In accordance with IFCC recommendations) **(Lustig, Papanastasiou-Diamandis, and Goldberg 1988).** The decrease in absorbance is proportional to AST activity in the specimen measured at 340 nm. The reaction scheme is as follows:





Reaction1 : Aspartate Aminotransferase Reaction (AST) and Malate Dehydrogenase (MDH). (1): Aspartate Aminotransferase; (2): Malate Dehydrogenase.

II.3.2. Alanine aminotransferase (ALT) Activity

Method developed by Wrobleski and La Due, optimized by Henry and Bergmeyer (in accordance with IFCC recommendations) **(Okorodudu and al. 1989).** The decrease in absorbance is proportional to ALT activity in the specimen measured at 340 nm.

The reaction scheme is as follows:

L-Alanine + 2-Oxoglutarate $\overset{(1)}{\rightleftharpoons}$ Pyruvate + L-Glutamate

Pyruvate + NADH + H□ $\overset{(2)}{\rightleftharpoons}$ L-Lactate + NAD□

Reaction02: Alanine Aminotransferase (ALT) and Lactate Dehydrogenase (LDH) reaction. (1) Alanine Aminotransferase; (2) Lactate Dehydrogenase.

II.3.3. GAMMA GT Activity

Method based on the work of Szasz, Rosalki, and Tarlow. The reaction scheme is as **(Szasz and al. 2019).** The rate of formation of p-nitroaniline is directly proportional to GGT activity in the specimen measured at 405 nm.

The reaction scheme is as follows:

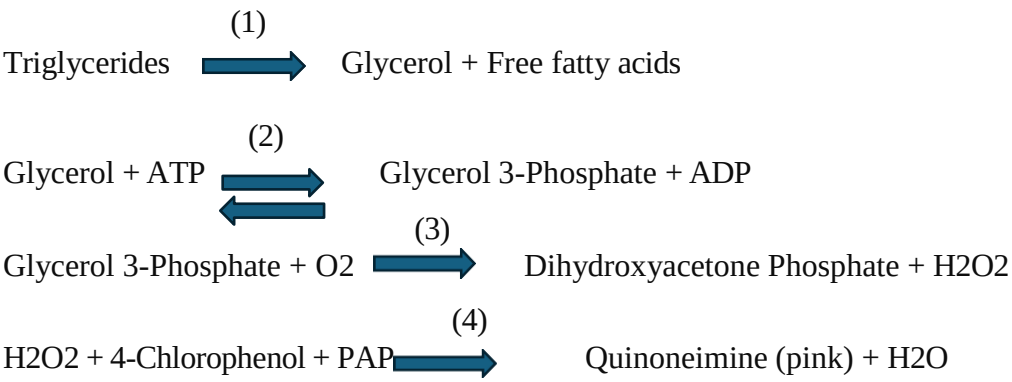
L-G-Glutamyl-3-carboxy-4-nitroanilide + Glycylglycine $\overset{(1)}{\rightleftharpoons}$ L-G-Glutamyl-glycylglycine + p-nitroaniline.

Reaction03: Enzymatic Reaction Scheme for GGT Activity Measurement. (1): (Gamma-Glutamyl Transferase)

II.3.4. Triglycerides quantification

Fossati and Prencipe method associated with the Trinder reaction **(Fossati, Prencipe, and Berti 1983; Trinder 1969).** The absorbance of the colored complex (quinoneimine) is proportional to the amount of triglycerides in the specimen measured at 500 nm.

The reaction scheme is as follows:

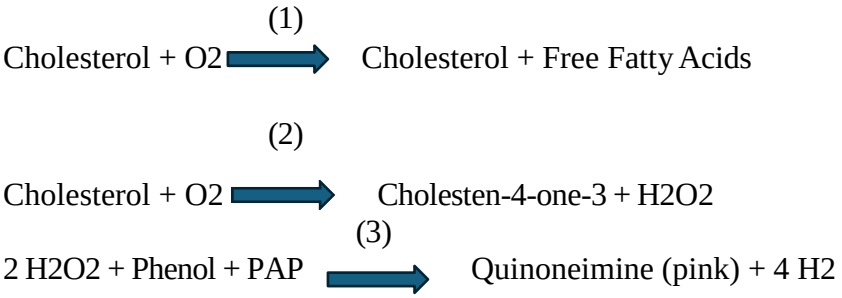


Reaction04: Enzymatic Reaction Scheme for Triglyceride Measurement. (1): lipase; (2): glycerol kinase; (3): Glycerol-3-Phosphate Oxidase; (4): Peroxidase.

II.3.5. Cholesterolimia quantification

Quantitative measurement of serum cholesterol was performed using the enzymatic methode enzymatic method described by Allain et al **(Allain and al. 1974).**

According to the Following Reaction Scheme:



Reaction05: Enzymatic reactions of cholesterol analysis. (1): Cholesterol esterase.

(2): cholesterol oxidase; (3): peroxidase.

II.3.6. Glucose quantification

Glucose is oxidized by GOD to gluconic acid and H2O2, which reacts with POD in the presence of chloro-4-phenol and PAP to form a red quinoneimine. The absorbance of the colored complex is proportional to the glucose concentration in the specimen measured at 500 nm **(Lott and Turner 1975).**

The reaction scheme is as follows:

$$\text{Glucose} + O_2 \xrightarrow{(1)} \text{Gluconic acid} + H_2O_2.$$

$$H_2O_2 + \text{Chloro-4-phenol} + \text{PAP} \xrightarrow{(2)} \text{Red Quinoneimoine} + H_2O.$$

Reaction06: Enzymatic Reaction of Glucose Oxidase (GOD) and Peroxidase (POD). (1) : Glucose Oxidase; (2): peroxidase.

II.4 Malondialdehyde (MDA)

Using the thiobarbituric acid-reactive substances method previously described by **(Ohkawa and al.1979).** lipid peroxidation (LPO) was evaluated by measuring the MDA levels in tissue homogenate. Briefly, in 5 ml of 1.15% cold KCl, 500 mg of tissue was homogenized. Then, to 0.5 ml of homogenate, 0.5 ml of 20% TCA and 1 ml of 0.67% TBA were added and shaken well. The mixture was incubated for 15 min at 100°C and cooled immediately on ice; then, 4 ml of *n* -butanol was added to the mixture. Centrifugation was performed at 3000 rpm and 4°C for 15 min, and the absorbance of the supernatant was measured at 530 nm.

II.5 Statistics

Various statistical tests including unpaired t-tests as well as one-way analysis of variance followed by Bonferroni 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.

III. Results

To date there is no treatment available for NAFLD. Therefore, it is necessary to develop therapeutic interventions to restrict or decrease the progression of liver fat accumulation. Our work aims to develop a therapeutic strategy by activating β2-agonist signaling pathway to reduce the progression of hepatic steatosis. The results on the preventive effect of β2-agonists will be presented in the following section and will include several parameters: weight and growth evolution or examination of the biochemical parameters.

III.1. β2-agonist administration does not alter the growth of animals

The graph depicts the growth of animals before treatment in two groups: the control group (CTL) and the high-fat diet group (HFD). Both groups show consistent growth curves, suggesting that the diet does not significantly affect the animals' growth (Figure 9A). Furthermore, the administration of formoterol and salbutamol does not exhibit any significant effect on the growth of animals across the different groups (CTL: Control, HFD: High Fat Diet, FOR: High Fat Diet Formoterol, SAL: High Fat Diet Salbutamol) (Figure 9B). Thus, neither the diet nor the administration of formoterol and salbutamol significantly alters the growth of the animals.

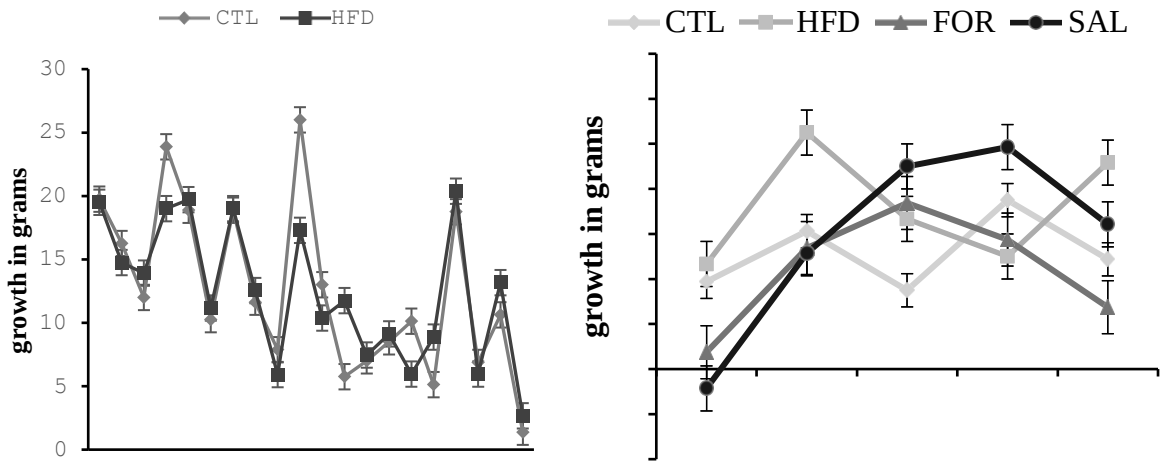


Figure 9: Graphical representation of animal growth rates before and after treatment administration in different groups (CTL: Control, HFD: High Fat Diet, FOR: High Fat Diet Formoterol, SAL: High Fat Diet Salbutamol). Values are means ± SE (n=6-8). Figure 2A: t test; Figure 2B: One-way ANOVA and Bonferroni as post hoc test.

III.2. 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 10). Adipose tissue weight was significantly ($p<0,001$) different between HFD and others

groups after two weeks of treatment. Noted that adipose tissue weight in the group HFD was significantly increased by 286% compared CTL group. In addition, adipose tissues weight was decreases in group FOR and SAL respectively 55% and 65% compared with HFD group. The following observations were noted on this occasion:

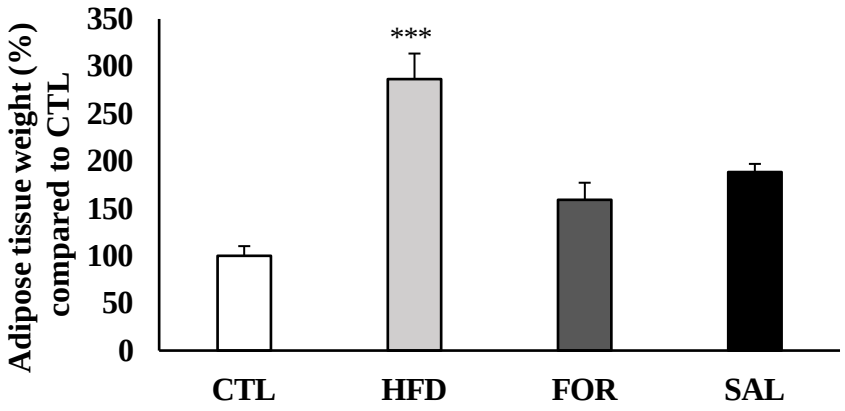


Figure 10: Histogram representing the percentage of adipose tissue weight in different groups (CTL: Control, HFD: High Fat Diet, FOR: High Fat Diet Formoterol, SAL: High Fat Diet Salbutamol) compared to the control group (CTL: Control). Values are means ± SE (n=6-8). ***P<0.001 relative to control group: One-way ANOVA and Bonferroni as post hoc test.

III.3. β2-agonist administration does not alter weight of different organs

Since the above experiments indicated that β2-agonist administration leads to decrease tissue adipose in animals' model HFD, we further examined the effect of formoterol and salbutamol administration on different organs such as liver, spleen, kidneys, lungs, and heart. Surprisingly, as shown in the presented (Figure 11a, 11b, 11c and 11d), there was no significant difference effect of β2-agonist administration on the tissue weights in different groups compared to the negative control. The histograms and statistical analysis clearly show 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 the tissue weights of the studied groups.

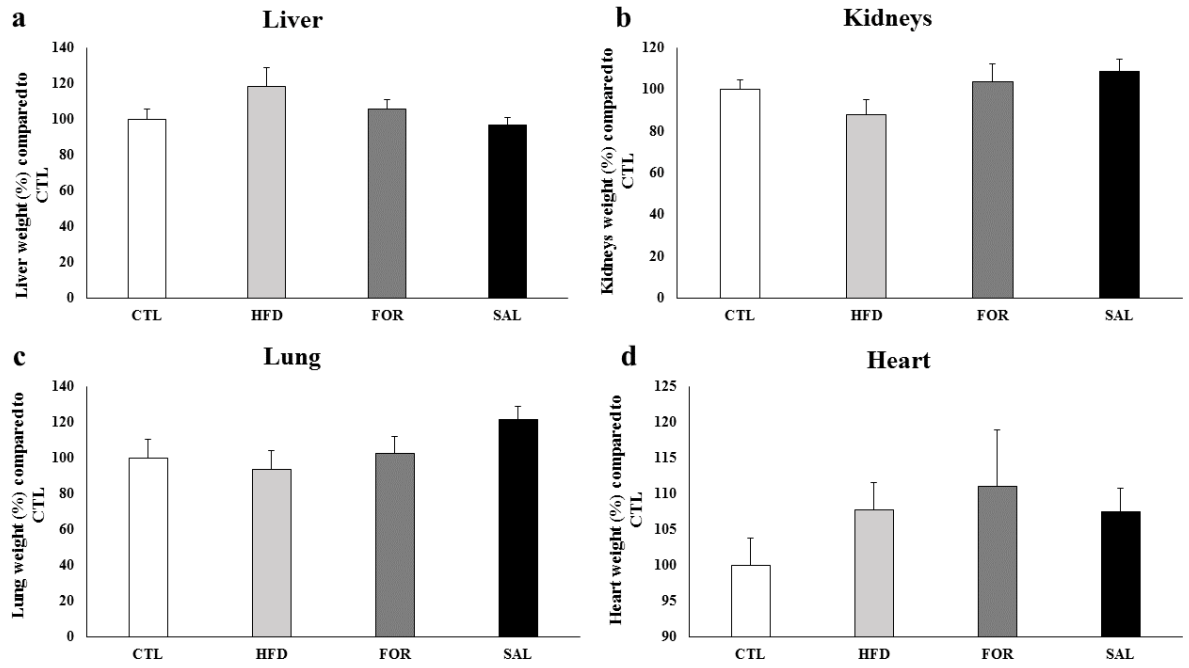


Figure 11: Histograms depicting the percentage weight of the liver, kidneys, lungs, and heart in different groups (CTL: Control, HFD: High Fat Diet, FOR: High Fat Diet Formoterol, SAL: High Fat Diet Salbutamol) relative to the control group (CTL: Control). Values are means ± SE (n=6-8) relative to control group: One-way ANOVA and Bonferroni as post hoc test.

III.4. β2-agonist administration leads to decrease liver enzymes in animals HFD

Aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), gamma-glutamyl transferase (ɣGT) and other markers of liver injury may be useful surrogate measures of NAFLD.

We have expected by spectrophometry the level of alanine aminotransferase (ALAT) in different groups. As expected, the level of ALAT in HFD treated with formoterol or salbutamol groups shows a substantial decrease respectively by 36% ($p<0.05$) and 42% ($p<0.05$) compared to HFD without treatment group (Table 8). Moreover, there is no significant difference of ALAT level between negative control and other groups (Table 8). The FOR group has levels around 75%, while the SAL group shows levels slightly below the control at around 90%. Similarly, we have also observed that aspartate aminotransferase (ASAT) is decreased in HFD treated with formoterol or salbutamol groups compared with non-treated HFD group (Table 8). As ALAT level, we didn't expect any difference of ASAT level between negative control groups with HFD treated or HFD alone. Surprisingly, we assessed the level of ɣGT in different groups. As shown in (Table 8), there is no significance difference of level ɣGT in different groups.

Table 8: Effect of β2-agonist administration on the liver enzymes. Values are means ± SE (n=6-8). $ P<0.05 relative to HFD: One-way ANOVA and Bonferroni as post hoc test.

	CTL	HFD	FOR	SAL
ASAT	100%	129,7	51,9	54,0
ALAT	100%	197,6	79,0	82,3
GAMMA-GT	100%	146,6	54,8	65,8

III.5 β2-agonist administration leads to decrease Triglyceride levels

The hallmark of NAFLD is triglyceride accumulation in the cytoplasm of hepatocytes. This arises from an imbalance between lipid acquisition (i.e., fatty acid uptake and de novo lipogenesis) and removal (i.e., mitochondrial fatty acid oxidation and export as a component of VLDL particles **(Kawano and Cohen 2013).**

To determine whether high fat diet induces steatosis through triglyceride over synthesis, we measured the rate of triglyceride in serum of different groups. The HFD group shows an increase of triglyceride level by approximately 60% compared to control group (Figure12). We don't find significant difference between HFD treated with formoterol and HFD group. However, triglyceride level is significantly ($p<0,05$) decreased in group HFD treated with salbutamol (Figure 12). Moreover, there is no significant difference on triglyceride level between negative control and HFD-treated with formoterol or salbutamol.

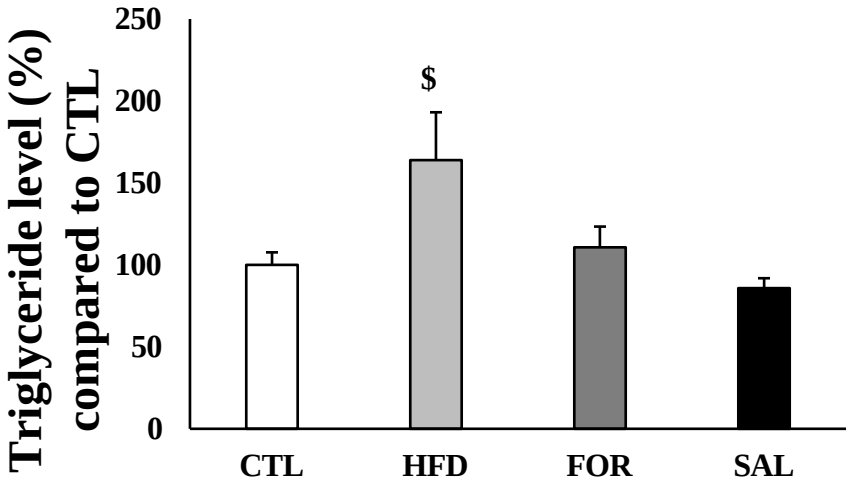


Figure 12: Histogram representation % of triglyceride levels in the different groups (CTL: Control, HFD: High Fat Diet, FOR: High Fat Diet Formoterol, SAL: High Fat Diet Salbutamol) relative to the control group (CTL: Control). Values are means ± SE (n=6-8). $P<0.05; $P<0.01; $$$P<0.001 relative to HFD group: One-way ANOVA and Bonferroni as post hoc test.

III.6 β2-agonist administration does not alter cholesterol level

Steatosis is considered the hepatic component of Metabolic syndrome (MetS) **(Flisiak-Jackiewicz and al. 2021).** Metabolic syndrome is characterized by hypertriglyceridemia and decreased High-Density Lipoprotein (HDL) cholesterol **(Eckel and al. 2010).** β2-agonist administration with formoterol or salbutamol does not alter total cholesterol level (Figure 13). We don't find the difference of total cholesterol level between groups CTL, HFD, HFD-FOR and HFD-SAL.

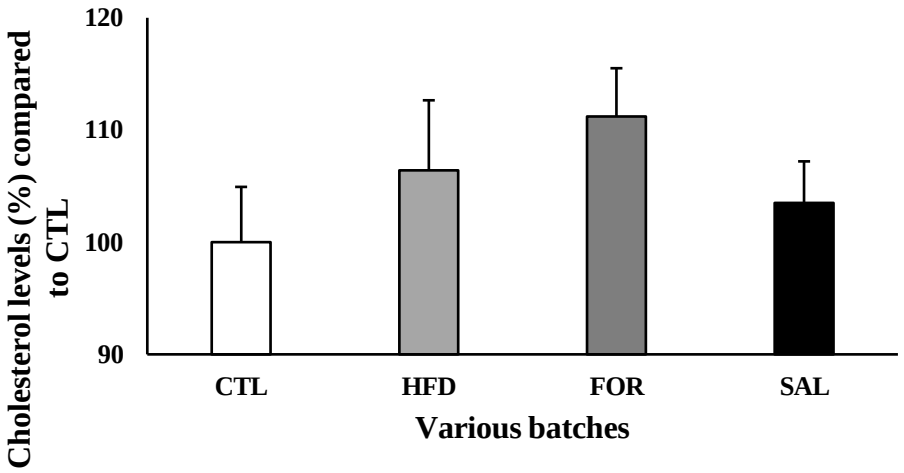


Figure 13: Histogram representation % of cholesterol levels in the different groups (CTL: Control, HFD: High Fat Diet, FOR: High Fat Diet Formoterol, SAL: High Fat Diet Salbutamol) relative to the control group (CTL: Control). Values are means ± SE (n=6-8) relative to CTL group: One-way ANOVA and Bonferroni as post hoc test.

III.7 β2-agonist administration does not alter glucose level

Steatosis is characterized by the accumulation of triglycerides in the liver, which increases insulin's inhibitory effect on the production of glucose and triglycerides by the liver **(Saltiel and Kahn 2001).** The results presented above indicate that β2-agonist administration leads to decrease Triglyceride level.

Surprisingly, β2-agonist administration with formoterol or salbutamol does not alter glucose level (Figure 14). The level of glucose is high in HFD (>30% compared to control). However, we don't find the difference of glucose level between groups negative control high fat diet, high fat diet treated with formoterol and high fat diet treated with salbutamol.

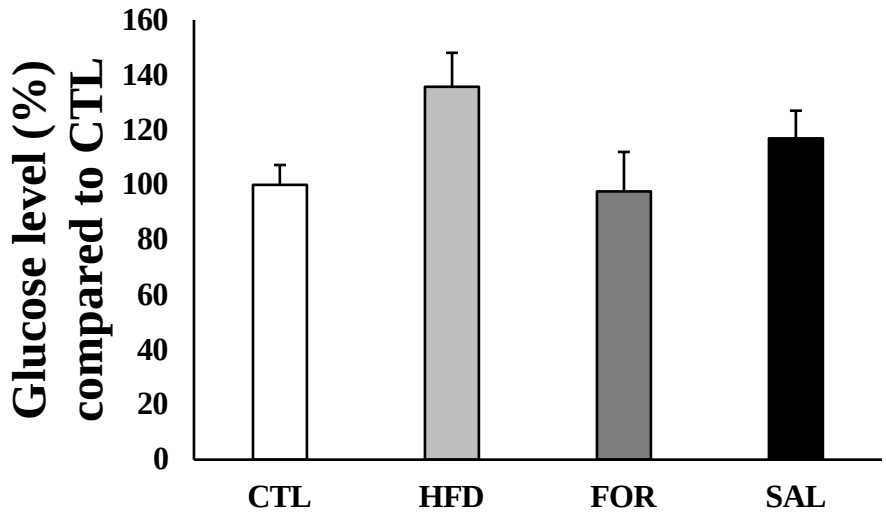


Figure 14: Histogram representation % of glucose levels in the different groups (CTL: Control, HFD: High Fat Diet, FOR: High Fat Diet Formoterol, SAL: High Fat Diet Salbutamol) relative to the control group (CTL: Control). Values are means ± SE (n=6-8) relative to CTL group: One-way ANOVA and Bonferroni as post hoc test.

III.8 β2-agonist administration leads to decrease malondialdehyde (MDA) activity.

According to the previously reported that oxidative stress seems to be one of the most important mechanisms leading to hepatic injury in NAFLD, playing a fundamental role in the progression from SS to NASH **(Spahis and al. 2017).**

As expected, MDA activity in HFD groups shows a substantial increase by 21 % ($p<0.05$) compared to negative control (Figure 15). However, we have observed that MDA activity is decreased in HFD treated with formoterol or salbutamol groups compared with non-treated HFD group, respectively by 36 % ($p<0.05$) and 32 % ($p<0.05$) compared to HFD. Moreover, there is no significant difference on MDA activity between negative control and HFD-treated with formoterol or salbutamol.

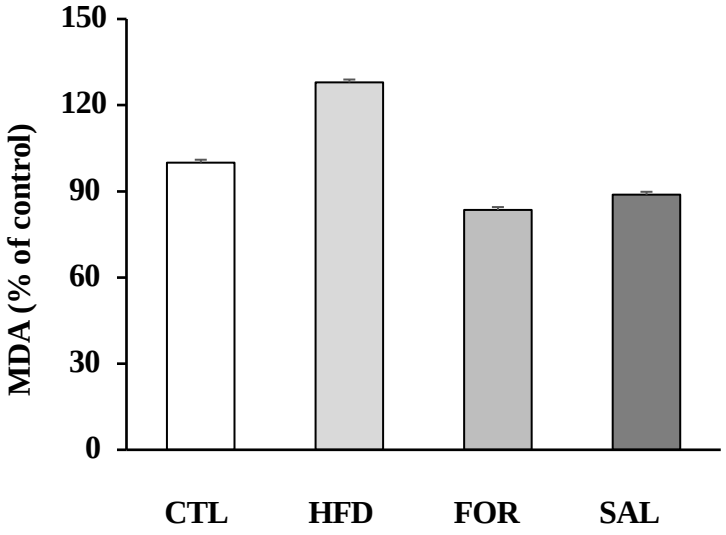


Figure 15: Histogram representation % of MDA activity in the different groups compared to control (CTL: Control, HFD: High Fat Diet, FOR: High Fat Diet Formoterol, SAL: High Fat Diet Salbutamol) relative to the control group (CTL: Control). Values are means ± SE (n=6-8) relative to CTL group: One-way ANOVA and Bonferroni as post hoc test.

IV. Discussion

The current study focuses on developing therapeutic interventions to reduce liver fat accumulation in non-alcoholic fatty liver disease (NAFLD). By administration of β2-agonist formoterol or salbutamol, we aimed to reduce the progression of hepatic steatosis. The results obtained demonstrate the preventive effects of β2-agonists, specifically formoterol or salbutamol, on several parameters: growth evolution and biochemical parameters. The analysis of animal growth showed no significant difference between the control (CTL) and high-fat diet (HFD) groups before treatment, indicating that the diet alone didn't significantly impact growth rates at this stage of development. Furthermore, the administration of formoterol or salbutamol didn't alter the growth of animals in either the control or HFD groups. These findings are an accordance with previous studies indicating that β2-agonists don't affect overall growth in the context of obesity-induced NAFLD **(Collins and al., 2011).**

Surprisingly, there were no significant effects on the weights of the liver, kidneys, lungs, and heart across different groups. Therefore, these results are consistent with Cartañà et al. 1994; Page et al. 2004 study, which reported that beta-2 agonists do not affect the weights of the kidneys and liver. However, other studies have found a slight decrease in liver weight after beta-2 agonist administration **(Choo and al. 1992; Reeds and al. 1986)**. This suggests that while β2-agonists effectively reduce adipose tissue, they may not significantly alter the weight of other major organs.

Despite the absence of significant effects on organ weights observed in our study, the impact of β2 agonists on adipose tissue is considerable which requires further analysis. Our results showed a significant increase in adipose tissue weight in the HFD group compared to the CTL group. However, administration of formoterol or salbutamol significantly reduced adipose tissue weight by 55% and 65%, respectively, compared to the HFD group. These results are consistent with other studies that have demonstrated the lipolytic effects of β2-agonists, which can reduce adipose tissue accumulation in obese models **(Hostrup and al., 2020; Lee and al., 2013)** Specifically, formoterol has been shown to increase resting energy expenditure and fat utilization, which suggests its potential for reducing adipose tissue accumulation **(Lee and al. 2013)**. Hostrup et al. have reported an increase in resting energy expenditure by 11-13% and fat oxidation by 23-38% in a dose-dependent manner **(Hostrup and al., 2020)**. The mechanism of this effect is described by Hostrup et al., 2020 as the β2-adrenergic stimulation by agonists such as salbutamol and formoterol triggers lipolysis in adipose tissue by activating hormone- sensitive lipase (HSL) and perilipin A via the cAMP/PKA pathway (**Hostrup and al., 2020**). Perilipin A, which protects lipid droplets, loses this function when phosphorylated, thus

allowing the translocation of HSL to the lipid droplets to hydrolyze triglycerides and diglycerides (**Hostrup and al., 2020**).

The study results demonstrate that a high-fat diet (HFD) significantly elevated the levels of hepatic enzymes such as AST (aspartate aminotransferase) and ALT (alanine aminotransferase), indicating liver damage associated with non-alcoholic fatty liver disease (NAFLD). Elevated AST and ALT levels are commonly used biomarkers for liver injury, as they are released into the bloodstream when liver cells are damaged **(Hoek 2004)**. In this work, we have demonstrated that treatment with β2-agonists, specifically formoterol (FOR) or salbutamol (SAL), significantly reduced the levels of these enzymes in animals' high fat diet. This reduction suggests that both formoterol or salbutamol leads to protective effects on the liver and can improve liver function in the context of HFD-induced liver damage. In this study, we found that AST levels significantly decreased in both HFD treated-formoterol group or HFD treated-salbutamol group compared to group high fat diet. These reductions were statistically significant, indicating that the treatments were effective in mitigating liver damage. Consistent with previous observations of AST results, ALT levels are drastically decreased with β2-agonists formoterol or salbutamol treatment compared to the HFD group. Further research is needed to fully elucidate the pathways through which these β2-agonists exert their beneficial effects on liver health. These reductions of livers enzymes, aminotransferases, confirmed the protective effect of the treatments on liver function. The findings suggest that formoterol and salbutamol may be effective therapeutic agents for improving liver function and reducing liver damage in NAFLD. Additionally, the study found no significant difference between the groups in terms of GAMMA-GT levels. Although the GAMMA-GT levels were lower in the HFD treated-formoterol group compared to the HFD group, this difference (p=0.06) did not reach statistical significance but may still be of interest. A previous study found that elevated GGT levels indicate liver damage but emphasized that it is less specific compared to ALAT and ASAT (**Kauppinen, 1984**), which may explain the lack of significant difference in our results regarding GAMMA-GT levels.

Given the crucial role of triglycerides in non-alcoholic fatty liver disease (NAFLD), our study focused on evaluating the impact of salbutamol on triglyceride levels in a high-fat diet model (Alves- Bezerra et Cohen 2017)**.** Our study showed that the HFD treated-salbutamol group had a significant reduction in triglyceride levels compared to the HFD group, aligning with **(Kalinovich and al. 2020)** who found that β2-agonists (clenbuterol) reduce triglyceride levels. Additionally, triglyceride levels in the HFD group increased compared to the CTL group. This

increase is consistent with other research indicating that triglyceride levels rise in cases of NAFLD **(Smiderle and al., 2021).** This is particularly relevant in the context of NAFLD, where triglyceride accumulation in the liver is a key factor **(Lanthier, 2020).** In contrast, glucose levels did not show significant differences between the groups, suggesting that β2-agonists primarily affect lipid metabolism rather than glucose metabolism. While we haven't observed a significant difference, there seems to be a trend of increased glucose levels in the HFD group compared to the others. It's possible that with a longer duration of the diet, the animals could develop insulin dependence, potentially reaching significance. This trend contrasts with the findings on cholesterol levels, where no significant differences were observed between the groups. This finding suggests that while β2-agonists may effectively reduce triglycerides and improve liver enzyme levels, their impact on cholesterol levels may be limited.

It well established that the augmented generation of ROS can induce lipid peroxidation leading to inflammation and fibrogenesis through the activation of HSC. Moreover, ROS inhibit hepatocytes' secretion of VLDL, inducing liver fat accumulation **(Spahis and al. 2017).** In addition, oxidative stress seems to be one of the most important mechanisms leading to hepatic injury in NAFLD, playing a fundamental role in the progression from S simple steatosis to NASH **(Spahis and al. 2017)**. MDA count was higher in the HFD group when paralleled to controls. Moreover, MDA count is decreased in HFD-treated with both formoterol or salbutamol. Increase in MDA levels corresponding to increased grades stressed upon increase in lipid peroxidation and progression of the disease. This finding must be validated by other targets implicated in oxidative stress. In this context, previous report indicates that formoterol Acting via β2-Adrenoreceptor Restores Mitochondrial Dysfunction Caused by Parkinson's Disease-Related UQCRC1 Mutation and Improves Mitochondrial Homeostasis **(Kalinovich and al. 2020).**

V. Conclusion

Our study demonstrates that formoterol ors albutamol, as β2-agonists, can significantly reduce adipose tissue weight, improve liver enzyme levels, and decrease triglyceride levels in a high-fat diet-induced non-alcoholic fatty liver disease (NAFLD) model. These results suggest that β2-agonists could offer an innovative therapeutic approach for the management of non-alcoholic fatty liver disease by directly targeting the cellular and molecular processes underlying the pathogenesis of the disease. To further these findings, future studies should include an in-depth histological analysis using histomorphometric techniques to quantify changes in liver tissue structure. Additionally, an evaluation of molecular markers such as the quantification of β2-adrenergic receptor expression and key enzymes involved in lipid and energy metabolism is crucial. It is important to characterize the functional effects of β2-agonists on intracellular signaling pathways. These approaches will provide a promising basis for the development of effective treatment strategies against non-alcoholic fatty liver disease.

Abstract:

Non-alcoholic fatty liver disease (NAFLD) is a common condition associated with obesity and metabolic syndrome, affecting approximately 25% of the global population. Our study explores the development of an effective treatment for non-alcoholic fatty liver disease (NAFLD), a condition for which there is currently no specific treatment. We studied the use of β2-agonists, such as formoterol, as a potential treatment for NAFLD, targeting lipid metabolism and inflammation. Rats fed a high-fat diet were divided into four groups: negative control, high-fat diet, treatment with formoterol, and treatment with salbutamol. Treatments were administered daily by injection for two weeks. Tissue and serum samples were analyzed to assess treatment effects. Treatment with formoterol and salbutamol significantly reduced adipose tissue weight and levels of liver enzymes (AST, ALT, GAMMA-GT), suggesting hepatic protection. β2-agonists show promising therapeutic potential for NAFLD, reducing hepatic fat accumulation and improving liver function. These findings offer prospects for future treatments targeting lipid metabolism and inflammation. Further research is needed to confirm these benefits.
Key words : Non-alcoholic fatty liver disease, β2-agonistes, formoterol, salbutamol .

Résumé:

la stéatose hépatique non-alcoolique (NAFLD), une maladie courante liée à l'obésité et au syndrome métabolique, affectant environ 25 % de la population mondiale. Notre étude explore le développement d'un traitement efficace pour la stéatose hépatique non-alcoolique (NAFLD), une maladie pour laquelle il n'existe actuellement aucun traitement spécifique. Nous avons étudié l'utilisation des β2-agonistes, tels que le formotérol, comme traitement potentiel pour la NAFLD, ciblant le métabolisme lipidique et l'inflammation. Des rats nourris avec un régime riche en graisses ont été divisés en quatre groupes : contrôle négatif, régime riche en graisses, traitement avec formotérol, et traitement avec salbutamol. Les traitements étaient administrés quotidiennement par injection pendant deux semaines. Les échantillons de tissus et de sérum ont été analysés pour évaluer les effets des traitements. Le traitement avec formotérol et salbutamol a réduit significativement le poids du tissu adipeux et les niveaux des enzymes hépatiques (AST, ALT, GAMMA-GT), suggérant une protection hépatique. Les β2-agonistes montrent un potentiel thérapeutique prometteur pour la NAFLD, réduisant l'accumulation de graisse hépatique et améliorant la fonction hépatique. Ces résultats offrent des perspectives pour des traitements futurs ciblant le métabolisme lipidique et l'inflammation. Des recherches supplémentaires sont nécessaires pour confirmer ces bénéfices.
Mots clé: Stéatose hépatique non-alcoolique , β2-agonistes, Formotérol, salbutamol

ملخص:

الستياتوز الدهني غير الكحولي)NAFLD(هو مرض شائع يرتبط بالسمنة ومتالزمة أليض، يؤثر على حوالي من سكان العالم. تستكشف دراستنا تطوير عالج فعّال لمرض الكبد الدهني غير الكحولي (NAFLD) ، وهو حاليًا ال يوجد له عالج محدد. لقد درسنا استخدام مثبطات β2 مثل الفورموتيرول، كعالج محتمل لـ(NAFLD)، مستهدفين أيض الدهون واللتهاب. تم تقسيم الجرذان التي تغذت على نظام غذائي غني بالدهون إلى أربع مجموعات مجموعة شاهدة، مجموعة نظام غذائي غني بالدهون، مجموعة معالجة بالفورموتيرول، و، مجموعة معالجة بالسالبوتامول. تم إعطاء العالج يوميًا بالحقن لمدة أسبوعين. تم تحليل عينات أألنسجة والمصل لتقييم تأثيرات العالجات. أظهر عالج الفورموتيرول والسالبوتامول تقلي ًال ملحو ًظا في وزن أألنسجة الدهنية ومستويات إلنزيمات الكبدية)AST, ALT, GAMMA-GT(مما يشير إلى حماية الكبد. تظهر مثبطات β2 إمكانات عالجية واعدة ل(NAFLD) ، بتقليل تراكم الدهون في الكبد وتحسين وظيفة الكبد. توفر هذه النتائج آفاقًا لعالجات مستقبلية تستهدف أيض الدهون واللتهاب. هناك حاجة إلى بحوث إضافية لتأكيد هذه الفوائد...
الكلمات المفتاحية: الستياتوز الدهني،غير الكحولي, مثبطات β2 , الفورموتيرول ,السالبوتامول

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