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Department of Biology

Evaluation of some physiological and biochemical Markers in patients with Diabetic Mellitus Type 2 associated with Non-alcoholic fatty liver disease

A thesis

Submitted to the Council of the College of Science, University of Kerbala, in Partial Fulfilment of the Requirements for Master Degree in Biology

Written by:

Iman Falih Hadi AL-Ghoraby

B.Sc. Biology, University of Kerbala (2017)

Supervised by:

Asst.prof.Dr. Ibtisam A.Al-ALl

College of Science

University of kerbala

Asst.prof.Dr. Hameed Hussein Al-jameel

College of Medicine

University of kerbala

2025 A.D

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صَدقَ اللهُ العلِيُّ العَظيم

سورة المجادلة : الآية﴿ ١١﴾

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We are the examining committee, certify that we have read this thesis entitled"**Evaluation of some physiological and biochemical Markers in patients with Diabetic Mellitus Type 2 associated with Non-alcoholic fatty liver disease** "and examined the student" **Eman Falih Hadi**" in its contents, and that according to our opinion, it is accepted as a thesis for the degree of Masters of science in Biology with estimation (Excellent).

Chairperson of the discussion committee

Signature:

Dr. Jasem Hanoon Hashim Alawadi

Scientific degree: professor

Address: College of Science-University of Kerbala

Date: / /2025

Committee member

Signature:

Name: Dr. Ekhlas Abid Hamza

Scientific degree: Professor

Address: College of Veterinary Medicine -University Al-Qasim Green

Date: / / 2025

Committee member

Signature:

Name: Dr. Muayad Naeem Kareem

Scientific degree: Lecturer

address: College of science – University of kerbala

Date: / / 2025

Member and supervisor

Signature:

Name: Dr. Ibtisam A. Al-ALI

Scientific degree: Asst.professor

Address: College of science – University of kerbala

Date: / / 2025

Member and supervisor

Signature:

Name:Dr.Hameed Hussein Al- jameel

Scientific degree: Asst.professor

Address: College of Medicine- University of kerbala

Date: / / 2025

Approval for the council of college

Signature:

Name: Dr. Hassan Jameel Jawad

Scientific degree: Professor

Address: Dean of College of Science- University of kerbala

Date: / / 2025

Supervisor certification

We certify that this M.Sc. thesis titled:

Evaluation of some Physiological and biochemical Markers in patients with Diabetic Mellitus Type 2 associated with Non-alcoholic fatty liver disease

was carried out under our supervision at the College of Science / University of Kerbela, as a partial fulfillment of the requirement for the degree of Masters of science in Biology.

Supervised.

Signature:

Asst.prof.Dr.Ibtisam Abbas Nasser

College of Science

University of kerbala

Date: / /

Signature:

Asst.prof.Dr.Hameed Hussien Tawafan

College of Medicine

University of kerbala

Date: / /

Chairman of Biology department

In view of the available recommendations, I forward this dissertation for debate by examining committee.

Signature:

Lecturer

Dr. Muyaad Naeem Kareem

College of Science –University of kerbala

Date: / /

Dedication

I dedicate the fruits of my efforts to the first teacher, the Prophet Muhammad (peace be upon him and his family)

To my master and lord Imam Ali ibn Abi Talib and his infallible sons (peace be upon him and them)

To my master and lord Imam Al-Hujjah Al-Mahdi (may Allah hasten his reappearance)

To my lady and mistress Umm Al-Baneen (peace be upon her)

To the souls of my mother and father who did everything they could without limits so that I could reach this stage and words of thanks and gratitude cannot do them justice

To the pure souls who left us and their prayers still accompany us to my grandfathers, may Allah have mercy on them to my beloved brother and sisters and the secret of my strength

To my husband and companion and my daughter Hadiya Al-Rahman and all my husband's family who were and still are like true family for their encouragement and continuous support

To all students of knowledge and learning and to all friends and to all those who cannot buy a book. I would also like to thank my fellow students I dedicate the fruits of my efforts to them, especially my dear friends Taiba, Zeina, Fatima, and Hanin, for their support and advice.

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Iman Falih

Summary

Metabolic diseases, including non-alcoholic fatty liver disease(NAFLD), metabolic syndrome, and type 2 diabetes, have widespread health impacts. Early identification and treatment of metabolic syndrome and related fatty liver disease can improve metabolic markers and prevent liver and cardiovascular complications.

The current study included 108 blood samples whom aged 35 to 77 years old. From October 2023 to April 2024, 70 blood samples were collected from patients with type 2 diabetes and fatty liver disease, 30 males and 40 females from the consulting clinics of Al-Hindiyah General Hospital and Imam Al-Hassan Hospital in Karbala Governorate. The control group included 38 healthy 16 males and 22 females, blood samples of the same age and gender. (Alcoholism, malignancy, gastrointestinal surgery, and liver disorders such as chronic hepatitis C, autoimmune liver disease, and Wilson's disease that may cause fatty liver were excluded). Blood was drawn from veins post 8-12 hours of fasting after agreement with patients. Glucose, insulin serum, Homeostasis Model Assessment of Insulin Resistance(HOMA-IR), Alanine Transaminase(ALT), Aspartate Transaminase (AST), triglycerides(TG), cholesterol, Low density lipoprotein (LDL), Very low density lipoprotein (VLDL), High density lipoprotein (HDL), Platelets(PLT), vitamin D, albumin, and uric acid levels were examined, and Asprosin and Adiponectin serum levels were measured. The mean alanine aminotransferase levels (22.21 ±10.1 U/L), in the patient group were significant higher than the mean level of the control group (7.63±3.29 U/L). The mean aspartate aminotransferase in the patient group was (31.46±14.25 U/L), significantly higher than the control group (22.12±5.23 U/L). The diabetic group with fatty liver had significantly higher mean cholesterol (212.81±101.3mg/dl) and triglyceride levels (279.96±124.76mg/dl) than the control group (172.78±77.52mg/dl and 171.57±46.21 mg/dl respectively).

The patients had significantly higher insulin levels and insulin resistance than the controls indicating metabolic abnormalities in type 2 diabetes, insulin resistance leads to increased blood sugar and the body's inability to use insulin effectively, which increases metabolic disturbances. As for Asprosin and Adiponectin Hormones, the patient group had a significantly higher mean level (150.56 ± 61.6 ng/ml) than the control group (127.64±46.9 ng/ml), and patients had higher Adiponectin (5.27±2.6 µg/ml) than the control group (4.18±2.08 µg/ml). Liver enzymes, especially alanine, were non-significant moderate negatively correlation with Asprosin and weak with Adiponectin. The data indicates a strong correlation between Asprosin, low-density lipoprotein, and insulin resistance. Lipid profile parameters indicated modest to moderate positive relationships between cholesterol, triglycerides, high-density lipoprotein, very low-density lipoprotein and Asprosin, with a negative correlation observed only for low-density lipoprotein.

Age, Body mass index(BMI), and disease duration had significant relationships and associations with the biomarkers. The results showed that these associations may be weak, moderate, or strong. Liver enzymes have a negative relationship with age, lipid profile has a positive relationship with age, insulin, sugar and liver fibrosis index have a positive relationship with age and body mass.

The study concludes that insulin resistance and elevated Asprosin levels can be used as biomarkers to predict type 2 diabetes and fatty liver, as well as a non-invasive test for metabolic-related NAFLD, since the basis of NAFLD is insulin resistance. Adiponectin has superior effectiveness compared to Asprosin in distinguishing between sick and healthy persons.

List of Contents

List of Tables

List of Figures

List of Abbreviation

BMI	Body mass index
DM	Diabetes mellitus
FFA	Free fatty acid

GSH	Glutathione
LDL	Low density lipoprotein
HDH	High density lipoprotein
TG	Triglyceride
ROS	Reactive oxygen species
T2DM	Type 2 diabetes mellitus
VLDL	Very low density lipoprotein
NAFLD	Nonalcoholic fatty liver disease
MetS	Metabolic syndrome
ALT	Alanine Transaminase
AST	Aspartate Transaminase
HOMA IR	Homeostasis Model Assessment of Insulin Resistance
MAFLD	Metabolic Dysfunction-Associated Fatty Liver Disease
NASH	Non-alcoholic steatohepatitis
HCC	Hepatocellular carcinoma
LDs	Lipid droplets
ALD	Alcoholic liver disease

TNF-α	Tumor necrosis factor alpha
IL-1β	Interleukin-1Beta
IL-6	Interleukin-6
IR	Insulin resistance
DNL	de novo lipogenesis
RNS	Reactive nitrogen species
HSCs	hepatic stellate cells
CAP	controlled attenuation parameter
CT	Computed Tomography
MRI	Magnetic Resonance Imaging
MASLD	Metabolic dysfunction-associated steatosis liver disease
APRI	AST to platelet Ratio Index

Chapter One
Introduction

1.1. Introduction

Fatty liver disease, caused by fat accumulation in the liver, is often linked to metabolic syndromes such as obesity, diabetes, hypertension, and dyslipidemia. Fatty liver disease may be classified into two types: Nonalcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease(AFLD). While most fatty liver patients have no symptoms, a few may develop end-stage liver disease, liver cancer, or pulmonary or cardiovascular disorders (Papadimitriou *et al*.,2024).

The bidirectional epidemiological and pathophysiological link between NAFLD and metabolic disorders led to an update of the terminology and the diagnostic criteria. For this purpose, Metabolic Dysfunction-Associated Fatty Liver Disease' (MAFLD) has been proposed as the more appropriate term because it better reflects the pathogenetic basis of the disease and allows a more comprehensive and standardized approach to patient management (Fouad *et al*., 2020). This term refers to a condition characterized by the presence of hepatic steatosis (detected by imaging techniques, blood biomarkers/scores, or liver histology) in conjunction with type 2 diabetes mellitus (T2DM) and overweight/obesity, regardless of alcohol consumption or exclusion of other etiologies of chronic liver disease, which were previously required for the diagnosis of NAFLD (Kawaguchi *et al*., 2022).

People with type 2 diabetes are more likely to develop nonalcoholic fatty liver disease (NAFLD). However, the disease's significant frequency and clinical consequences are just now becoming more well-recognized, and the two conditions are linked to faster development of cirrhosis (Lomonaco *et al*., 2021). According to reports, the global incidence of NAFLD in persons with type 2 diabetes ranges from (60 to 86%) (Riazi *et al*.,2022).

Recent clinical practice recommendations for NAFLD (Rinella, *et al*., 2023) urge that all persons with type 2 diabetes be screened using the ((Fibrosis-4; (FIB-4) score)). According to studies conducted utilizing transient electrography or Magnetic Resonance Imaging MRI-based approaches, the prevalence of NAFLD exceeds 70%. Even more troubling, almost half of all individuals with concomitant type 2 diabetes and NAFLD have steatohepatitis, and one in every six has mild to severe fibrosis (Castera *et al*., 2023). However, it appears that numerous causes are at play. These include genetic factors that may influence insulin action or hepatocyte lipid metabolism, as well as a web of acquired factors driven by insulin resistance, such as glucotoxicity and lipotoxicity; other lipids are present in a variety of lipoprotein fractions, including low-density lipoproteins (LDL) and HDL, which are linked to dysfunctional adipose tissue and ectopic fat accumulation in the liver in people with obesity and diabetes (Polyzos *et al*., 2019).

Insulin resistance is crucial since it induces changes in glucose and lipid metabolism, intracellular inflammatory pathways, mitochondrial dysfunction, and endoplasmic reticulum stress. A consistent finding among different causes is more severe hyperinsulinemia, atherogenic dyslipidemia, and insulin resistance in adipose tissue, the liver, and the muscle. Worldwide, according to research, NAFLD is predicted to affect 23-25% of the population, which is expected to rise in the following decades (Lazarus *et al*., 2022). Worldwide, NAFLD affects different regions at different rates; for example, 32% of the population lives with the disease in the Middle East, 30% in South America, 24% in Europe and North America, and 13% in Africa (Mitra *et al*., 2020). Furthermore, 20% of those with NAFLD had NASH verified as a prevalence (Lazarus *et al*., 2022). On the other hand, there are discrepancies within epidemiological research on topics like the number of comprehensive studies conducted in poor nations, the diagnostic methods used for

nonalcoholic fatty liver disease (NAFLD), and the use of ultrasonography or serum markers. There may be differences in the worldwide and regional incidence of nonalcoholic fatty liver disease (NAFLD). However, those with type 2 diabetes and a high body mass index (BMI) have a disproportionately heavy load of the illness (Ye *et al*., 2020).

1.2. Aim of the study

The current study aims to investigate the physiological and biochemical factors contributing to the progression of Non-Alcoholic Fatty Liver in patients with type 2 diabetes. This will be achieved through the following objectives:

1- Determine the biochemical markers (Glucose, Insulin, PLT, ALT, AST, HOMA-IR, Vitamin D ،Uric acid serum level and lipid profile (TG, LDL, VLDL and HDL) among NAFLD patients and type 2 diabetes and their possible relationship with the degrees of fatty liver.

2- Evaluating the markers measured across patients and healthy individuals to understand and investigate the relationship among the original markers (Adiponectin, Asprosin Hormones, and liver damage index FIB-4).

3- Study the sensitivity of some markers (Adiponectin, Asprosin Hormones) to wards detecting the disease in people

Chapter Two

Literatures Review

2.1. Nonalcoholic Fatty Liver Disease

NAFLD is defined as excessive fat (steatosis) in the liver when no secondary cause of hepatic steatosis can be found. NAFLD is the liver manifestation of metabolic syndrome (a cluster of conditions including abdominal obesity, impaired glucose regulation or diabetes, hypertension, hypercholesterolemia, and hypertriglyceridemia- all of which are associated with increased cardiovascular risk). The NAFLD spectrum encompasses isolated steatosis (non-alcoholic fatty liver (NAFL), steatosis with inflammation non-alcoholic steatohepatitis (NASH), ballooning associated with fibrosis, cirrhosis (irreversible liver scarring) and hepatocellular carcinoma (HCC), a primary liver tumor that usually develops in the setting of chronic liver (Li *et al*., 2022). NAFLD is the most common chronic liver disease, impacting over 1 billion individuals globally (Golabi et al., 2023).

The range of fatty liver illnesses not caused by alcohol consumption, viral, autoimmune, drug-induced, or genetic, causes, previously known as nonalcoholic fatty liver disease (NAFLD), has been renamed metabolic (dysfunction) associated fatty liver disease (MAFLD) (Eslam *et al*., 2020)By a continuous line of ideas, this innovative nomenclature appropriately highlights the condition's "positive" aspects, namely its close relationship with metabolic diseases, rather than designating it for what it is not (Fouad *et al*., 2020).

2.2. Pathophysiology of NAFLD

Many factors interact to cause MAFLD and its progression. These include genetics (specific polymorphisms), environment (bad food, insufficient exercise), epigenetic modifications, endocrine disruptors, obesity (insulin resistance, adipokine dysregulation), lipotoxicity, stress on the endoplasmic reticulum, oxidative stress, and a dysbiosis of the gut microbiota (Juanola *et al*.,2021).

The primary route implicated in NAFLD development is the influx of free fatty acids (FFAs) and triglyceride (TG) buildup in hepatocytes, which creates a lipotoxic environment inside liver cells and disrupts normal lipid homeostasis. (Geng *et al*., 2021). Extra liver fat appears harmless at first because it primarily contains TG and cholesterol esters packed by a phospholipid monolayer in the form of lipid droplets (LDs). However, some hazardous lipid metabolites and intermediates are formed during the digestion of these LDs, affecting cell homeostasis by creating a lipotoxic milieu. Such a cellular milieu stresses the cell and stimulates inflammatory responses, promoting fibrogenesis in the hepatic tissue and acting as a driving force for disease progression (Fabregat *et al*., 2018).

Diet can cause NASH by affecting hepatic lipid buildup, antioxidant activity, postprandial triglyceride metabolism, and insulin sensitivity. Diet can impact hepatic lipid accumulation by affecting the connection between the liver, adipose tissue, and gut, regardless of energy consumption. Recent research found that 15% of liver fat comes from food, which may increase if fat consumption surpasses 30% of daily energy demands (Estruch *et al*., 2018). Saturated fatty acids disrupt mitochondrial function, impairing phospholipid metabolism and insulin resistance, altering respiratory chain activity, increased ROS production and apoptosis (Meex and Blaak, 2021). Modifying one's lifestyle and implementing weight loss methods play an essential part in treating MAFLD. According to many studies, the amount of fat inside the liver may be affected by following a low-calorie diet. Consumption of high-quality foods is more essential than calorie consumption alone. The ideal diet is low in fat (particularly saturated fat), high in fruit and vegetables, and has a low glycemic index. Patients with MAFLD are encouraged to follow a diet similar to the Mediterranean diet, which is considered the gold standard (Eslam *et al*., 2020). The ketogenic diet also helps people with MAFLD who have insulin resistance.

Concerning the safety and consequences in the long run, however, further research is required (Luukkonen *et al*., 2020).

Aerobic and resistance training are also essential to MAFLD therapy (Lessiani *et al*., 2016). Sarcopenia, the gradual loss of lean body mass, is a concern for overweight and obese patients with MAFLD who are on restrictive diets to lose weight. Resistance training is recommended in such a patient to counteract the effects of calorie restriction and promote more rapid fat loss while maintaining muscle mass. Overweight individuals have a more challenging time tolerating aerobic exercise, which lowers their compliance and efficacy (Niederseer *et al*., 2021).

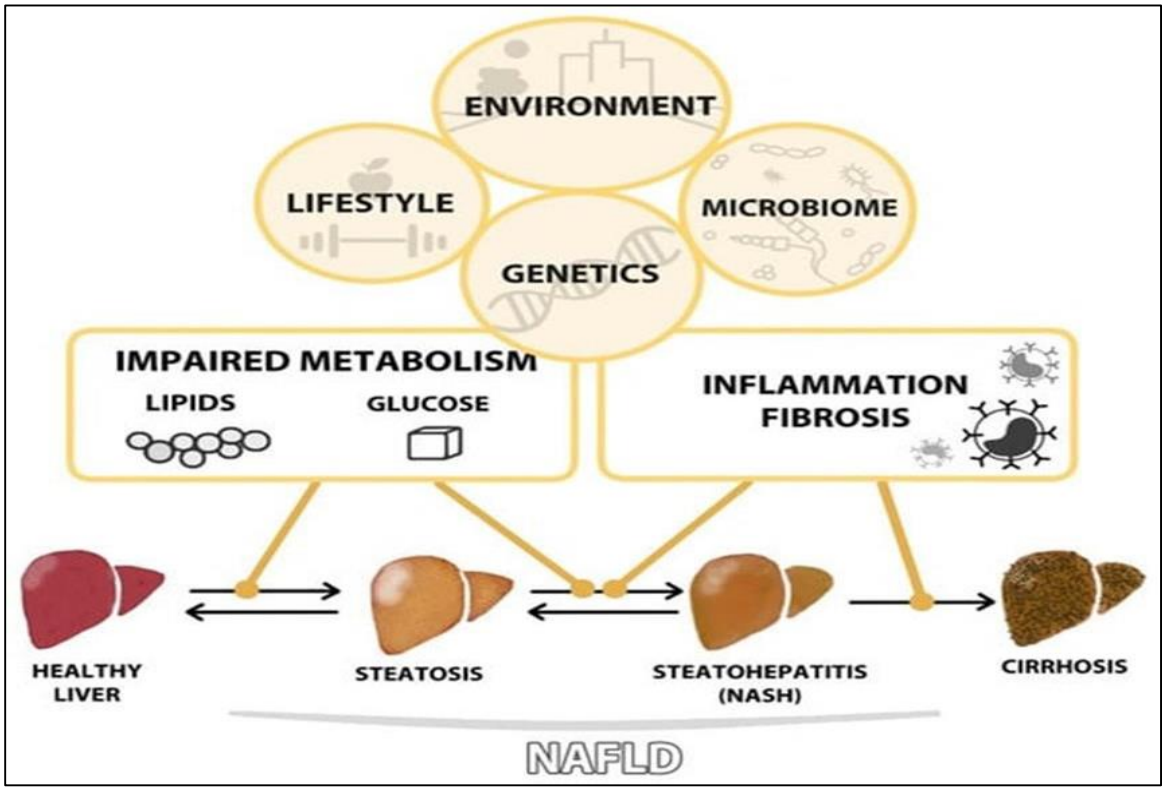

Figure (2.1) Factors contributing to the development of the disease non-alcoholic fatty liver disease (Younossi *et al* ., 2018)

2.3. Relationship between diabetic and liver disease

According to the World Health Organization (WHO), diabetes is a set of metabolic illnesses characterized by persistent hyperglycemia, which can cause damage to numerous organs particularly the eyes, kidneys, nerves, heart, and blood vessels, finally leading to malfunction (Sarkar *et al*.,2019). Diabetes has been classified into two forms, among others: type 1 diabetes (T1D) and type 2 diabetes (T2D). This classification system differs between patients with T1D and T2D based on various factors such as age at disease onset, excessive weight, insulin resistance (IR), metabolic syndrome (MS), degree of loss of pancreatic β-cell function, presence of specific autoantibodies associated with β-cell destruction, presence of a systematic subclinical inflammatory state, concentration of C-peptide in the blood and requirement for insulin treatment(Leslie *et al*., 2016). Known as a chronic metabolic autoimmune illness, T1D is characterized by immune cells infiltrating the pancreatic islets of Langerhans which eventually destroys the β-cells that produce insulin(Redondo *et al*., 2022). Type 2 diabetes is a chronic metabolic illness defined by high blood glucose levels caused by insulin resistance in the body's cells, as well as the pancreas' relative weakness in insulin release. This illness disrupts the control of glucose and fat metabolism and is frequently linked to obesity and an unhealthy lifestyle as the disease progresses. Type 2 diabetes promotes the development of NAFLD via a variety of pathways. Insulin resistance and obesity cause fat buildup in the liver (steatosis), which leads to hepatitis (NASH) and tissue fibrosis. Metabolic abnormalities, as well as the metabolic syndrome, increase the likelihood of acquiring the illness(Younossi *et al*., 2018).

T2D is the most important clinical predictor of negative outcomes such as advanced liver fibrosis and mortality, and it is the main risk factor for both Metabolic dysfunction-associated steatotic liver disease (MASLD) and Metabolic dysfunction-associated steatohepatitis (MASH). Because of their same pathophysiology, which includes insulin resistance, inflammation, and dyslipidemia, T2D and MASLD frequently coexist and have a reciprocal connection in which each disease makes the other worse(Lonardo *et al*., 2016).

2.4. Epidemiology of NAFLD in Type 2 Diabetes

Nonalcoholic fatty liver disease (NAFLD), recently renamed metabolic dysfunction-associated fatty liver disease (MAFLD) (Eslam *et al*., 2020) or metabolic dysfunction-associated steatotic liver disease (MASLD) (Rinella *et al*., 2023), represents by far the most common chronic liver condition worldwide. Recent data showed that approximately one in three people from the general adult population (Ciardullo *et al*., 2021; Ciardullo *et al*., 2022). and one in four adolescents (Asero *et al*., 2023) is affected, making it one of the most common non-communicable diseases. Its development and progression are tightly linked with metabolic dysregulation and insulin resistance. It is therefore not surprising that its prevalence is even higher in patients with type 2 diabetes (T2D), where it reaches 60–75% (Younossi *et al*., 2019; Lomonaco *et al*., 2021). Several studies have demonstrated the strong bidirectional relationship between NAFLD and T2D. On the one hand, NAFLD increases the risk of developing T2D among non-affected individuals (Mantovani *et al*., 2022) and the risk of micro- and macro-vascular complications in patients with previous T2D (Huang *et al*., 2023); on the other, patients with diabetes tend to progress faster to its more advanced forms including nonalcoholic steatohepatitis (NASH, MASH), advanced liver fibrosis, cirrhosis, and

hepatocellular carcinoma (Kwok *et al*., 2015). In a recent study in patients with NAFLD/MASLD studied with paired liver biopsies, even after adjustment for potential confounders, the presence of T2D was associated with a 70% increase in the relative risk of fibrosis progression (Castera *et al*., 2023). While previously considered a relatively uncommon finding, recent studies performed in unselected patients with T2D found that 15–38% of patients might have advanced liver fibrosis or cirrhosis (F3–F4).

Importantly, the degree of liver fibrosis is the most important histologic predictor of future development of liver-related events, as shown by several cohort studies and meta-analyses (Dulai *et al*., 2017). The higher disease prevalence and the faster rate of progression account for the ~three times higher risk of dying from liver disease shown in patients with T2D compared with age- and sex-matched controls (Zoppini *et al*., 2014). Nonetheless, awareness of this condition and its potential prognostic implications is limited both among affected individuals (Ciardullo *et al*., 2021) and among healthcare professionals (Polanco-Briceno *et al*., 2016).

2.5. Gut microbiota alterations and NAFLD

The human gut is one of the most varied ecosystems, with around 100 trillion bacteria (roughly 1.5 kg in total weight). The gut microbiota plays a vital role in maintaining the host's metabolism, physiology, nutrition, and immune-related functions, such as nutrient harvesting, energy regulation, vitamin synthesis, fermentation of non-digestible fibers, bile acid metabolism, and inflammatory response modulation (Martín-Mateos and Albillos, 2021). Recent studies have shown that compared to healthy persons, those with NAFLD/NASH have drastically different gut microbiota regarding phylum, class, family, and genus (Aron-

Wisnewsky *et al*., 2020). (Gomez-Perez *et al*.,2023) also found that individuals with various levels of liver fibrosis had different gut microbiota abundance.

Disruption of such host-microbe interaction and harmony leads to a range of chronic illnesses, including alcoholic liver disease (ALD) and NAFLD. The hepatic portal vein connects the liver to the gut physically and physiologically. This link allows for bidirectional communication between the liver and its byproducts and the gut microbiota and its metabolites (Albillos *et al*., 2020).

According to various pre-clinical models, a high fat/calorie diet alters the gut microbiome, causing dysbiosis, which successively ruptures the intestinal barrier integrity, allowing the microbial and its metabolite to translocate to the liver, resulting in high-end toxin exposure and hepatocyte injury. The liver is a regenerative organ that responds well to adequate treatment (So *et al*., 2019). However, persistent insult to the hepatocytes occurs when the liver is in its most susceptible state at subclinical pathological levels, such as fat buildup, resulting in severe inflammation and important physiological abnormalities. This was further validated in retrospective clinical research that linked gut dysbiosis with the intestinal barrier and the development of NAFLD (Kobayashi *et al*.,2022).

2.6. The hepatic Nero-inflammatory process in NAFLD

NAFLD is a proinflammatory disorder, and inflammation plays a key role in defining the severity of the disease and its associated consequences, According to(Bessone et al., 2019) many physiologically active factors impact the development of NAFLD. These factors include cytokines that promote inflammation, such as TNFα, IL-1β, and IL-6, and molecules that inhibit inflammation, such as adipokines and adiponectin. Obesity, IR, and related inflammatory diseases are believed to be linked via these biomarkers. The presence of proinflammatory cytokines in the liver

may lead to many histological changes, such as necrosis and the death of hepatocytes, the migration of neutrophils, the activation of stellate cells in the liver, and the development of Mallory bodies. Additionally, macrophages in adipocyte tissue help generate inflammatory cytokines, and being overweight causes macrophages to change their phenotype from anti-inflammatory M2 to pro-inflammatory M1.

Essential for the normal physiological functioning of the liver, these mediators are produced by all cell types in the liver (Ozougwu, 2017).Inflammation, cell death, cholestasis, and fibrosis in the liver are caused by cytokines. The regrowth of damaged liver tissue is another function of these cells. A critical component of liver injury is the generation of proinflammatory cytokines, such as IL-6 and TNF-α. Both of these cytokines have a multiplicative effect on cytokine production, which in turn may attract more inflammatory cells and kick off the liver's healing process (Arra and Abu-Amer., 2023).

2.7. Insulin resistance causes steatosis

Steatosis is caused by Insulin Resistance At least two out of three people with type 2 diabetes who are overweight or obese will develop hepatic steatosis, according to research that used transient elastography or Magnetic Resonance Imaging (MRI) based approaches (Stefan and Cusi., 2022). Although the exact processes are still not fully understood, doctors may have a better grasp of insulin resistance's effects by thinking about the ways insulin regulates the liver's glucose and lipid metabolism during eating and when fasting (Teo *et al*., 2021).

During fasting, insulin levels are low, and muscles get their energy mostly from fat stores in body (Mambrini.,2024). The breakdown of triglycerides stored in adipose tissue and their subsequent release into the plasma as free fatty acids (FFAs)

is facilitated by a low insulin level. Maintaining normal fasting plasma glucose and hepatic glucose synthesis is achieved by gluconeogenesis and liver glycogen breakdown, stimulated by reduced insulin and increased glucagon secretion (Packard *et al*., 2020). Following a meal, there is a metabolic shift from using fat for energy to glucose, since the increase in plasma insulin concentration prevents lipolysis in adipose tissue (which lowers plasma FFA levels) and encourages glucose uptake and use in muscles and the liver. The term "metabolic flexibility" describes the change from a fasting to a fed state and the subsequent energy substrate transfer from free fatty acids to glucose (Gastaldelli and Cusi., 2019).

Metabolic inflexibility, the interruption of the glucose-lipid (FFA) energy transition induced by insulin-resistant, malfunctioning adipose tissue, is a hallmark of insulin-resistant states such as obesity, non-alcoholic fatty liver disease (NAFLD), and type 2 diabetes. Because of this disturbance, the liver and muscles can rely on FFAs—a persistent and chronic excess supply of fat—instead of glucose for their daily energy needs. Lipotoxicity is the medical term for this overabundance of free fatty acids (FFAs) that characterizes insulin resistance (Fromenty and Roden., 2023). The extent to which this excess is present is often directly correlated with the degree of hepatic triglyceride excess (Santos-Baez and Ginsberg., 2021).

One explanation for the buildup of intrahepatic triglycerides is that the liver is trying to protect itself against endothelial reticulum stress, reactive oxygen species (ROS), and more harmful lipid intermediates like diacylglycerols and ceramides. When steatosis progresses to steatohepatitis, these symptoms become worse. It should be noted that even within the normal range, even modest increases in liver triglycerides are linked to insulin resistance, atherogenic dyslipidemia, decreased plasma adiponectin, and an unsavory metabolic profile (Gastaldelli *et al*., 2021).

Despite ongoing debate, it does not seem that worse steatosis is related to the severity of steatohepatitis (i.e., necroinflammation or fibrosis).

Liver steatosis results from an imbalance between the body's insulin responsive cells and the energy-releasing FFAs produced by the over-lysis of white adipose tissue, which is exacerbated by elevated rates of hepatic de novo lipogenesis(DNL). This is how FFAs are eliminated from the body: the liver uses mitochondrial β-oxidation, re-esterification into triglycerides, storage in lipid droplets, or release into the bloodstream as very low-density lipoprotein (VLDL) cholesterol. Atherogenic dyslipidemia, which includes an elevated number of apolipoprotein B particles, hypertriglyceridemia, and low HDL cholesterol, is caused by the over secretion of VLDL cholesterol in insulin-resistant individuals with type 2 diabetes or NAFLD (Hirano *et al*.,2025).

2.8. NAFLD Diagnosis

New non-invasive diagnostic technologies, including ultrasonography, CT, and MRI, have improved safety, availability, sensitivity, and specificity compared to gold-standard methods (Rinaldi *et al*., 2021). Liver ultrasonography is the gold standard for diagnosing suspected HS due to its extensive availability and exceptional accuracy, according to scientific organizations and authorities (Byrne *et al*., 2018).

Ultrasonography also helps doctors quickly diagnose hepatocellular carcinoma, gallstones, localized liver disorders, metastases, and other abnormal liver function tests. Ultrasonography is also readily accessible, unlike costlier imaging procedures like magnetic resonance-based techniques (Ballestri *et al*., 2019). Recent studies have shown that semi-quantitative indices, like the ultrasonographic fatty liver indicator used in conventional ultrasonography, can help with the accurate

detection of mild hepatic steatosis, which is defined as less than 30% of steatotic hepatocytes on histology, according to previous research (Brunt *et al*., 2020). This would correspond to at least 10% of hepatic steatosis, as determined histologically (Ballestri *et al*., 2017). The "Fibroscan" technology offers a unique and accurate approach for detecting and quantifying hepatic steatosis, known as controlled attenuation parameter (CAP).

Fibroscan's transient elastography may accurately identify liver stiffness, a reliable indicator of liver fibrosis (Lombardi *et al*., 2020). The fatty liver index is determined by measuring blood triglyceride levels, waist circumference, and γglutamyl-transpeptidase. (Leung *et al*., 2016) The NAFLD Fat score assesses metabolic syndrome, type 2 diabetes, insulin levels, and the AST/ALT ratio to predict NAFLD and liver fat content.

Liver biopsy is the gold standard for detecting and quantifying hepatic fibrosis in NAFLD patients. Percutaneous or trans jugular liver biopsy are invasive procedures that have low, but not negligible, complication rates. Bravo et al reported a hospitalization incidence of up to 1-3% after percutaneous liver biopsy, with complications including discomfort, vasovagal hypotension, and, less often, hemorrhage, infection, and organ injury (Bravo *et al*.,2001) Consequently, noninvasive procedures are recommended as first-line examinations. Liver biopsy is finally reserved for individuals in whom additional confirmation of the etiology of chronic liver disease is necessary or when the degree of liver fibrosis or a diagnosis of cirrhosis cannot be properly determined using noninvasive measures. NASH is defined by histological findings of steatosis, hepatocyte ballooning, and lobular inflammation with or without fibrosis. Histologically, fibrosis can be categorized as F1 (mild) or F4 (cirrhosis) (Hirano *et al*.,2022). Several non-invasive scoring evaluations have been established to evaluate the degree of hepatic fibrosis in the

context of risk stratification for cirrhosis and/or HCC. These include the NAFLD fibrosis score, which incorporates invasive clinical and laboratory data such as age, BMI, glucose levels, AST/ALT ratio, platelet count, and albumin. Other scoring methods include the Fib-4 score (age, AST, ALT, platelets), the AST/ALT ratio, the APRI (AST/platelet ratio), and the BARD score (BMI, AST, ALT, diabetes) (McPherson *et al*., 2010).

2.9. Biomarkers for diagnosis of NAFLD

A liver biopsy is the most accurate way to diagnose NAFLD and NASH cirrhosis. However, in clinical practice, liver biopsy's invasiveness, low acceptance, and high cost make it challenging to utilize for general population screening, Conventional ultrasonography is widely used to test and diagnose NAFLD (Castera *et al*., 2019). However, due to the enormous number of T2DM patients, routine liver ultrasonography screening is exceedingly expensive. Furthermore, many rural health facilities and community hospitals do not have ultrasound equipment or qualified ultra-sonographers. As a result, several prior investigations have raised hopes for early screening of NAFLD patients using multiple blood indicators, However, no serum marker has become an established diagnosis (Alqahtani and Schattenberg.,2021)

Blood lipids and liver enzymes are well-known serum biochemical markers of a regular medical checkup. According to earlier research, liver enzyme alterations do not always reflect the severity of hepatic steatosis, making them unreliable for use in NAFLD screening. NAFLD is highly correlated with dyslipidemia, which is characterized by increases in triglycerides (TG), cholesterol (TC), low-density lipoprotein-cholesterol(LDL-C), and decreases in high-density lipoprotein cholesterol (HDL-C), (Pacifico *et al*., 2014).

2.10. Vitamin D

Vitamin D (VD) is a sterol derivative produced by UV light in the skin and may also be received through diet or dietary supplements. VD is a fat-soluble hormone that regulates calcium and phosphorus metabolism and various physiological processes such as immunological modulation, inflammation, and insulin production and secretion (Chen *et al*., 2022).

In recent years, vitamin D supplementation has received much attention for improving blood glucose management, reducing inflammation, increasing insulin production, and improving muscular performance in T2DM patients (Di Filippo *et al*., 2022). According to reports, over 50% of T2DM patients have vitamin D insufficiency, with roughly 1/3 to 2/3 of them having reduced bone density, which increases the risk of falls, fractures, and mortality in senior people (Rodrigues *et al*., 2022). The pathophysiology underlying the relationship between NAFLD and low vitamin D levels is unknown; however, vitamin D's protective anti-fibrotic and anti-inflammatory action on hepatic stellate cells has been proposed. Vitamin D inhibits free fatty acid-induced insulin resistance in peripheral tissues and hepatocytes. As a result, insufficient vitamin D levels may cause intrahepatic lipid buildup, which is responsible for NAFLD pathogenesis (Konstantakis *et al*., 2016).

2.10. Asprosin hormone

Asprosin is a novel protein hormone. It is a polypeptide and cleavage product of a 140-amino acid long C-terminal produced by white fat tissue, which promotes glucose release by the liver. In T2DM, Asprosin levels are elevated and positively correlated with insulin resistance. Asprosin may serve as a biomarker to predict T2DM onset and monitor treatment effects (Cui *et al*., 2024).

discovered Asprosin through neonatal progeroid syndrome, commonly expressed in mature white adipose tissues (Hoffmann *et al*., 2020). White adipose tissue is the primary source of its release into the circulation, which affects the liver. Asprosin has been discovered to modulate liver glucose levels in addition to working as an orexigenic hormone that raises adiposity and stimulates hunger.

Recent studies have shown that Asprosin can produce inflammation and stress in the endoplasmic reticulum. Furthermore, Asprosin levels in the circulation are increased in some metabolic illnesses such as type 2 diabetes mellitus (T2DM), obesity, polycystic ovarian syndrome, gestational diabetes mellitus, and cardiometabolic diseases (Shabir *et al*., 2021) MAFLD is commonly associated with insulin resistance, metabolic syndrome, and endoplasmic reticulum stress. The relationship between Asprosin and MAFLD, however, has received little attention in the study. Asprosin appears to have a close association with MAFLD. Understanding this link can help with the early detection of MAFLD with Asprosin, potentially delaying the development of liver fibrosis, cirrhosis, and end-stage liver disease. Asprosin may be a viable target for treating MAFLD and other metabolic illnesses, leading to the development of novel therapeutic strategies.

Obesity and obesity-related disorders, including T2DM, are quickly increasing internationally as people's living standards improve and their lifestyles become unhealthier. Therefore, insulin resistance and hyperinsulinemia are associated with obesity (Szukiewicz, 2023). The Asprosin hormone, which has a central effect, increases hunger, resulting in weight gain and obesity (Farrag *et al*., 2023). Obesity is a pathological condition that raises the risk of a variety of ailments, including cardiovascular disease, type 2 diabetes, some forms of cancer, and fatty liver disease (Bentsa, 2024). Adipose tissue stores energy and secretes peptides,

lipids, Adipokines, and cytokines that regulate the metabolism of the heart, liver, central nervous system, and muscles.

2.10. Adiponectin hormone

Adiponectin, also called adipocyte complement-related protein (Acrp), Adiponectin is a protein hormone discovered in 1995 comprised of 244 amino acids with a molecular weight of twenty-eight kDa released by adipocytes that regulate metabolic balance and protect the vasculature (Li *et al*., 2024). has several functions, including increasing insulin sensitivity, inhibiting cell death, and reducing inflammation (Achari and Jain., 2017). It targets numerous organs and cell types, such as liver, kidney, cardiac myocytes, and pancreatic β cells. Obesity lowers adiponectin levels, which may contribute to a chronic state of inflammation that causes insulin resistance, type 2 diabetes, coronary artery disease, myocardial infarction, nonalcoholic steatohepatitis, and kidney disease (Aljafary and Al-Suhaimi., 2022).

Low levels of adiponectin in NAFLD patients are linked to the severity of hepatic steatosis, necroinflammation, and fibrosis. Adiponectin is a key factor in the development of basic hepatic steatosis to NASH (Vachliotis *et al*., 2023). Thus, several investigations show that serum adiponectin levels can be used to diagnose the necro-inflammatory grade and fibrosis in NAFLD, as well as a possible NAFLD treatment target (Heydari *et al*., 2020).

Chapter Three
Materials & Methods

3. Material and Methods

3.1. Experimental Design

This study was conducted at Al-Hindiya General Hospital and Imam Hassan Hospital in Karbala Governorate between October 2023 and April 2024. 108 blood samples were collected from participants aged 35 - 77 years old. Of these, seventy samples were obtained from30 males and 40 females visitors to the hospital's advisory clinics who were diagnosed with type 2 diabetes and non-alcoholic fatty liver disease. The comparison group consisted of thirty-eight blood samples collected from healthy individuals 16 males and 22 females of similar age and gender distribution (Figure 3.1).

The questionnaire data collected for all participants included measurements of their height, weight, and body mass index BMI=Kg/m^2 (Seo *et al*., 2019), calculated by dividing weight by the square of height in meters. Physician assistants took these measurements. Additionally, comprehensive medical histories and physical examinations were conducted on all patients who consented to gather information on the duration of their diabetes, medication use, Smoking status and any chronic conditions, questionnaire data are indicated in (Appendix.1.). The patients also confirmed to have non-alcoholic fatty liver disease using imaging techniques such as ultrasound by (Siemens SONY sonar) to examine their livers.

The exclusion criteria were:

(a) history of alcohol ingestion, (b) malignancy, (c) previous gastrointestinal tract surgery, (d) presence of any liver disease that can cause fatty liver, such as chronic hepatitis C, autoimmune liver disease, and Wilson’s disease.

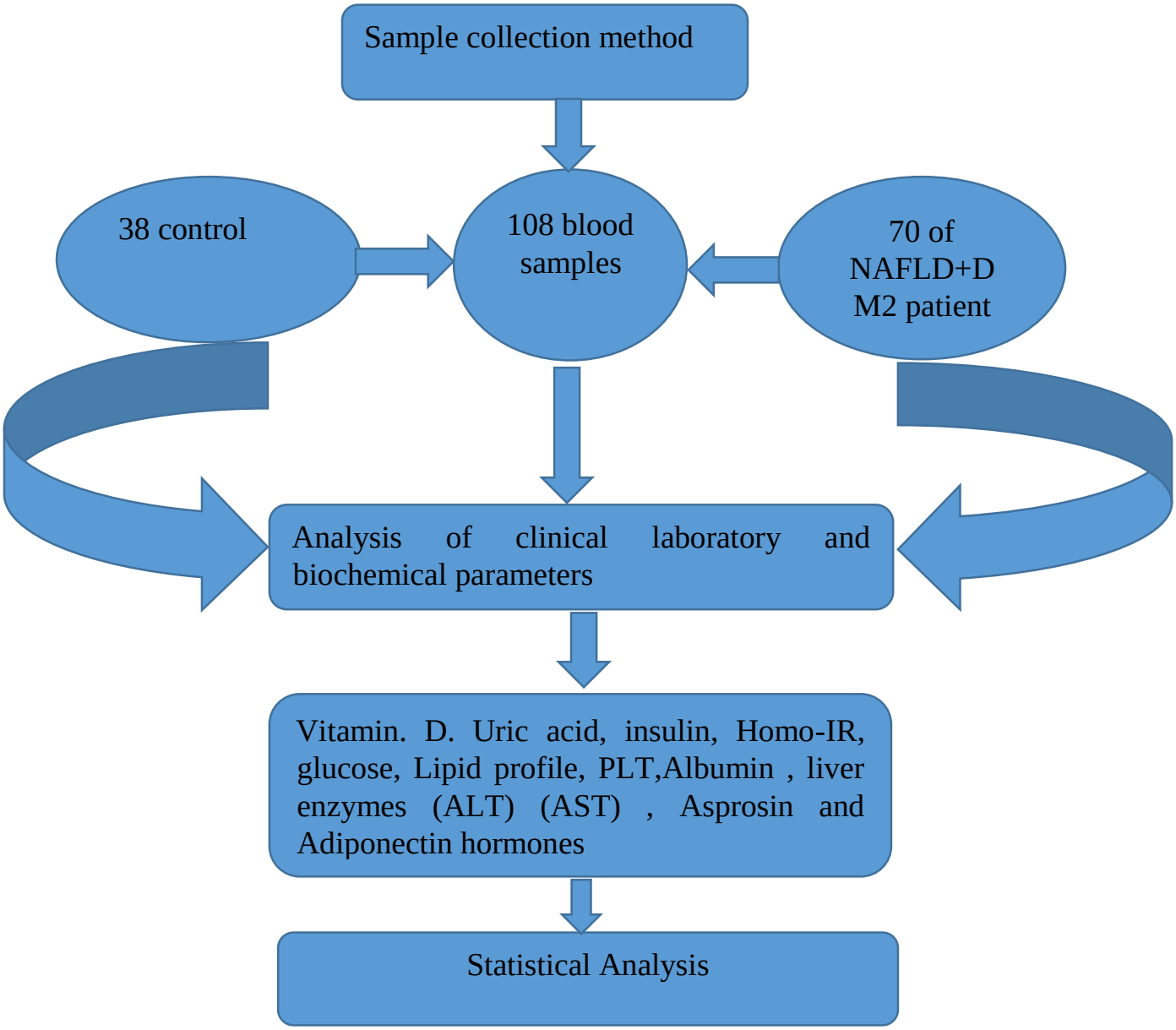

Figure (3.1) Study design

3.2. Laboratory equipment and apparatuses

The equipment and apparatuses utilized in this study are shown in table (3-1).

Table (3-1): Equipment and apparatuses used through about the study

NO	Equipment and apparatuses	Company	Country
1	Refrigerator	Concord	Japan
2	Centrifuge	Hawksley	England
3	Incubator	Gallenkamp	England
4	Body weight Balance	BL Savories s3100	Germany
5	AU480 Chemistry Analyzer	Beckman coulter	California
6	XP-300 (CBC)	SYSMEX	Japan
7	vortex Tube		Japan
8	Eppendorf Tube	Bio Basic Inc.	Belgium
9	Gel tube	Harshman	Germany
10	Disposable syringes	Medical ject	S.A.R
11	EDTA tube	Harshman	Germany
12	Gloves	Apollo	Malaysia
14	Micro pipet	Human	Germany
15	Disposable micro pipette	Walter	Germany

16	Tip Micro pipet	Human	Germany
17	Microplate Reader ELISA	Para Medical	Italy
18	Sony (sonar)	Siemens	Japan

3.3. The biological materials that utilized in study are shown in table (3-2)

Table (3-2): the biological materials used.

No.	Material	Company-origin
1	Human Asprosin ELISA Kit	Sunlong Biotech-China
2	Human Adiponctin ELISA Kit	Sunlong Biotech-China

3.4. Collection of blood samples

Using medical syringes, 5 ml of venous blood was withdrawn from diagnosed patients with fatty liver disease, diabetes, and healthy individuals. The 4 ml of blood samples were placed into gel tubes labeled with a unique barcode and kept at room temperature for 30 minutes. The serum was then separated using a centrifuge EBA280 from Hettich, which was spun at 4500 rpm for 4 minutes. The separated serum was divided into sections labeled with the sample's name and collection date using Eppendorf tubes. Each collected sample was analyzed Chemically using a protocol specific to devices and equipment in the Hindiya Hospital laboratory. The remaining serum was stored in a deep freezer at a temperature of -20°C until use, to examine hormones using ELISA technology. Finally, the remaining 1 ml of blood was put in EDTA anticoagulant tubes and placed in a roll mixer apparatus before being

transported to a Sysmex device for a whole blood test to measure the platelet count.

3.5.1. Analysis of clinical laboratory and biochemical parameters

All participants were required to fast for 8 to 12 hours before their blood samples were collected. The samples were analyzed for glucose, triglycerides, serum ALT, AST, serum cholesterol, vitamin D, albumin, uric acid, and insulin. The LDL-cholesterol ratio was calculated using the formula LDL= LDL=Total Cholesterol-(HDL+TG/5), and the very low-density lipoprotein ratio was determined by the formula VLDL=TG/5. To measure the Insulin resistance used test HOMA IR = [(fasting blood sugar mg/dl) x (blood insulin µU/mL)] / 405. The testing was performed using AU480 Chemistry Analyzer device from Beckman coulter company in the laboratory of the Al-Hindiya General Hospital. A SYSMEX XP-300 device from CBC Company was used to examine the complete blood count and measure the platelet count. Asprosin and adiponectin were tested using the Sandwich-ELISA method and the ELISA Kit in the Al-Amin Center for Research / Holy Shrine of Imam Ali in Najaf Governorate.

Multiple analysis kits produced by the Chinese company Sunlong Biotech Co., Ltd. and utilizing the sandwich method were employed using an ELISA microplate reader from the Italian company Para Medica. The steps for analyzing each hormone were carried out. A human ELISA kit was used to assess the concentrations of Adiponectin and Asprosin in human serum.

3 .5.2. Principle Human Asprosin ELISA

This kit includes a Micro Elisa strip plate pre-coated with an antibody specific to Asprosin. Standards or samples are added to the appropriate strip plate wells and combined with the specific antibody. Next, a Horseradish

Peroxidase-conjugated antibody specific for Asprosin is added to each well and incubated. Unbound components are then washed away. The TMB substrate solution is added, causing the wells containing Asprosin and HRP-conjugated Asprosin antibody to appear blue, then turn yellow after the stop solution is added. The optical density is measured spectrophotometrically at 450 nm, with the OD value proportional to the Asprosin concentration. The Asprosin concentration in the samples can be calculated by comparing the OD to the standard curve (Sunlong Biotechnology, 2023). Materials provided with the Asprosin kit are indicated in (Appendix 2).

Procedure

1. Dilution of Standards

First, the samples were diluted in small tubes. Then, 50 microliters from each tube were pipetted into the wells of a microplate. Each tube was used for two wells, resulting in ten wells.

2. One well in the Microelisa strip plate was left empty to serve as a blank control. 40µL of sample dilution buffer and 10µL of the sample were added to the sample wells. The samples were carefully loaded onto the bottom of the wells without touching the well walls. The contents were then gently mixed by shaking.
3. Incubation: After being sealed with the closure plate membrane, the sample was incubated for 30 minutes at 37 degrees Celsius.
4. Dilution: The concentrated washing buffer was diluted with distilled water at 1:30 for 96-well plates and 1:20 for 48-well plates.

5. Washing: The closure plate membrane was gently removed, the contents aspirated, and the membrane filled with the wash solution. After sitting for 30 seconds, the wash solution was discarded. This washing process was repeated five times.
6. A 50 µl HRP-conjugate reagent was added to each except the blank control well.
7. Incubation as described in Step 3.
8. Washing as described in Step 5.
9. Coloring: 50 µl of Chromogen Solution A and 50 µl of Chromogen Solution B were added to each well. The contents were gently mixed by shaking and incubated at 37°C for 15 minutes while avoiding exposure to light during the color development process.
10. Termination: fifty µl of stop solution was added to each well to halt the reaction. The color in the wells should transition from blue to yellow.
11. The absorbance optical density at 450nm was measured using a Microtiter Plate Reader. The OD value of the blank control well was set to zero. The assay should be performed within 15 minutes after adding the stop solution.

3.5.3. Principle Human Adiponectin ELISA

The Micro Elisa strip plate is pre-coated with an antibody specific to Adiponectin. Samples or standards are added to the wells and combined with the antibody. A horseradish peroxidase-conjugated antibody specific for Adiponectin is then added and incubated. After washing, a TMB substrate solution is added, causing only the wells containing Adiponectin and the conjugated antibody to turn blue, which then turns yellow upon adding a stop solution. The optical density is measured at 450 nm, and the OD value is

proportional to the Adiponectin concentration, which can be determined by comparing the sample ODs to a standard curve (Sun long Biotechnology, 2023). Materials provided with the Adiponectin kit are indicated in (Appendix .3.)

Procedure

The process for determining adiponectin hormone concentrations is the same as outlined in the Asprosin ELISA kit protocol (Appendix .4.)

3.6. Principle Fasting blood sugar test

An enzyme UV test (hexokinase method) was used to quantitatively determine glucose in human serum on Beckman Coulter AU analyzer.

Methodology

Glucose is phosphorylated by hexokinase (HK) in the presence of adenosine triphosphate (ATP) and magnesium ions to produce glucose-6-phosphate and adenosine diphosphate (ADP). Glucose-6-phosphate dehydrogenase (G6P-DH) specifically oxidizes glucose-6-phosphate to gluconate-6-phosphate with the concurrent reduction of NAD+ to NADH. The increase in absorbance at 340nm is proportional to the glucose concentration in the sample (Beckman Coulter,2025). Active ingredients are indicated in (Appendix.5.)

Specimen

Type of Specimen Serum.

Reagent Preparation

The reagents are ready for use and could be placed directly on board the instrument.

Calculation

The Beckman Coulter analyzers automatically compute the glucose concentration of each sample.

Reference Intervals

Serum /Plasma (fasting) Adult 4.1 – 5.9 mmol/l (74 – 106 mg/dl)

3.7. Principle of alanine aminotransferase (ALT)

Kinetic UV test for quantitatively determining alanine aminotransferase, EC 2.6.1.2 (ALT), in human serum. On Beckman Coulter AU analyzer.

Methodology

Method based on the recommendations of the "International Federation for Clinical Chemistry" (IFCC). ALT transfers the amino group from alanine to 2-oxoglutarate to form pyruvate and glutamate. The addition of pyridoxal phosphate to the reaction mixture ensures maximum catalytic activity of ALT. The pyruvate enters a lactate dehydrogenase (LDH) catalyzed reaction with NADH to produce lactate and NAD+. The decrease in absorbance due to the consumption of NADH is measured at 340 nm and is proportional to the ALT activity in the sample. Endogenous pyruvate is removed during the incubation period (Beckman Coulter,2025). Active ingredients are indicated in (Appendix.6.)

Calculation

The Beckman Coulter analyzers automatically compute the ALT activity of each sample.

Reference Intervals

Male (Adult) < 50 U/L (0.85 μkat/l)

Female (Adult) < 35 U/L (0.60 μkat/l)

3.8. Principle of aspartate aminotransferase (AST)

Kinetic UV test for the quantitative determination of aspartate aminotransferase, EC 2.6.1.1 (AST), in human serum.

Methodology

Method based on the recommendations of the "International Federation for Clinical Chemistry" (IFCC). In this method, aspartate aminotransferase (AST) catalyzes the transamination of aspartate and 2-oxoglutaratFe, catalytic activity of AST. The oxaloacetate is reduced to L-malate by malate dehydrogenase (MDH), while NADH is simultaneously converted to NAD+. The decrease in absorbance due to the consumption of NADH is measured at 340 nm and is proportional to the AST activity in the sample. Endogenous pyruvate is removed by the LDH-reaction during the incubation period (Beckman Coulter,2025). Active ingredients are indicated in (Appendix.7.)

Calculation

The Beckman Coulter analyzers automatically compute the AST activity of each sample.

Reference Intervals

Male (Adult) < 50 U/L (0.85 μkat/l)

Female (Adult) < 35 U/L (0.60 μkat/l)

3.9. Principle of Triglyceride

Enzymatic color test for the quantitative determination of triglyceride in human serum on Beckman Coulter AU analyzers.

Methodology

This Triglyceride procedure is based on a series of coupled enzymatic reactions. The triglycerides in the sample are hydrolyzed by a combination of microbial lipases to give glycerol and fatty acids. The glycerol is phosphorylated by adenosine triphosphate (ATP) in the presence of glycerol kinase (GK) to produce glycerol-3phosphate. The glycerol-3-phosphate is oxidized by molecular oxygen in the presence of GPO (glycerol phosphate

oxidase) to produce hydrogen peroxide (H2O2) and dihydroxyacetone phosphate.

The formed H2O2 reacts with 4aminophenazone and N.N-bis(4-sulfobutyl)-3,5-dimethylaniline, disodium salt (MADB) in the presence of peroxidase (POD) to produce a chromophore, which is read at 660/800nm. The increase in absorbance at 660/800nm is proportional to the triglyceride content of the sample (Beckman Coulter,2025). Active ingredients are indicated in (Appendix.8.)

Calculation

The Beckman Coulter analyzers automatically compute the triglyceride concentration of each sample.

Reference Intervals

Normal < 1.70 mmol/l (150 mg/dl)

Borderline high 1.70 - 2.25 mmol/l (150 - 199 mg/dl)

High 2.26 - 5.64 mmol/l (200 - 499 mg/dl)

Very high ≥ 5.65 mmol/l (500 mg/dl)

3.10 Principle of Cholesterol

Enzymatic color test for the quantitative determination of cholesterol in human serum on Beckman Coulter AU analyzers.

Methodology

The Cholesterol reagent utilizes an enzymatic method to measure cholesterol in human serum and plasma. In this procedure cholesterol esters in a sample are hydrolyzed by cholesterol esterase (CHE). The free cholesterol produced is oxidized by cholesterol oxidase (CHO) to cholestene-3-one with the simultaneous production of hydrogen peroxide (H2O2), which oxidative couples with 4-aminoantipyrine and phenol in the presence of peroxidase

(POD) to yield a chromophore. The red quinoneimine dye formed can be measured spectrophotometrically at 540/600 nm as an increase in absorbance (Beckman Coulter,2025). Active ingredients are indicated in (Appendix.9.) and

Reference Intervals are indicated in (Appendix.10). and European Atherosclerosis Society recommendations are indicated in (Appendix.11).

Calculation

The Beckman Coulter analyzers automatically compute the cholesterol concentration of each sample.

Total cholesterol levels in plasma should be corrected by multiplying the result obtained by 1.03 to be equivalent to serum levels of total cholesterol.

3.11. Principle of uric acid

Enzymatic color test for the quantitative determination of uric acid in human serum on Beckman Coulter analyzers.

Methodology

Uric acid is converted by uricase to allantoin and hydrogen peroxide. The Trinder reaction is utilised to measure H2O2. The formed H2O2 reacts with N,N-bis(4-sulfobutyl)-3,5-dimethylaniline, disodium salt (MADB) and 4-aminophenazone in the presence of peroxidase to produce a chromophore, which is read biochromatically at 660/800nm. The amount of dye formed is proportional to the uric acid concentration in the sample (Beckman Coulter,2025). Active ingredients are indicated in (Appendix.11.)

Reference Intervals

Serum Male 208.3 - 428.4 μ mol/l(3.5 - 7.2 mg/dl)

Female 154.7 - 357.0 μ mol/l (2.6 - 6.0 mg/dl)

Urine, 24h Average diet 1488 - 4463 μ mol/d (250 - 750 mg/dl)

3.12. Principle of albumin

Photometric cooler test for the quantitative determination of albumin in human serum on Beckman Coulter analyzers.

Methodology

A colored complex is formed when bromocresol green reacts with albumin. The absorbance of the albumin-BCG complex is measured bichromatically (600/800nm) and is proportional to the albumin concentration in the sample (Beckman Coulter,2025). Active ingredients are indicated in (Appendix.12.)

Calculation

The Beckman Coulter analyzers automatically compute the albumin concentration of each sample.

Reference Intervals

Serum (Adults) 35 – 52 g/L (3.5 – 5.2 g/dl)

Serum (Newborn 0 – 4 day) 28 – 44 g/L (2.8 – 4.4 g/dl)

3.13. Statistical Analysis

Information from the questionnaire and all test results from study group samples were entered into a data sheet. The data analysis for this work was generated using the graphical Pad prism9. Descriptive statistics were performed on the data of each group. Values were illustrated by n (%) for categorical, Scale variables were presented by mean ± standard deviation for normal data. The distribution of the data was checked using the Shapiro-Wilk test as a numerical means of assessing normality, and using T-test to compare the averages of two groups.

Significant differences in categorical variables among the parameters were confirmed through analytical statistical tests. Results of all hypothesis tests with p values <0.05 (two-sided) were considered to be statistically significant. optimal

threshold with high specificity and sensitivity for study cases was detected using receiver operating characteristic (ROC) analysis (Fawcett, T. 2006).

Chapter four

Results and Discussion

4. Results and Discussion

4.1. Demographic Characteristics of the patients with diabetes mellitus and fatty liver and Control group.

Table (4-1) summarizes the demographic characteristics of the patients with diabetes mellitus and fatty liver (n=70) compared to the control group (n=38). The patient's group had an average age of 54.28 years, while the control group had a mean age of 48.88. T2DM patients with NAFLD exhibited a significantly elevated mean BMI of 39.77 kg/m2 compared to the control group of 31.24 kg/m^2. Medical history analysis revealed a higher incidence of hypertension in the patient group, with 23 individuals affected compared to only three in the control group. The control group included two individuals with chronic diseases, compared to the patient group, which included 8 individuals with chronic conditions (heart disease, lung disease, obesity, etc.) and 39 individuals with diabetes and fatty liver. The duration of diabetes mellitus among participants was categorized into three subgroups: 34 patients had the condition for 1-3 years, 16 had it for 4-6 years, and 20 had it for more than seven years. Additionally, the smoking status results showed that most patients in both groups were non-smokers.

Table (4-1) Distribution of the Samples According to demographic information

Variables		Patients Group n=70	Control Group n=38
Age (Mean)		54.28	48.88
Sex (No.)	Male	30	16
	Female	40	22
BMI (Mean)		39.77	31.24
Medical history (No.)	HT	23	3
	Other chronic diseases	8	2
	Only DM +Fatty liver	39	/
Duration of DM (Years)	1-3	34	/
	4-6	16	/
	>7 Years	20	/
Smoking status (No.)	Smoker	10	4
	Non-smoker	60	34

4.2. Level of liver enzyme

Figure (4-1) shows the distribution level of liver enzymes, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in two groups: patients with diabetes mellitus and fatty liver, compared to the control group. Results indicated that patients with diabetes and fatty liver have higher levels of both ALT and AST compared to the control group. The mean level of ALT in the patient's group was 22.21 U/L, almost three times significantly higher than in the control group (7.63 U/L). The mean level of AST in the patient's group was 31.46 U/L, which is about 40% significantly higher than the control group (22.12 U/L).

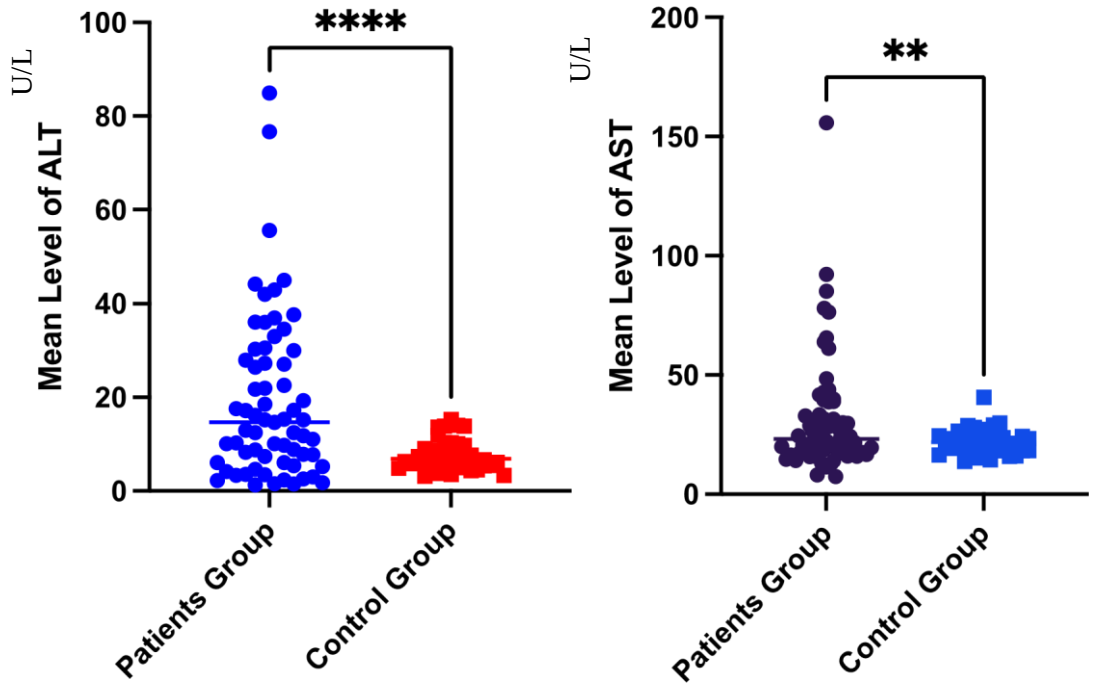

Figure (4-1) Distribution level of liver enzymes included alanine aminotransferase (ALT)U/L and aspartate aminotransferase (AST)U/L among patients group of diabetes mellitus with fatty liver compared to control group. (T-test was *: significant at $p \leq 0.05$, **: significant at $p \leq 0.01$, ***: significant at $p \leq 0.001$).

The ALT and AST enzymes are mostly stored in hepatocytes, which are cells of the liver. Liver illness often manifests as elevated ALT and AST readings, which are markers of damage to these cells. Hepatic steatosis, a condition associated with fat accumulation in the liver, may also be detected using the ALT/AST ratio as a composite test (Long *et al*., 2016).

Using Chinese volunteers who did not have obesity, (Zou *et al*.,2020) conducted a longitudinal research in China for five years. Scientists found that NAFLD progression was correlated with an increased alanine aminotransferase to aspartate aminotransferase ratio. This was more significantly linked to hyperglycemia, hypertension, and elevated blood lipids (Zou *et al*., 2020).

Previous studies have also shown that type 2 diabetics, in comparison to non-type 2 diabetics, had higher levels of liver enzyme activity, which is consistent with the current results (Kumar *et al*., 2023). Consequently, the groups of patients with diabetes and fatty liver have higher liver enzymes, which may suggest liver injury.

4.3. Level of Lipid profile

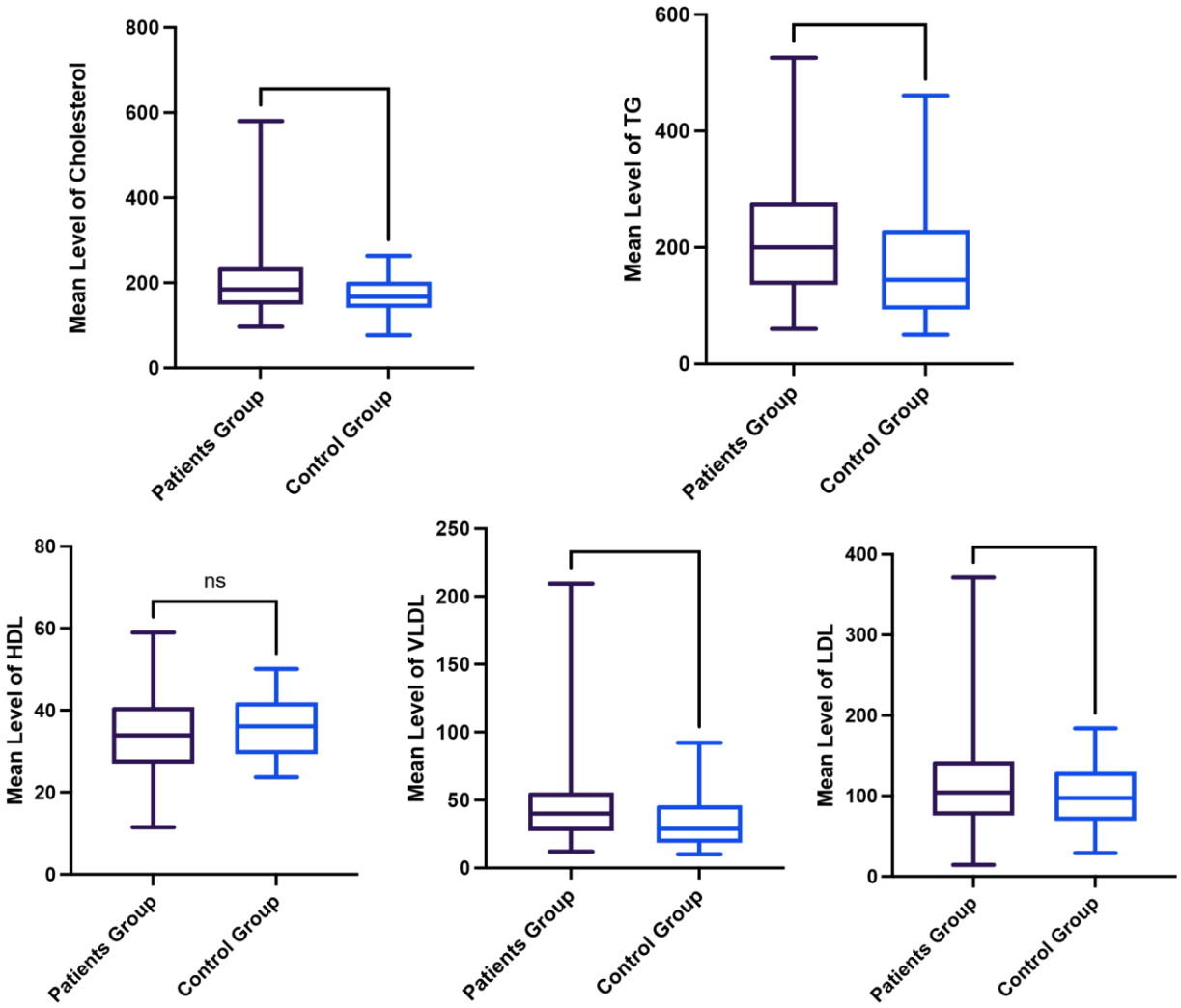

Figure (4-2) Distribution level of Lipid profile panel (TG mg/dl, Cholesterol mg/dl, LDL mg/dl and HDL mg\dl) among patients group of diabetes mellitus with fatty liver compared to control group. (T-test was *: significant at $p \leq 0.05$, **: significant at $p \leq 0.01$, ***: significant at $p \leq 0.001$)

Figure (4-2) demonstrates the mean level and standard deviation of five lipid profile markers in two groups: patients with diabetes mellitus and fatty liver and a control group. The results indicated a potential abnormality in the lipid profile of the

patients with diabetes and fatty liver compared to the control group. Specifically, the patient's group had higher mean levels of cholesterol (212.81 mg/dl) High total cholesterol may be caused by the accumulation of fat in the liver (fatty liver) in addition to insulin resistance. A previous study found (Min *et al*.,2012) that fatty liver is associated with increased levels of total cholesterol.

triglycerides (279.96 mg/dl), and LDL (119.81 mg/dl) compared to the control group. Higher significantly triglyceride levels indicate metabolic disorders associated with insulin resistance, a hallmark of diabetes and fatty liver disease ،this is consistent with a previous study (Root *et al*.,2014). that confirmed that patients with fatty liver disease suffer from significantly high triglycerides.

The results indicated that high LDL levels are associated with an increased risk of atherosclerosis and heart disease, and are common in patients with diabetes and fatty liver. This is consistent with the results of the previous study (Lee *et al*., 2021).

The results of the current study show that the average level of high-density lipoprotein (HDL) in the patient group was higher than in the control group, and this difference was not statistically. However, results may vary based on several factors, including the demographics of the participants, the severity of the disease, and the treatments used.

the VLDL level (55.99 mg/dl) was higher statistically. in the diabetes mellitus with fatty liver group compared to the control group (34.55 mg/dl). Increased VLDL levels indicate a disorder of lipid metabolism and are associated with fatty liver This is consistent with the previous study (Adiels *et al*.,2006). Insulin resistance appears to contribute to liver fat accumulation through elevating adipose tissue-free fatty acid release, increasing hepatic fatty acid and triglyceride production, decreasing

hepatic fatty acid breakdown (Fabbrini *et al*., 2010). Accumulating indication displayed that lipid profile was notably related to a raised risk of NAFLD in the general inhabitants (Pacifico *et al*., 2014b; Wu *et al*., 2016). This proposed that dyslipidemia in individuals with diabetes, even if they were not obese, could be identified as a marker of the presence of non-alcoholic fatty liver disease.

4.4. Level of Metabolic markers

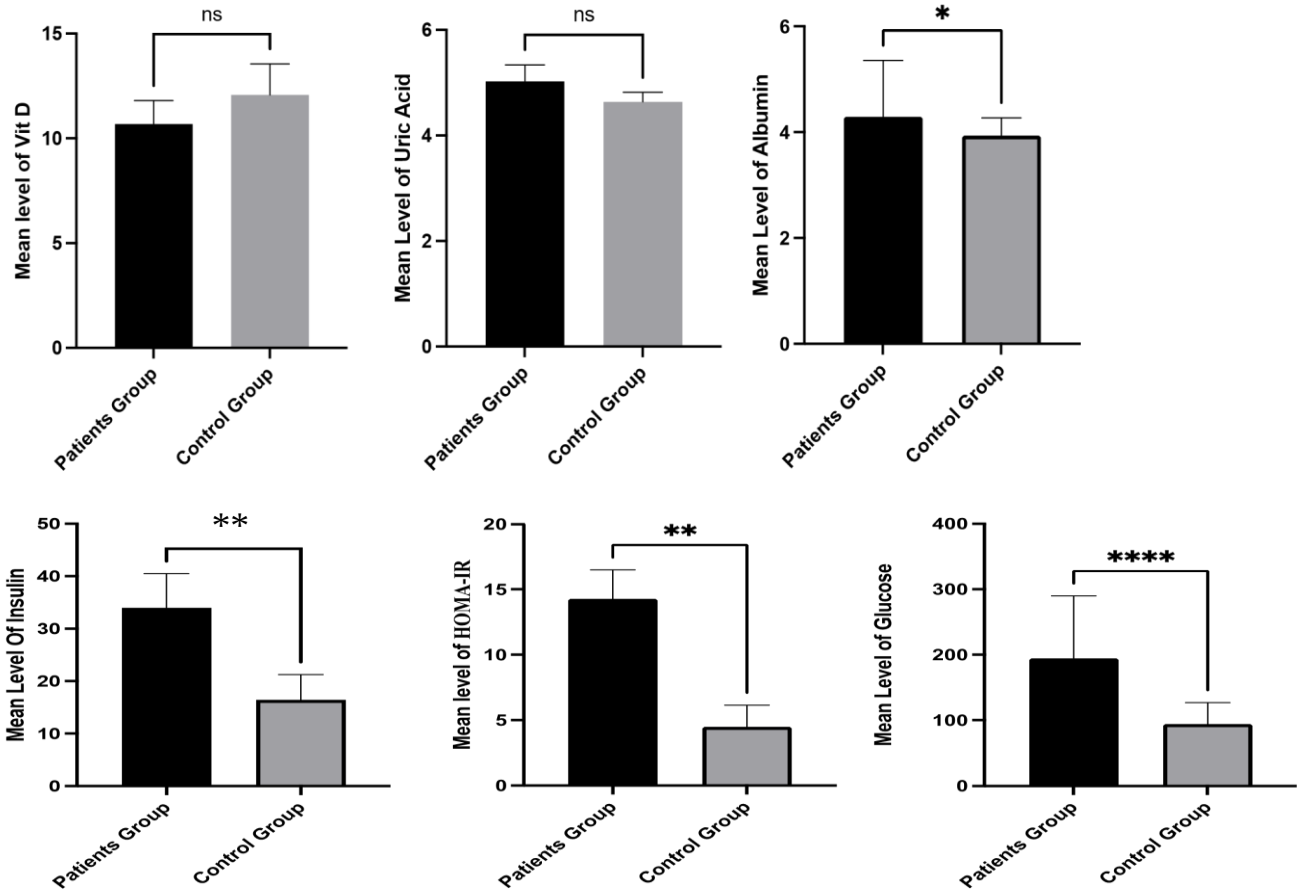

Figure (4.3) Mean differences of Vit D (ng/ml), Uric acid(mg/dl), Insulin(U/ml), Homo-IR(U/ml), Glucose(mg/dl) and Albumin(g/dl) among patients group of diabetes mellitus with fatty liver compared to control group. (T-test was *: significant at $p \leq 0.05$, **: significant at $p \leq 0.01$, ***: significant at $p \leq 0.001$)

Figure (4-3) presents the mean level and standard deviation (SD) of five metabolic syndrome markers in two groups: patients with diabetes mellitus associated with fatty liver and a control group. Results showed a potential abnormality in the metabolic profile of patients with diabetes and fatty liver compared to the control group, as presented in Figure (4-3).

Vitamin D is implicated in controlling cell proliferation and differentiation, in addition to its role in calcium and bone homeostasis (Khammissa *et al*., 2018). In addition, growing evidence indicates a potential causal association between vitamin D deficit and non-alcoholic fatty liver disease (Kitson and Roberts., 2012). The current results reveal that the patient group had a lower vitamin D concentration than the control group. These results align with the previous results showing that individuals with biopsy-confirmed NAFLD had lower vitamin D levels compared to healthy controls (Pacifico *et al*., 2019).

Other research, on the other hand, suggests that the differences may be small and not statistically significant, particularly if patients are taking nutritional supplements or are getting enough sun exposure (Bikle ,2014) The lack of statistical significance may be attributable to considerable individual variations in genetics, diet, lifestyle, physical activity, age, gender, and sunshine exposure.

Predicting the existence or progression of NAFLD by detecting blood uric acid levels has been suggested in previous studies. It also implies that serum uric acid might be involved in NAFLD progression. Researchers in the lab have demonstrated that uric acid may cause insulin resistance by interfering with the insulin signaling system, which in turn affects fat storage and hepatic steatosis (Lombardi *et al*.,2016).

albumin level is slightly higher in the patient group compared to the control group. Albumin is a major protein in plasma that is produced primarily in the liver, and therefore any liver disorder can affect its levels. In advanced cases of chronic liver diseases such as cirrhosis, it decreases significantly due to the liver's weak ability to manufacture. However, in the early or intermediate stages of fatty liver, the liver is still able to produce, so a clear decrease may not be observed. Patients may have slight increases in albumin production in response to the chronic inflammation associated with diabetes due to their insulin resistance. This is consistent with the previous study (Kaefer *et al*.,2010).

Regarding the Homo-IR, diabetes mellitus with a fatty liver group has a higher mean level (14.28) compared to the control group (4.49). These findings suggest potential metabolic dysregulation in such patient groups, possibly associated with various health risks. Insulin resistance is the critical factor driving this effect. Hyperinsulinemia, a consequence of insulin resistance, would increase the fatty acid substance of hepatocytes. The subsequent circumstance is an increase in the intensity of fatty acids and triglycerides in the hepatocytes, which initiates steatosis (Kalra *et al*., 2013). A previous study confirmed a close relationship between high insulin and worsening inflammation and oxidative stress in patients with diabetes and NAFLD (Marušić *et al*.,2021).

4.5. Level of Asprosin and Adiponectin Hormones

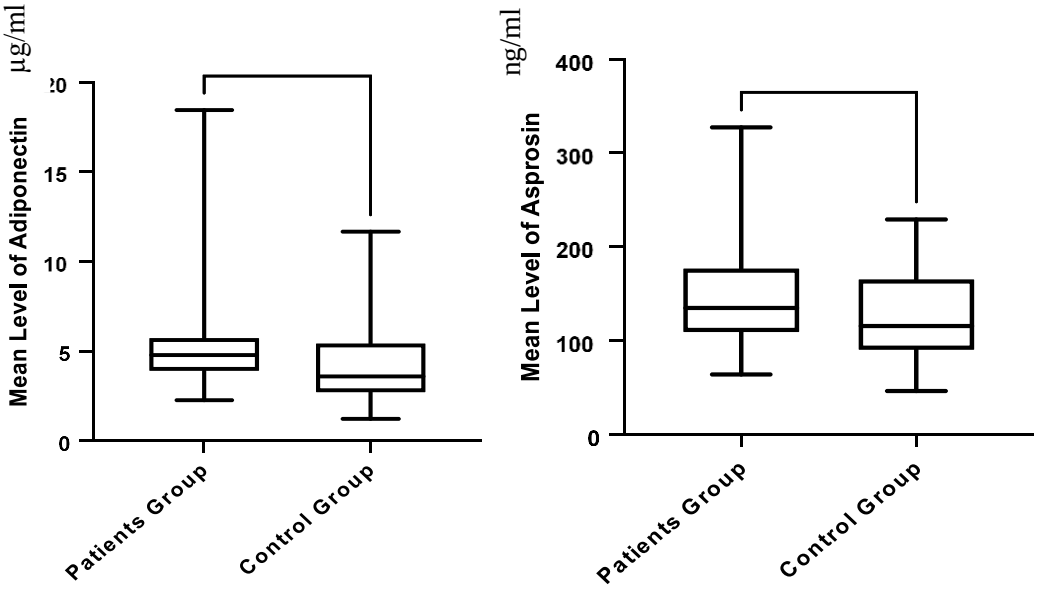

Figure (4-4) Distribution level of Asprosin and Adiponectin hormones among patients group of diabetes mellitus with fatty liver compared to control group.

Figure (4-4) shows the mean level and standard deviation (SD) of two hormone markers in two groups: patients with diabetes mellitus and fatty liver and a control group. Results indicated a wide difference in hormone levels between the two groups, as presented in Figure (4-4). The patient group had a higher mean level (150.56) of Asprosin than the control group (127.64). Similarly, Adiponectin was increased in the patient's group (5.27) compared to the control group (4.18). The adipose tissue plays a vital role in regulating metabolism and maintaining energy balance (Booth *et al*., 2016). Various molecules secreted by adipose tissue can either improve or delay insulin action. Insulin resistance, a significant factor in developing type 2 diabetes (T2DM), is a prominent effect of excess body fat. Therefore, obesity is directly associated with a range of metabolic conditions, including T2DM and metabolic syndrome (O'Neill & O'Driscoll, 2015).

Asprosin is a recently discovered hormone secreted by adipocytes. It acts to lower dietary glucose by initiating the distribution of liver glucose stores. The drop in Asprosin level has been found to protect against hyperinsulinism allied with metabolic syndrome, involving type 2 diabetes mellitus, Polycystic ovarian syndrome, and NAFLD (O'Neill & O'Driscoll, 2015). An elevated level of Asprosin was found to be linked with insulin resistance in patients with T2DM as well as in mice. Neutralizing Asprosin led to a decrease in appetite and body weight in obese mice. Zhang et al. recounted a higher circulating level of Asprosin in T2DM patients, accompanied by fasting blood glucose and triglyceride levels (Zhang *et al*., 2019).

In patients with type 2 diabetes, high levels of Asprosin are positively correlated with insulin resistance. Asprosin could potentially be used as a biomarker to predict the onset of Type 2 diabetes and to monitor the effects of treatment (Shabir et al., 2021). Additionally, elevated levels of Asprosin have been reported in metabolic dysfunction-accompanying fatty liver disease (MAFLD) in comparison to diabetic individuals without MAFLD (Cui *et al*., 2024).

Adiponectin is a protein nearly entirely derived from white adipose tissue and can affect insulin secretion and insulin action (Adiyaman *et al*., 2020). In this study, higher adiponectin levels were recorded in NAFLD patients, which was inconsistent with previous research results (Ke *et al*., 2020). The divergent result may be attributed to the difference in the types of patients included in the study.

Although studies supporting elevated adiponectin levels are scarce, study noted an increase in adiponectin levels in patients with cirrhosis associated with non-alcoholic fatty liver due to increased production in response to metabolic and inflammatory changes (Ismaiel *et al*., 2025) . We conclude probably that an increase may occur as a result of compensatory factors to reduce insulin resistance, reduce

chronic inflammation, advanced pathological changes, drug effects, or genetic differences.

4.6. Analysis of Clinical Factors in Patients with Differing Stages of Liver Fibrosis.

Table (4-2) Comparison of Clinical factors in Patients with Different Stages of Liver Fibrosis.

Variables	**IB-4 risk category**			**P value**
	Low **<1.3**	**Indeterminate** **1.31-2.6**	**High** **>2.6**	
Albumin(g/dl)	4.17±0.33	**4.45±1.48**	**4.09±0.28**	0.02
Vit.D (ng/ml)	19.82±10.64	20.98±11.97	19.29±12.25	0.9
ALT(U/L)	34.44±45.46	17.26±15.51	15.62±24.28	0.23
AST (U/L)	26.33±17.76	31.17±17.08	40.1±41.94	0.501
U. A (mg/dl)	4.25±1.19	4.78±1.62	4.86±1.44	0.41
Cholesterol (mg/dl)	200.45±83.68	220.41±146.09	213.15±120.71	0.84
TG (mg/dl)	298.67±237.96	292.66±253.11	219.92±232.88	0.95
HDL (mg/dl)	32.1±12.41	40.87±13.61	35.08±12.78	0.37
VLDL(mg/dl)	59.73±47.59	58.53±50.62	43.98±46.58	0.64
LDL (mg/dl)	108.62±55.37	121.01±124.05	134.09±81.68	0.22

Glucose(mg/dl)	170.77±64.12	219.44±111.17	170.23±81.57	0.16
Insulin (U/ml)	26.4±23.09	27.56±25.84	61.47±105.57	0.75
HOMA-IR(U/ml)	11.1±9.81	14.73±14.09	23.6±33.38	0.38
PLT (µL /10^3x)	268.9±74.33	259.94±52.03	229.54±29.92	0.51
ASP (ng/ml)	4.63±1.48	8.78±2	**15.03±2.31**	**<0.001**
ADP(µg/ml)	23.14±7.4	43.88±9.99	**75.14±11.54**	**<0.001**

As shown in Table 2, serum Asprosin and adiponectin concentrations in patients with high-risk FIB-4 scores were higher than those with low and indeterminate risk for advanced fibrosis. At the same time, the albumin level was significantly higher in the intermediate-risk group than in the high-risk FIB-4 group. There were no group differences in terms of other clinical parameters.

The present results partially align with recent studies that have reported elevated adiponectin levels in chronic hepatitis B patients showing advanced liver fibrosis and inflammation (da Silva *et al*., 2018; Hsu *et al*., 2015). A potential reason for the elevated levels of adiponectin observed in patients in contrast to healthy controls could be that adiponectin is a potent inhibitor of hepatic stellate cell activation. Therefore, a lack of adiponectin may lead to more severe liver fibrosis (Tardelli *et al*., 2017). The current data corroborated previous findings using Fibroscan's elastography technology, which was employed to detect CAP and quantitatively assess MAFLD. The analysis revealed a significant positive correlation between asprosin and CAP, an indicator of hepatic steatosis, suggesting

that Asprosin can be a non-invasive measure for forecasting the likelihood of rising metabolicassociated fatty liver disease (Lv *et al*., 2024).

4.7. The correlation coefficient (r) between biomarkers among patients with diabetes mellitus with fatty liver compared to the control grope

Considering the important role of the measured parameters in Diabetes Mellitus with Fatty Liver patients, the Pearson analysis of such patients was used to show the response relationship between parameters. The correlation study demonstrated many significant relationships among the measured parameters. P values were (< 0.05).

Liver Enzymes, namely ALT showed a non-significant moderate negative correlation (r = -0.602) with Asprosin and a weak correlation with adiponectin. Results were also showed a significant correlation between Asprosin and LDL, Insulin and HOMA-IR, p values were <0.05, as presented in Figure (4-5).

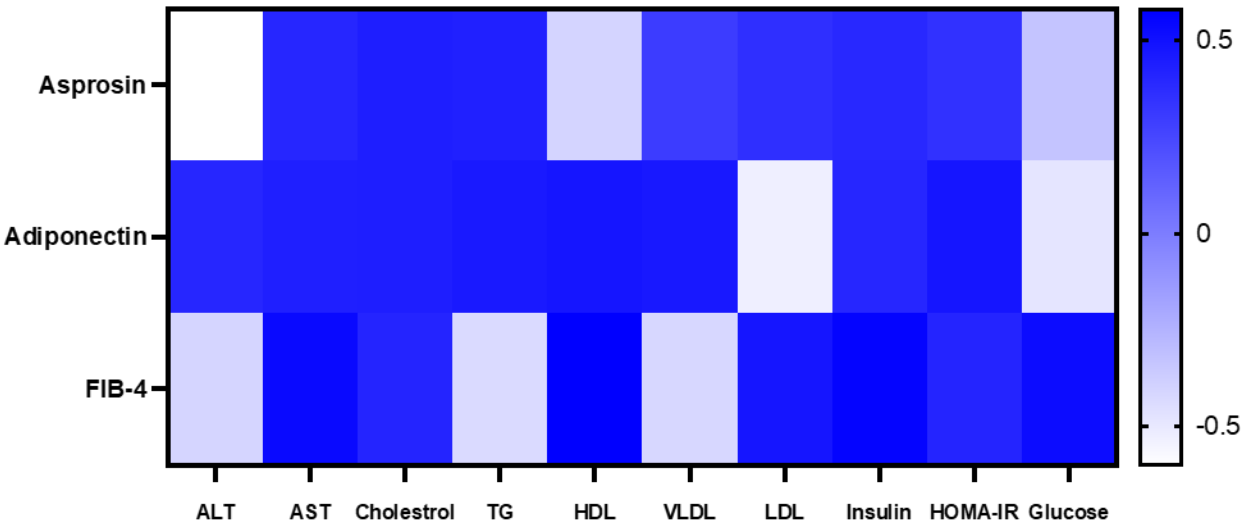

Figure (4-5) The correlation coefficient (r) between biomarkers among patients with diabetes mellitus with fatty liver compared to the control grope.

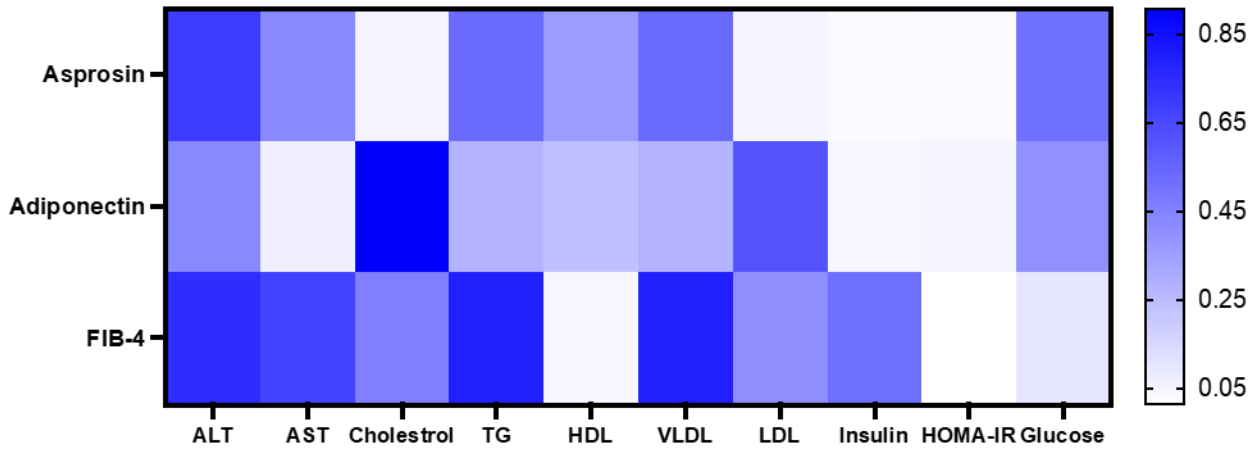

Figure (4-6) The significant *P* value between biomarkers among patients' diabetes mellitus group with fatty liver compared to the control group

The lipid profile markers showed positive correlations for cholesterol, triglycerides, VLDL, and HDL but a negative correlation for LDL with Adiponectin Adiponectin had weak positive correlations with most markers, except LDL and glucose, and a significant correlation with insulin. The liver fibrosis marker FIB-4 had negative correlations with ALT and VLDL, suggesting better liver function with lower FIB-4 scores. FIB-4 also showed a significant correlation with Homo-IR at $p<0.005$, as presented in Figure (4-6).

Similar results were noted, with elevated levels of Asprosin in the blood found in fatty liver disease patients who also had HOMA-IR, indicating a strong connection between the two conditions in non-alcoholic fatty liver disease, according to paired analysis, metabolic changes are less likely to be the primary cause of the Asprosin abnormalities detected than fatty liver disease. Additionally, Asprosin and HOMA-IR were shown to be positively correlated. For the fatty liver group, HOMA-IR and Asprosin were shown to be independently associated in the multivariate linear regression analysis. Previous studies have shown that HOMA-IR, which depends on body mass index and lipid levels, has great predictive value in fatty liver disease

(Gutierrez *et al*., 2017) These results point to the possibility that abnormal HOMA-IR is indicative of liver changes, and that the increased Asprosin, which is closely linked to HOMA-IR, is mostly caused by liver-specific changes.

It seems from the results that insulin resistance is a common trait of both fatty liver disease (FLD) and type 2 diabetic mellitus (T2DM). It was shown that FLD was associated with insulin resistance in many tissues, including the liver, muscle, and fat. Several studies have shown that insulin is not as effective in suppressing glucose synthesis in the liver, which suggests insulin resistance in the liver, and that glucose disposal drops by about half, which means insulin sensitivity in the bloodstream is lowered (Bugianesi *et al*., 2005).

Some new research suggests that FLD could increase the likelihood of type 2 diabetes on its own (Targher and Byrne., 2013). Across the FLD-T2DM spectrum, Asprosin levels may change, which may indicate insulin resistance. Although Asprosin does contribute to insulin resistance in FLD patients, the exact pathophysiological mechanism by which this occurs remains unknown. A study conducted by Lee et al. recently found that recombinant Asprosin can worsen inflammation, hinder the secretion of insulin in response to glucose, cause cell death through TLR4/JNK signaling, and contribute to a vicious cycle of inflammation and dysfunction in β-cells driven by hyperlipidemia in individuals with type 2 diabetes (Lee *et al*., 2019). Results showed a strong correlation between FLD and insulin resistance as well as the Toll-like receptor 4 /c-Jun N-terminal kinase TLR4/JNK-mediated inflammatory response (Jung *et al*., 2018). Additionally, Asprosin dysregulation may function via this pathway. Although triglyceride levels were favorably associated with Asprosin levels in fatty liver disease patients, Asprosin 2levels were not independently connected to TG levels. Never the less, adiponectin was shown to be independently related with TG levels (Divella *et al*., 2019).

4.8. The significance of the P value for biomarkers among patients with diabetes mellitus with fatty liver based on the Duration of disease, Age and BMI.

The significance of the P value for biomarkers among patients with diabetes mellitus with fatty liver based on the Duration of disease, Age and BMI.

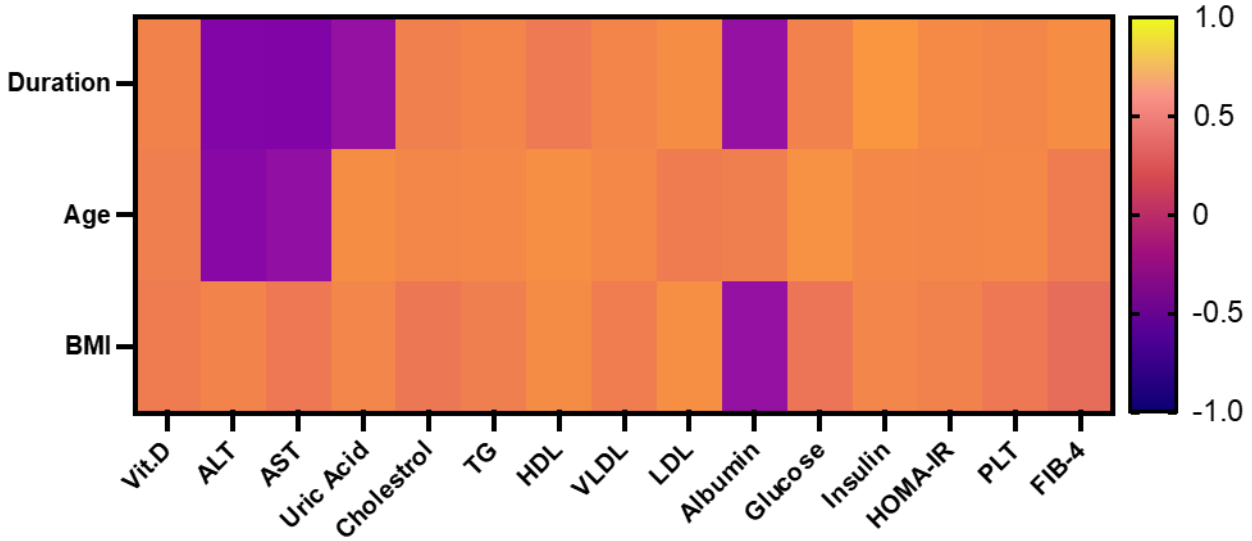

Figure (4-7) The correlation coefficient (r) between biomarkers among patients with diabetes mellitus with fatty liver based on the Duration of disease, Age and BMI

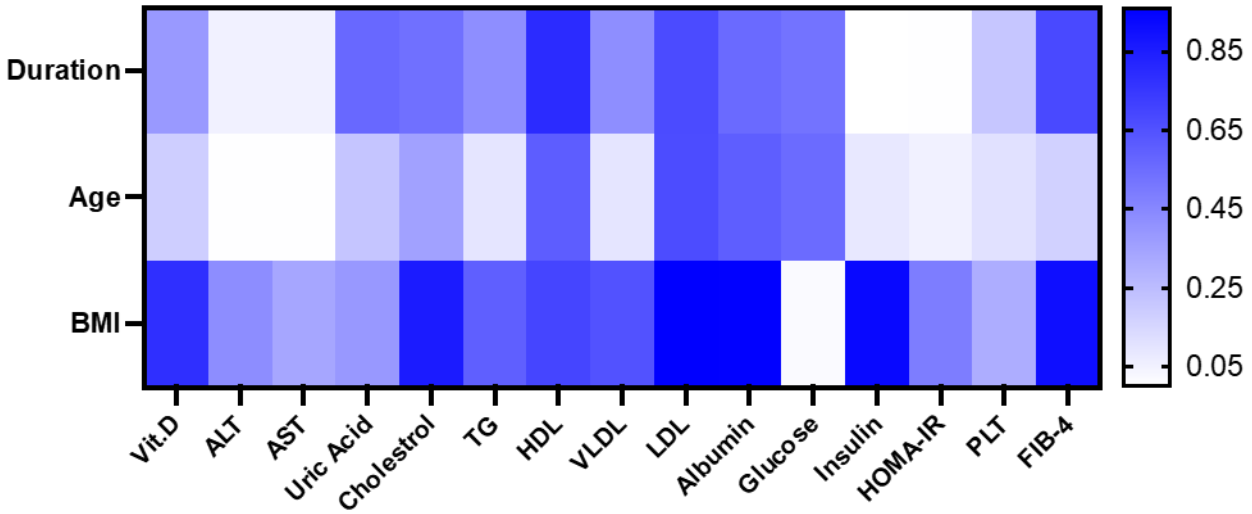

Figure (4-8) The significance of the *P* value for biomarkers among patients with diabetes mellitus with fatty liver based on the Duration of disease, Age and BMI.

Figures (4-7) & (4-8) summarized the correlation coefficients (r) and P values between various biomarkers in a group of patients with diabetes mellitus and fatty liver. The correlations are assessed based on three factors: duration of disease, age, and BMI. The strength of the correlations can be categorised as weak (0.3-0.5), moderate (0.5-0.7), or firm (0.7-1.0). In this dataset, most correlations are weak, suggesting limited linear relations between the variables. Results indicated that there was a significant negative correlation ($r < -0.4$) between Age and both ALT (alanine aminotransferase) and AST (aspartate aminotransferase) levels. This suggests that ALT and AST levels tend to decrease with increasing age in this patient group This is consistent with previous studies (Gan et al., 2011). The reason for the decrease is likely due to The normal liver may shrink in size or function, both of which contribute to the typical decline in liver enzyme levels that occurs with aging.

In humans, oxidative damage builds up over time and causes the liver to gradually shrink in size and blood supply (Dong *et al*., 2010).

The present study found that lipid biomarkers, including triglycerides, VLDL, LDL, total cholesterol, and HDL, were positively correlated with all three factors examined: disease duration, age, and body mass index. This suggests that these lipid levels rise with longer disease progression, older age, and increased adiposity. These findings align with prior research that has established the close connections between obesity, dyslipidemia, and fatty liver disease (Corbin *et al*., 2012).

The research indicated that there were modest positive relationships between age and BMI and HOMA-IR, Uric Acid, Glucose, Insulin, and Vitamin D ،this is consistent with previous study (Chung *et al*., 2016).

There is a slight positive connection between HOMA-IR and illness duration as well. By allowing free fatty acids to accumulate in the liver, insulin resistance plays a crucial role in the development of non-alcoholic fatty liver disease (Bugianesi

et al., 2005). Rising insulin resistance in industrialized countries is a major factor contributing to the alarming increase in the incidence of non-alcoholic fatty liver disease (NAFLD) (Brunt, 2005).

A higher risk of obesity was associated with elevated blood uric acid levels, according to an earlier research (Wang *et al*., 2022). There are a number of possible explanations for why blood uric acid levels are associated with obesity. Hyperuricemia, poor uric acid metabolism, and increased serum uric acid production may be connected with obesity or excess body fat, which is caused by insulin resistance. Hyperuricemia and obesity are mutually reinforcing conditions because serum uric acid promotes fat storage in the liver and the periphery. Both components may be more prominent when there are problems with glycolipid and uric acid metabolism. It is essential for preventive medicine to thoroughly assess the interaction between blood uric acid and body mass index due to the tight biological link between the two variables (Kızılay *et al*., 2019).

This study's results also revealed a modest negative connection between albumin and age. Several health issues have been linked to variations in blood albumin levels that occur with aging. Specifically, when there is fat around the abdomen, high triglyceride levels, and raised blood sugar, metabolic syndrome is associated with decreased serum albumin concentrations (Cho *et al*., 2012). Albumin is an anti-inflammatory and antioxidant protein produced by the liver (Liu and Chien., 2023). Patients with liver cirrhosis have abnormalities in serum albumin structure and function, as well as impaired albumin production (Domenicali *et al*., 2014). Researchers have shown that individuals with NAFLD who also have hypoalbuminemia have a worse prognosis (Spinella *et al*., 2016; Kawanaka *et al*., 2021).

4.9. ROC curve and AUC analysis for the Aspersion and Adiponectin in patients with diabetes mellitus with fatty liver compared to control groups

Results of the receiver operating curve (ROC) had shown that both markers have good performance for predicting such cases, data are presented in Table (4.3), Figure (4.9) & (4.10)

For Aspersion levels: (sensitivity 58%, specificity 63.2%) at a level = 128.83, For Adiponectin levels: (sensitivity 80%, specificity 56.6%) at a level = 3.723. The p value of the AUC was <0.001 and highly statistically significant. results of the Sensitivity & Specificity were confirmed using Youden's J statistics to the parameters.

Table (4-3) Receiver operating characteristic curve showing sensitivity and specificity of Aspersion and Adiponectin in patients with diabetes mellitus with fatty liver compared to control groups.

Test Result Variable(s)	**Aspersion**	**Adiponectin**
AUC	60.4%	64.8%
Sensitivity %	58%	80%
Specificity %	63.2%	52.6%
Youden index	0.212	0.326
Cut- off points	128.8345	3.723
CI (95%)	(0.473-0.714)	(0.528-0.768)
PPV	67.5	57%
NPV	53.3	50%
P value	0.018**[S]**	**<0.001[S]**
S= Significant, PPV= Positive protective value, NPV= Negative predictive value, AUC= Area under curve, CI= confidence interval		

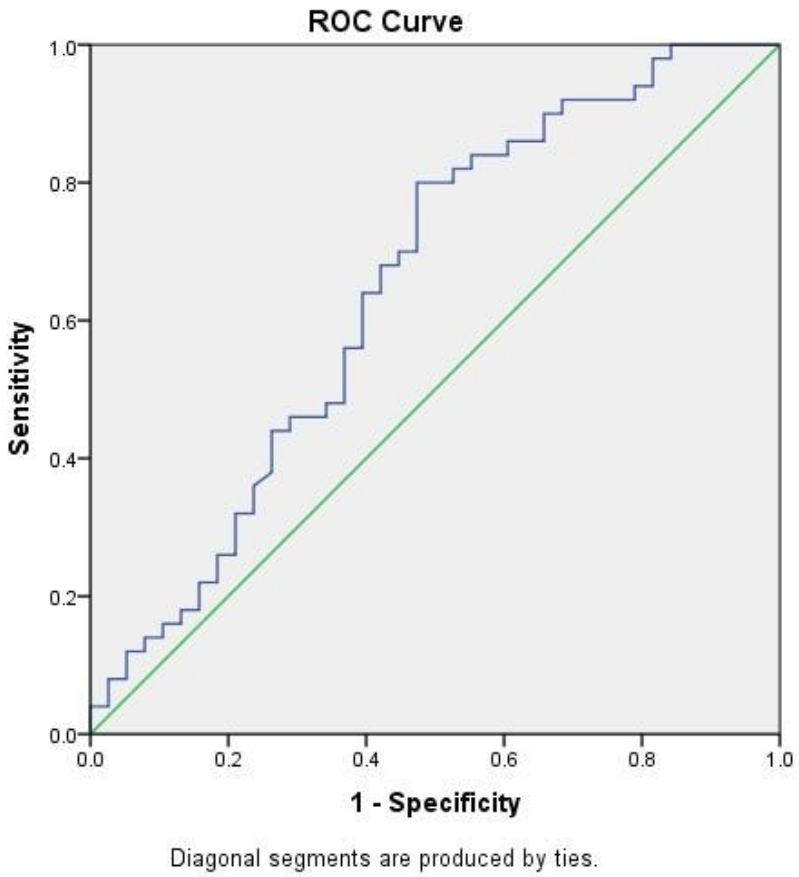

Figure (4.9) ROC curves for Adiponectin in diabetes mellitus with fatty liver to analyze the optimal diagnostic points for predicting such cases compared to control group.

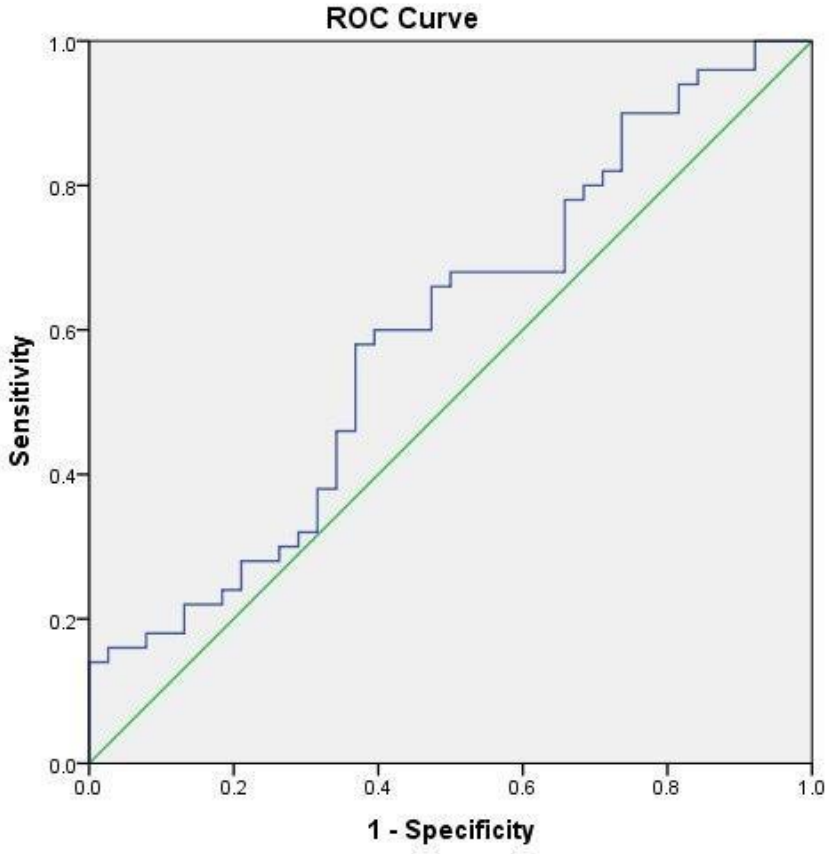

Figure (4.10) ROC curves for Asperosin in diabetes mellitus with fatty liver to analyze the optimal diagnostic points for predicting such cases compared to control group.

Based on recent studies, we review the role of the biomarkers Asprosin and adiponectin in patients with metabolic fatty liver disease associated with type 2 diabetes. A study showed that Asprosin levels were significantly higher in patients with type 2 diabetes who suffer from fatty liver (Cui *et al*., 2024). Research also indicates that Asprosin contributes to the development of fatty liver disease by promoting fat accumulation and inflammation in the liver.

Many researches confirmed the need to validate the role of these markers and determine if a threshold level or changes in serum levels can be utilized clinically in the assessment and follow up of patients with fatty liver.

These findings were established the cut-offs and indicated the reference ranges of such cases to get a broader context on their levels.

Full understanding of adiponectin's function in NAFLD development remains elusive. Adipose tissue secretes several essential Adipokines, one of which is adiponectin (Boutari & Mantzoros, 2020; Shabalala *et al*., 2020). In fact, adiponectin controls glucose and lipid metabolism via activating AMP-activated protein kinase, which it uses to promote hepatic fatty acid oxidation and decrease hepatic fatty acid synthesis (Gastaldelli *et al*., 2021). By stimulating the nitric oxide generation of hepatic stellate cells, which constitutively express both AdipoR1 and AdipoR2, adiponectin may potentially reduce liver fibrosis (Dong *et al*., 2015).

A further mechanism by which adiponectin inhibits fibrosis is via increasing the release of TIMP metallopeptidase inhibitor-1 (TIMP-1) by hepatic stellate cells, which in turn slows their migration (Ramezani-Moghadam *et al*., 2015). A comprehensive assessment of four randomised clinical trials, including data on 187 individuals with NASH treated up to 12 months, also found that there were concurrent increases in plasma adiponectin levels and improvements in histology of NASH (Polyzos and Mantzoros, 2016).

It may be useful to monitor these indicators over time to evaluate the patient's response to various therapeutic interventions such as lifestyle modifications or drug treatments. Asprosin and adiponectin biomarkers show promise in the evaluation of type 2 diabetes patients with metabolic fatty liver, but their use should be part of a comprehensive diagnostic and therapeutic approach that takes into account the individual factors of each patient.

Conclusions and Recommendations

Conclusions

1-Individuals with diabetes exhibit significantly elevated liver enzyme alanine aminotransferase levels compared to those without the condition.

2-Type two diabetes is closely associated with the development of non-alcoholic fatty liver disease, primarily due to the underlying mechanism of insulin resistance.

3-Adiponectin and Asprosin can serve as non-invasive markers for to predict metabolic fatty liver disease.

3-Disease duration, age, and BMI are strongly linked with lipid levels, whereas Asprosin levels relate to insulin resistance.

4-physiological markers had weak linear relationships with illness duration, age, and BMI.

5-Adiponectin demonstrates greater efficacy than Asprosin in differentiating between individuals who are ill and those who are healthy.

Recommendations

1-In groups with type 2 diabetes and at risk of developing non-alcoholic fatty liver disease (obesity,Metabolic Syndrome(MetS)), screening for NAFLD by liver enzymes and/or ultrasound is recommended as part of a routine work-up.

2-All patients with NAFLD and type 2 diabetes should be assessed for features of MetS.

3-NAFLD patients with age >50 and multiple MetS components need to be assessed for advanced fibrosis by noninvasive methods, such as s APRI, FIB-4 or
CAP.

4-Study of the relationship between high levels of adiponectin and glucose in patients with non-alcoholic fatty liver disease and type 2 diabetes.

5- Based on this study, we recommend using Asprosin Hormone as a predictor of diabetes.

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Appendices

Appendix .1

Questionnaire Form of Non-alcoholic Fatty Liver patients											
s	Fasting period	phone number	Full name	Age	Height(cm)	weight (Kg)	Body mass(kg/m2)	Duration of diabetes	Smoking status	Medical history	Do you drink alcohol
1											
2											

Appendix 2. Materials provided with the Asprosin kit

Materials provided with the kit

	Materials provided with the kit	96 determinations	Storage
1	User manual	1	R.T.
2	Closure plate membrane	2	R.T.
3	Sealed bags	1	R.T.
4	Microelisa stripplate	1	2-8℃
5	Standard：540 pg/ml	0.5ml×1 bottle	2-8℃
6	Standard diluent	1.5ml×1 bottle	2-8℃
7	HRP-Conjugate reagent	6ml×1 bottle	2-8℃
8	Sample diluent	6ml×1 bottle	2-8℃
9	Chromogen Solution A	6ml×1 bottle	2-8℃
10	Chromogen Solution B	6ml×1 bottle	2-8℃
11	Stop Solution	6ml×1 bottle	2-8℃
12	wash solution	20ml (30X)×1bottle	2-8℃

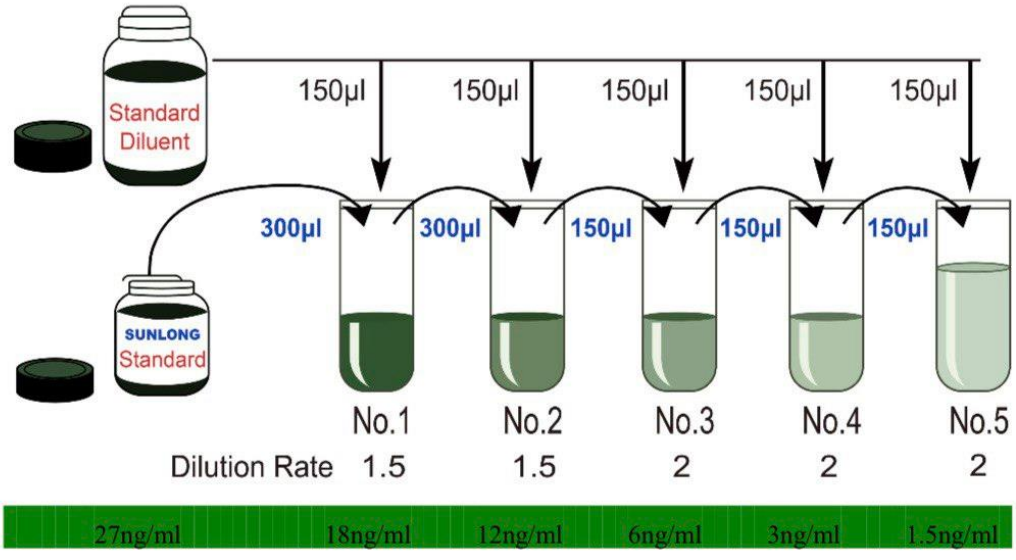
Appendix 3.

Materials provided with the kit

	Materials provided with the kit	96 determinations	Storage
1	User manual	1	R.T.
2	Closure plate membrane	2	R.T.
3	Sealed bags	1	R.T.
4	Microelisa stripplate	1	2-8℃
5	Standard: 27ng/ml	0.5ml×1 bottle	2-8℃
6	Standard diluent	1.5ml×1 bottle	2-8℃
7	HRP-Conjugate reagent	6ml×1 bottle	2-8℃
8	Sample diluent	6ml×1 bottle	2-8℃
9	Chromogen Solution A	6ml×1 bottle	2-8℃
10	Chromogen Solution B	6ml×1 bottle	2-8℃
11	Stop Solution	6ml×1 bottle	2-8℃
12	Wash Solution	20ml (30X)×1bottle	2-8℃

Appendix.4. Asprosin ELISA kit protocol

18ng/ml	Standard No.1	300µl Original Standard + 150µl Standard diluents
12ng/ml	Standard No.2	300µl Standard No.1 + 150µl Standard diluents
6ng/ml	Standard No.3	150µl Standard No.2 + 150µl Standard diluent
3ng/ml	Standard No.4	150µl Standard No.3 + 150µl Standard diluent
1.5ng/ml	Standard No.5	150µl Standard No.4 + 150µl Standard diluent



Appendix 5. Reactive ingredients of TG

The final concentration of reactive ingredients:

reactive ingredients	Concentration
PIPES buffer (pH 7.6)	24.0 mmol/L
ATP	≥ 2.0 mmol/L
NAD+	≥ 1.32 mmol/L
Mg2+	2.37 mmol/L
Hexokinase	≥ 0.59 kU/L
G6P-DH	≥ 1.58 kU/L

Appendix .6 Reactive ingredients of ALT

Final concentration of reactive ingredients:

reactive ingredients	Concentration
Tris buffer, pH: 7.15 (37C)	100 mmol/L
L-Alanine	500 mmol/L
2-Oxoglutarate	12 mmol/L
LDH	≥ kU/L
NADH	0.20 mmol/L
Pyridoxal Phosphate (P-5-P)	0.1 mmol/L (when Cat. No. 60106 or OSR60180 is used)

Appendix .7 Reactive ingredients of AST

Final concentration of reactive ingredients:

reactive ingredients	**Concentration**
Tris buffer, pH 7.65 (37°C)	80 mmol/L
L-aspartate	240 mmol/L
2-Oxoglutarate	12 mmol/L
LDH	≥ 0.9 kU/L
MDH	≥ 0.6 kU/L
NADH	0.20 mmol/L
Pyridoxal phosphate (P-5-P)	0.1 mmol/L (when Cat. No. 60106 or OSR60180 is used)

Appendix .8 Reactive ingredients of Colugos

Final concentration of reactive ingredients:

reactive ingredients	**concentration**	**reactive ingredients**	**Concentration**
PIPES buffer (pH 7.5)	50 mmol/L	Lipases	1.5 kU/L (25 μkat/L)
Mg2+	4.6mmol/L	Glycerol kinase	0.5 kU/L (8.3 μkat/L)
MADE	0.25 mmol/L	Peroxidase	0.98 kU/L (16.3 μkat/L)
4-Aminoantipyrine	0.5 mmol/L	Ascorbate Oxidase	1.48 kU/L (24.6 μkat/L)
ATP	1.4 mmol/L	Glycerol-3-phosphate oxidase	1.48 kU/L (24.6 μkat/L)

Appendix .9 Reactive ingredients of Cholestrol

Final concentration of reactive ingredients:

Reactive ingredients	**Concentration**
Phosphate buffer (pH 6.5)	103 mmol/L
4-Aminoantipyrine	0.31 mmol/L
Phenol	5.2 mmol/L
Cholesterol esterase	≥ 0.2 kU/L (3.3 μkat/L)
Cholesterol oxidase	≥ 0.2 kU/L (3.3 μkat/L)
Peroxidase	≥ 10.0 kU/L (166.7 μkat/L)

Appendix .10 Reference Intervals

National Cholesterol Education Program Adult Treatment Panel III recommendations:

< 5.2 mmol/L (200 mg/dL)	Desirable
5.2 – 6.2 mmol/L (200 – 239 mg/dL)	Borderline High
≥ 6.2 mmol/L (240 mg/dL)	High

Appendix .11 European Atherosclerosis Society recommendations:

Cholesterol Triglyceride	< 5.2 mmol/L (< 200 mg/dL) < 2.3 mmol/L (< 200 mg/dL)	No Lipid metabolism disorder
Cholesterol	5.2 – 7.8 mmol/L (200 – 300 mg/dL)	Lipid metabolisdisordeif HDL-Cholesterol is < 0.9 mmol/L (< 35 mg/dL)
Cholesterol Triglyceride	> 7.8 mmol/L (> 300 mg/dL) > 2.3 mmol/L (> 200mg/dL)	Lipid metabolism disorder.

Appendix.12. Reactive ingredients

Final concentration of active ingredients of uric acid:

Active ingredients	**concentration**
Phosphate Buffer (pH 7.5)	42 mmol/L
MADB	0.15 mmol/L
4-Aminophenazone	0.30 mmol/L
Peroxidase	≥ 5.9 kU/L (98 µkat/L)
Uricase	≥ 0.25 kU/L (4.15 µkat/L)

Appendix .13. Reactive ingredients

Final concentration of reactive ingredients of Albumin:

reactive ingredients	Concentration
Succinate buffer	(pH 4.2) 100 mmol/L
Bromocresol green	0.2 mmol/L

الخلاصة

تؤثر الأمراض الأيضية، بما في ذلك مرض الكبد الدهني غير الكحولي ومتلازمة التمثيل الغذائي ومرض السكري من النوع٢، على الصحة العالمية. تتضمن متلازمة التمثيل الغذائي عدم تحمل الجلوكوز ومقاومة الأنسولين وخلل شحميات الدم والحالات المؤيدة للالتهابات وارتفاع ضغط الدم الشرياني.

شملت الدراسة ١٠٨عينة دم تتراوح أعمارهم بين ٣٠-٧٧ عامًا. من تشرين الاول ٢٠٢٣ إلى نيسان٢٠٢٤ تم جمع٧٠ عينة من الذكور والإناث من مرضى السكري من النوع٢ ومرض الكبد الدهني غير الكحولي ، ٣٠ ذكرا و٤٠ انثى من العيادات الاستشارية في مستشفى الهندية العام ومستشفى الإمام الحسن بمحافظة كربلاء. وشملت مجموعة المقارنة 38 عينة دم صحية من نفس العمر والجنس ١٦ذكرا و٢٢ انثى. تم استبعاد إدمان الكحول والأورام الخبيثة وجراحة الجهاز الهضمي وأمراض الكبد المناعية الذاتية واضطرابات الكبد مثل التهاب الكبد المزمن ويلسون التي قد تسبب الكبد الدهني. تم سحب الدم من الأوردة بعد الصيام (١٢-٨) ساعة. تم فحص الجلوكوز والانسولين ومقاومة الانسولين وملف الدهون وانزيمات الكبد الانين امينوتراسفيراز والاسبارتيت امينو تراسفيراز والالبومين واليورك اسد وفيتامين دي وهرمون الاديبونكتين والاسبروسين.

كان متوسط مستويات الانين امينوتراسفيراز في مجموعة المرضى ٢٢,٢١ ±١٠,١ U/L، أي أعلى بثلاثة أضعاف تقريبًا من مستوى متوسط المجموعة الضابطة٧,٦٣وحدة / لتر. كان متوسط الاسبارتيت امينو تراسفيراز في مجموعة المرضى 31.46±14.25 U/L، أي أكثر بنسبة ٤٠٪ من المجموعة الضابطة 22.12±5.23 U/L. تعد نسبة ألانين إلى أسبارتات أمينوترانسفيراز مؤشرًا نموذجيًا لتليف الكبد الدهني في ارتفاع ضغط الدم وفرط شحميات الدم وفرط سكر الدم، كان هذا الارتباط أقوى. أظهر مؤشر التراكم أيضًا أن ملف الدهون هو علامة على مرض الكبد الدهني غير الكحولي ويزيد من المخاطر.

كان لدى المجموعة المصابة بمرض السكري مع الكبد الدهني متوسط أعلى من الكوليسترول ٢١٢,٨١±١٠١,٣mg/dl ومستويات الدهون الثلاثية ٢٧٩,٩٦±١٢٤,٧ mg/dl(من مجموعة التحكم) ١٧٢,٧٨±٧٧,٥٢mg/dl،ومتوسط مستوى مجموعة التحكم ١٧١,٥٧±٤٦,٢١ mg/dl.

كان لدى المرضى مستويات إنسولين ومقاومة للأنسولين أعلى بكثير من الضوابط. كان متوسط مستوى الأنسولين ١٧١,٥٧±٤٦,٢١ mg/dl، وهو أعلى بكثير من مجموعة التحكم ١٦,٤١±٩,٨١U/ml. كان لدى مرضى السكري المصابين بالكبد الدهني متوسط مستوى أعلى لمقاومة الانسولين ١٤,٢٨±٨,٥٣U/ml من

مجموعة التحكم ٤,٤٩± ٢,٢٤U/ml مما يشير إلى وجود تشوهات أيضية. ما بالنسبة لهرموني الأسبرسين والأديبونيكتين، كان لدى المجموعة المريضة متوسط مستوى أعلى من الاسبروسين ١٥٠,٥٦ ± ٦١,٦ /ng من المجموعة الضابطة ١٢٧,٦٤±٤٦,٩ ng/mlكما كان لدى المرضى مستوى أديبونيكتين ٥,٢٧±٢,٦ µg/ml أعلى من المجموعة الضابطة ٤,١٨±٢,٠٨ µg/ml .

كانت إنزيمات الكبد، وخاصة الألانين، مرتبطة سلبًا بالأسبرين ومرتبطة بشكل ضعيف بالأديبونيكتين شير البيانات الى وجود ارتباط كبير بين الاسبروسين والبروتين الدهني منخفض الكثافة والانسولين ومقاومة الانسولين. مؤشرات ملف الدهون اشارت الى وجود علاقات إيجابية بين الكوليسترول والدهون الثلاثية والبروتين الدهني عالي الكثافة والمنخفض جدا والاسبروسين، حيث لوحظ ارتباط سلبي ففط للبروتين الدهني منخفض الكثافة مع الاسبروسين. وقد أظهرت النتائج أن العمر ومؤشر كتلة الجسم ومدة المرض كانت لها علاقات وارتباطات مهمة مع المؤشرات الحيوية. وقد أظهرت النتائج أن هذه الارتباطات قد تكون ضعيفة أو متوسطة أو قوية. كما أن إنزيمات الكبد لها علاقة سلبية مع العمر، ومؤشر الدهون له علاقة إيجابية مع العمر، والأنسولين والسكر ومؤشر تليف الكبد لها علاقة إيجابية مع العمر وكتلة الجسم. وخلصت الدراسة إلى أنه يمكن استخدام مقاومة الأنسولين وارتفاع مستويات الأسبروسين كمؤشرات حيوية للتنبؤ بمرض السكري من النوع ٢ والكبد الدهني، فضلاً عن اختبار غير جراحي لمرض الكبد الدهني غير الكحولي المرتبط بالتمثيل الغذائي، حيث أن أساس مرض الكبد الدهني غير الكحولي هو مقاومة الأنسولين. ويتميز الأديبونيكتين بفعالية متفوقة مقارنة بالأسبرين في التمييز بين الأشخاص المرضى والأصحاء.

جامعة كربلاء

كلية العلوم

قسم علوم الحياة

تقييم بعض المعايير الفسلجيه والكيموحيويه في مرضى السكري النوع الثاني المرتبط بمرض الكبد الدهني غير الكحولي

رسالة مقدمة الى

مجلس كلية العلوم/جامعة كربلاء وهي جزء من متطلبات نيل

درجة الماجستير في علوم الحياة

كتبت بواسطة:

ايمان فالح هادي الغرابي

بكالوريوس علوم حياة /جامعة كربلاء (٢٠١٧)

بأشراف

أ.م. د. ابتسام عباس ناصر

جامعة كربلاء

كلية العلوم

أ.م. د. حميد حسين طوفان

جامعة كربلاء

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