Study of Adiponectin Promotor Methylation Status in Patients with Non Alcoholic Fatty Liver Disease

Thesis

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Study of Adiponectin Promotor Methylation status in patients with Non Alcoholic Fatty Liver Disease

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LIST OF CONTENTS

Titles	*Page No.*
Introduction	1
Aim of the Work	4
Review of Literature	5
▪ **Chapter I:** NON ALCOHOLIC FATTY LIVER DISEASE	5
▪ **Chapter II:** ADIPONECTIN	22
▪ **Chapter III**: ADIPONECTIN AND NON-ALCOHOLIC FATTY LIVER DISEASE	42
Patients and Methods	46
Results	54
Discussion	85
Conclusion & Recommendations	91
Summary	92
References	95
Arabic Summary	1

List of Abbreviations

Abbreviation	Meaning
ACC	acetylcoenzyme A carboxylase
Adipo Q	adiponectin
A-FABP	adipocyte fatty acid binding protein
AKT	Protein Kinase B
ALT	Alanine amino trasaminase
AMPK	adenosine monophosphate-activated protein kinase
APPL1	Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1
ARH	para-ventricular hypothalamus and arcuate nuclei
AST	Aspartate trasaminase
AUC	Area under curve
BAT	Brown adipose tissue
Bax	B-cell lymphoma protein 2 (Bcl-2)-associated X
Bcl-2	B-cell lymphoma protein 2
BMI	Body mass index
CAD	coronary artery disease
CBC	Complete Blood Count
CHF	Chronic heart failure
C-myc	c-myelocytomatosis
COL1A	type 1 alpha collagen
COPD	Chronic Obstructive Pulmonary Disease
CRP	C-reactive protein
CT	computed tomograph
CVD	cardio vascular disease (CVD
DNMTs	DNA methyl transferases
DPP-4	dipeptidyl peptidase-4
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinases
FAK	focal adhesion kinases
FFA	free fatty acid
FIB-4	fibrosis index
FXR	farnesoid X receptor
GDM	Gestational diabetes mellites
GGT	gamma-glutamyltranspeptidase
GLP-1	Glucagon-like peptide-1

HbA_{1c}	glycated hemoglobin
HBsAg	Hepatitis B surface antigen
HCC	hepatocellular cancer
HCV Ab	Hepatitis C virus antibody
HDL	high density lipoprotein
HMW	high molecular weight
HSCs	hepatic stellate cells
ICAM	Intercellular adhesion molecule
IL-6	interleukin 6
INR	international normalized ratio
IRS	Insulin receptor substrate
LDL	low density lipoprotein
LMW	low molecular weight,
MAPK	Mitogen-Activated Protein Kinase
MI	myocardial infarction
miRNA	Micro Ribonucleic acid
MMP1	matrix metalloproteinase 1
MMW	middle molecular weight
MRI	magnetic resonance imaging
MSP	methylation specific polymerase chain reaction
mTOR	Mammalian Target Of Rapamycin
NAFLD	Non-alcoholic fatty liver disease
NAFLD	Nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NEFA	non-esterified fatty acid
NF-κB	nuclear factor kappaB
NO	nitric oxide
NPV	Negative predictive value
OCA	Obeticholic acid
OSA	obstructive sleep apnea
P21	cyclin-dependent kinase inhibitor 1
p300	Protein 300
P53	Protein 53-cellular tumour antigen
PAQR	progestin and adipoQ receptors
PBMCs	Peripheral blood mononuclear cells
PC	prothrombin concentration
PCOS	polycystic ovarian syndrome
PCSK9	Proprotein convertase subtilisin/kexin type 9
PEPCK	Phospho Enol Pyruvic Carboxy Kinase

PI3K	phosphatidylinositol 3 kinase
PPAR	peroxisome proliferator activated receptor
PPV	Positive predictive value
PT	prothrombin time
PTT	partial thromboplastin time
RCT	randomised control trial
ROC curve	Receiver operating characteristic
ROS	reactive oxygen species
STAMPEDE	Surgical Treatment and Medications Potentially Eradicate Diabetes Efficiently
T2DM	Type 2 diabetes mellites
TE	transient elastography
TIMP-1	tissue inhibitor of metalloproteinase -1
TLR4	Toll-like receptor 4
TNFα	tumor necrosis factor α
UCP1	uncoupling protein 1
UV	ultraviolet
VCAM	vascular cell adhesion molecule
VLDL	very low density lipoprotein
WAT	white adipose tissue
WHR	waist hip ratio
Wnt	Wingless and Int-1

LIST OF TABLES

Table No.	Title	Page No.
1.	Reaction setup for SYBR Green Methylation specific PCR(qPCR)	51
2.	Primer sequence and annealing temperature of *Adiponectin* promotor	52
3.	Age distribution among the studied groups	54
4.	Demographic characteristics (Gender, Residence and Occupation) among the studied groups	55
5.	Frequency distribution and percentage of clinical history among the studied groups	57
6.	Frequency distribution and percentage of clinical manifestations among the studied groups	60
7.	Mean± SD and Median of Weight, Height, BMI, Waist circumference and Waist/Hip ratio in all studied groups	61
8.	Mean ± SD and median of HB, platelets, WBCs counts and liver function tests among the studied groups.	65
9.	Mean ± SD and median of lipid profile & blood glucose profile in all studied groups	68
10.	Frequency distribution and percentage of Liver biopsy findings among simple steatosis and NASH groups	72
11.	Comparison between the study groups regarding *adiponectin* promotor methylation	73
12.	Correlation coefficient and P values between *adiponectin* promotor methylation and different parameters in studied groups	75
13.	Relation between *adiponectin* promotor methylation and clinicodemographic parameters in simple steatosis group	79
14.	Relation between *adiponectin* promotor methylation and liver biopsy findings in simple steatosis group	80
15.	Relation between *adiponectin* promotor methylation and clinico-demographic parameters in NASH group	81

16.	Relation between *adiponectin* promotor methylation and liver biopsy findings in NASH group (n=29).	82
17.	Validity of *adiponectin* promotor methylation (AUC, Sensitivity, specificity) in detection of NAFLD	84

LIST OF Figures

Fig. No.	Title	Page No.
1.	Pathophysiological mechanisms involved in the development and complications of nonalcoholic fatty liver disease (NAFLD)	11
2.	Domains and structure of adiponectin	23
3.	Adiponectin multimer formation	24
4.	Overall structures of AdipoR1 and AdipoR2	25
5.	Schematic representation of adiponectin signal transduction pathway implicating across talk with the insulin signaling pathway	28
6.	Summary of tissue-specific functions of adiponectin and its agonizing effect on insulin resistance	30
7.	Adiponectin possesses anti-inflammatory properties	31
8.	Adiponectin protects the vasculature	33
9.	Adiponectin regulation of food intake	36
10.	Adiponectin inhibits fibrosis	38
11.	Schematic depiction of the clinical outcomes of hypoadiponectinemia	41
12.	Box-plot showing comparison between the studied groups regarding Age	54
13.	Comparison between the study groups regarding gender	55
14.	Comparison between the study groups regarding residence	56
15.	Comparison between the study groups regarding occupation	56
16.	Comparison between the study groups regarding Frequency distribution and percentage of smoking	57
17.	Comparison between the study groups regarding Frequency distribution and percentage of Schistosomiasis	58
18.	Comparison between the study groups regarding Frequency distribution and percentage of DM	58
19.	Comparison between the study groups regarding Frequency distribution and percentage of HTN	59
20.	Box-plot showing comparison between the studied groups regarding weight.	64
21.	Box-plot showing comparison between the studied groups	64

	regarding BMI	
22.	Box-plot showing comparison between the studied groups regarding GGT	67
23.	Box-plot showing comparison between the studied groups regarding INR	67
24.	Box-plot showing comparison between the studied groups regarding Total Cholesterol	70
25.	Box-plot showing comparison between the studied groups regarding LDL	70
26.	Box-plot showing comparison between the studied groups regarding HDL	71
27.	Box-plot showing comparison between the studied groups regarding FBS	71
28.	Comparison between simple steatosis & NASH groups regarding grades of fibrosis	72
29.	Box-plot showing comparison between the studied groups regarding *Adipo Q* methylation	73
30.	Agarose gel electrophoresis for methylation specific PCR products of *adiponectin* gene	74
31.	Scatter plot showing positive correlation between *Adipo Q* and Height in simple steatosis group.	77
32.	Scatter plot showing positive correlation between *Adipo Q* and PC in NASH group	77
33.	Scatter plot showing positive correlation between *Adipo Q* and BMI in NASH group	78
34.	Scatter plot showing positive correlation between *Adipo Q* and AST in NASH group	78
35.	Relation between *adiponectin* promotor methylation and fibrosis grades in NASH group	83
36.	ROC curve of *Adipo Q* as a predictor between NAFLD patients and control group	84

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a disorder in which fat accumulates in the liver after all other causes of hepatic steatosis have been ruled out, such as liver disease caused by other factors, excessive alcohol intake, and other conditions that might induce hepatic steatosis. NAFLD has a wide clinical spectrum, ranging from non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH) ***(Perumpail et al., 2017).*** Hepatic fat accounts for more than 5–10% of entire liver weight in NAFLD. 20% of patients may acquire progressive hepatic fibrosis, which can lead to liver cirrhosis or failure, as well as hepatocellular cancer. ***(Borai et al., 2017).***

NAFLD is a silent disease that affects the Egyptian people. It affected 20%–30% of the general population worldwide, but it can affect up to 50% of the population in obese people or those with diabetes mellitus. According to previous studies, steatosis is found in 70% of obese patients and 35% of non-obese patients, while NASH is found in 18.5 percent of obese patients and 3% of non-obese patients. Fatty liver disease might affect up to 75 percent of patients with type 2 diabetes mellitus (DM2) (***Binobaid et al., 2018).*** The total incidence of NAFLD in adolescents has reached about 10%, with 17% of teenagers and 40%–70% of obese youngsters suffering from the disease ***(Deeb et al., 2018)***.

NAFLD pathogenesis has been linked to a number of risk factors, including advanced age, obesity, insulin resistance, and hyperlipidemia, as well as the involvement of pro- and anti-inflammatory cytokines ***(Mohamed et al., 2016).***

Many studies have highlighted the importance of adipose tissue as

an active endocrine organ that produces adipokines such as adiponectin (Adipo Q), leptin, resistin, and visfatin, all of which are involved in the pathogenesis and progression of NAFLD ***(Xie et al., 2018).***

Hepatocytes respond to adiponectin by increasing free fatty acid oxidation while decreasing gluconeogenesis, FFA inflow, de novo lipogenesis and hepatocytes apoptosis. It has anti-inflammatory and anti-fibrotic properties by acting on hepatic stellate cells (HSC), kupffer cells, and perhaps sinusoidal cells. It works to reduce inflammation by inhibiting pro-inflammatory cytokines (TNF- α and IL-6) and activating anti-inflammatory cytokines (IL-10) ***(Polyzos et al., 2016).***

Adiponectin's anti-fibrotic activity is primarily done by inhibiting HSC activation and proliferation. TGF-, connective tissue growth factor, and collagen are all downregulated by adiponectin, which favors matrix breakdown ***(Saxena and Anania, 2015).***

Previous research has shown that DNA hypermethylation of the adiponectin promoter inhibits adiponectin expression and, as a result, decreases its activity and exacerbates metabolic disorders in obese people ***(Kim et al., 2015).*** Adiponectin DNA methylation levels in subcutaneous adipose tissue are linked to obesity-related anthropometric measurements, according to another study ***(Houde et al., 2015).***

Hypoadiponectinemia was found in women with GDM. Significant changes in locus-specific DNA methylation were detected in maternal fat and blood cells, also the methylation of DNA in GDM offspring was changed ***(Ott et al., 2018).***

Liver biopsy is the gold standard for diagnosing NASH, but it is invasive, expensive, and linked to uncommon but potentially dangerous consequences and sampling errors; thus, it is ineffective as a screening

tool ***(Borai et al., 2017).*** As a result, detecting adiponectin promoter methylation could be useful in NAFLD diagnosis, prognosis, and treatment.

Aim of the Work

This study was designed to:

- Detect the adiponectin promotor methylation status in patients with NAFLD.
- Evaluate the correlation between the adiponectin promotor methylation status and the clinicopathological characteristics of NAFLD patients.

CHAPTER (I)

NON ALCOHOLIC FATTY LIVER DISEASE

I- Introduction

NAFLD (nonalcoholic fatty liver disease) is a clinicopathological syndrome characterized by fat accumulation in hepatocytes due to genetic, environmental, and metabolic stress-related factors. Fat accumulation reaches 5% of hepatic wet weight, or alterations in fatty content occurred in more than 1/3 of hepatocytes per unit area without alcohol overconsumption. The condition can progress from nonalcoholic fatty liver to nonalcoholic steatohepatitis (NASH), fatty hepatic fibrosis and cirrhosis ***(Liu et al., 2020)***.

The incidence and prevalence of NAFLD is increasing due to increased obesity and diabetes rates around the world. Many recent studies have found that people with NAFLD can develop liver cancer even if they don't have cirrhosis. These outcomes have a profound impact on the personal and societal costs of disease. As a result, developing effective strategies for detecting and treating this illness is a public health priority ***(Albhaisi and Sanyal, 2018).***

II- Epidemiology

NAFLD is the most common cause of liver disease in developed countries, affecting 25–33% of the general population and up to 75% of obese persons. NAFLD is connected to obesity, insulin resistance, type 2 diabetes and cardiovascular disease. NAFLD is a critical contributor in the progression of type 2 diabetes and the metabolic syndrome ***(Layal et al., 2020).***

The total incidence of NAFLD in adolescents has reached about 10%, with 17% of teenagers and 40%–70% of obese children suffering from the disease ***(Deeb et al., 2018)***. The Middle East (31.79%) and South America (30.45%) have the greatest prevalence rates of NAFLD, whereas Africa has the lowest prevalence rate (13.48%) ***(Younossi et al., 2016),*** the true prevalence of NAFLD and related illnesses is unknown, owing to the absence of effective and relevant diagnostic tests ***(Araújo et al., 2018).***

In Egypt, a cross-sectional study showed that the prevalence of NAFLD in children and adolescents was 15.8%. The prevalence increased with age, from less than 20% under the age of 20 years to more than 40% in over the age of 60 years, NAFLD is more common in men (31%) than in women (16%) ***(Hassan etal., 2020).***

III- Risk Factors and Etiology:

➢ Obesity

The most frequent and well-documented risk factor for NAFLD is obesity (high BMI and visceral obesity). In fact, NAFLD is linked to the full spectrum of obesity, from being overweight to being obese or severely obese. In this situation, NAFLD will affect the vast majority (>95%) of individuals with severe obesity who have bariatric surgery ***(Subichin et al., 2015).***

➢ Metabolic syndrome and type 2 diabetes mellitus

Metabolic syndrome is a term used to describe a group of symptoms that Three out of five of the following variables are required by current diagnostic criteria: Hyperglycemia (fasting glucose of 100 mg/dL or above), high-density lipoprotein-cholesterol of less than 40 mg/dL in men and less than 50 mg/dL in

women, triglycerides of 150 mg/dL or more, Abdominal obesity(waist circumference more than 35 inches for women and more than 40 inches for men) and hypertension (systolic blood pressure of 130 mmHg or greater or diastolic blood pressure of 85 mmHg or greater) ***(Kanwar and Kowdley, 2016).***

As previously stated, the prevalence of NAFLD has been rising in tandem with the prevalence of metabolic syndrome. The liver of Type 2 diabetes mellitus (T2DM) patients has 80% fat contents higher than non-diabetic patients (matched for age, gender, and body weight) ***(Lonardo et al., 2015).*** T2DM patients are also at a very high risk of developing NASH and other fatty liver-related complications ***(Sung et al., 2014).***

- **Ethnic differences**

Hispanic patients have been reported to have the highest rate of NAFLD development. NAFLD has also been on the rise in the Asian population, and strangely, it can be detected in those with a normal BMI ***(Kalia and Gaglio, 2016).*** In a study conducted in the United States, researchers discovered that African Americans have less steatosis than whites, as well as a higher prevalence of NAFLD in Asians and Hispanics ***(Benedict and Zhang, 2017)***

- **Gender and age**

NAFLD is more common in men, and it has been proven to increase in individuals who are young to middle-aged, with a drop after the age of 50-60. NAFLD, on the other hand, has been demonstrated to spare premenopausal women, with a spike in prevalence after the age of 50, peaking at 60-69 years, but some researchs suggest that NASH is histologically more severe in females than in males ***(Lonardo et al., 2015).***

Several investigations using magnetic resonance spectroscopy have shown steatosis in infants born to mothers with gestational diabetes, leading to the suggestion that NAFLD originates in utero ***(Goyal and Schwimmer, 2016).*** Obesity in children is a significant risk factor for the development of NAFLD. ***(Benedict and Zhang, 2017).***

- **Diet, smoking and life style**

Diet is an independent risk factor for the development of NAFLD. Diets heavy in fats and red meat, refined carbohydrates, and pastries have been linked to a higher risk of metabolic syndrome and eventual NAFLD ***(Satapathy and Sanyal et al. 2015)***

It has been demonstrated that dietary changes, such as energy restriction and adjustment of dietary macronutrients, such as carbohydrate restriction, fat restriction, or enrichment with monounsaturated fatty acids, can improve metabolic syndrome ***(Godos et al., 2017).***

Cigarette smoking was discovered to be an independent risk factor for the onset of NAFLD due to the development of insulin resistance and metabolic syndrome in a retrospective investigation ***(Benedict and Zhang, 2017).***

In terms of lifestyle, studies have linked sedentary behavior to the chance of NAFLD and NASH developing and severity ***(Satapathy and Sanyal et al. 2015).*** There is also a great impact of exercise on hepatic fatty infiltration; this advantage can be seen even when there is little or no weight loss ***(Keating et al., 2012).***

- **Polycystic ovarian syndrome**

NAFLD was found to be more common in people who had polycystic ovarian syndrome (PCOS), which is a common endocrine condition in reproductive-aged women characterised by obesity and insulin resistance. As a result, women with PCOS are more likely to develop T2DM and NAFLD ***(Macut et al., 2016).***

- **Obstructive sleep apnea**

Patients with obstructive sleep apnea (OSA), which is characterised by total or partial airway obstruction caused by pharyngeal collapse during sleep, were shown to have a significant prevalence of NAFLD. Several studies have found a link between OSA and NASH, with chronic intermittent hypoxia potentially causing liver damage, inflammation, and fibrogenesis ***(Paschetta et al., 2015).***

IV- Pathogenesis of NAFLD

Although the exact cause of NAFLD is unknown, insulin resistance, oxidative stress, and inflammation all have a role in its development and progression ***(Obika and Noguchi, 2012).*** Insulin resistance is also a symptom of fatty liver, hepatic fat accumulation can cause hepatic insulin resistance, which can happen before changes in peripheral insulin actions and can cause peripheral insulin resistance ***(Tarantino et al., 2012).***

Insulin controls glucose absorption, oxidation, and storage in insulin-sensitive tissues such as the liver, skeletal muscle and adipose tissue. Insulin resistance in the periphery decreases glucose absorption from the circulation into skeletal muscle and adipose tissue, and serum non-esterified fatty acid (NEFA)

levels may be raised as a result of insulin's failure to control lipolysis ***(Joseph et al., 2012).***

Insulin resistance is linked to an increase in the cellular level of fatty acids and their metabolites in the liver (diacylglycerides and ceramides) ***(Rocha et al., 2011).*** In the presence of high circulating levels of NEFA, hyperinsulinemia (produced by insulin resistance) increases hepatic fatty acid absorption and promotes lipogenesis ***(Gathercole et al., 2011).***

Hepatic steatosis is also caused by abnormalities in mitochondrial - oxidation, increased fatty acid production, and reduced secretion of triacylglyceride-rich very low density lipoproteins ***(Nagarajan et al., 2012).***

Endotoxin exposure and virus infections cause steatohepatitis by increasing hepatic lipid storage, causing hepatocellular damage, and promoting oxidative stress and inflammation in the liver. In addition, lipotoxicity, cytokine release, and other pro-inflammatory mediators all play a role in this process. Furthermore, when NASH progresses, inflammation might obstruct insulin signalling even more ***(Finelli and Tarantino, 2017).***

NASH is characterised by hepatocyte nuclear ballooning, apoptosis, and Mallory's hyaline and inflammatory foci on histological examination ***(Liu et al., 2010).***

Normally, adipose tissue serves as a buffer for excess energy by absorbing and storing fatty acids, it also acts as an endocrine organ (fig.1) by releasing adipokines. In Obesity and NAFLD adipose tissue function impaired; Secretion of most adipokines increases and adiponectin secretion decreases ***(Parker, 2018),*** despite Adiponectin protects the liver from inflammation and fibrosis by limiting excess lipid accumulation ***(Petta et al., 2016).***

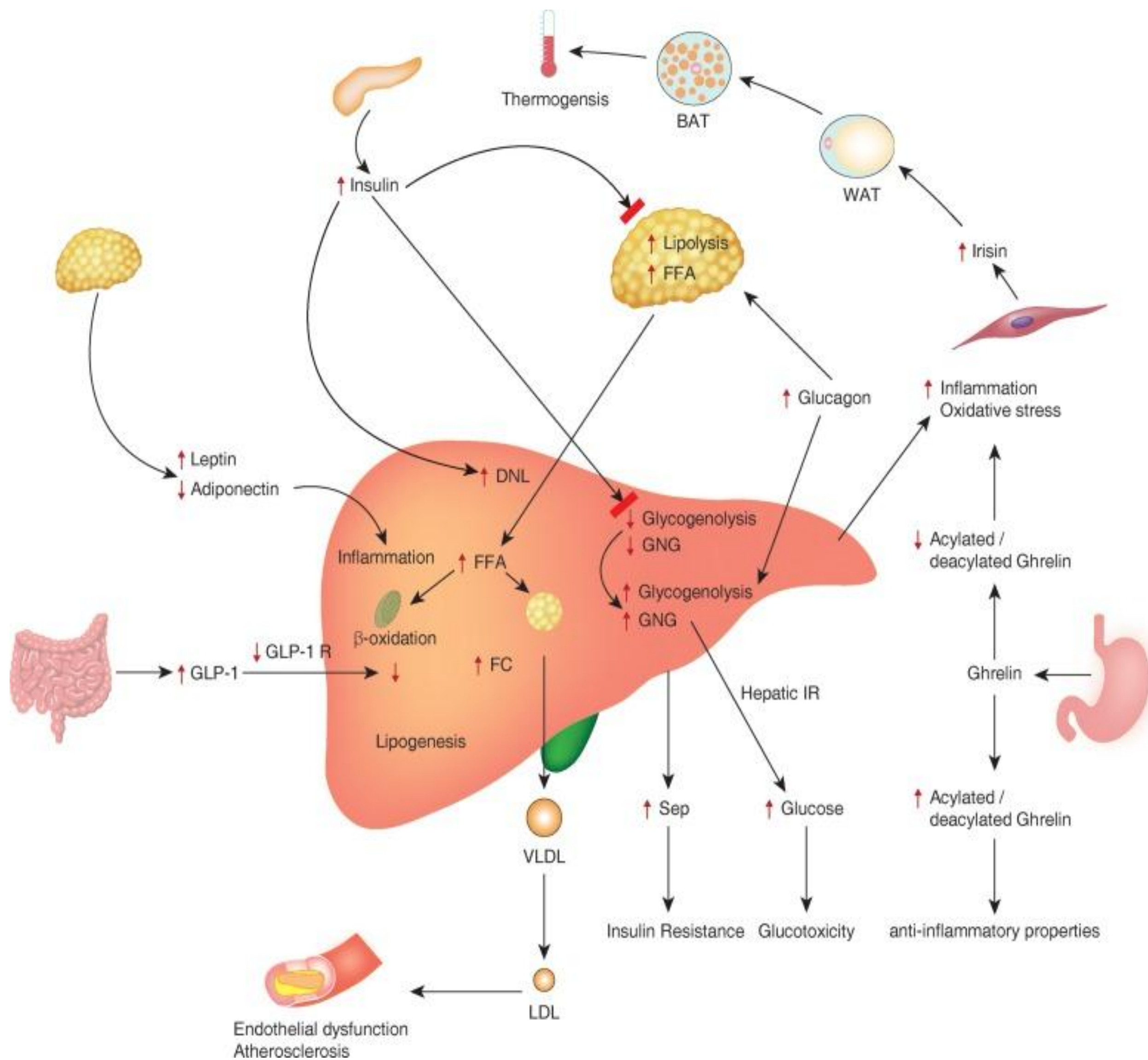


Figure (1): Pathophysiological mechanisms involved in the development and complications of nonalcoholic fatty liver disease (NAFLD).BAT, brown adipose tissue; DNL, de novo lipogenesis; FC, free cholesterol; FFA, free fatty acid; GLP-1, glucagon-like insulinotropic peptide; GNG, gluconeogenesis; IR, insulin resistance; LDL, low density lipoprotein; SeP, selenoprotein P; VLDL, very low density lipoprotein; WAT, white adipose tissue ***(Pappachan et al., 2017).***

V- Symptoms and Signs of NAFLD

NAFLD affects the majority of persons who have few or no symptoms. Fatigue, malaise, and dull right-upper-quadrant stomach ache are common complaints. Mild jaundice is possible, but not common ***(Ahmed et al., 2017).***

VI- Diagnosis of NAFLD:

A considerable majority of people with NAFLD are asymptomatic, and the diagnosis is frequently assumed when liver functions are abnormal on biochemical testing and hepatic imaging (ultrasonography, CT, or MRI of the liver) suggests fatty liver ***(Albhaisi and Sanyal, 2018).***

- **Invasive methods:**

The gold standard for diagnosing NAFLD is still a liver biopsy ***(Albhaisi and Sanyal, 2018).***

Because NAFLD is so common, performing a liver biopsy to diagnose fibrosis-cirrhosis is impossible. Due to sampling errors and intra- and inter-observer variability, the accuracy of liver biopsy to detect fibrosis has also been questioned, potentially leading to over- or under-staging. The principal disadvantages of liver biopsy include the cost, procedure-related problems, and intra- and inter-observer variability in histology reporting. As a result, it is rarely suggested in clinical practice, unless additional differential diagnosis must be ruled out ***(Castera and Pinzani, 2010).***

- **Non-invasive methods:**

Throughout the last decade, there has been an increase in interest in several new methods for the non-invasive evaluation of fibrosis. Two distinct but

complementary approaches are the identification of serum biomarkers and the computation of liver stiffness using ultrasound-based elastography with transient elastography (TE) ***(Castera and Pinzani, 2010).***

TE, the fibrosis index (FIB-4), and NAFLD fibrosis scores are the best non-invasive diagnostics in NAFLD patients ***(Friedrich-Rust et al., 2016).*** The most accurate method for diagnosing cirrhosis is TE. In a recent meta-analysis based on 64 studies with a total of 13,046 NAFLD patients, the summary AUROC values of TE, FIB-4, and the NAFLD fibrosis score for diagnosing severe fibrosis-cirrhosis were 0.88, 0.84, and 0.84, respectively, for detecting severe fibrosis-cirrhosis ***(Boursier et al., 2016).***

The NAS is a grade that is calculated by adding the numerical values for steatosis (0-3), hepatocellular ballooning (0-2), and lobular inflammation (0-3). As a result, the NAS scales from 0 to 8. The NAS is one of the few NAFLD grading systems that have been externally validated ***(Michael and Murray, 2020).***

The standard imaging modalities utilized in clinical practice for the diagnosis of NAFLD include ultrasonography, CT, and MRI of the liver. Sonography can detect NAFLD when hepatic steatosis exceeds 33%. TE is an ultrasound-based imaging tool for determining the degree of fibrosis. TE has been shown to have a sensitivity and specificity of 79–92% and 75–92 % respectively, for diagnosing various stages of fibrosis ***(Kwok et al., 2014).*** Ultrasound-based controlled attenuation parameter value of the TE method can predict the degree of steatosis in NAFLD patients ***(Karlas et al., 2017).***

The gold standard for non-invasive measurement of hepatic steatosis is the use of MRI protein density fat fraction. By non-invasively assessing the degree of

fibrosis, newer MRI techniques, such as MR elastography, can diagnose and estimate the prognosis of people with NAFLD ***(Kinner et al., 2016).***

Liver enzymes are mostly normal in persons with NAFLD. TNF-a, IL-6, CRP, pentraxin, ferritin, serum prolidase enzyme activity, soluble receptor for advanced glycation end product and cytokeratin-18 have all been proposed in the past as useful biochemical indicators for predicting the severity of NAFLD/NASH. None of these markers have demonstrated sufficient sensitivity or specificity for regular clinical diagnosis ***(Oh et al., 2016)***.

VII- Treatment of NAFLD

There is no single medication for NAFLD that has been demonstrated to be completely successful. The primary goals of treatment are to improve steatosis and prevent disease progression. The corner-stones of NAFLD management are intensive lifestyle change and treatment of risk factors. Medical and surgical therapies are used as adjuvants to first-line therapy ***(Pappachan et al., 2017)***

A- Lifestyle interventions

Multiple studies have indicated that sustained and effective weight loss through calorie restriction and increased physical exercise improves liver function and histology ***(Katsagoni et al., 2017).*** NAFLD biochemical and histological markers could be improved with exercise and nutrition treatments. Multiple studies have demonstrated that a low-carbohydrate, high-fat diet is beneficial in correcting all of the aberrant clinical and biochemical markers of metabolic syndrome and NAFLD ***(Noakes and Windt, 2017)*.**

These dietary changes have also been linked to weight loss in patients. However, even without significant weight reduction, lifestyle therapies have been

shown to improve NAFLD, but Patient compliance concerns are always a challenge for therapeutic approaches ***(Pappachan et al., 2017).***

B- Medication

1) Insulin sensitizing agents

Insulin sensitizing drugs are likely to change the pathophysiological mechanisms of NAFLD because it is linked to insulin resistance and metabolic syndrome. Metformin and the thiazolidinedione category of anti-diabetic drugs are the most extensively researched ***(Pappachan et al., 2017).***

- **Metformin**

Despite the fact that metformin was linked to considerable reductions in insulin resistance (IR) and liver transaminases (AST and ALT), the medicine failed to improve histological indicators such steatosis, inflammation, hepatocellular ballooning, and fibrosis ***(Li et al., 2013).*** Metformin should be explored for patients with T2DM or even prediabetic conditions with NAFLD because of its antidiabetic effects. Metformin has been demonstrated to be safe, even in individuals with cirrhosis, and may protect people with T2DM and chronic liver disorders from developing HCC ***(Bhat et al., 2015).***

- **Thiazolidinediones**

These medicines improve blood glucose control by modulating tissue insulin sensitivity via the peroxisome proliferator activated receptor (PPAR) signalling pathway. The medicines rosiglitazone and pioglitazone have been investigated

extensively in this family of medications for the treatment of T2DM. Rosiglitazone use has dropped dramatically in recent years due to increased cardiovascular problems, while pioglitazone becomes the most commonly prescribed medication. Pioglitazone improves Hepatic insulin sensitivity and fatty acid oxidation and it inhibits hepatic lipogenesis ***(Pappachan et al., 2017).*** Pioglitazone improves biochemical and histological indices of NAFLD, but it is related to weight gain ***(Singh et al., 2015).***

Antioxidants

Antioxidants have been investigated extensively in the pathophysiology of NAFLD since oxidative stress is a crucial factor in the disease's etiology ***(Singh et al., 2015).*** In this group, vitamin E is the most researched antioxidant. It results in significant improvements in all histology parameters, including steatosis, hepatocyte ballooning, lobular inflammation and fibrosis ***(Xu et al., 2015).***

Patients with NASH are given 800 International Units of vitamin E daily, especially in non-diabetic cases ***(Carr et al., 2016).*** Despite the use of many medicines such as N-acetylcysteine, betaine, probucol, and viusid in various trials, their usage is not advised in clinical practise due to conflicting/insufficient evidence on their benefits ***(KASL, 2013).***

2) Incretin-based therapy

Glucagon-like peptide-1(GLP-1) analogues (e.g., exenatide, liraglutide, lixisenatide, dulaglutide, and semaglutide) and dipeptidyl peptidase-4 (DPP-4) inhibitors (e.g., sitagliptin, saxagliptin, vildagliptin, alogliptin and linagliptin) are two main classes of incretin-related medications that have been widely researched for use in NAFLD. Both kinds of medicines increase pancreatic insulin secretion in response to meals, as well as having extra-pancreatic effects on many organs,

making them extremely beneficial in the treatment of T2DM ***(Pappachan et al., 2015).***

GLP-1 analogues cause weight loss, but DPP-4 inhibitors have little effect on weight. This class of agents is special in handling overweight/obese T2DM patients because of the remarkable benefits on both conditions ***(Pappachan et al., 2014).***

Recent evidence suggests that GLP-1 analogue medication benefits individuals with NASH, particularly those with T2DM, with improvements in liver histology and lower liver transaminase levels compared to baseline ***(Carbone et al., 2016).***

3) <u>Lipid lowering agents</u>

Treatment with lipid-lowering medications is beneficial, especially in patients with dyslipidemia and NAFLD ***(Pappachan et al., 2017).***

❖ Statins

Cases treated with statins showed some improvements in serum aminotransferase levels and ultrasonological abnormalities ***(Eslami et al., 2013).*** In individuals with concomitant disorders such as hyperlipidemia, diabetes mellitus, and metabolic syndrome, statins can ameliorate the poor outcomes associated with NASH. A recent modest randomised control trial (RCT) demonstrated that rosuvastatin monotherapy improved biopsy-proven NASH and resulted in metabolic syndrome resolution within 12 months of treatment ***(Kargiotis et al., 2015).***

❖ **Fenofibrate**

Fenofibrate was also reported to diminish liver steatosis associated with a high-fat diet, T2DM, and metabolic syndrome in experimental models of NAFLD. A few modest clinical investigations have also shown positive results ***(Kostapanos et al., 2013).***

❖ **Proprotein convertase subtilisin/kexin type 9 (PCSK9)**

PCSK9 is a hepatocyte-secreted molecule that reduces LDL absorption by targeting the receptor for degradation and it increases lipogenesis ***(Ruscica et al., 2016).***

PCSK9 levels in the blood have been reported to be higher in NAFLD patients. PCSK9 inhibitors have been established to be highly successful in lowering hypercholesterolemia in patients with significant improvements in cardiovascular risk ***(Lipinski et al., 2016).***

These medications are frequently reserved for patients with statin intolerance and familial forms of lipid diseases that are not successfully controlled by full dosages of conventional cholesterol lowering therapies due to the high cost of treatment ***(Pappachan et al., 2017).***

4) <u>Drugs for weight loss</u>

Weight-loss medications have the ability to change the pathogenic pathways of NAFLD and may be beneficial in some patients. The majority of these drugs have only a minor weight-loss benefit, and others have been pulled from the market due to unfavourable side effects ***(Pappachan et al., 2017).***

❖ Orlistat

Because this medicine inhibits pancreatic lipase, it causes fat malabsorption and weight loss. Although orlistat has been shown to have some favourable effects in individuals with NASH in two prior RCTs, it is unclear if the benefit was due to weight loss or a direct effect of the drug ***(Younossi et al., 2014).***

❖ Lorcaserin

When Lorcaserin (appetite suppressor) is paired with lifestyle adjustments, this has been linked to a 4% weight loss in 12 months ***(Apovian et al., 2015).*** Some trials demonstrated a moderate reduction in ALT levels and improved cardiovascular outcomes in NAFLD patients treated with the drug ***(Mehal et al., 2014).***

❖ Naltrexone/bupropion combination

This medication combination has been linked to a 5-percentage-point weight decrease. With a larger dose of the combination, individuals who lost more than 10% of their body weight in 12 months showed modest reductions in hepatic aminotransferase levels ***(Winokur et al., 2015).***

❖ Phentermine/topiramate

This combination has also been linked to significant weight loss and the improvement of NAFLD ***(Barb et al., 2016).***

❖ Liraglutide

The United States Food and Drug Administration and the European Medicines Agency recently approved high-dose liraglutide therapy (3 mg daily) for the main management of obesity in people without diabetes. In a large clinical trial,

treated patients lost about 8.5% of their weight as compared to placebo ***(Pi-Sunyer et al., 2015).***

However, a recent phase 2 clinical researchers found that giving individuals 1.8 mg liraglutide improved their liver histology significantly ***(Armstrong et al., 2016).*** As a result, high-dose liraglutide treatment could have the same benefit.

❖ **Other novel agents**

Pentoxyphylline** is a nonselective competitive phosphodiesterase inhibitor that inhibits Tumor Necrosis Factor alpha (TNF –α) and enhances cyclic adenosine monophosphate. This new drug has been shown to have favorable benefits in both animal research and human clinical trials ***(Ye et al., 2016).

Although prebiotics and probiotics have been reported to be beneficial in the treatment and prevention of obesity and NAFLD, there is insufficient evidence from high-quality clinical research to advocate their use in routine clinical practice ***(Tarantino and Finelli, 2015).***

Obeticholic acid (OCA)** is a farnesoid X receptor (FXR) agonist and synthetic bile acid that was recently created for the treatment of primary biliary cirrhosis and has showed promise in the management of NAFLD ***(Makri et al., 2016). FXR is an essential nuclear receptor in the human body that regulates bile acid, glucose, and cholesterol homeostasis ***(Barb et al., 2016).*** OCA has been shown to be effective in the management of NAFLD in both animal and human studies ***(Makri et al., 2016).***

C- Bariatric surgery

In a recent meta-analysis, obese patients who underwent bariatric surgery showed significant improvements in both histological and biochemical NAFLD

markers ***(Bower et al., 2016).*** Weight loss surgery was recommended in 2015 by the Japanese Society of Gastroenterology in collaboration with the Japan Society of Hepatology as an effective treatment option for patients with NAFLD/NASH complicated by severe obesity for improving fatty changes in the liver and inflammation associated with NASH ***(Watanabe et al., 2015).***

Although there is no clear global agreement among professional organizations on the indications for proposing metabolic surgery in patients with NAFLD, rapidly expanding research may eventually lead to such an agreement. The most recent findings from the STAMPEDE (Surgical Treatment and Medications Potentially Eradicate Diabetes Efficiently) clinical trial, which showed significant improvements in metabolic syndrome indices following bariatric surgery, are an excellent example of such high-quality research ***(Schauer et al., 2017).***

D- Liver transplantation

NASH-related end-stage liver disease is the third biggest cause of hepatic transplants in the United States, and due to the obesity epidemic, it is predicted to become the most common cause in 1–2 decades ***(Charlton, 2013).*** Furthermore, the Japanese Society of Gastroenterology, in collaboration with the Japan Society of Hepatology, advises patients with severe NASH hepatic failure to get a liver transplant ***(Watanabe et al., 2015).*** These individuals' overall survival rates following hepatic transplantation are nearly identical to those who receive transplants for liver failure caused by other hepatic diseases. In the absence of intensive post-transplant lifestyle adjustments, nearly one-third of patients who get a liver transplant for NASH will experience disease recurrence in the transplanted liver ***(Canbay et al., 2016).***

CHAPTER (II)

ADIPONECTIN

I- Introduction

Adipokines are bioactive chemicals that govern energy balance, insulin sensitivity, appetite management, inflammatory response, and vascular homeostasis, among other physiological functions. Pro-inflammatory cytokines like adipocyte fatty acid binding protein (A-FABP) and anti-inflammatory cytokines like adiponectin, as well as vasodilator and vasoconstrictor molecules, are all among them ***(El husseny et al., 2017).***

The Adipo Q gene (which spans 17 kb on chromosome 3q27 and has been identified as a region containing a susceptibility gene for type 2 diabetes and metabolic syndrome) encodes human adiponectin. Because of its close link to obesity and insulin resistance, adiponectin has attracted scientists' interest in recent years ***(Achari and Jain, 2017).***

White Adipose Tissue secretes adiponectin, an adipocytokine with 244 amino acids ***(Geagea et al., 2018).*** Human and murine osteoblasts, liver parenchyma cells, myocytes, epithelial cells, and placental tissue are among the tissues that express *adiponectin* ***(Achari and Jain, 2017).*** Adiponectin has many functions in the body, but the most significant is that it regulates energy metabolism ***(Dobrzyn et al., 2018)***

II- Structural Features and Synthesis of Adiponectin

Human adiponectin, which is encoded by the *Adipo Q* gene on chromosome 3q27 and spans 17 kb, is a multimeric protein hormone with a wide range of physiologic functions. The encoded protein has a collagenous domain at the N-terminus and a globular region at the C-terminus that retains biological characteristics following cleavage ***(Zhao et al., 2014).***

Adiponectin is made up of 244 amino acids and has a similar structure to collagen and TNF-α ***(Fisman et al., 2014).*** It comes in three different molecular weights: low, intermediate, and high (trimer, hexamer and 12 to 18 multimer adiponectin respectively) ***(Geagea et al., 2018).***

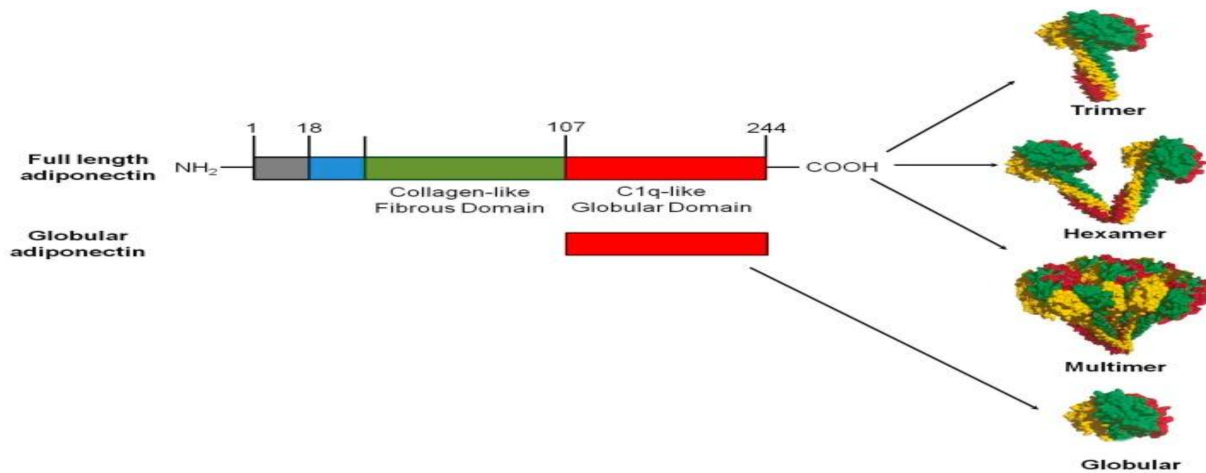


Figure (2): Domains and structure of adiponectin. Each adiponectin subunit in the basic trimeric building block represented in a different color ***(Achari and Jain, 2017).***

III- Post Translational Modification of Adiponectin

Pre-secretion, post-translational processes (e.g., hydroxylation and glycosylation) in the collagenous domain of adiponectin at the four lysines have been demonstrated to improve the efficacy of sub-physiological levels of insulin, resulting in gluconeogenesis inhibition in liver cells ***(Udomsinprasert et al., 2018).***

Monomeric adiponectin is hydroxylated and glycosylated on conserved lysine residues in the collagenous domain in the endoplasmic reticulum. Initial globular head attractions create trimers, which are stabilized by interactions in the collagenous domain. Disulfide bonds develop between single cysteine residues in the N-terminal hypervariable region of trimers (LMW) to create hexamers (MMW). Additional disulfide bonding in the same location converts hexameric adiponectin to bigger multimers of 12 to 18 monomers ***(Fang and Judd, 2018).***

The globular domains of adiponectin form three primary complexes in the circulation, including trimers, hexamers, and high-molecular-weight (HMW) multimers. A precise folding and assembly of adiponectin appears to be a critical stage in regulating its complicated circulatory distribution (***Udomsinprasert et al., 2018).***

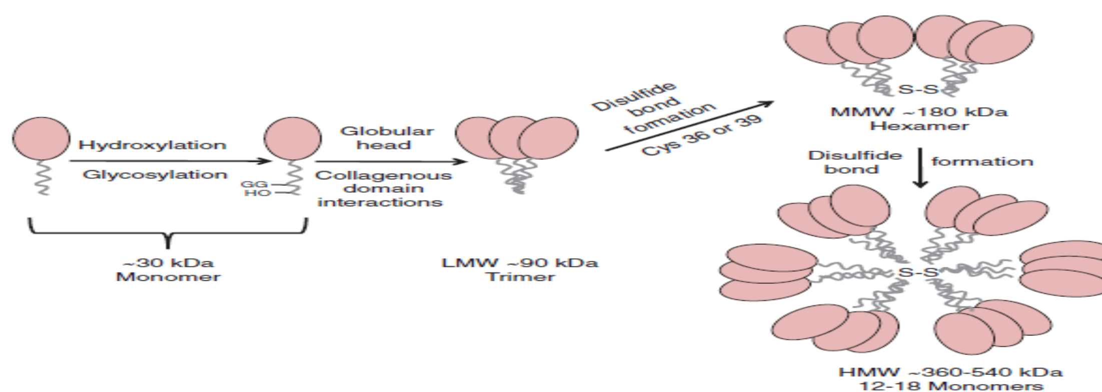


Figure (3): Adiponectin multimer formation ***(Fang and Judd, 2018).***

IV- Adiponectin Receptors

***Types:** AdipoR1, AdipoR2 and T-cadherin

Structure:** Both receptors (AdipoR1, AdipoR2) have seven transmembrane domains and are members of the PAQR family, which has a transmembrane topology opposite that of G-protein coupled receptors, with the N-terminus in the cytoplasm and the C-terminus facing extracellular space ***(Tanabe et al., 2015).

The most interesting feature of their structures is a huge cavity comprising three conserved histidine residues coupled to a zinc ion that is included in the seven-transmembrane helices in both AdipoR1 and AdipoR2. The zinc-binding motif has been linked to adiponectin-stimulated AMPK and PPAR activation ***(Tanabe et al., 2015).***

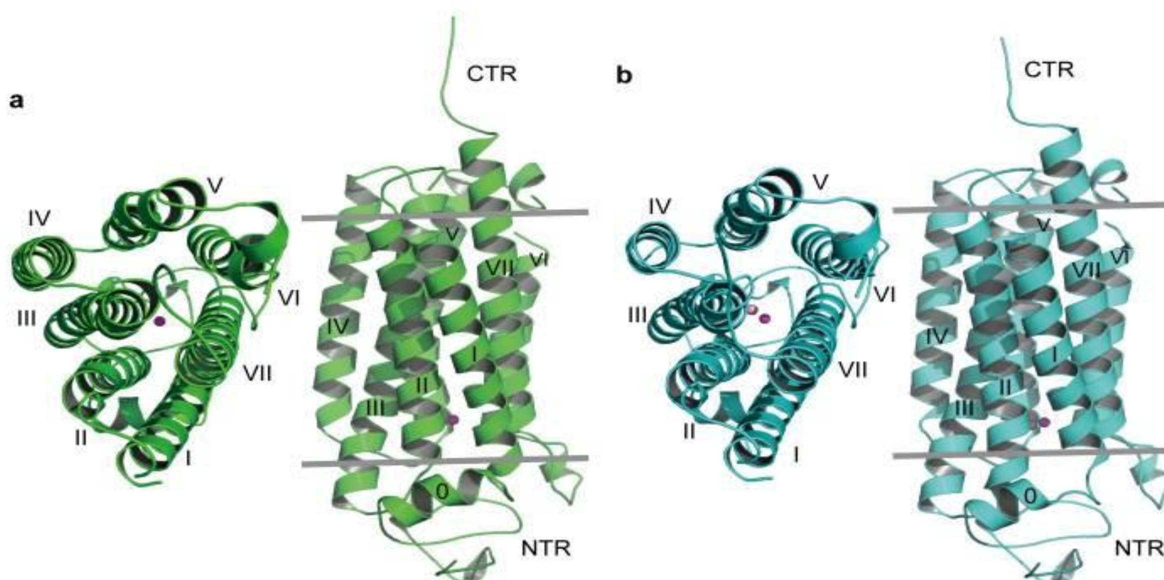


Figure (4): Overall structures of AdipoR1 and AdipoR2**; a,** structure of AdipoR1. **b,** structure of AdipoR2. The structures are viewed from the extracellular side (left) and parallel to the membrane (right). The NTR (N-terminus), helix 0, transmembrane helices I–VII, and the CTR(C-terminus) of AdipoR1 (**a**) and AdipoR2 (**b**) are indicated ***(Tanabe et al., 2015).***

Site:** AdipoR1 is most common in skeletal muscle, liver, heart, and renal tissues ***(Kim et al., 2018), while AdipoR2 expression is primarily localized to the liver ***(Ruan and Dong, 2016).***

Adiponectin presents in the bloodstream as low-molecular-weight (LMW) homotrimers ,hexamers and high-molecular-weight (HMW) multimers with 12–18 monomers, but a smaller form of adiponectin called globular domain adiponectin exists in trace amounts ***(Achari and Jain, 2017).*** However, HMW adiponectin is the most abundant type in the bloodstream and is used as a diagnostic for disease-related adipocyte malfunction (***Udomsinprasert et al., 2018).***

V- Adiponectin plasma level

Adiponectin is one of the adipocytokines with high circulating levels in healthy people, accounting for about 0.01% of total circulating protein and ranging from 5 to 30 g/mL (***Udomsinprasert et al., 2018).***

Adiponectin levels differ across genders, with females having higher levels than males. Sexual hormones, such as estrogen and testosterone, may play a role in modulating adiponectin plasma levels, according to some studies; but the mechanism of action needs to be investigated further. These sexual features may help to explain why men are more likely than women to develop insulin resistance and atherosclerosis ***(Geagea et al., 2018).***

The exact mechanisms that control adiponectin levels in the human body are unknown. Multiple factors, including genetics, processes affecting adiponectin clearance, and post-translational changes associated with modulating adiponectin gene expression, have been reported to play essential roles in regulating adiponectin levels in the human body ***(Shehzad et al., 2012).*** Furthermore,

adiponectin receptor modulation is regarded to be critical for adiponectin's essential physiological action (***Udomsinprasert et al., 2018).***

A physiological level of circulating adiponectin is critical for metabolic disorders defense and may be linked to other chronic diseases such as chronic obstructive pulmonary disease ***(Bianco et al., 2013)***, chronic kidney disease ***(Lim et al., 2015).*** Hypoadiponectinemia, on the other hand, has previously been linked to metabolic changes such as insulin resistance, dyslipidemia, and atherosclerosis ***(Chen et al., 2017).***

VI- Adiponectin Signal Transduction Pathway

AdipoR1 and ADipoR2 receptors can bind to adiponectin, and their signalling is predominantly driven by AMPK phosphorylation ***(Kadowaki and Yamauchi, 2011).*** TSC2 (Tuberous Sclerosis Complex 2) is phosphorylated by AMPK, which inhibits protein synthesis and cell growth ***(Jardé et al., 2011).***

Adiponectin inhibits cell growth by phosphorylating PI3k and AKT, which activates AMPK and suppresses the mTOR pathway. Adiponectin also induces cell cycle arrest by down-regulating C-myc, cyclin D, and Bcl levels, as well as increasing the expression of P53 (cellular tumour antigen), P21 (cyclin-dependent kinase inhibitor 1) and Bax ***(Khan et al., 2013).***

A cascade of signaling events is triggered when insulin and adiponectin receptors are activated by their respective ligands. The PI3K/AKT pathway (fig.5) is responsible for the majority of insulin's metabolic effects, which include increased protein synthesis, lipogenesis, glucose uptake and utilization, and glycogen synthesis, as well as decreased lipolysis and gluconeogenesis. In the case of adiponectin, APPL1 interacts with AdipoR1 or AdipoR2 and mediates the

effects of adiponectin on the activation of various pathways, including PPAR-, AMPK. AdipoR1 and AdipoR2 are both linked to ceramidase activity, which is triggered by adiponectin binding. APPL1, a major IRS1/2 binding partner, boosts IRS1/2 binding to the insulin receptor and improves insulin signalling transduction. A main method by which adiponectin sensitizes insulin action in insulin target tissues is through crosstalk between insulin and adiponectin signalling pathways ***(Ruan and Dong, 2016)***

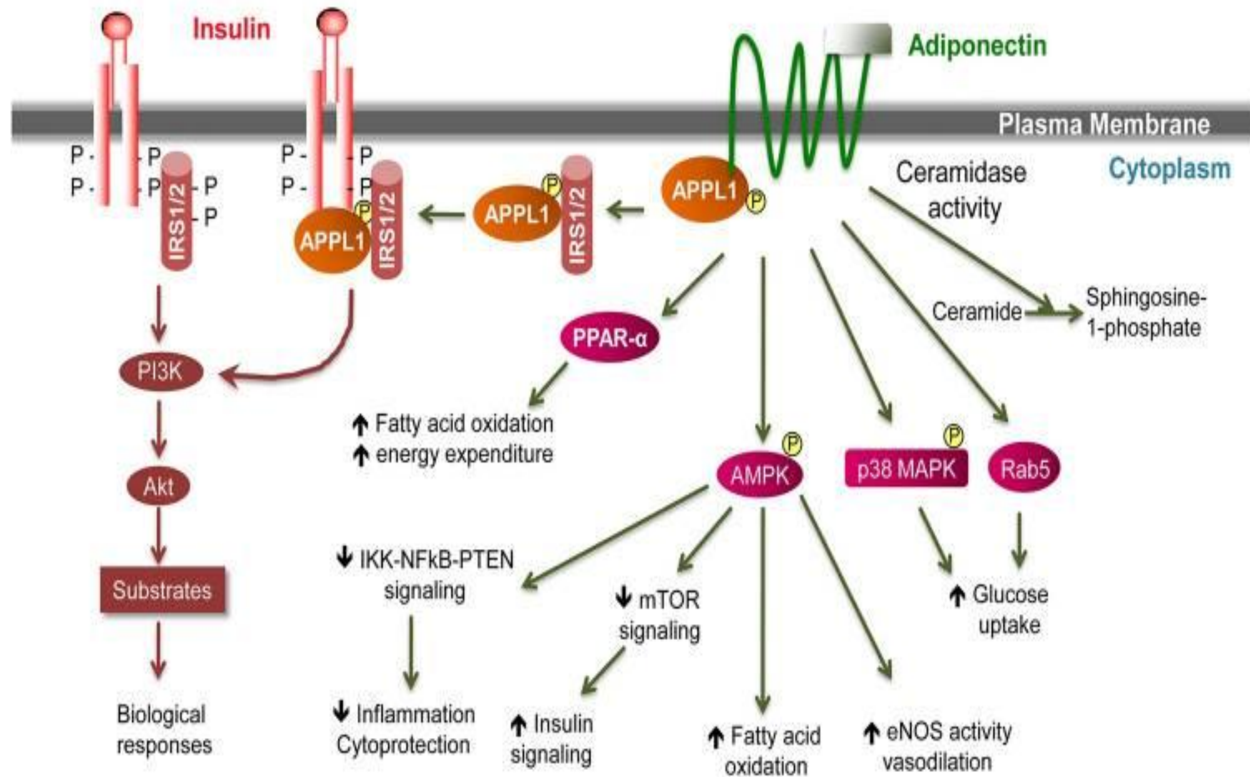


Figure (5): Schematic representation of adiponectin signal transduction pathway implicating across talk with the insulin signaling pathway ***(Ruan and Dong, 2016)***.

VII- Physiological function of adiponectin

1- Insulin sensitivity

Insulin resistance is defined as a cellular response to insulin that is interfered. Significant clinical evidence supports adiponectin's involvement as an insulin-sensitizing hormone. Both globular and full-length adiponectin stimulating AMPK in skeletal muscle, as well as full-length adiponectin stimulating AMPK in the liver, are responsible for these effects ***(Fang and Judd, 2018).***

Adiponectin's insulin-sensitizing activities primarily target the liver and skeletal muscle. Adiponectin improves the actions of insulin in the liver by activating AMPK phosphorylation, which leads to increased phosphorylation of acetylcoenzyme A carboxylase (ACC), and decreased of Phospho Enol Pyruvic Carboxy Kinase (PEPCK) and Glucose-6-Phosphatase activity. However, adiponectin's capacity to decrease gluconeogenic gene expression and hepatic glucose production may be mediated in part by AMPK ***(Miller et al., 2011).***

The capacity of a diponectin to phosphorylate APPL1 contributes to its insulin sensitizing capabilities in the liver and other insulin target organs ***(Ruan and Dong, 2016).*** Insulin and adiponectin binding to their respective receptors stimulates the interaction of APPL1 with IRS1/2, and this interaction is enhanced by both insulin and adiponectin binding to their respective receptors ***(Ryu et al., 2014)***. Insulin resistance occurs when tissues do not respond well to insulin stimulation, leading to hyperlipidemia, dyslipidemia, hyperglycemia, inflammation, and lower plasma adiponectin levels ***(Fang and Judd, 2018).***

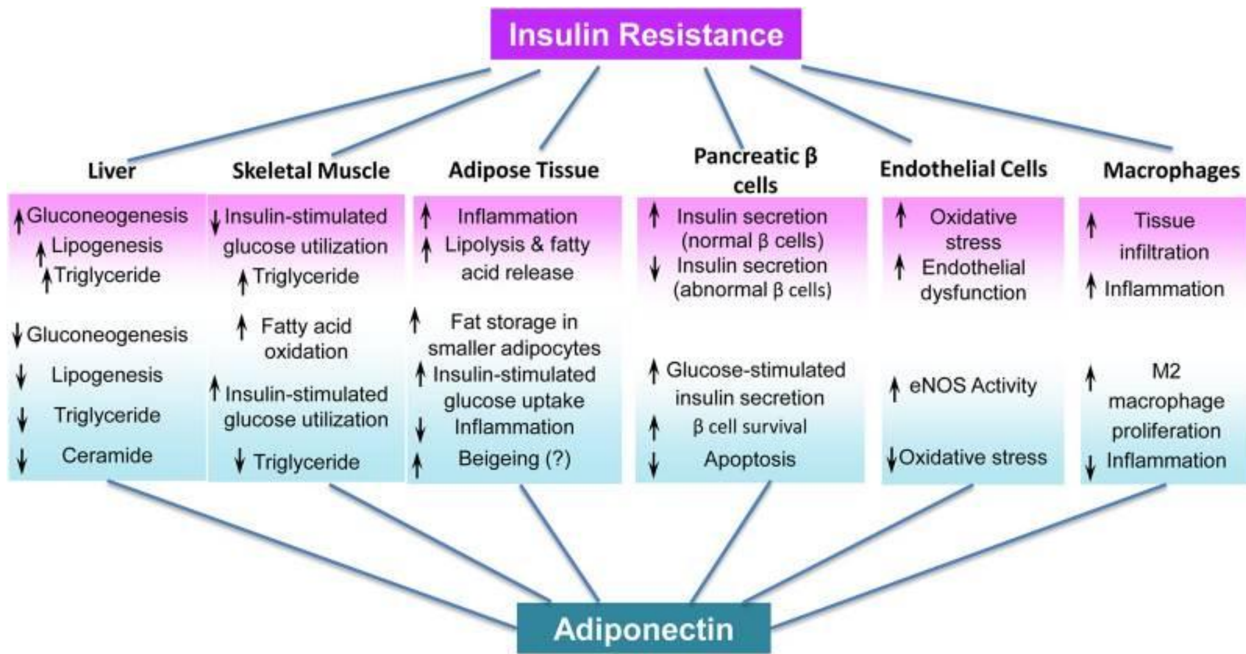


Figure (6): Summary of tissue-specific functions of adiponectin and its agonizing effect on insulin resistance ***(Ruan and Dong, 2016)***

2- Anti-inflammatory

Adiponectin has anti-inflammatory characteristics in a variety of disease states, including type 2 diabetes, NAFLD and cardiovascular disease as reported by many researches. There have been inverse relationships between plasma and adipose tissue levels of adiponectin and C-reactive protein (CRP), an acute-phase marker of inflammation, in patients with coronary artery disease (CAD). Obese women who lose weight have lower levels of CRP and interleukin 6 (IL-6), another pro-inflammatory cytokine. During this time of weight loss, adiponectin concentrations rise ***(Fang and Judd, 2018).***

Macrophages are the principal target of adiponectin's anti-inflammatory activities. Adiponectin suppresses myeloid progenitor cell development and regulates macrophage activity ***(Ohashi et al., 2014).*** In macrophages, adiponectin reduces the production of inflammatory chemokines while increasing the

production of the anti-inflammatory cytokine interleukin 10 (IL-10). Adiponectin inhibits the activation of pro-inflammatory classically activated (M1) macrophages and enhances the growth of anti-inflammatory alternatively activated (M2) macrophages ***(Fang and Judd, 2018).***

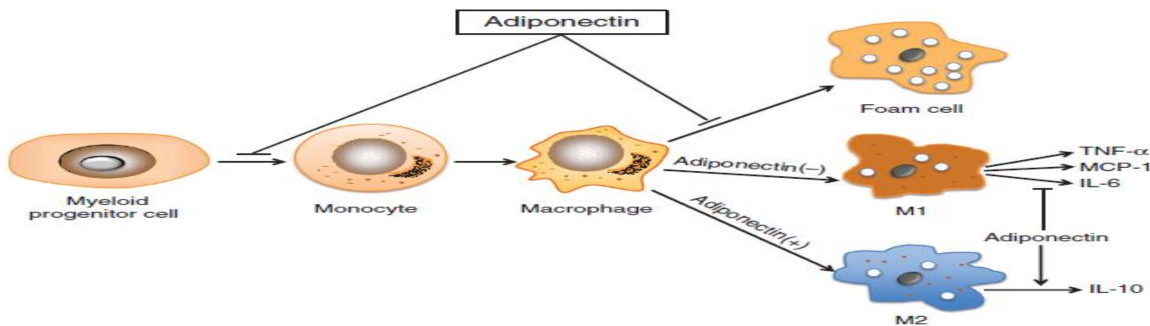


Figure (7): Adiponectin possesses anti-inflammatory properties. Adiponectin decreases inflammation by targeting differentiation and function of macrophages. Adiponectin inhibits differentiation of myeloid progenitor cells into monocytes, inhibits formation of foam cells from macrophages, and promotes the polarization of macrophages to an M2 anti-inflammatory state ***(Fang and Judd, 2018).***

3- Vasculo-protective anti-atherosclerotic effects

Adiponectin reduces the endothelium inflammatory response, macrophage to foam cell transition, and vascular smooth muscle proliferation, which protects the vasculature when the endothelial barrier is weakened. Adiponectin deficiency is linked to the onset and progression of obesity-related vascular disorders, such as atherosclerosis and coronary artery disease (CAD) ***(Fang and Judd, 2018).***

High levels of plasma adiponectin are linked to a lower risk of myocardial infarction (MI), while low levels are linked to CAD. The fact that adiponectin levels are linked to cardiovascular risk regardless of glycemic or lipid status shows that adiponectin has direct preventive effects on vascular health rather than indirect effects via insulin sensitivity and diabetes ***(Fang and Judd, 2018).***

When blood leukocytes (mainly monocytes) stick to injured endothelium in arteries, move and mature into macrophages in the intima, and ingest lipid, foam cells are formed. Smooth muscle cells from the media migrate into the intima, where they multiply and drive the production of extracellular matrix components and lipids from dead and dying cells, resulting in plaque formation. Plaques can be physically disrupted, causing thrombus development and a reduction in blood flow ***(Libby et al., 2011).***

By controlling endothelial inflammation and exerting direct anti-atherogenic effects on the vasculature, adiponectin regulates numerous steps in the atherogenic process, including preventive functions in the onset and progression of atherosclerosis. Adiponectin reduces the expression of adhesion molecules and inhibits the synthesis of pro-inflammatory cytokines in endothelial cells, therefore modulating endothelial inflammation ***(Komura et al., 2013).***

Adiponectin protects blood vessels in a variety of ways (fig.8), including anti-inflammatory effects, increasing nitric oxide (NO) production, suppressing endothelial activation, inhibiting adhesion molecules (VCAM 1, ICAM 1, E-selection), inhibiting foam cell formation, inhibiting smooth muscle migration/proliferation, and plaque stabilization. In both endothelial cells and smooth muscle cells, T-cadherin enhances adiponectin accumulation ***(Fang and Judd, 2018).***

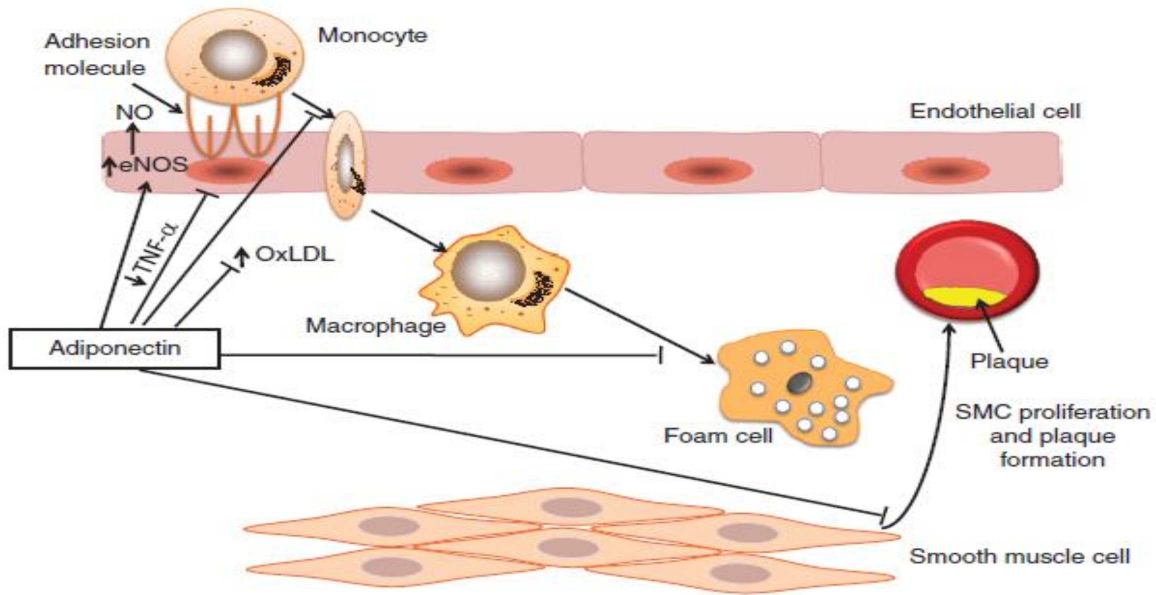


Figure (8): Adiponectin protects the vasculature ***(Fang and Judd, 2018).***

4- Cardioprotection

Low serum adiponectin levels are linked to metabolic syndrome, which is a cardio vascular disease (CVD) risk factor. Low adiponectin levels have been linked to the development of CAD, MI, hypertension, left ventricular hypertrophy, and other cardiovascular dysfunctions in clinical and epidemiological investigations ***(Denzel et al., 2010).*** Adiponectin levels are inversely related to the risk of CVD in a healthy ageing population ***(Cote M et al., 2011).***

Patients with low plasma adiponectin concentrations have a higher risk of MI than those with high levels of it. Adiponectin has potent anti-inflammatory effect on coronary arteries, preventing the onset of CAD ***(Fang and Judd, 2018).***

Adiponectin protects the heart by reducing inflammation and protecting it from the damage caused by a variety of mediators ***(Nanayakkara et al., 2012)***.

5- Colon homeostasis and protection

The intestinal epithelium layer serves as a protective barrier against undigested intestinal contents and bacteria in the gut, which is important for colon homeostasis ***(Burisch and Munkholm, 2015).*** Colonic illnesses such as inflammatory bowel disease and colon cancer can be caused by decreased proliferation and enhanced apoptosis-mediated disruption of this epithelial layer. Adiponectin protects the colon by increasing the survival of the intestinal epithelial layer and acting as an anti-inflammatory agent ***(Obeid et al., 2017).***

Adiponectin suppresses goblet cell apoptosis and increases the differentiation of epithelial cells into goblet cells, in addition to its proliferative effects on intestinal epithelial cells. Mucus is produced by goblet cells and serves an important function in maintaining intestinal homeostasis by covering epithelial cells and preventing bacterial invasion and inflammation ***(Kaser et al., 2010).***

6- Wound healing

Hemostasis, inflammation, tissue proliferation, and tissue remodeling are all processes in the healing of damaged dermis and epidermis. Keratinocytes has important role in tissue growth and re-epithelialization. Adiponectin receptors are expressed in normal human keratinocytes, and adiponectin stimulation promotes their proliferation and migration. Adiponectin controls proliferative signaling via the AdipoR1/R2-ERK pathway ***(Shibata et al., 2012).***

7- Browning of white adipose tissue

Brown adipose tissue (BAT) generates heat via activating uncoupling protein 1(UCP1) in the mitochondria, which uncouples electron transport and ATP generation ***(Fang and Judd, 2018).*** BAT is mostly found in the inter-scapular fat

pad, where it differs from white adipose tissue (WAT) by having small lipid droplets and a notable mitochondrial concentration ***(Saely et al., 2012).***

Recent research has shown that a subset of WAT cells can be transformed to brown-like adipocytes by a process called "browning" or "beiging"***(Wu et al., 2012).*** Environmental and hormonal factors, such as persistent cold exposure, vascular endothelial growth factors, adiponectin, and others, trigger this conversion process ***(Scherer, 2016).***

Later research found that continuous cold exposure causes significant adiponectin buildup in subcutaneous WAT in mice. This cold-induced adiponectin increase was linked to subcutaneous WAT browning, which was not seen in animals lacking adiponectin. Browning of subcutaneous WAT appears to be induced by adiponectin through both direct and indirect pathways ***(Hui et al., 2015).***

Adiponectin stimulates the growth of M2 macrophages, which stimulate the synthesis of catecholamines in subcutaneous WAT, enhancing thermogenic remodeling ***(Qiu et al., 2014).***

8- Central regulation of food intake and energy expenditure

In humans, adiponectin is found in the CSF in modest amounts and mostly in the trimeric form. The para-ventricular hypothalamus and arcuate nuclei (ARH) both contain AdipoR1 and R2. Because serum concentrations of adiponectin rise during fasting, it is regarded as a starvation hormone. Adiponectin levels in serum and CSF, as well as Adi-poR1 expression in the ARH, rise during a fasting period. Adiponectin signaling in the ARH activates AMPK, resulting in a rise in neuropeptide Y, which promotes food intake while suppressing energy expenditure. Refeeding lowers serum and CSF adiponectin levels & AdipoR1

expression, resulting in lower AMPK activation, lower food intake and energy expenditure ***(Fang and Judd, 2018).***

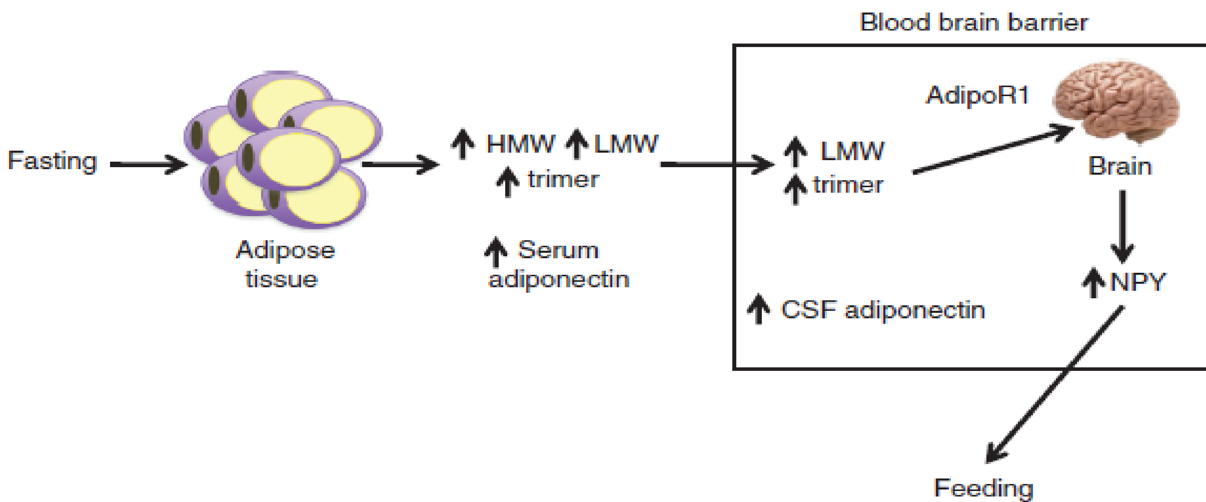


Figure (9): Adiponectin regulation of food intake ***(Fang and Judd, 2018).***

9- Anti-fibrotic mediator

Fibrosis is caused by an overabundance of extracellular matrix (ECM) proteins. Adiponectin is a protein that has anti-fibrotic effects ***(Park et al., 2015).*** Adiponectin also inhibits matrix metalloproteinase 1 (MMP1) while increasing tissue inhibitor of metalloproteinase (TIMP-1), both of which are necessary for ECM deposition **(*Ramezani-Moghadam et al., 2015*).**

Both AdipoR1 and R2 are involved in adiponectin's antifibrotic effects. Increased AdipoR2 expression stimulates PPAR activation that lead to suppressed generation of TGF-induced Reactive Oxygen Species ***(Fang and Judd, 2018).*** AdipoR2 is also involved in the regulation of oxidative stress and inflammation ***(Matsunami et al., 2010).*** TGF-1-induced expression of type 1 alpha collagen (COL1A) was reduced by adiponectin through suppressing the transcription co-activator p300 ***(Lim et al., 2012).***

Adiponectin may target focal adhesion kinases (FAK), which are important for focal adhesion assembly as well as hepatic stellate cells (HSCs) adherence and maturation ***(Kumar et al., 2014).*** Adiponectin also helps in liver injury repair by controlling hepatocyte proliferation ***(Correnti et al., 2015)***.

adiponectin causes inhibition of Fibrosis in the liver, skin, heart, lungs, and kidney. Activated HSCs and Kupffer cells are key players in the formation of fibrosis in the liver. Adiponectin reduces TLR4 signalling in Kupffer cells with boosting M2 macrophage polarization. Adiponectin also has a direct effect on HSCs, reducing polarization, migration, collagen/ECM deposition and focal adhesion assembly, while raising HSC death susceptibility. In skin, Adiponectin suppresses profibrotic TGF signaling at the fibroblast by boosting AMPK and reducing canonical Wnt signaling both locally and systemically ***(Fang and Judd, 2018).***

The anti-fibrotic effects of adiponectin are significant in the progression of illness. Adiponectin levels in the blood are linked to the development of steatosis, inflammation, and fibrosis in the liver. In NAFLD, hypo-adiponectinemia is linked to the severity of hepatic fibrosis and advanced fibrosis. As a result, plasma adiponectin concentrations have been suggested as a good biomarker for the development of several liver disorders ***(Fang and Judd, 2018).***

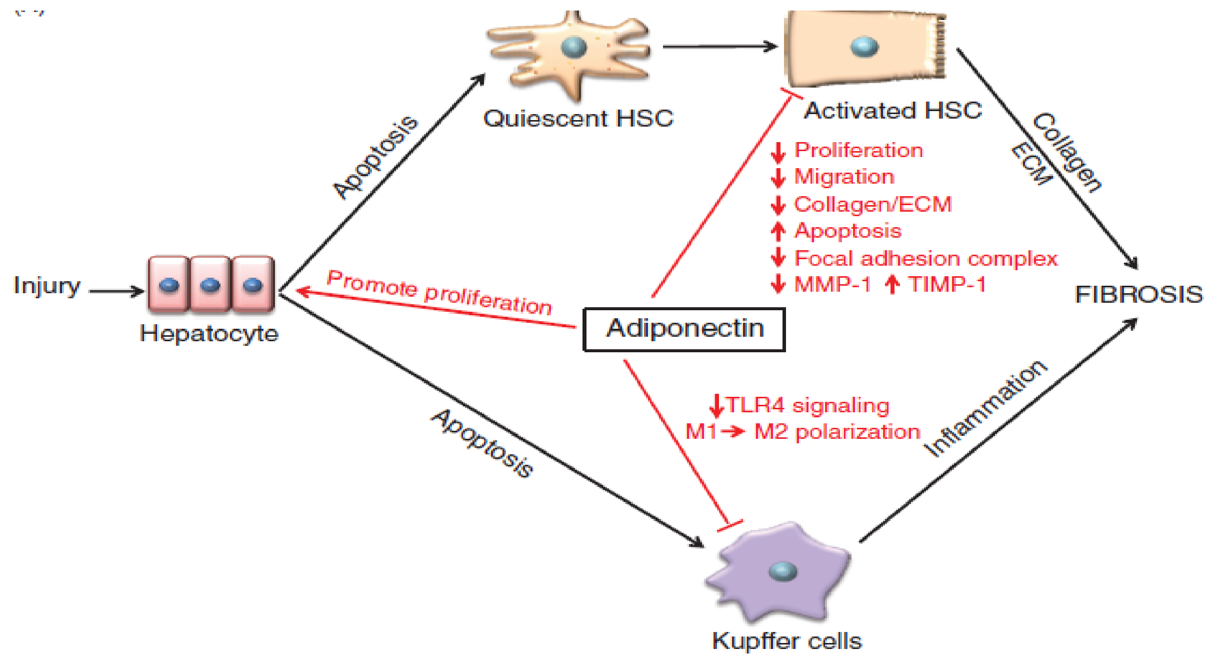


Figure (10): Adiponectin inhibits fibrosis. Fibrosis is the accumulation of excessive ECMs *(Fang and Judd, 2018).*

VIII- Diseases associated with impaired adiponectin plasma level

Adiponectin protects against the onset of a variety of pathophysiological conditions; hypo-adiponectinemia or hyper-adiponectinemia can be significant risk factors for the onset of a variety of diseases ***(GALINDO et al., 2017).***

1) Diseases related to hyper-adiponectinemia

❖ Chronic kidney Disease

Chronic kidney disease is characterized by a progressive loss of renal function over time and is a risk factor for cardiovascular disease. Due to a reduced rate of adiponectin clearance by the kidney, patients with chronic renal disease had higher plasma adiponectin levels than healthy people ***(Heidari et al., 2015).***

❖ Lung Disease

In Chronic Obstructive Pulmonary Disease (COPD) patients, hyper-adiponectinemia is linked to weight loss, systemic inflammation, and hyperinflation ***(Garcia and Sood, 2012).***

❖ Chronic Heart Failure

Chronic heart failure (CHF) and people with CVD benefit from adiponectin. However, in some studies, high levels of plasma adiponectin were found in patients with CHF, and increased plasma adiponectin levels correlated directly with the severity or mortality in CHF ***(De la Cruz et al., 2017).***

2) Diseases associated with hypo-adiponectinemia:

In overweight and obese people, adiponectin expression in adipose tissue and plasma concentration are both reduced. plasma adiponectin concentration is also inversely linked with body mass index and triglyceride concentration ***(Tahergorabi et al., 2016).***

❖ Diabetes Mellitus Type 2 and Resistance to Insulin

Adiponectin levels have been linked to the existence of diabetes in recent studies. The degree of obesity, fasting plasma glucose, and insulin levels all have an inverse association with adiponectin levels. A diabetes locus has also been discovered on chromosome 3q27, the same chromosome that contains the adiponectin gene. Changes in the adiponectin gene that result in decreased adiponectin production could be a cause of T2DM pathogenesis ***(Geagea et al., 2018).***

❖ **Hypertension**

Hypo-adiponectinemia and hypertension have a positive relationship. Hypertension is more likely in obese people with low plasma adiponectin levels ***(Kim et al., 2013).***

❖ **Dyslipidemia**

Dyslipidemia is a lipid metabolism condition characterized by high serum triglycerides, high serum low density lipoprotein (LDL) cholesterol, and low high density lipoprotein (HDL) cholesterol levels. There is a positive relationship between Serum adiponectin level and plasma HDL cholestrol level. There is a significant inverse relationship between this hormone and plasma triglyceride, very low density lipoprotein (VLDL) and LDL levels as reported from studies ***(Izadi et al., 2013).***

❖ **Metabolic Syndrome**

It is a set of conditions that raise the risk of diabetes, stroke, and heart disease, such as abdominal obesity, insulin resistance, dyslipidemia, hyperglycemia, and hypertension, the combination of low serum adiponectin levels and high visceral fat area significantly predicted the development of metabolic syndrome (**Cho *et al., 2017).***

❖ **Cardiovascular Disease and Atherosclerosis**

Adiponectin serves a preventive role in the pathogenesis of vascular illnesses by boosting the synthesis of nitric oxide, as well as inhibiting inflammation and oxidative stress. Low levels of adiponectin play an essential role in the development of atherosclerosis and cardiovascular disease. As a result,

adiponectin shortage causes endothelium-dependent vasodilation to deteriorate ***(Lee and Kwak, 2014).***

❖ **Cancer**

Low plasma levels of adiponectin are directly correlated with the risk of developing many types of cancer, and hypo-adiponectinemia plays a vital role in the genesis and progression of obesity-related cancer ***(Macis et al., 2014).*** It also has anti-inflammatory properties and may play a role in cancer cell death and proliferation ***(Geagea et al., 2018).***

❖ **Non Alcoholic Fatty liver (NAFLD)**

By excluding alcohol abuse, this disease is characterized by accumulation of excess fat in liver cells, which can progress to fibrosis and cirrhosis. Adiponectin protects the liver from inflammation and fibrosis by limiting excess lipid accumulation ***(Petta et al., 2016).***

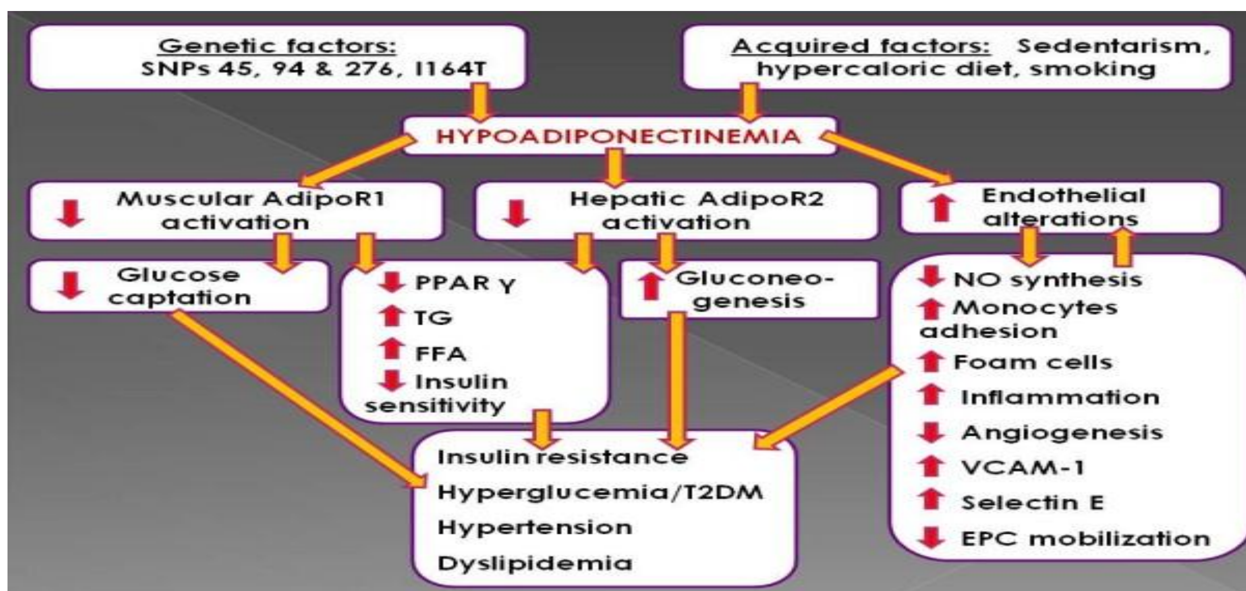


Figure (11): Schematic depiction of the clinical outcomes of hypoadiponectinemia ***(Fisman and Tenenbaum, 2014).***

CHAPTER (III)

ADIPONECTIN AND NON-ALCOHOLIC FATTY LIVER DISEASE

Nonalcoholic fatty liver disease (NAFLD) is a clinical illness characterized by fatty hepatic parenchymal cell degeneration in the absence of a history of frequent alcohol drinking. There are many pathogenic variants of NAFLD starting as Simple fatty liver, nonalcoholic steatohepatitis (NASH), and NASH-related cirrhosis, which can progress to hepatocellular cancer (HCC) ***(Cheung and Sanyal, 2010).***

NAFLD is often referred to as a set of inherited and environmental illnesses linked to metabolic stress, and it frequently coexists with metabolic syndrome (MS), which includes type 2 diabetes, obesity, dyslipidemia, and hypertension ***(Cohen et al., 2011),*** so it is considered a manifestation of MS in the liver ***(Chen et al., 2016).***

Adiponectin (a 30-kDa protein mainly produced by adipose tissue), is the most influencing adipokine in the pathogenesis of NAFLD ***(Adolphet al., 2017).*** It has anti-steatotic, anti-inflammatory and anti-fibrotic effects. Especially in liver, adiponectin prevents lipid deposition by enhancing fatty acids oxidation through peroxisome proliferator-activated receptor alpha ***(Achari and Jain, 2017)***. Also it decreases pro-inflammatory cytokines produced by Kupffer cells and hepatic stellate cells, preventing their transformation into myofibroblasts, so liver fibrosis could be decreased ***(Adolphet al., 2017)***

Adiponectin reduces Kupffer cell release of inflammatory mediators, decreases HSC proliferation and migration, and makes them more susceptible to caspase-mediated death ***(Ramezani-Moghadam et al., 2015).*** Adiponectin activates AdipoR1 causing an increase in AMPK signalling, which inhibits HSC proliferation and migration, in part by blocking TGF-β, so it protects against fibrosis ***(Fang and Judd, 2018).***

Epigenetic factors, which can impact gene expression without modifying the DNA sequence, have as a critical link between environmental exposures, genetic determinants, and disease risk. Epigenetics offer a new perspective on the etiology of NAFLD ***(Zimmer and Lammert, 2011).***

The chromatin structure, alterations, and initiation of transcription are all controlled by epigenetic modulation of gene expression, which changes the accessibility of genes to transcription factors and their cofactors, that controls the rate at which a gene is highly transcribed ***(de Conti et al., 2017).*** There is a great relationship between changes in chromatin states, cellular phenotype and organ function ***(Eslam et al., 2017).***

Modulation of DNA methylation in NAFLD

Modification of DNA methylation is one of the epigenetic mechanisms that can influence the development and progression of NAFLD ***(Lee et al., 2014).*** The earliest discovery of epigenetic regulation of gene expression was DNA methylation, which is a biochemical change of cytosine in DNA with a methyl group (one-carbon moiety) ***(Maschietto et al., 2016).*** DNA methyl transferases (DNMTs) catalyse the DNA methylation reaction, which necessitates the addition of a methyl group to cytosine with guanine as the next nucleotide, resulting in CpG sites ***(Portela et al., 213).***

CpG islands (CGIs) in vertebrates are short interspersed DNA sequences that are GC-rich, CpG-rich, and mostly nonmethylated, deviating greatly from the usual genomic pattern. The majority, if not all, CGIs are transcription initiation sites, with thousands of them located far from already identified promoters. CGIs are adapted for promoter activity by shared DNA sequence characteristics that destabilise nucleosomes and attract proteins that establish a transcriptionally permissive chromatin state. CGI promoters are silenced by extensive CpG methylation or polycomb recruitment, both of which rely on their unique DNA sequence composition. CGIs are thus universally capable of influencing local chromatin structure and simplifying gene activity regulation ***(Deaton and Bird, 2011)***.

CpG dinucleotide clustering (often referred to as CpG islands and CpG island shores) occurs more frequently in the promoter regions of genes than in other DNA locations ***(Kundaje et al., 2015).*** Hypermethylation of CpG islands is usually associated with gene silence, but hypermethylation of heterochromatin is associated with genomic instability ***(de Mello et al., 2017).***

Humans have three isoforms of DNMT (DNMT1, DNMT3A, and DNMT3B), irregular methylation patterns of genomic DNA is one of the primary epigenetic alterations that can cause aberrant gene expression in NAFLD. Furthermore, epigenetic changes in mitochondrial DNA methylation are known to occur throughout the progression of NAFLD ***(Gautam, 2018).***

Differentially methylation genes have been found to identify patients with advanced NASH from those with mild steatosis ***(Murphy et al., 2013).*** In advanced NAFLD, a large number of tissue repair genes were hypomethylated in the liver, whereas genes for metabolic pathways including one-carbon metabolism were

hypermethylated, according to a recent epigenetic clinical investigation. DNA methylation changes in multiple CpG sites within fibrosis-linked genes have been observed in moderate NAFLD patients as compared to those with severe stage ***(Zeybel et al., 2015).***

The methylation status of particular CpGs in DNA appears to be useful in predicting the progression of NAFLD to NASH fibrosis. Furthermore, DNA methylation profiles in genes connected to lipid homeostasis, fibrosis, and carcinogenesis should help researchers figure out how DNA methylation plays a role in the development of NAFLD ***(Gautam, 2018). Ott et al., 2018*** demonstrated that increased *adiponectin* promotor methylation is one of epigenetic regulation that leads to decreased *adiponectin* gene transcription and expression, so adiponectin anti-inflammatory and ant fibrotic benefits are decreased that results in NAFLD development and progression.

PATIENTS AND METHODS

PATIENTS:

This cross sectional study was carried out between July 2018 and July 2021 after approval of the study scheme by the research ethical committee of Faculty of Medicine, Benha University and obtaining informed consent from the included subjects. This study was carried out on 49 subjects of both sexes selected from Endemic Medicine Department-Faculty of Medicine, Cairo University Hospital.

The subjects were categorized into 3 groups:

A. **Simple steatosis group**: included 5 patients, diagnosed by clinical, radiological and histopathological examinations (NAS score < 4)
B. **NASH group**: included 29 patients, diagnosed as NAFLD patients by clinical, radiological and histopathological examinations(NAS score ≥ 4)
C. **Control group**: included 15 apparently healthy subjects, age and sex matched to patients group, with normal liver.

inclusion and exclusion criteria: all participants aged above 18 years, and all patients were suspected to have NAFLD on the basis of increased hepatic brightness by abdominal ultrasound with the exclusion of other causes of secondary steatosis such as negative viral markers (HCV Ab, HBsAg, HBc total), negative ANA, transferrin saturation <45% coupled with normal ferritin levels, normal serum levels of ceruloplasmin, and negative history for significant alcohol consumption (>20 g/d for females and 30 g/d for males) and for use of medications that can cause fatty liver.

All individuals were subjected to:

1. Full history with attention to:

   a) Special habits including, tobacco smoking, diets that are high in fats and red meat.

   b) Hepatological symptoms and signs including, fatigue, malaise, jaundice.

2. General and local abdominal examinations.
3. Investigations obtained from patients sheets include:

   - Routine Laboratory investigations

     • Liver function tests: serum albumin, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) alkaline phosphatase (ALP), gamma glutamyle transferase(GGT) ***(Reitman and Frankel, 1957).***

     • Complete Blood Count (CBC).

     • Fasting blood sugar, glycated hemoglobin (HbA1c), 2hours postprandial blood sugar (2hrs PP- BS) ***(Trinder, 1969).***

     • Kidney function tests: serum urea ***(Tobacco et al., 1979)*** and serum creatinine ***(Bowers and Wong, 1980).***

     • Coagulation profile: plasma prothrombin concentration (PC) Prothrombin time (PT), partial thromboplastin time (PTT) and International normalized ratio (INR) ***(Hirsh et al., 1995).***

     •lipid profile (HDL, LDL, total cholesterol, triglycerides).

     • Urine analysis

   - Radiological investigations include abdominal ultrasonography.

4. Liver biopsy for histopathology (from patients' group) obtained by gun method. Core biopsy size is about 1.5 cm. to be valuable biopsy, portal tracts number was regarded. NAFLD depended upon specific staging for ballooning steatosis, necroinflamatory status and fibrosis using NAFLD activity score (NAS) and SAF score (steatosis, activity and fibrosis).
5. Molecular biology investigations: SYBR Green methylation specific polymerase chain reaction (qMSP) for detection of adiponectin promotor methylation status.

METHODS:

❖ ***Sampling*:**

Peripheral blood sample (5ml) was withdrawn from each subject, and then divided into3 parts:

- First part (2ml): it was collected into EDTA vacutainers. From each blood sample (1 ml) was transferred immediately into Eppendorf tubes to be stored at -80°C, for later processing. While the remaining part (1ml), was used for assessment of Complete blood count.
- Second part (1ml): it was used to measure prothrombin time and activity by adding 9 parts blood to one part tri-sodium citrate.
- Third part (2ml): it was collected into plain vacutainers. After centrifugation, the separated sera were used for estimation of ALT, AST, serum albumin, Lipid profile, HBsAg, HCV Ab.

❖ ***Detection of the adiponectin promotor methylation status according to the following steps:***

I. **Extraction of DNA from Peripheral blood samples:** using QIAmp DNA blood mini kit ***(QIAGEN, Germany)*** according to manufacturer's instructions.

II. **Bisulfite treatment:** extracted DNA was bisulfite treated using The EZ DNA Methylation-Gold Kit ***(ZYMO RESEARCH, USA),*** according to manufacturer's instructions.

III. **SYBR Green methylation specific polymerase chain reaction (qMSP):** HotStarTaq Master Mix Kit ***(QIAGEN, Germany)*** and Specific primer sets for either methylated and non-methylated products of adiponectin gene promoter. The PCR product was separated by gel electrophoresis, stained with ethidium bromide and visualized by UV irradiation, for detection of specific bands.

I. Extraction of DNA from peripheral blood samples: using QIAmp DNA blood mini kit 51104 *(QIAGEN, Germany)* according to manufacturer's instructions.

❖ **Procedure**

1. Protease (20 µl) was Pipetted into a 1.5 ml microcentrifuge tube and 200 µl blood sample were added.
2. Buffer AL (200 µl) was added and mixed thoroughly by vortexing.
3. Samples were incubated at 56°C for 10 minutes and briefly centrifuged to remove drops from the lid.
4. Ethanol (96–100%) (200 µl) was added and Mixed thoroughly by vortexing, then briefly centrifuged to remove drops from the lid.
5. The mixture was pipetted onto the QIAamp Mini spin column (in a 2 ml collection tube) and centrifuged at 6000 x g for 1 min. the flow-through and collection tube were discarded.

6. The QIAamp Mini spin column was placed in a new 2 ml collection tube and 500 µl Buffer AW1 were added, followed by Centrifugation at 6000 x g for 1 min. The flow-through and collection tube were discarded.

7. The QIAamp Mini spin column was placed in a new 2 ml collection tube and 500 µl Buffer AW2 were added and Centrifuged at full speed (20,000 x g) for 3 min. The flow-through and collection tube were discarded.

8. The QIAamp Mini spin column was placed in a new 2 ml collection tube, and centrifuged at full speed for 1 min.

9. The QIAamp Mini spin column was placed in a new 1.5 ml microcentrifuge tube and 200 µl of Buffer AE. The sample was incubated at room temperature for 1 min and centrifuged at 6000 x g for 1 min to elute the DNA.

II. **Bisulfite treatment: extracted DNA was bisulfite treated using** The **EZ DNA Methylation-Gold Kit D5005** ***(ZYMO RESEARCH, USA),*** **according to manufacturer's instructions.**

- **Procedure**

1. CT Conversion Reagent (130 µl) was added to 20 µl of DNA sample in a PCR tube. Samples were mixed by flicking the tubes, and then the liquid was flash centrifuged to the bottom of the tube.

2. The sample tubes were placed in T100 thermal cycler ***(Bio-Rad, USA)*** and the following steps were performed:

    - 98°C for 10 minutes
    - 64°C for 2.5 hours

3. M-Binding Buffer (600 µl) was added to a Zymo-Spin IC Column and the column was placed into a provided collection tube.

4. Samples (from Step 2) were loaded into the Zymo-Spin IC Column containing the M-Binding Buffer. Tubes were mixed by inverting the column several times.

5. Samples were centrifuged at 12,000 x g for 30 seconds and the flow-through was discarded.

6. M-Wash Buffer (100 µl) was added to the column and centrifuged at 12,000 x g for 30 seconds.

7. M-Desulphonation Buffer (200 µl) was added to the column and incubated at room temperature for 15 minutes. After the incubation, samples were centrifuged at 12,000 x g for 30 seconds.

8. M-Wash Buffer (200 µl) was added to the column and centrifuged at 12,000 x g for 30 seconds. Another 200 µl of M-Wash Buffer were added and centrifuged for an additional 30 seconds.

9. Columns were placed into a 1.5 ml microcentrifuge tube and 10 µl of M-Elution Buffer were added directly to the column matrix and centrifuged for 30 seconds at 12,000 x g to elute the DNA.

III. SYBR Green Methylation specific PCR (qMSP): using HotStarTaq Master Mix Kit-203446 ***(QIAGEN, Germany)*** and Specific primer sets for either methylated or non-methylated products of adiponectin gene promoter according-to-manufacturer'sinstructions.

❖ **Procedure**

1. The PCR reaction mix was prepared in a total volume of 25µl) as in the **table (1)** using two primer pairs were used for *adiponectin* gene. **ADPN-M** for methylated products while **ADPN-U** for unmethylated products. **(Table 2)**

Table (1): Reaction setup for SYBR Green Methylation specific PCR (qMSP)

Component	**Volume/reaction**
HotStarTaq Master Mix	12.5 µl
Forward Primer	0.5 µl

Reverse Primer	0.5 µl
Template DNA	2 µl
Water, nuclease-free	9.5 µl
Total volume	25 µl

Table (2): Primer sequence and annealing temperature of *Adiponectin* promotor

Gene	Forward Primer	Reverse Primer	Annealling temperature	Product size	Reference
ADPN-M	**5'-AATTACAAACACCTACCATCACG-3'**	**3'-AGCTAGAGGGTTTAATGATTTTAAT-5'**	**50 ºC**	**140kb**	***(Haghiac et al., 2014)***
ADPN-U	**5'-AAATTACAAACACCTACCATCACAC-3'**	**3'-AGTTAGAGGGTTTAATGATTTTAATG-5'**	**51 ºC**	**142kb**	

2.The PCR tubes were placed in T100 thermal cycler ***(Bio-Rad, USA)*** and the run was started according to the following program:

-Initial activation step: for 13min at 95ºC

- 35 cycles of denaturation at 94 ºC for 30 seconds, annealing temperature for 30 seconds then extension at 72 ºC for 1 minute.

- Final extension step at 72 ºC for 2 min.

3. The PCR product was separated by gel electrophoresis, stained with ethidium bromide and visualized by UV irradiation, for detection of specific bands.

4. *Adiponectin* methylation percentage was calculated according to the following equation: $adiponectin$ methylation percentage $= \frac{1}{1+2^{(-\Delta Ct)}} \times 100\%$

While ΔCt = adiponectin Ct – GAPDH Ct ***(Lu et al., 2007).***

***Statistical analysis*:**

The collected data was tabulated, and statistically analyzed using SPSS program (Statistical Package for Social Sciences) software version 26.0, Microsoft Excel 2016.

Descriptive statistics were done for numerical data as mean±SD (standard deviation) while they were done for categorical data as number and percentage.

Inferential analyses were done for quantitative variables using independent t-test in cases of two independent groups with parametric data and Mann Whitney U in cases of two independent groups with non-parametric data. Inferential analyses were done for qualitative data using Chi square test for independent groups. Analysis of variance (ANOVA or F test) was used for continuous data to test for significant difference between more than two normally distributed groups. Assumptions of normality in each group and homogeneity of variances were verified using Shapiro-Wilk test and Levine's test, respectively. Kruskal-Wallis test: It is a non-parametric equivalent to ANOVA and used when ANOVA assumptions were violated to compare between more than two groups of skewed data. Post Hoc tests: Tukey honestly significant difference (Tukey- HSD) test was used as a post hoc test to adjust for multiple comparisons after significant ANOVA test to indicate which significant difference between pairs of groups whereas Bonferroni post hoc test was used after significant Kruskal- Wallis test.

The level of significance was taken at P value <0.05 is significant, otherwise is non-significant. The p-value is a statistical measure for the probability that the results observed in a study could have occurred by chance.

RESULTS

Table (3): Age distribution among the studied groups

Groups / Age (years)	*Group I (Simple steatosis group) (n=5)*	*Group II (NASH group) (n=29)*	*Group III (Control group) (n=15)*	F-Test	P-value*
Mean± SD	51.80± 8.98	42.52± 9.06	43.60±14.98	1.47	0.240
Median	53.0	40.0	47.0		
Range	40- 64 years	27- 60 years	19- 64 years		

p≤0.05: significant, p≤0.01: highly significant, SD: standard deviation, analysis done by One Way ANOVA Test

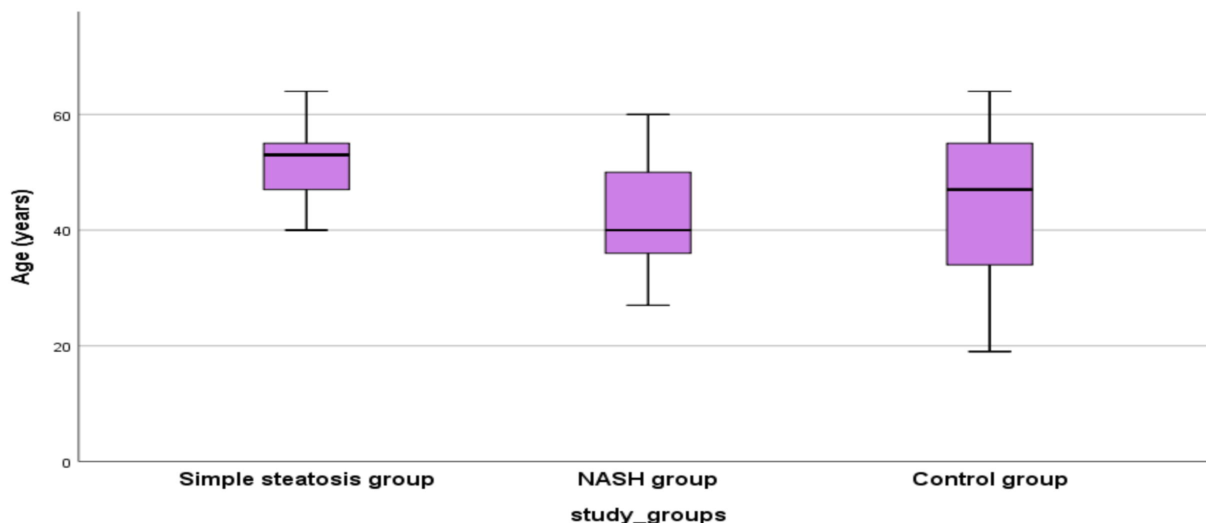


Figure (12): Box-plot showing comparison between the studied groups regarding Age.

Table (3) &figure (12) show that there was non-significant statistical difference regarding age between control group when compared to patient groups (simple steatosis and NASH groups) (F=1.47, P=0.240)

Table (4): Demographic characteristics (Gender, Residence and Occupation) among the studied groups

		Group I (Simple steatosis group) (n=5)		*Group II (NASH group) (n=29)*		*Group III (Control group) (n=15)*		**Test value**	**P-value**
		n	**%**	**n**	**%**	**n**	**%**		
Gender	**Male**	2	40.0%	11	37.9%	8	53.3%	X^2= 0.976	0.614
	Female	3	60.0%	18	62.1%	7	46.7%		
Residence	**Rural**	1	20.0%	13	44.8%	6	40.0%	X^2= 1.09	0.597
	Urban	4	80.0%	16	55.2%	9	60.0%		
Occupation	**Not working**	2	40.0%	18	62.1%	8	53.3%	X^2= 0.976	0.614
	Working	3	60.0%	11	37.9%	7	46.7%		

p≤0.05: significant, p≤0.01: high significant, comparison between groups done by Chi- Square test

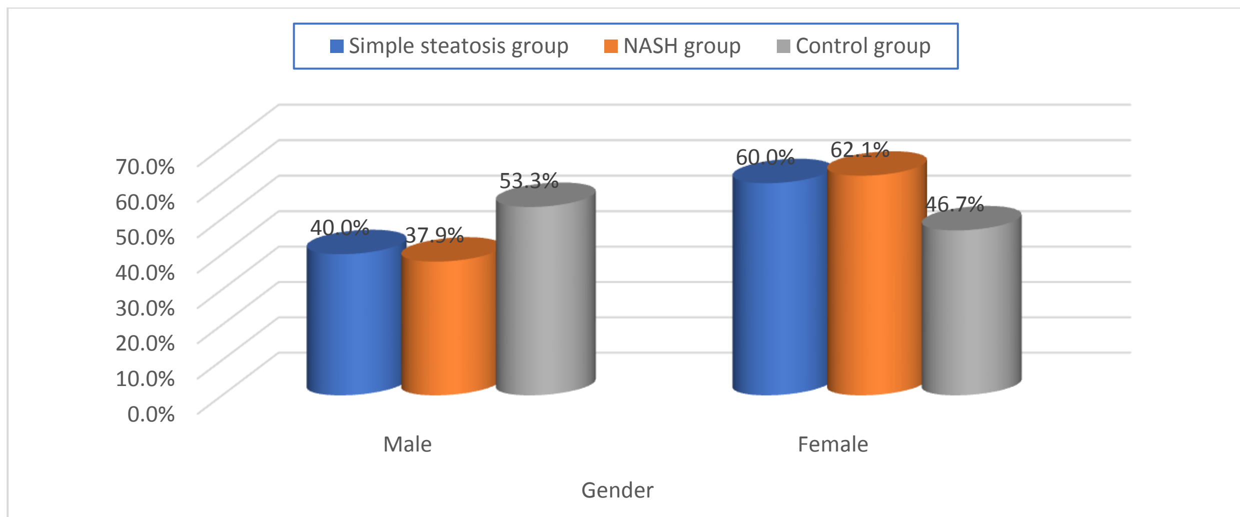


Figure (13): Comparison between the study groups regarding gender.

Table (4) &figure (13) show that there was non-significant statistical difference regarding Gender in control group as compared to patient groups (simple steatosis and NASH groups)(X^2==0.976, P=0.614)

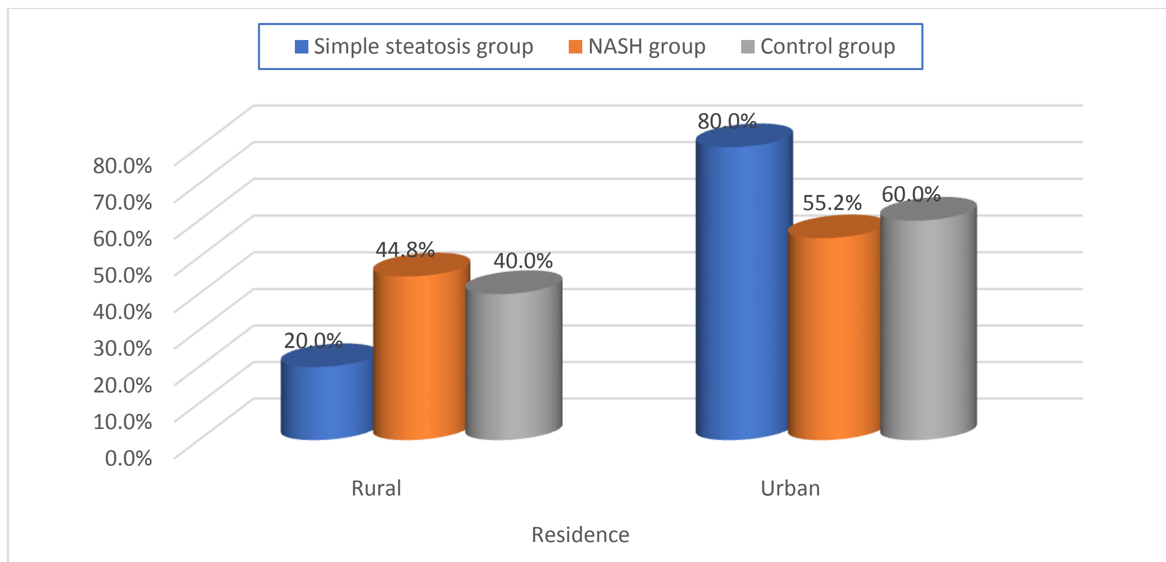


Figure (14): Comparison between the study groups regarding residence.

Table (4) &figure (14) show that there was non-significant statistical difference regarding residence in control group as compared to patient groups (simple steatosis and NASH groups)(X^2=1.09, P=0.597)

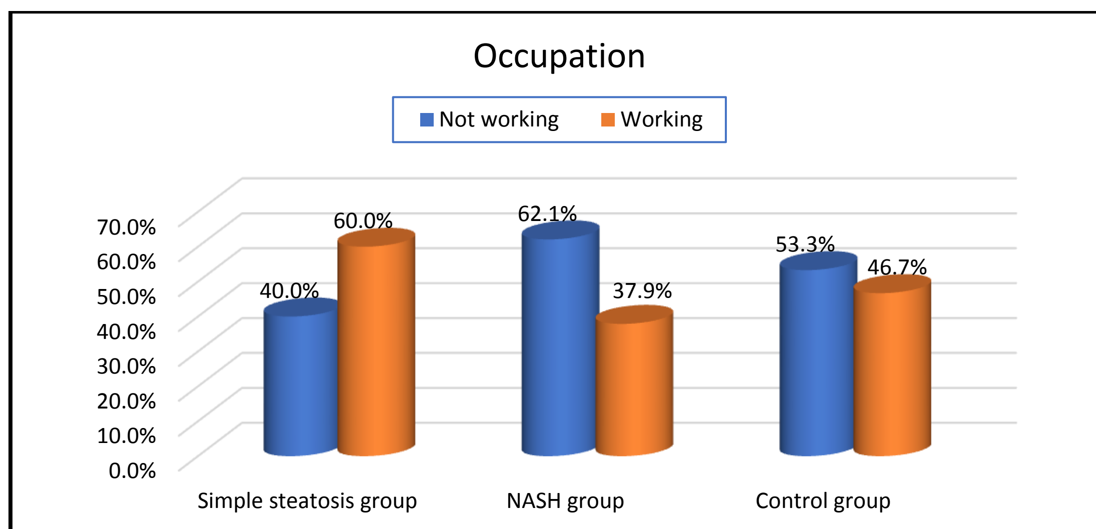


Figure (15): Comparison between the study groups regarding occupation.

Table (4) &figure (15) show that there was non-significant statistical difference regarding occupation in control group as compared to patient groups (simple steatosis and NASH groups)(X^2=0.976, P=0.614)

Table (5): Frequency distribution and percentage of clinical history among the studied groups

	Group I (Simple steatosis group) (n=5)		*Group II (NASH group) (n=29)*		*Group III (Control group) (n=15)*		**Test value**	**P-value**
	n	**%**	**n**	**%**	**n**	**%**		
Smoking	3	60.0%	25	86.2%	11	73.3%	X^2= 2.32	0.313
Schistosomiasis	0	0.0%	4	13.8%	0	0.0%	X^2= 3.00	0.223
DM	1	20.0%	10	34.5%	0	0.0%	X^2=6.77	**0.034**
HTN	0	0.0%	7	24.1%	0	0.0%	X^2= 5.63	0.060

$p \leq 0.05$: significant, $p \leq 0.01$: high significant, comparison between groups done by Chi- Square test.
DM: diabetes mellitus, HTN: hypertension.

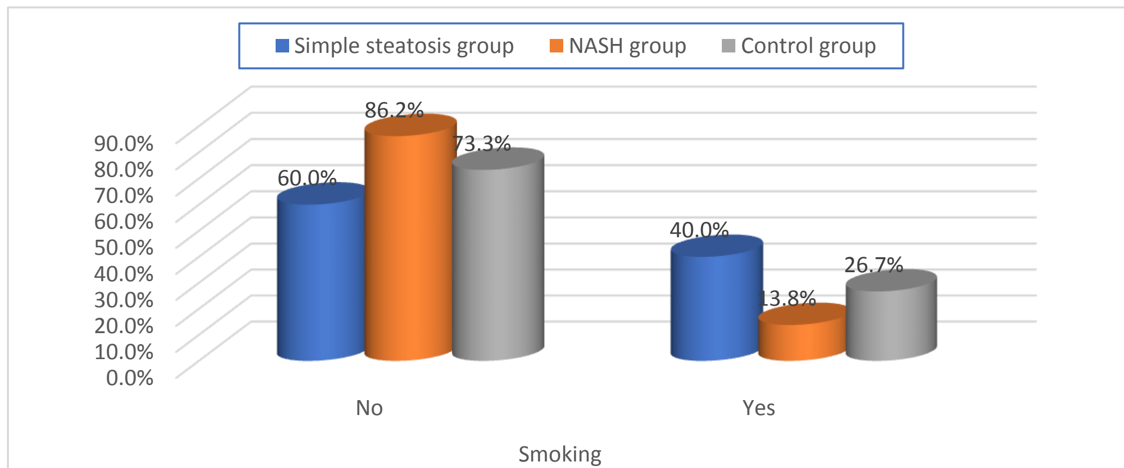


Figure (16): Comparison between the study groups regarding Frequency distribution and percentage of smoking

Table (5) &figure (16) show that there was non-significant statistical difference regarding smoking frequency distribution and percentage in control group as compared to patient groups (simple steatosis and NASH groups)(X^2=2.32, P=0.313)

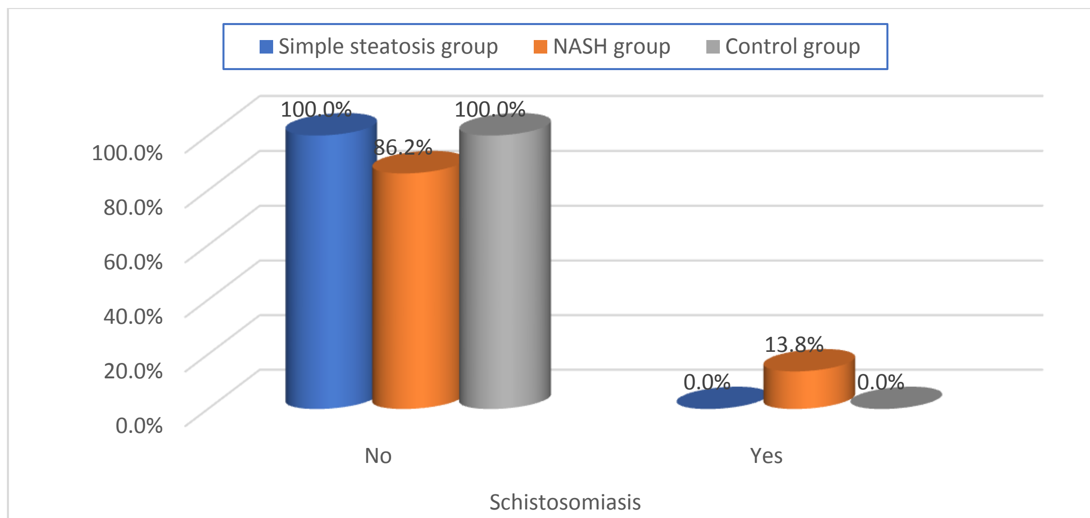


Figure (17): Comparison between the study groups regarding Frequency distribution and percentage of Schistosomiasis

Table (5) &figure (17) show that there was non-significant statistical difference regarding schistosomiasis frequency distribution and percentage in control group as compared to patient groups (simple steatosis and NASH groups)(X^2=3.00, P=0.223)

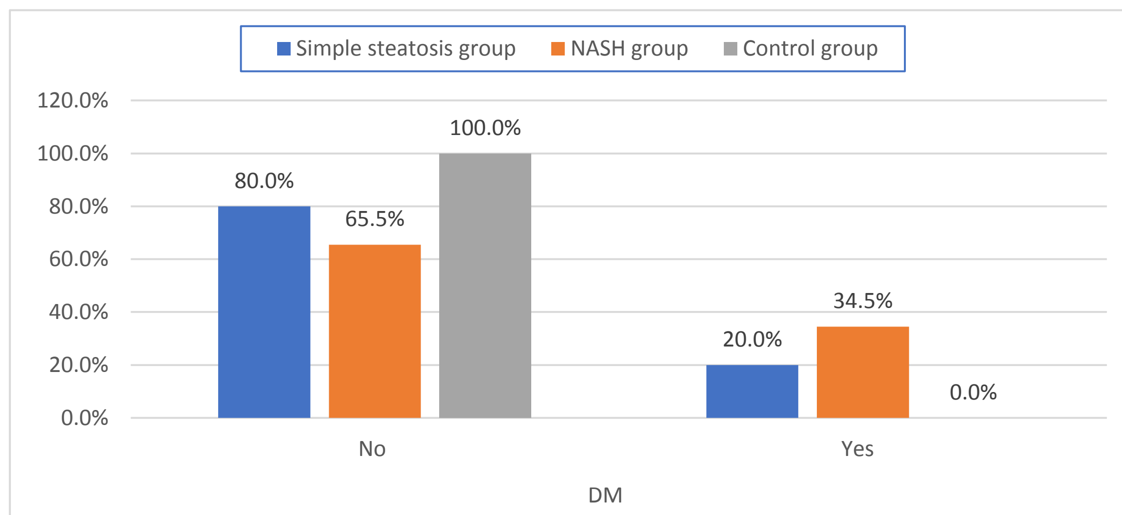


Figure (18): Comparison between the study groups regarding Frequency distribution and percentage of DM

Table (5) &figure (18) show that there was significant statistical difference regarding diabetes mellitus frequency distribution and percentage in control group as compared to patient groups (simple steatosis and NASH groups) (X^2=6.77, P=0.034)

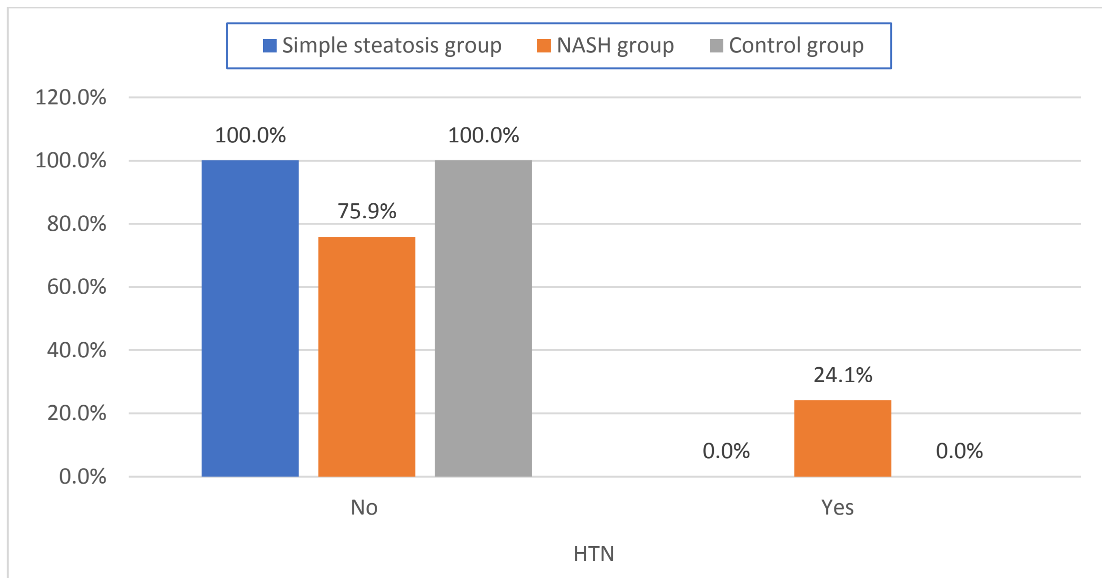


Figure (19): Comparison between the study groups regarding Frequency distribution and percentage of HTN

Table (5) &figure (19) show that there was non-significant statistical difference regarding hypertension frequency distribution and percentage in control group as compared to patient groups (simple steatosis and NASH groups)(X^2=5.63, P=0.060)

Table (6): Frequency distribution and percentage of clinical manifestations among the studied groups

		Group I (Simple steatosis group) (n=5)		*Group II (NASH group) (n=29)*		Test value	P-value
		n	%	n	%		
Pain	**No**	4	80.0%	18	62.1%	X^2= 7.75	**0.021**
	Yes	1	20.0%	11	37.9%		
Fatigue	**No**	4	80.0%	21	72.4%	X^2= 5.03	0.081
	Yes	1	20.0%	8	27.6%		
Nausea	**No**	5	100.0%	28	96.6%	X^2= 0.704	0.703
	Yes	0	0.0%	1	3.4%		
Anorexia	**No**	5	100.0%	29	100.0%	-	-
	Yes	0	0.0%	0	0.0%		

p≤0.05: significant, p≤0.01: high significant, comparison between groups done by Chi- Square test

Table (6) shows that complaint of right hypochondrial pain was statistically significantly higher among NASH patients (37.9%) as compared to simple steatosis group (X^2=7.75, P=0.021).

There were non-significant statistical differences regarding Fatigue and Nausea in simple steatosis group as compared to NASH group (X^2=5.03 and X^2=0.704 respectively, P> 0.05 in all).

Table (7): Mean± SD and Median of Weight, Height, BMI, Waist circumference and Waist/Hip ratio in all studied groups

	Group I (Simple steatosis group) (n=5)			*Group II (NASH group) (n=29)*			*Group III (Control group) (n=15)*			Test value	P-value
	Mean	**± SD**	**Median**	**Mean**	**± SD**	**Median**	**Mean**	**± SD**	**Median**		
Weight (Kg)	92.40	17.10	88.00	106.27	20.25	103.50	61.93	5.28	62.00	F=34.07	**<0.001** P1=0.094 P2=**0.001** P3<**0.001**
Height (cm)	162.60	6.99	162.00	161.42	8.57	159.00	162.73	6.70	163.00	KW= 0.149	0.862
BMI (Kg/ m2)	34.71	4.06	33.25	40.93	7.64	39.25	23.49	2.75	22.68	F= 38.18	**<0.001** P1=**0.045** P2 =**0.001** P3<**0.001**
Waist circumference in male	113.00	2.83	113.00	126.20	13.59	124.00	92.63	5.13	94.00	KW=14.99	**0.001** P1=0.843 P2 =**0.047** P3<**0.001**
Waist circumference in female	112.00	1.73	113.00	125.92	13.80	125.00	80.14	8.76	80.00	KW=4.597	**<0.001** P1=0.184 P2 =0.142

											P3<**0.001**
Waist/Hip ratio in male	93.41	3.43	93.41	103.36	11.30	103.33	94.61	7.07	96.41	KW= 4.597	0.100
Waist/Hip ratio in female	96.91	7.84	100.00	102.97	20.41	98.57	78.88	6.54	79.21	KW=9.03	**0.011** P1=0.965 P2 =**0.042** P3=**0.004**

p≤0.05: significant, p≤0.01: high significant, SD: standard deviation, analysis done by One Way ANOVA Test, Kruskal Wallis test, P:patient groups Vs. controls, P1:group (I) Vs. group (II), P2: group (I) Vs. group (III), P3: group (II) Vs. group (III), ` BMI: Body mass index.

Table (7) shows that there were significant statistical differences regarding weight in simple steatosis group and NASH group as compared to control group (P2=0.001, P3<0.001). However there was non-significant statistical difference in simple steatosis group as compared to NASH group (P1=0.094) .

There was non-significant statistical difference regarding Height among all studied groups (P> 0.05 in all).

There were significant statistical differences regarding body mass index in simple steatosis group as compared to NASH and control groups (P1=0.045, P2 =0.001). There was also significant statistical difference in NASH group as compared to control group (P3<0.001).

there were significant statistical differences regarding Waist circumference in male in simple steatosis group and NASH group as compared to control group (P2 =0.047, P3<0.001). However there was non-significant statistical difference in simple steatosis group as compared to NASH group (P1=0.843) .

There was significant statistical difference regarding waist circumference in female in NASH group as compared to control group (P3<0.001). However there were non-significant statistical differences in simple steatosis group as compared to NASH & control groups (P1=0.184, P2 =0.142) .

There was non-significant statistical difference regarding waist/hip ratio in male among all studied groups (P> 0.05 in all).

There were significant statistical differences regarding waist/hip ratio in female in simple steatosis group and NASH group as compared to control group (P2 =0.042, P3=0.004). However there was non-significant statistical difference in simple steatosis group as compared to NASH group (P1=0.965) .

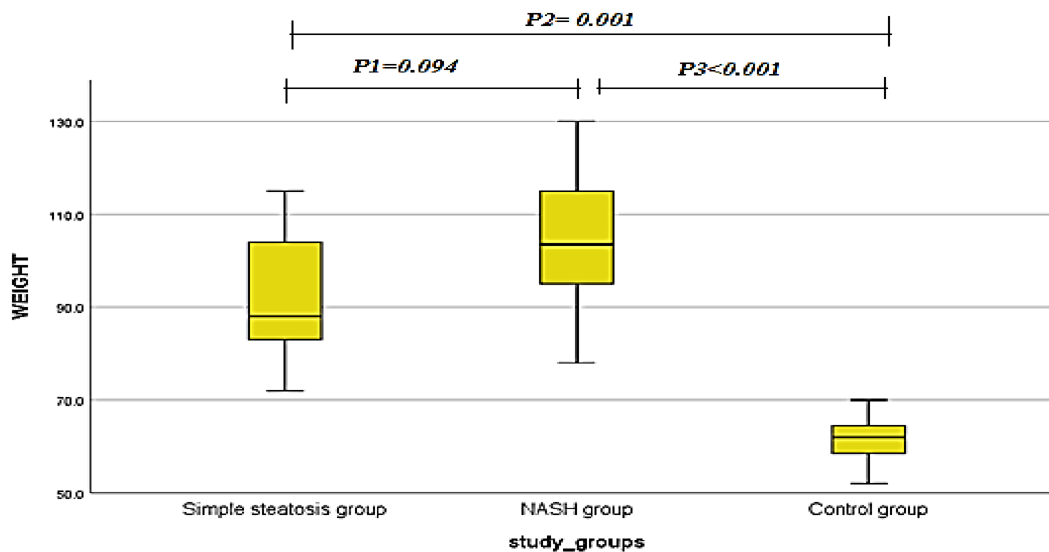


Figure (20): Box-plot showing comparison between the studied groups regarding weight.

Figure (20) shows that there were significant statistical differences regarding weight in simple steatosis group and NASH group as compared to control group (P2=0.001, P3<0.001). However there was non-significant statistical difference in simple steatosis group as compared to NASH group (P1=0.094)

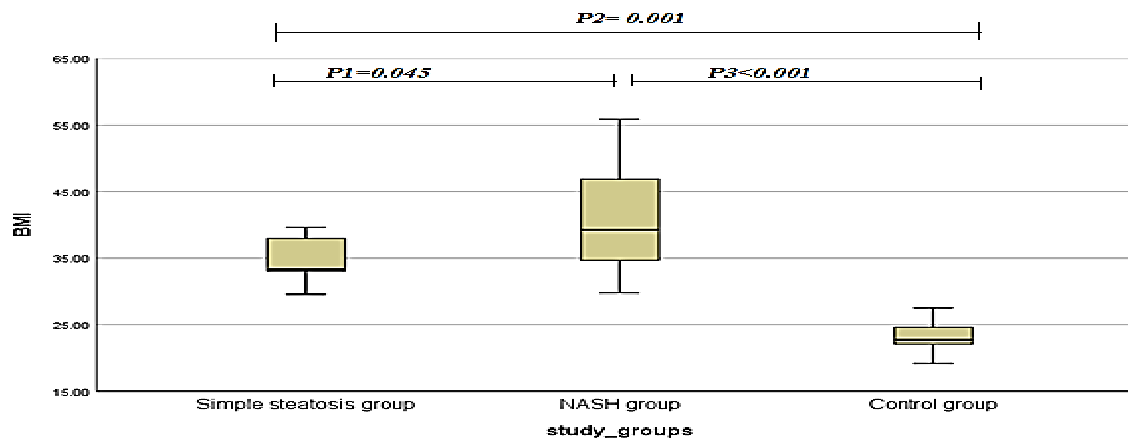


Figure (21): Box-plot showing comparison between the studied groups regarding BMI.

Figure (21) shows that There were significant statistical differences regarding Body mass index in simple steatosis group as compared to NASH and control groups (P1=0.045, P2 =0.001). There was also significant statistical difference in NASH group as compared to control group (P3<0.001).

Table (8): Mean ± SD and median of HB, platelets, WBCs counts and liver function tests among the studied groups.

Parameter	*Group I (Simple steatosis group) (n=5)*			*Group II (NASH group) (n=29)*			*Group III (Control group) (n=15)*			Test value	P-value*
	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median		
Hb	12.36	1.54	12.60	12.95	1.55	13.00	12.05	1.47	12.50	F=1.74	0.188
Platelets	246.20	62.17	223.00	294.52	69.20	280.00	249.14	94.98	229.00	F=2.06	0.139
WBCS	6.36	2.39	6.60	8.09	2.25	7.60	7.28	3.00	7.30	F=1.26	0.293
Total bilirubin	.42	.11	.40	.51	.30	.46	.57	.27	.60	KW= 0.638	0.727
Total protein	7.24	.57	7.20	7.33	.65	7.00	7.42	.45	7.30	F= 0.303	0.740
Albumin	4.24	.81	4.50	4.30	.59	4.40	3.83	.75	4.15	F= 1.01	0.375
AST	39.20	35.77	25.00	33.97	18.55	27.50	20.73	4.80	21.00	KW= 4.78	0.092
ALT	38.80	25.76	34.00	32.39	21.86	27.00	21.15	5.54	19.00	KW= 2.55	0.280
GGT	115.00	92.06	78.00	96.68	74.32	54.00	20.60	8.02	24.00	KW= 9.93	**0.007** P1=0.736 P2 =**0.009** P3=**0.002**
ALP	95.40	19.10	107.00	100.05	47.10	93.00	330.00	0.45	330.00	KW= 3.42	0.181
PC	105.15	18.35	101.30	94.86	7.93	92.40	92.44	5.95	93.20	KW= 2.11	0.349
INR	1.17	.	1.17	1.05	.05	1.05	.96	.08	1.00	KW= 15.63	**<0.001** P1=0.077 P2 =**0.001** P3=**0.001**

p≤0.05: significant, p≤0.01: high significant, SD: standard deviation, analysis done by One Way ANOVA Test, Kruskal Wallis test, P:patient groups Vs. controls, P1:group (I) Vs. group (II), P2: group (I) Vs. group (III), P3: group (II) Vs. group (III),

Table (8) shows that there was non-significant statistical difference regarding HB level, platelets, WBCs counts in all studied groups (P=0.188, P=0.139 and P=0.293, respectively).

There was non-significant statistical difference regarding serum level of total bilirubin, total protein and albumin in all studied groups (P=0.727, P=0.740 and P=0.375, respectively).

There was non-significant statistical difference regarding serum level of AST, ALT and ALP in all studied groups (P=0.092, P=0.280 and P=0.181, respectively).

There was significant statistical difference in serum level of GGT in simple steatosis group and NASH group as compared to control group (P2 =0.009, P3=0.002). However there was non-significant statistical difference in simple steatosis group as compared to NASH group (P1=0.736).

There was non-significant statistical difference regarding prothrombin concentration (PC) in all studied groups (0.349)

There was significant statistical difference in international normalized ratio (INR) in simple steatosis group and NASH group as compared to control group (P2 =0.001, P3=0.001). However there was non-significant statistical difference in simple steatosis group as compared to NASH group (P1=0.077).

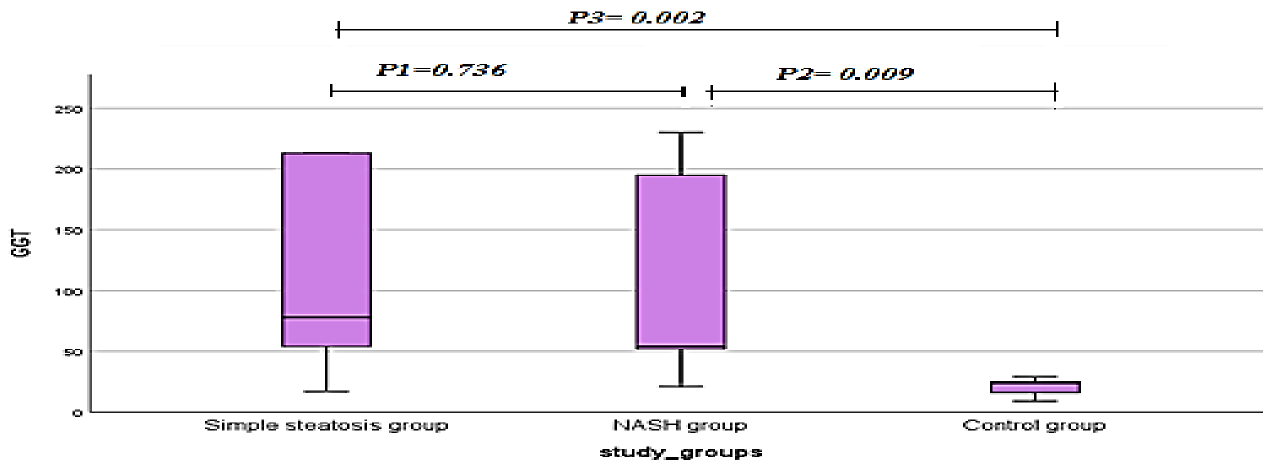


Figure (22): Box-plot showing comparison between the studied groups regarding GGT.

Figure (22) shows that There was significant statistical difference in serum level of GGT in simple steatosis group and NASH group as compared to control group (P2 =0.009, P3=0.002). However there was non-significant statistical difference in simple steatosis group as compared to NASH group (P1=0.736).

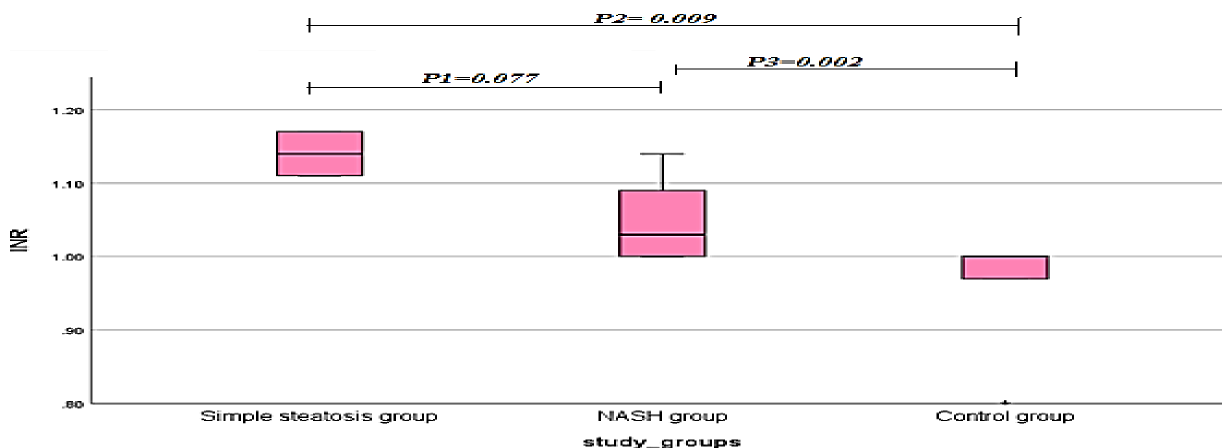


Figure (23): Box-plot showing comparison between the studied groups regarding INR.

Figure (23) shows that There was significant statistical difference in international normalized ratio (INR) in simple steatosis group and NASH group as compared to control group (P2 =0.001, P3=0.001). However there was non-significant statistical difference in simple steatosis group as compared to NASH group (P1=0.077).

Table (9): Mean ± SD and median of lipid profile & blood glucose profile in all studied groups.

	Group I (Simple steatosis group) (n=5)			*Group II (NASH group) (n=29)*			*Group III (Control group) (n=15)*			**Test value**	**P-value**
	Mean	**± SD**	**Median**	**Mean**	**± SD**	**Median**	**Mean**	**± SD**	**Median**		
Total Cholesterol	235.50	68.53	245.00	219.87	39.80	212.00	157.13	14.74	155.00	F= 8.797	**0.001** P1=0.465 P2 =**0.003** P3<**0.001**
LDL	175.67	16.77	167.00	146.84	35.89	141.00	84.67	9.50	85.00	F= 6.09	**0.009** P1=0.171 P2 =**0.003** P3=**0.005**
HDL	53.00	10.00	52.00	42.12	13.63	40.00	60.50	4.20	61.00	F= 4.39	**0.021** P1=0.121 P2 =0.409 P3=**0.011**
TGs	179.75	109.48	145.00	175.65	72.11	169.50	105.00	12.70	99.00	KW= 3.20	0.202
FBS	107.00	45.70	93.50	125.38	45.20	111.00	87.25	15.17	87.50	KW= 6.698	**0.035** P1=0.482 P2 =0.162 P3=**0.020**
2hrs PP- BS	185.33	91.53	174.00	168.00	79.19	133.00	132.00	5.66	132.00	KW= 0.494	0.781
HbA1C	8.00	0.0	8.00	8.07	2.24	8.55	4.23	.93	4.50	KW= 6.32	**0.042** P1=0.230 P2 =0.769 P3=**0.012**

p≤0.05: significant, p≤0.01: high significant, SD: standard deviation, analysis done by One Way ANOVA Test, Kruskal Wallis test, P:patient groups Vs. controls, P1:group (I) Vs. group (II), P2: group (I) Vs. group (III), P3: group (II) Vs. group (III),

Table (9) shows that there was significant statistical difference in serum level of total cholesterol in simple steatosis group and NASH group as compared to control group (P2=0.003, P3<0.001). However there was non-significant statistical difference in simple steatosis group as compared to NASH group (P1=0.465).

There was significant statistical difference in serum level of LDL in simple steatosis group and NASH group as compared to control group (P2=0.003, P3=0.005). However there was non-significant statistical difference in simple steatosis group as compared to NASH group (P1=0.171).

There was significant statistical difference regarding HDL in NASH group as compared to control group (P3=0.011). However there were non-significant statistical differences in simple steatosis group as compared to NASH & control groups (P1=0.121, P2 =0.409) .

There was non-significant statistical difference regarding serum level of Triglycrides in all studied groups (P=0.202).

There was significant statistical difference regarding fasting blood glucose in NASH group as compared to control group (P3=0.020). However there were non-significant statistical differences in simple steatosis group as compared to NASH & control groups (P1=0.482, P2 =0.162) .

There was non-significant statistical difference regarding serum level of 2hrs post prandial blood glucose in all studied groups (P=0.781).

There was significant statistical difference regarding glycated Hemoglobin in NASH group as compared to control group (P3=0.012). However there were non-significant statistical differences in simple steatosis group as compared to NASH & control groups (P1=0.230, P2 =0.769) .

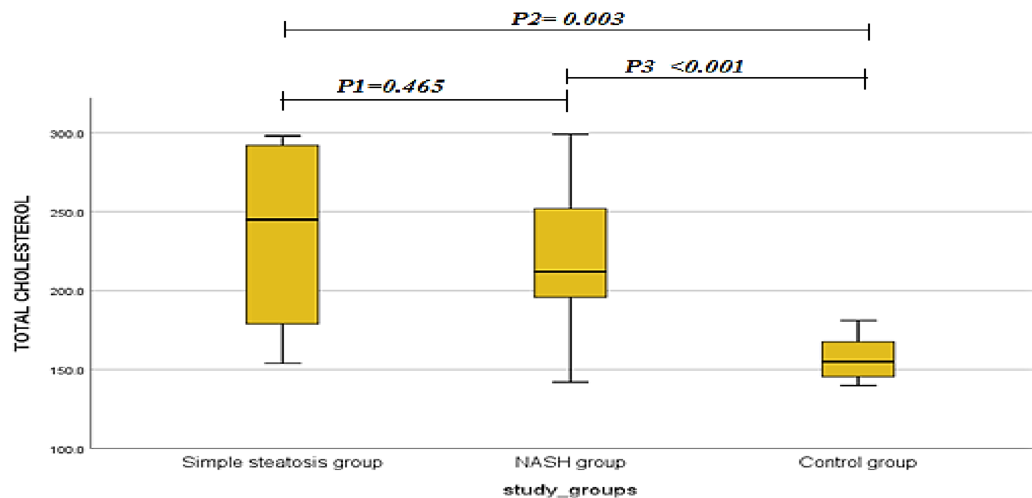


Figure (24): Box-plot showing comparison between the studied groups regarding Total Cholesterol

Figure (24) shows that there was significant statistical difference in serum level of total cholesterol in simple steatosis group and NASH group as compared to control group (P2=0.003, P3<0.001). However there was non-significant statistical difference in simple steatosis group as compared to NASH group (P1=0.465).

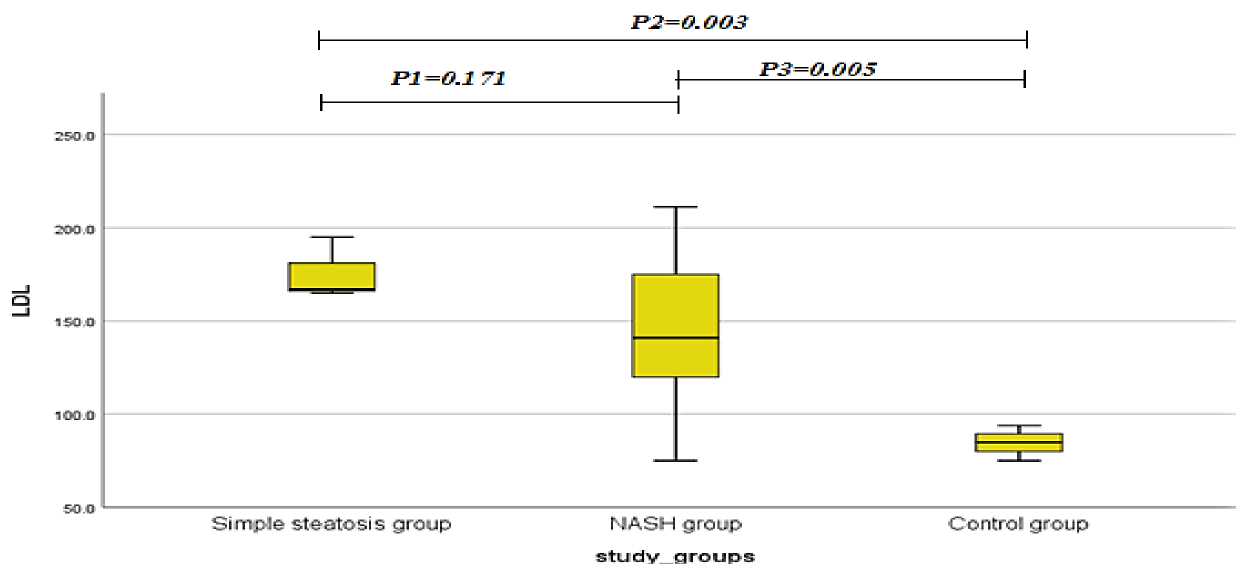


Figure (25): Box-plot showing comparison between the studied groups regarding LDL

Figure (25) shows that There was significant statistical difference in serum level of LDL in simple steatosis group and NASH group as compared to control group (P2=0.003, P3=0.005). However there was non-significant statistical difference in simple steatosis group as compared to NASH group (P1=0.171).

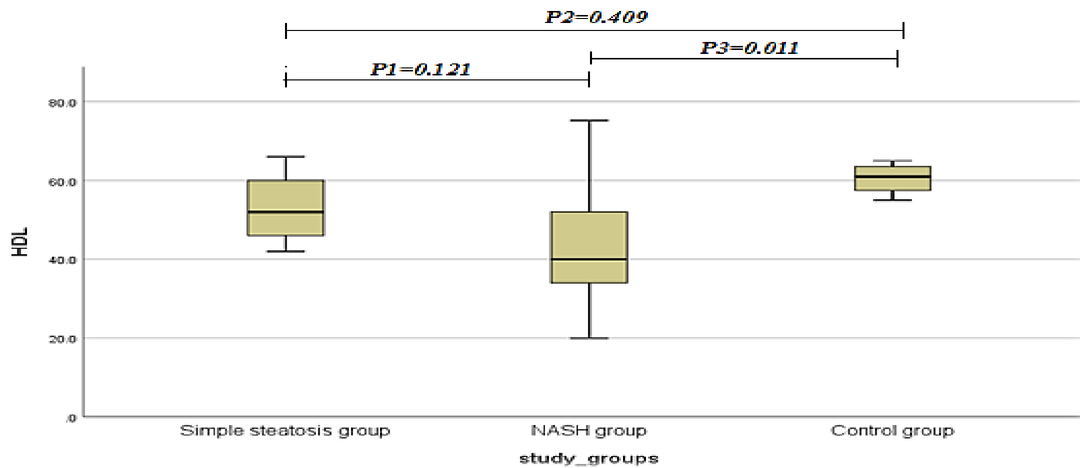


Figure (26): Box-plot showing comparison between the studied groups regarding HDL

Figure (26) shows that There was significant statistical difference regarding HDL in NASH group as compared to control group (P3=0.011). However there were non-significant statistical differences in simple steatosis group as compared to NASH & control groups (P1=0.121, P2 =0.409) .

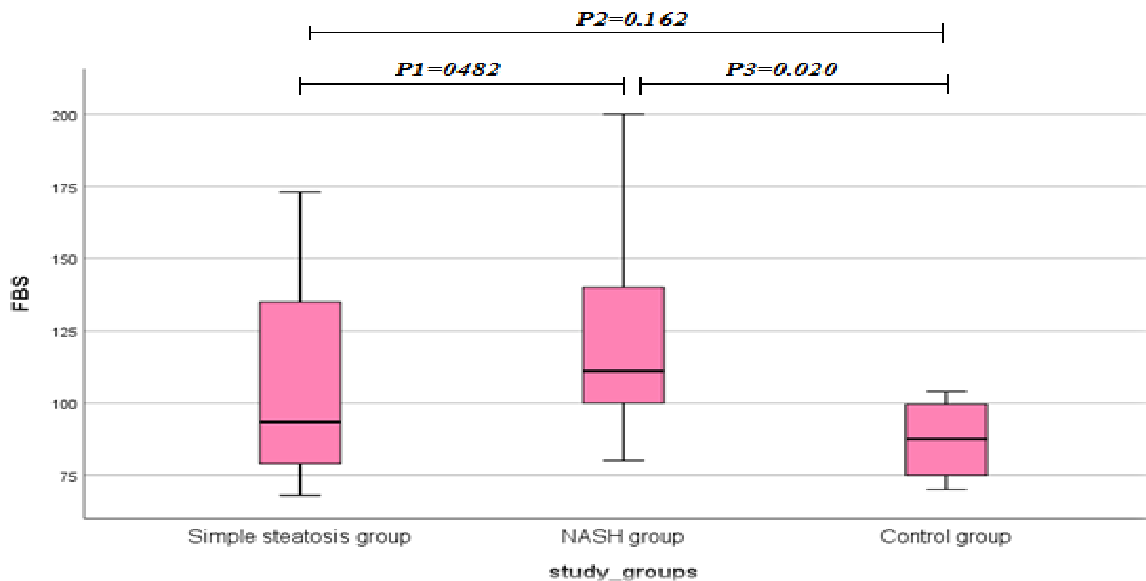


Figure (27): Box-plot showing comparison between the studied groups regarding FBS

Figure (27) shows that There was significant statistical difference regarding fasting blood glucose in NASH group as compared to control group (P3=0.020). However there were non-significant statistical differences in simple steatosis group as compared to NASH & control groups (P1=0.482, P2 =0.162) .

Table (10): Frequency distribution and percentage of Liver biopsy findings among simple steatosis and NASH groups

		Group I (Simple steatosis group) (n=5)		Group II (NASH group) (n=29)		Test value	P-value
		n	%	n	%		
Fibrosis	F1	1	20.0%	0	0.0%	X^2= 59.4	<0.001
	F2	4	80.0%	21	72.4%		
	F3	0	0.0%	4	13.8%		
	F4	0	0.0%	3	10.3%		

p≤0.05: significant, p≤0.01: high significant, comparison between groups done by Chi- Square test

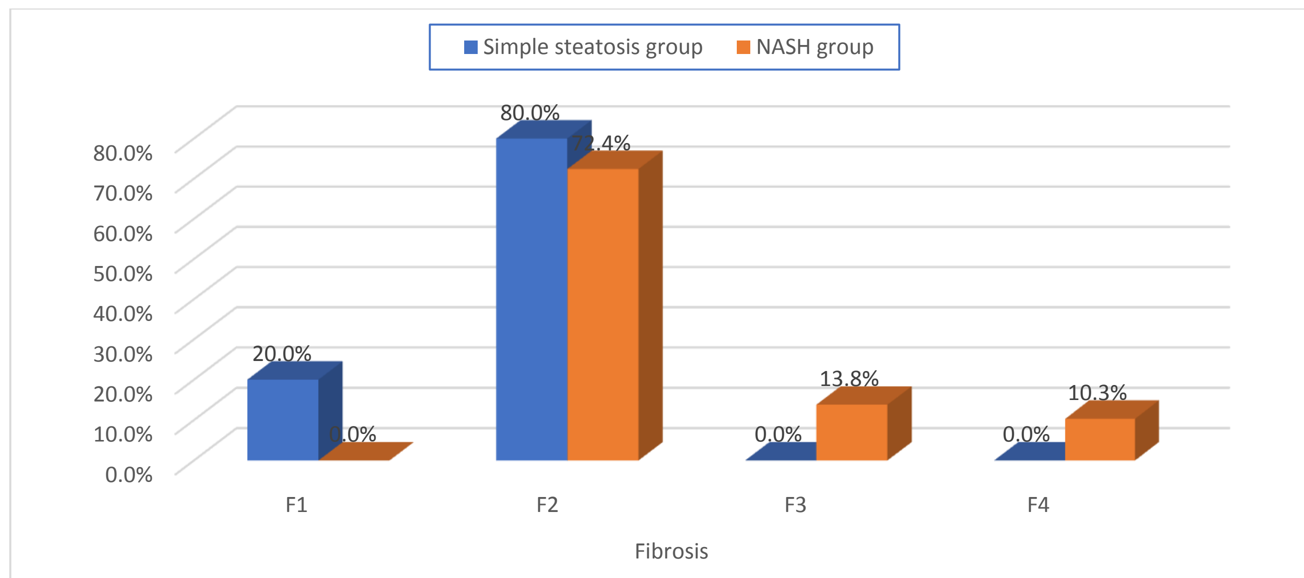


Figure (28): Comparison between simple steatosis & NASH groups regarding grades of fibrosis

Table (10)& figure(28) show that there was significant statistical difference regarding fibrosis grades in NASH group as compared to simple steatosis group (P<0.001).

Table (11): Comparison between the study groups regarding *adiponectin* promotor methylation

Groups / Adipo Q methylation	*Group I (Simple steatosis group) (n=5) (1)*	*Group II (NASH group) (n=29) (2)*	*Group III (Control group) (n=15) (3)*	F-Test	P-value*
Mean± SD	52.45± 20.21	53.89± 18.94	40.03± 10.98	3.35	**0.044** P1=0.862 P2 =0.165 P3=**0.014**
Median	60.65	53.74	39.73		
Range	19.19 – 71.78	13.89-93.38	10.97 – 53.46		

$p \le 0.05$: significant, $p \le 0.01$: high significant, SD: standard deviation, analysis done by One Way ANOVA Test, P: patient groups vs. controls, P1: group (I) vs. group (II), P2: group (I) vs. group (III), P3: group (II) vs. group (III),

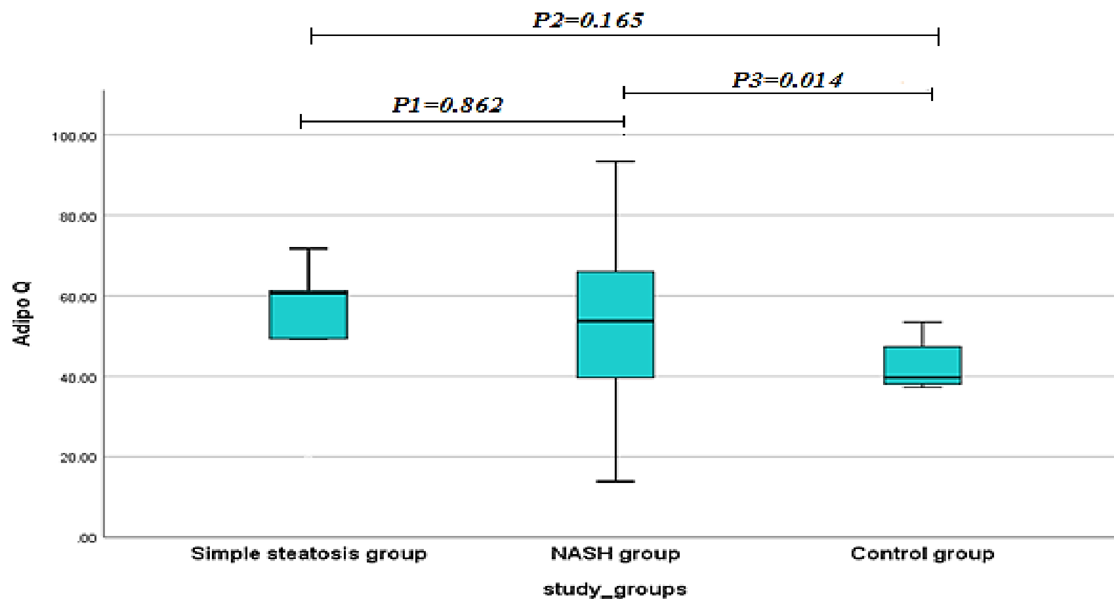


Figure (29): Box-plot showing comparison between the studied groups regarding Adipo Q methylation

Table (11) & figure(29) show that there was significant statistical difference regarding *adiponectin* promotor methylation in NASH group as compared to

control group (P3=0.014).However there was non-significant difference in simple steatosis group as compred to NASH and control groups (P1=0.862, P2 =0.165).

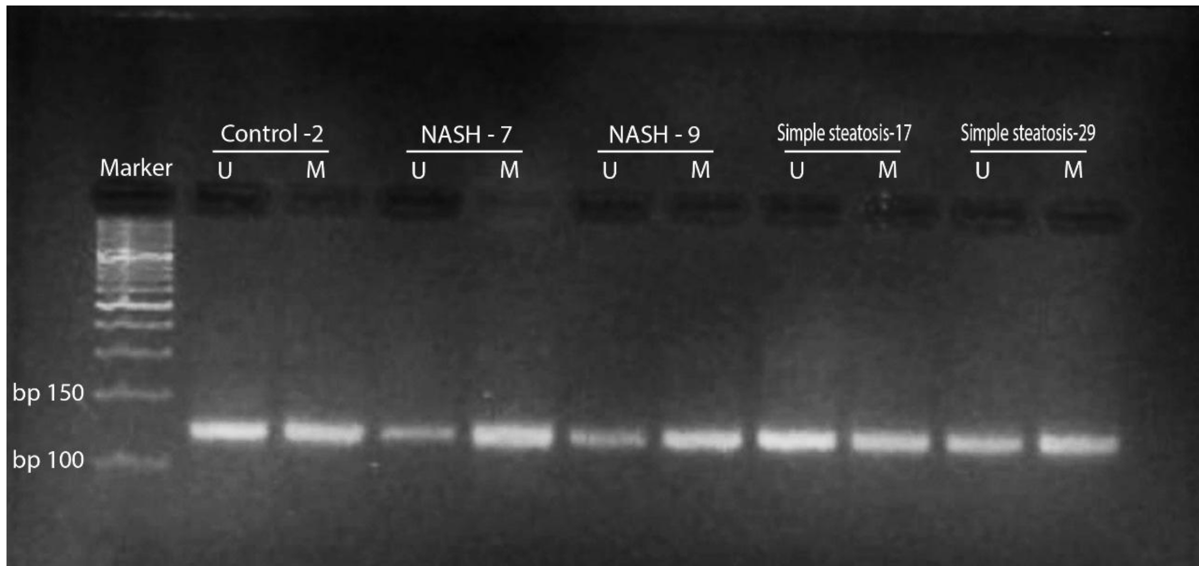


Figure (30) shows Agarose gel electrophoresis for methylation specific PCR products of *adiponectin* gene using unmethylated and methylated primers. Lane 1: DNA marker; lane 2, 3: control; lane 4, 5 & 6, 7: NASH patients; lane 8, 9 &10, 11: simple steatosis patients.

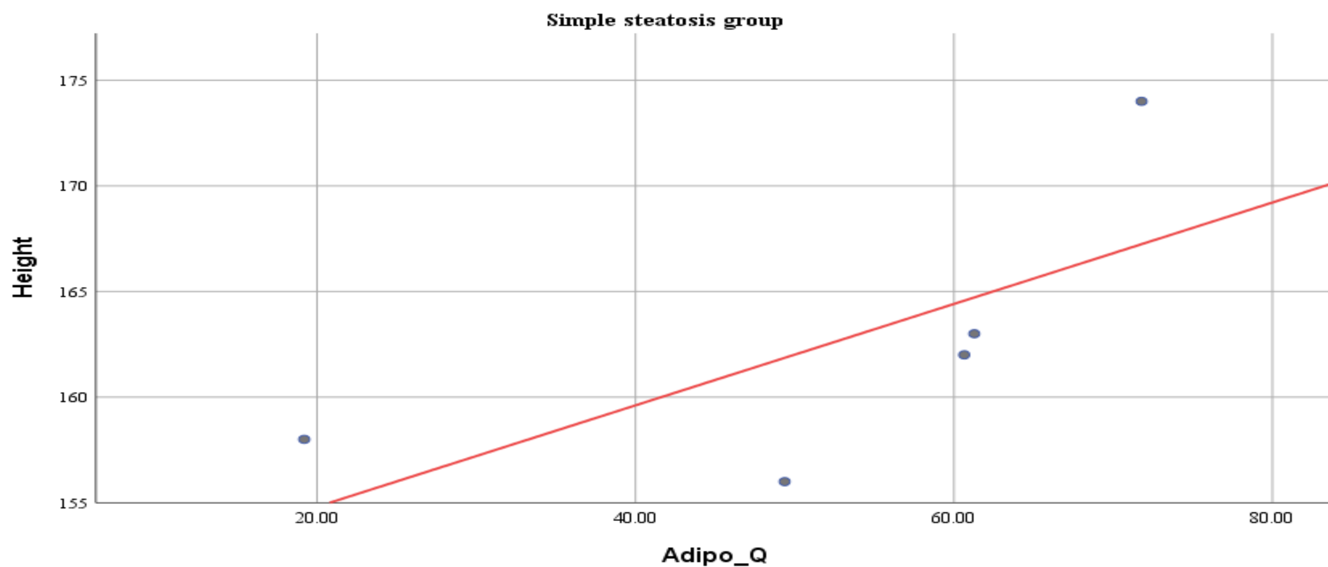
Table (12): Correlation coefficient and P values between *adiponectin* promotor methylation and different parameters in studied groups

Parameters	Adipo Q					
	Group I (Simple steatosis group) (n=5) (1)		*Group II (NASH group) (n=29) (2)*		*Group III (Control group) (n=15) (3)*	
	r	**P-value**	**r**	**P-value**	**r**	**P-value**
Age	-.600-	.285	.230	.231	.111	.694
Height	.900	**.037**	-.143-	.486	-.328-	.232
Weight	.800	.104	-.170-	.405	.081	.775
BMI	.300	.624	.015	**.016**	.538	**.039**
Waist circumference	.500	.667	-.170-	.561	.599	.117
Waist/hip ratio	-.600	0.286	-.056	0.819	-.275	0.321
HGB	.100	.873	.055	.777	-.249-	.390
PLT	-.800-	.104	-.117-	.547	.011	.970
WBCS	-.300-	.624	-.154-	.424	-.182-	.533
Total bilirubin	.632	.368	-.193-	.355	.015	.943
Total protein	-.527-	.361	-.263-	.185	-.500-	.391
Albumin	-.100-	.873	.099	.610	-.600-	.400
AST	-.200-	.747	.021	**.027**	.694	**.018**
ALT	.300	.624	-.084-	.671	-.017-	.956
GGT	-.154-	.805	.169	.421	.500	.391
ALP	-.783-	.118	.059	.801	.018	.930
PC	.700	.188	.460	**.024**	.400	.600
INR	-1.000-	-	-.317-	.123	.401	.222

Parameters	Adipo Q					
	Group I (Simple steatosis group) (n=5) (1)		Group II (NASH group) (n=29) (2)		Group III (Control group) (n=15) (3)	
	r	P-value	r	P-value	r	P-value
TOTAL Cholesterol	-.200-	.800	-.070-	.718	.347	.399
LDL	-.500-	.667	.018	.930	-.500-	.667
HDL	-.200-	.800	-.062-	.768	-.800-	.200
TGs	.400	.600	.266	.171	.211	.789
FBS	-.800-	.200	.293	.123	.800	.200
PP	-.500-	.667	.258	.273	.400	.600
HbA1c	.018	.930	-.262-	.531	-.500-	.667

*$p \leq 0.05$ is considered statistically significant, * r= Spearman's rho*

Table (12) shows that there was significant positive correlation between *adiponectin* promotor methylation and Height in simple steatosis group(r= 0.900, P=0.037). There was significant positive correlation between *adiponectin* promotor methylation and body mass index and serum level of AST in control group(r=0.538, P=0.039) and (r= 0.694, P=0.018) respectively. There was significant positive correlation between *adiponectin* promotor methylation and body mass index, serum level of AST and prothrombin concentration in NASH group(r=0.015, P=0.016) (r=0.021, P=0.027) and (r=0.460, P=0.024) respectively. However there was non-significant correlation between *adiponectin* promotor methylation and other studied parameters in all studied groups (P> 0.05 in all).



Figure(31): Scatter plot showing positive correlation between Adipo Q and Height in simple steatosis group.

Figure (31) shows that there was significant positive correlation between *adiponectin* promotor methylation and height in simple steatosis group(r= 0.900, P=0.037).

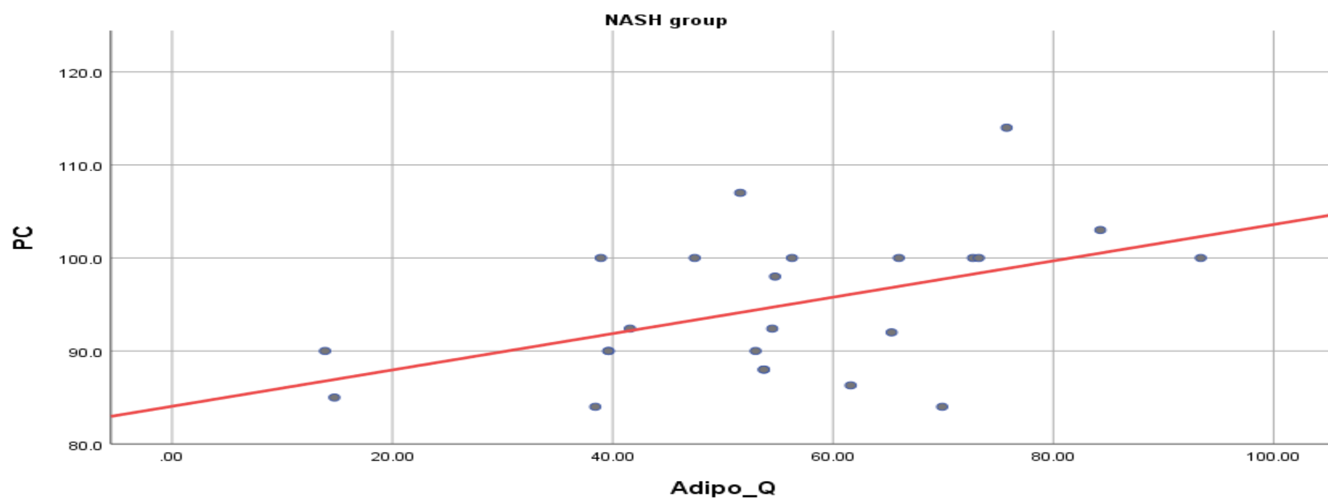


Figure (32): Scatter plot showing positive correlation between Adipo Q and PC in NASH group.

Figure (32) shows that there was significant positive correlation between *adiponectin* promotor methylation and prothrombin concentration in NASH group(r=0.460, P=0.024).

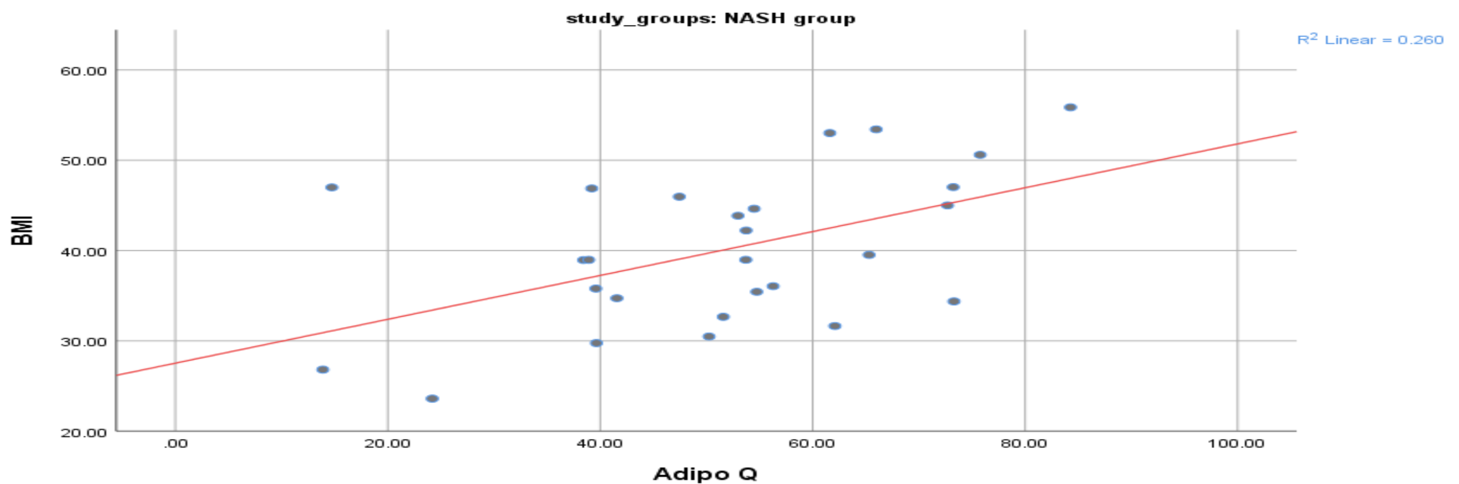


Figure (33): Scatter plot showing positive correlation between Adipo Q and BMI in NASH group

Figure (33) shows that there was significant positive correlation between *adiponectin* promotor methylation and body mass index in NASH group(r=0.015, P=0.016).

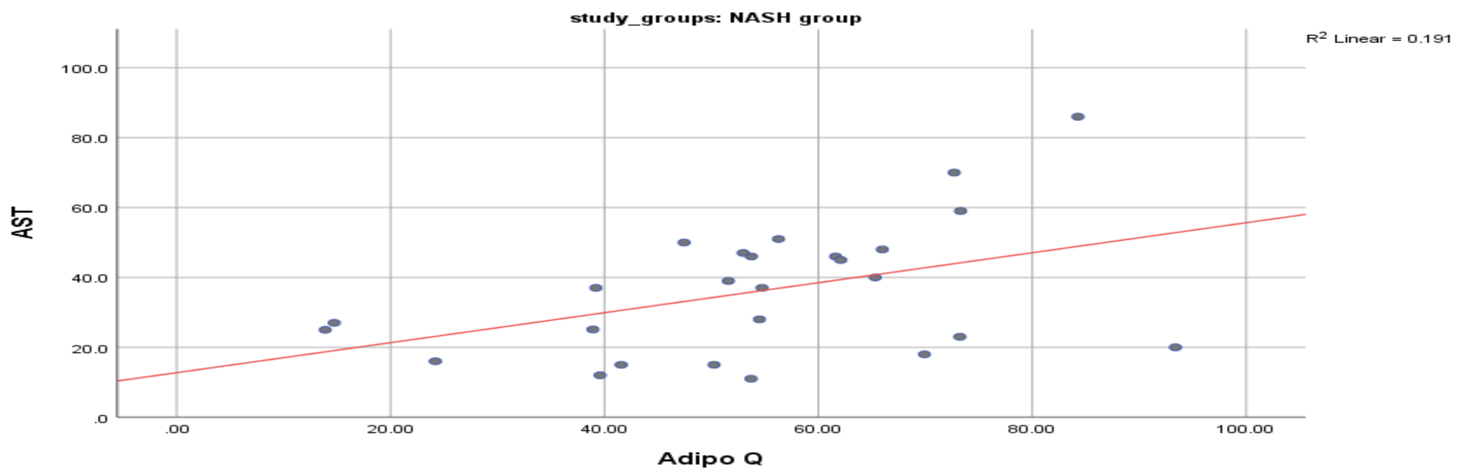


Figure (34): Scatter plot showing positive correlation between Adipo Q and AST in NASH group

Figure (34) shows that there was significant positive correlation between *adiponectin* promotor methylation and serum level of AST in NASH group(r=0.021, P=0.027).

Table (13): Relation between *adiponectin* promotor methylation and clinicodemographic parameters in simple steatosis group (n=5).

		MethCT			Test significance	p- value
		Mean	**SD**	**Median**		
Gender	**Male**	66.53	7.43	66.53	1.73	0.083
	Female	43.07	21.43	49.37		
Residence	**Rural**	61.28	.	61.28	0.707	0.480
	Urban	50.25	22.63	55.01		
Occupation	**Working**	19.19	0.0	19.19	1.41	0.157
	Not working	60.77	9.16	60.96		
Schistosomiasis	**No**	52.45	20.21	60.65	-	-
	Yes	-	-	-		
Smoking	**No**	57.10	6.70	60.65	0.0	1.00
	Yes	45.49	37.18	45.49		
History of DM	**No**	60.77	9.16	60.96	0.820	0.157
	Yes	19.19	.	19.19		
History of HTN	**No**	52.45	20.21	60.65	0.820	0.718
	Yes	57.10	6.70	60.65		
Pain	**No**	50.40	22.73	55.32	0.0	1.00
	Yes	60.65	.	60.65		
Fatigue	**No**	50.40	22.73	55.32	0.0	1.00
	Yes	60.65	.	60.65		
pruritus	**No**	52.45	20.21	60.65	-	-
Nausea	**No**	52.45	20.21	60.65	-	-

p≤0.05: significant, p≤0.01: high significant, SD= standard deviation, comparison between groups done by Student T test

Table (13) shows that there was non-significant relation between *adiponectin* promotor methylation and clinico-demographic parameters in simple steatosis group (P> 0.05 in all).

Table (14): Relation between *adiponectin* promotor methylation and liver biopsy findings in simple steatosis group (n=5).

		Adipo Q			Test of significance	p- value
		Mean	SD	Median		
Steatosis	**No grade**	66.53	7.43	66.53	1.73	0.083
	Mild grade	43.07	21.43	49.37		
	Moderate grade	-	-	-		
	Marked grade	-	-	-		
Ballooning	**No**	60.96	.44	60.96	2.0	0.368
	Mild	60.57	15.85	60.57		
	Moderate	-	-	-		
	Diffuse	19.19	0.0	19.19		
Inflammation	**Minimal**	50.25	22.63	55.01	0.707	0.800
	Mild	61.28	.	61.28		
	Moderate	-	-	-		
Fibrosis	**F1**	50.25	22.63	55.01	0.707	0.800
	F2	61.28	0.02	61.28		
	F3	-	-	-		
	F4	-	-	-		

p≤0.05: significant, p≤0.01: high significant, SD= standard deviation, comparison between groups done by Student T test

Table (14) shows that there was non-significant relation between *adiponectin* promotor methylation and Steatosis, Ballooning, Inflammation and Fibrosis in simple steatosis group (P> 0.05 in all).

Table (15): Relation between *adiponectin* promotor methylation and clinico-demographic parameters in NASH group (n=29).

		MethCT			Test significance	p- value
		Mean	**SD**	**Median**		
Gender	**Male**	66.53	7.43	66.53	1.73	0.083
	Female	43.07	21.43	49.37		
Residence	**Rural**	61.28	.	61.28	0.707	0.480
	Urban	50.25	22.63	55.01		
Occupation	**Working**	48.69	20.76	49.51	0.700	0.484
	Not working	55.25	18.70	54.48		
Schistosomiasis	**No**	52.45	20.21	60.65	-	-
	Yes	-	-	-		
Smoking	**No**	57.10	6.70	60.65	0.0	1.00
	Yes	45.49	37.18	45.49		
History of DM	**No**	60.77	9.16	60.96	0.820	0.157
	Yes	19.19	.	19.19		
History of HTN	**No**	52.45	20.21	60.65	0.820	0.718
	Yes	57.10	6.70	60.65		
Pain	**No**	50.40	22.73	55.32	0.0	1.00
	Yes	60.65	.	60.65		
Fatigue	**No**	50.40	22.73	55.32	0.0	1.00
	Yes	60.65	.	60.65		
pruritus	**No**	52.45	20.21	60.65	-	-
Nausea	**No**	52.45	20.21	60.65	-	-

p≤0.05: significant, p≤0.01: high significant, SD= standard deviation, comparison between groups done by Student T test

Table (15) shows that there was non-significant relation between *adiponectin* promotor methylation and clinico-demographic parameters in NASH group (P> 0.05 in all).

Table (16): Relation between *adiponectin* promotor methylation and liver biopsy findings in NASH group (n=29).

		Adipo Q			Test of significance	p- value
		Mean	SD	Median		
Steatosis	No grade	55.03	12.78	54.61	2.02	0.364
	Mild grade	57.43	21.64	57.91		
	Moderate grade	45.52	18.90	41.57		
	Marked grade	-	-	-		
Ballooning	No	62.79	10.18	62.09	4.52	0.211
	Mild	59.58	17.28	55.50		
	Moderate	46.37	10.86	40.60		
	Diffuse	47.66	25.89	45.39		
Inflammation	Minimal	38.42	0.0	38.42	1.32	0.276
	Mild	54.44	19.05	54.11		
	Moderate	-	-	-		
Fibrosis	F1	36.88	12.81	35.03	13.6	**0.003**
	F2	46.89	3.37	47.16		
	F3	70.01	16.34	75.77		
	F4	93.38	0.0	93.38		

p≤0.05: significant, p≤0.01: high significant, SD= standard deviation, comparison between groups done by Student T test

Table (16) shows that there was there was significant increase in *adiponectin* promotor methylation in NASH patients with higher fibrosis grades as compared to those with lower liver fibrosis grades(P=0.003).However there was non-significant relation between *adiponectin* promotor methylationin and Steatosis, Ballooning and Inflammation in NASH patients (P> 0.05 in all).

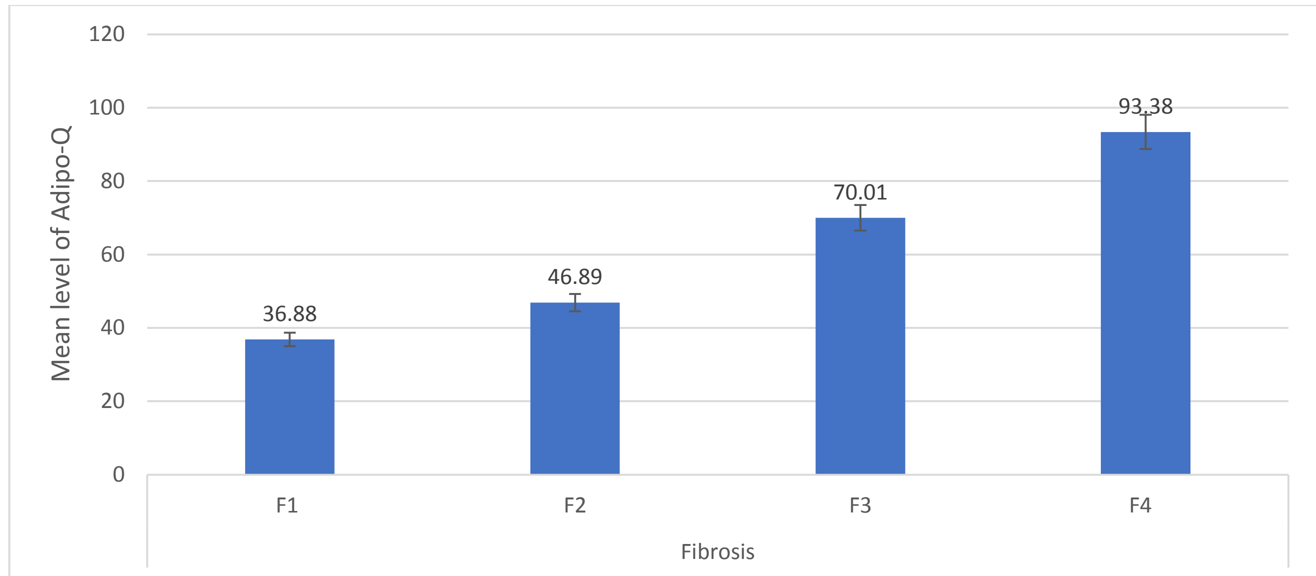


Figure (35): Relation between adiponectin promotor methylation and fibrosis grades in NASH group (n=29).

Figure (35) shows that there was there was significant increase in *adiponectin* promotor methylation in NASH patients with higher fibrosis grades as compared to those with lower liver fibrosis grades(P=0.003).

Table (17): Validity of *adiponectin* promotor methylation (AUC, Sensitivity, specificity) in detection of NAFLD

	AUC	Cut off Point	Sensitivity	Specificity	PPV	NPV	p- value
Adipo Q methylation	0.767	>53.46	55.9%	100%	100%	69.4%	**<0.001**

AUC: Area under Curve, PPV: Positive Predictive Value, NPV: Negative Predictive Value, ROC analysis

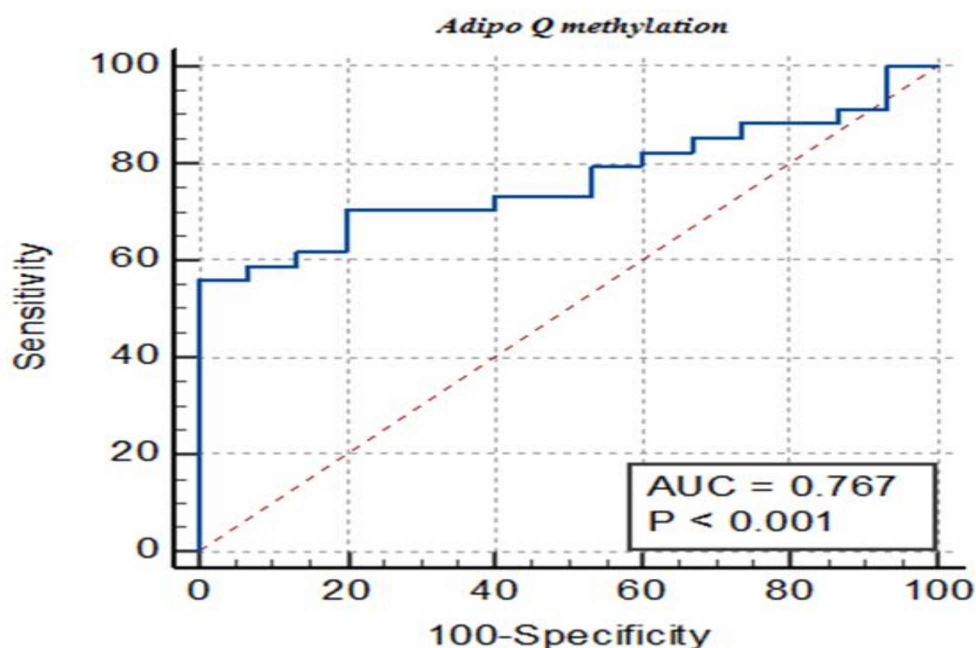


Figure (36): ROC curve of Adipo Q as a predictor between NAFLD patients and control group.

Table (17) and figure (36) show that AUC of *adiponectin* promotor methylation in detection of NAFLD was 0.767with Sensitivity 55.9%, Specificity was 100%, PPV was 100%, NPV was 69.4%, and Accuracy was 78.0%, with significant prediction (P<0.001).

DISSCUSSION

NAFLD is a metabolic syndrome characterized by hepatic fat accumulation in the absence of significant alcohol consumption ***(Zhou et al., 2018).*** It is a global health issue that affects over 25% of the world's population. The Middle East has the greatest prevalence rate of NAFLD (31.79%), while Africa has the lowest incidence rate (13.48 percent) ***(Hassan etal., 2020).*** NAFLD is defined by fat accumulation affects more than 5% of hepatocytes. It is a group of histological alterations that range from non-alcoholic fatty liver (NAFL) or simple steatosis to non-alcoholic steatohepatitis (NASH), which is marked by inflammation, hepatocyte degradation, and progressive fibrosis that leads to liver cirrhosis and hepatocellular cancer ***(Jukić et al., 2021)***.

Identification of new NAFLD biomarkers can improve prognosis due to early diagnosis of high risk patients and introduction of novel therapeutic strategies ***(Piazzolla and Mangia, 2020)***. Although Liver biopsy is the gold standard for diagnosing NAFLD, it is an invasive technique with possible risks and many complications. Hence, introducing novel noninvasive diagnostic methods is an important issue ***(Hasan et al., 2021).***

Adiponectin is an adipose tissue-derived hormone that regulates glucose and lipid metabolism to influence whole-body energy homeostasis. Adiponectin improves insulin sensitivity in metabolic tissues by boosting glucose consumption and fatty acid oxidation. Obesity and obesity-related metabolic disorders such as insulin resistance, type 2 diabetes, and cardiovascular disease are inversely linked with adiponectin blood levels ***(Dias et al., 2021).***

DNA methylation signatures and modifiers in NAFLD could be used to generate biomarkers for predicting the beginning and progression of NAFLD, as well as therapeutic agents for the disease ***(Hyun and Jung 2020).*** Previous study showed that DNA hypermethylation of the *adiponectin* promoter suppresses *adiponectin* expression ***(Kim et al., 2015).*** The role of *adiponectin* methylation has been also investigated in previous studies to show its relationship with pathogenesis of obesity ***(Houde et al., 2015)*** and gestational diabetes ***(Ott et al., 2018).***

The aim of this study was to investigate *adiponectin* promotor methylation status in patients with NAFLD and to evaluate the correlation between the *adiponectin* promotor methylation status and the clinicopathological characteristics of NAFLD patients.

In the present study, there was a non-significant difference regarding age and gender in either simple steatosis or NASH group as compared to control group. These results were opposed by ***Lonardo et al., 2015,*** who reported that NAFLD is more common in men. They also added that NAFLD prevalence has been proven to increase in young to middle-aged individuals, with a drop after the age of 50-60. This discrepancy may be attributed to different patient selection criteria as the control subjects of the current study were selected to match patients regarding age and gender distribution.

Though the difference was statistically nonsignificant, the comparison between simple steatosis and NASH groups regarding age revealed that it was higher in patients with simple steatosis. ***In 2020, Sarkar and colleagues*** found that about 22% of patients with NAFLD develop non-alcoholic steatohepatitis. Such finding is illustrated by ***NIH, 2020*** which reported that simple steatosis does not

usually progress into NASH or liver cirrhosis. So, patients with simple steatosis can reach older age without developing steatohepatitis, especially in case of absence of other risk factors.

It is also of note, that complaint of right hypochondrial pain was significantly higher among NASH cases compared to those with simple steatosis. This finding is in accordance with that reported by ***HealthwiseStaff in 2020.*** Such aching pain in the upper right area of the abdomen may be explained by aggravation of inflammation, fibrosis and liver damage as NASH progresses.

Importantly, comparison of liver fibrosis between NASH patients and simple steatosis cases revealed that the fibrosis grade was significantly higher in NASH group. This finding is supported by ***Zhou et al., 2018*** who reported that NAFLD comprises a wide variety of disorders, from mild uncomplicated steatosis to severe NASH. Simple steatosis is widely thought to be a benign condition, whereas progression towards NASH carries more risk for increasing fibrosis, liver cirrhosis and hepatocellular carcinoma. Increased hepatocyte damage, inflammation, and varying degrees of fibrosis characterize the pathophysiology of NASH.

The present results showed that the methylation status of *adiponectin* promotor was significantly higher (P=0.014) in NASH patients as compared to controls. This finding is supported by an earlier study reporting that liver tissue showed a significantly increased rate of *adiponectin* promoter methylation in NAFLD rats as compared to controls (75.0% vs. 28.3%, P < 0.05) ***(CHEN et al., 2016)***. They also suggested that the increased methylation of *adiponectin* promoter in the NAFLD group distorted the expression of *adiponectin* in the liver tissues resulting in gene dysfunction. In ***2015, Kim et al.*** showed that epigenetic regulation suppressed *adiponectin* expression through promotor hypermethylation that

interferes with binding of a variety of factors at specific transcription binding sites. In turn, decreased circulatory adiponectin led to biochemical abnormalities and exacerbation of metabolic diseases in obesity cases. Recently, ***Mohamed et al., 2021*** reported a highly significant assosciation between low adiponectin level and liver fibrosis. This is due to adiponectin's hepatoprotective and antifibrogenic properties in liver damage. It possesses anti-inflammatory and antifibrotic properties by neutralizing TNF-α and inhibiting HSC proliferation and migration respectively.

The current results showed that there was significant positive correlation between *adiponectin* promotor methylation and height in simple steatosis group. That positive correlation may be attributed to a very small sample size of simple steatosis group.

Additionally, the current results showed that there was significant positive correlation between *adiponectin* promotor methylation and body mass index, serum level of AST and prothrombin concentration in NASH group. These results are in parallel with those of ***Tahergorabi et al., 2016*** who found that plasma adiponectin level is inversely related to body mass index. Furthermore, ***Houde et al., 2015*** showed that *adiponectin* DNA methylation levels in subcutaneous adipose tissue are associated with obesity-related anthropometric measures. In addition, a recent study reported that serum adiponectin concentration had a significant inverse correlation with serum liver enzymes in NAFLD cases ***(Gunjal et al., 2020)***. Adiponectin maintains liver integrity and prevents inflammation and fibrosis by enhancing fat degeneration in hepatocytes ***(Hasan et al., 2021)***. Inversely, decreased circulating adiponectin promotes excess lipid accumulation with subsequent hepatocellular damage and release of intracellular enzymes ***(Gunjal et al., 2020)***. The aforementioned findings indicate that

adiponectin promoter methylation may modulate liver function and pathology in NAFLD cases through regulation of adiponectin gene expression. The positive correlation regarding height may be attributed to very small sample size of simple steatosis group.

interestingly, there was significant inc5.rease in *adiponectin* promotor methylation in NASH patients with higher fibrosis grades as compared to those with lower liver fibrosis grades(P=0.003). This finding is supported by ***Adolphet al., 2017***, who reported that adiponectin decreases pro-inflammatory cytokines produced by Kupffer cells and hepatic stellate cells, preventing their transformation into myofibroblasts and liver fibrosis could be decreased.

The methylation status of particular CpGs in DNA may be useful in predicting the progression of NAFLD to NASH fibrosis ***(Gautam, 2018)***. ROC curve analysis of the current study showed that *adiponectin* promotor methylation might be used for prediction of NAFLD (AUC was 0.767with Sensitivity 55.9%, Specificity was 100%, PPV was 100%, NPV was 69.4%, and Accuracy was 78.0%, with significant prediction P<0.001). Recently ***Marques and colleagues***, 2021, showed that serum adiponectin may be used to distinguish NASH from simple steatosis patients with good assay performance (AUC = 0.87, sensitivity = 100%, specificity = 63%, PPV = 63% and NPV=100%, accuracy of 77%, p < 0.0001). The current result of *adiponectin* promotor methylation regarding Roc curve analyais is supported by that of ***Marques et al., 2021*** because of the great impact of *adiponectin* promotor hypermethylation on decreasing *adiponectin* gene transcription and expression

The present study demonstrated that altering the DNA methylation of adiponectin promotor is an important mechanism for NAFLD development and progression.

CONCLUSION

It could be concluded that alteration of methylation status of *adiponectin* promotor seems to play a mechanistic role in pathogenesis of NAFLD and progression of liver fibrosis in NAFLD patients. It may be a promising marker for diagnostic and predictive application.

RECOMMENDATIONS

From the result of the present study we can recommend the following:

- Further studies using wider scale with large study sample size are needed.
- Further studies targeting use of *adiponectin* methylation in prognosis of severe liver fibrosis of NAFLD cases.
- Studying potential application of *adiponectin* methylation in treatment of NAFLD and NASH.

SUMMARY

Nonalcoholic fatty liver disease (NAFLD) is a condition that causes fatty hepatic parenchymal cell degeneration without a history of alcohol consumption. The pathogenic variations of NAFLD include simple steatosis, nonalcoholic steatohepatitis (NASH), and NASH-related cirrhosis, which may progress to hepatocellular carcinoma (HCC).

The introduction of reliable non or minimal invasive accurate biomarkers is urgently needed to avoid the hazardous complications of invasive liver biopsy technique.

Adiponectin has been shown to reduce hepatic and systemic insulin resistance, as well as liver inflammation and fibrosis. Adiponectin is a hormone that predicts the degree of steatosis and NAFLD. Despite the lack of a proven pharmacotherapy for NAFLD, current therapeutic efforts have focused on the indirect upregulation of *adiponectin* via the administration of various therapeutic agents and/or lifestyle changes.

The aim of this study was to investigate *adiponectin* promotor methylation status in patients with NAFLD and to evaluate the correlation between the *adiponectin* promotor methylation status and the clinicopathological characteristics of NAFLD patients

The study included 49 subjects of both sex selected from Department of Endemic Medicine, Faculty of Medicine, Cairo University Hospital.

The subjects were categorized into 3 groups:

- **simple steatosis group**: included 5 patients, diagnosed by clinical, radiological and histopathological examinations (NAS score < 4)

- **NASH group**: included 29 patients, diagnosed as NAFLD patients by clinical, radiological and histopathological examinations(NAS score ≥ 4)
- **Control group**: included 15 apparently healthy subjects, age and sex matched, with apparently normal liver.

All individuals were subjected to:

- Full history taking.
- General and local abdominal examinations.
- Investigations include:
    - Routine Laboratory investigations
    - Radiological investigations
- Liver biopsy for histopathology (from patients' group).
- Molecular biology investigations: SYBR Green methylation specific polymerase chain reaction (qMSP) for detection of *adiponectin* promotor methylation status.

Blood samples were collected into EDTA vacutainers from all individuals and stored at -80°C. *Adiponectin* promotor qMSP assay was performed as follows:

- Extraction of genomic DNA from peripheral blood samples.
- Bisulfite treatment of genomic DNA.
- SYBR Green Methylation specific PCR (qMSP) using specific primer sets for either methylated or non-methylated products of *adiponectin* gene promoter. The PCR product was separated by gel electrophoresis, stained with ethidium bromide and visualized by UV irradiation, for detection of specific bands. *Adiponectin*

methylation percentage was calculated according to the following equation:

$$diponectin \text{ methylation percentage} = \frac{1}{1 + 2^{(-\Delta Ct)}} \times 100\%$$

While ΔCt = adiponectin Ct – GAPDH Ct

The current study showed that patients with NASH had significantly higher results when compared to simple steatosis regarding, pain as clinical symptom and body mass index.

Importantly, there was a significant increase in *adiponectin* promotor methylation in NASH patients with higher fibrosis grades as compared to those with lower liver fibrosis grades

There was significant positive correlation between *adiponectin* promotor methylation and height, body mass index, serum level of AST and prothrombin concentration in NASH patients.

ROC curve analysis showed that AUC of *adiponectin* promotor methylation in detection of NAFLD was 0.767with Sensitivity 55.9%, Specificity was 100%, PPV was 100%, NPV was 69.4%, and Accuracy was 78.0%, with significant prediction ($P<0.001$).

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اوضح منحنى ROC أن استخدام حالة مثيلة منظم جين الأديبونكتين في توقع الاصابة بالكبد الدهني اللاكحولي اظهرت حساسية ٥٥.٩٪ ، و خصوصية ١٠٠٪ ، ودقة ٧٨.٠٪ مما يدل علي امكانية استخدامها كدلالة تشخيصية غير مخترقة لحالات الكبد الدهني اللاكحولي.

١- مجموعة الكبد الدهني البسيط: شملت ٥ مرضى ، تم تشخيصهم عن طريق الفحوصات السريرية والإشعاعية والنسيجية(NAS score < 4)

٢- مجموعة التهاب الكبد الدهني: شملت ٢٩ مريضًا ، تم تشخيصهم عن طريق الفحوصات السريرية والإشعاعية والنسيجية ($NAS\ score \geq 4$)

٢- المجموعة الضابطة: شملت ١٥ شخصًا من المتطوعين الأصحاء متقاربين فى السن والجنس مع مجموعة المرضى.

خضع جميع المرضى للتاريخ الكامل مع الانتباه إلى: العادات الخاصة بما في ذلك السمنة والحياة الخالية من الانشطة والعديد من التحاليل والاشعة المختلفة مع اخذ خزعة الكبد التشخيصية لعلم الأنسجة مع استخدام تفاعل البلمرة المتسلسل الخاص بالميثلة للكشف عن حالة المثيلة لمنظم جين الأديبونكتين.

تم جمع عينات الدم في انابيب تحتوي علي مضاد للتجلط (EDTA) من جميع الأفراد وتخزينها في -٨٠ درجة مئوية, تم استخدام هذه العينات للكشف عن حالة الميثيلة لجين الاديبونكتن بواسطة تفاعل البلمرة المتسلسل الخاص بالميثلة والهلام الكهربائي المصبوغ بمادة بروميد الإيثيديوم والذي تم تصويره بواسطة الأشعة فوق البنفسجية ، للكشف عن عصابات معينة وتم حساب نسبة ميثلة منظم جين الاديبونكتن طبقا لهدذه المعادلة

نسبة مثيلة الديبونكتين= $\frac{1}{1+2^{(-\Delta Ct)}} \times 100\%$

النتائج:

الأهم من ذلك ، كان هناك زيادة كبيرة في ميثلة منظم جين الأديبونكتين في مرضي التهاب الكبد الدهني الذين يعانون من درجات تليف كبدي أعلي مقارنة مع أولئك الذين يعانون من درجات تليف اقل في المجموعة المصابة بالتهاب الكبد الدهني اللاكحولي.

وأيضا كان هناك ارتباط إيجابي ذو دلالة احصائية بين ميثلة منظم جين الأديبونكتين والطول ، ومؤشر كتلة الجسم ، ومستوى AST وتركيز البروثرومبين في الدم في المجموعة المصابة بالتهاب الكبد الدهني اللاكحولي.

الملخص العربي

مقدمة البحث

مرض الكبد الدهني اللاكحولي هو حالة تسبب تنكس الخلايا الكبدية بسبب تراكم الدهون دون تاريخ من الإفراط في تناول الكحول. وقد يمر هذا المرض باربع مراحل باثولوجية تبدأ بالكبد الدهني البسيط ثم التهاب الكبد الدهني ثم تليف الكبد الدهني والذي يمكن أن يتطور إلى سرطان الخلايا الكبدية.

على الرغم من أن العينة الكبدية هي المعيار الذهبي لتشخيص هذا المرض وتوقع نتائجه ، إلا أنها تعرض المريض لكثير من المضاعفات لذلك توجد حاجة ماسة إلى اكتشاف مؤشرات حيوية موثوقة ودقيقة وغير وغزية أو طفيفة التوغل.

لقد ثبت أن الاديبونكتن يقلل من مقاومة الأنسولين في الكبد وفي اجهزة الجسم المختلفة ، وكذلك يقلل التهاب الكبد وتليفه. كذلك فإن هذا الهرمون يتنبأ بدرجة التنكس الدهني و ودرجة الكبد الدهني اللاكحولي. ونظرا لعدم وجود علاج دوائي مثبت لهذا المرض ، فقد ركزت الجهود العلاجية الحالية على التنظيم غير المباشر للأديبونكتين عن طريق إدارة العديد من العوامل العلاجية و / أو تغيير نمط الحياة.

الهدف من البحث

كان الهدف من هذه الدراسة هو:

- استكشاف حالة ميثلة منظم جين الاديبونكتن في مرض الكبد الدهني اللاكحولي.
- تقييم العلاقة بين حالة ميثلة منظم جين الاديبونكتن والخصائص الإكلينيكية لمرضى الكبد الدهني اللاكحولي.

مواد وطرق البحث

اشتملت الدراسة على ٤٩ شخصا من كلا الجنسين تم اختيارهم من قسم الطب المتوطن بكلية الطب بمستشفى جامعة القاهرة.

تم تصنيف الأشخاص إلى ثلاث مجموعات:

دراسة حالة ميثلة منظم جين الاديبونكتن في مرضى الكبد الدهني اللاكحولي

توطئة للحصول على درجة الدكتوراة

فى الكيمياء الحيوية الطبية والبيولوجيا الجزيئية

والمقدمة من

الطبيبة/ نعمة السيد عبد المقصود عبد السميع

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