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Nesfatin-1 Levels, Nucleobindin-2 Genetic Variants with their Roles in Pathogenesis of Obese Type 2 Diabetic Iraqi Women

A thesis

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By

Israa Khalil Ibrahim Al-Yassiri

B.Sc. in Department of pathological analyses- Al Safwa University College (2017)

Supervised By

Professor
Dr. Fadhil Jawad Al-Tu'ma

Assist. prof.
Dr. Maher Abbood Mukheef

Department of Chemistry and Biochemistry
College of Medicine-University of Kerbala

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﴿وَيَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُم مِّن الْعِلْمِ إِلاَّ قَلِيلاً﴾

صدق الله العلي العظيم

(سورة الاسراء - الآية ٨٥)

Dedication

To

The soul of my father

And to

My mother

My Husband

My family

My supervisors

The patients

All my friends

The soul of the late Assist. Prof. Dr. Shaymaa Zahraw Neda

Israa

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Israa

Supervisors Certification

We certify that this M.Sc. thesis titled: -

(Nesfatin-1 Levels, Nucleobindin-2 Genetic Variants with their Roles in Pathogenesis of Obese Type 2 Diabetic Iraqi Women)

was prepared by (**Esraa Khalil Ibrahim Al-Yassiri**) under our supervision at the College of Medicine, University of Kerbala, as a partial fulfillment of the requirement for the Degree of Master in **(Clinical Chemistry).**

Professor
Dr. Fadhil Jawad Al-Tu'ma

Assist. Prof.
Dr. Maher Abbood Mukheef

Department of Chemistry and Biochemistry
College of Medicine – University of Kerbala

In the view of the available recommendation, I forward this M.Sc. thesis for debate by the examining committee .

Assist. Prof. Dr. Atheer Hameed Al-Ghanmi
Head of Department of Chemistry and Biochemistry
College of Medicine / University of Kerbala

Examining Committee Certification

We, the examining committee, certify that we have read this M.Sc. thesis entitled: -

(Nesfatin-1 Levels, Nucleobindin-2 Genetic Variants with their Roles in Pathogenesis of Obese Type 2 Diabetic Iraqi Women)

We have examined the postgraduate student **(Israa Khalil Ibrahim Al-Yassiri)** in the content of this thesis and, in our opinion; it meets the standard for the degree of master in **(Clinical Chemistry)** and it is adequate with **(Excellent)** degree.

Signature:

Prof. Dr. Rana Fadhil Mousa
College of Veterinary Medicine
University of Kerbala
Date: 18 / 6 / 2023

(Member)

Signature:

Assist. Prof. Dr. Suzanne Jubair Abbas
College of Pharmacy
University of Kerbala
Date: 18 / 6 / 2023

(Member)

Signature:

Prof. Dr. Fadhil Jawad Al-Tu'ma
College of Medicine
University of Kerbala
Date: 19 / 6 / 2023

(Member / Supervisor)

Signature:

Assist. Prof. Dr. Maher Abbood Mukheef
College of Medicine
University of Kerbala
Date: 19 / 6 / 2023

(Member / Supervisor)

Signature:

Prof. Dr. Ferdous Abbas Jabir Alturaihy
College of Medicine - University of Al-Qadisiyah
Date: 20 / 6 / 2023
(Chairman)

Approved by the College of Medicine - University of Kerbala

Signature:

Date: 20 / 6 / 2023
Prof. Dr. Riyadh Dayhood Al-Zubaidi
Dean of the College of Medicine

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List of Abbreviations

Abbreviation	Complete Names
AB	Anti-Body
ARMS	Amplification-Refractory Mutation System
ASPCR	Allele-Specific Polymerase Chain Reaction
AT	Adipose Tissue
β-Cell	Beta-Cell
BMI	Body Mass Index
bp	Base Pear
C	Cytosine
CHD	Coronary Heart Disease
Chol.E	Cholesterol Esterase
Chol.Oxi	Cholesterol Oxidase
Chol	Cholesterol
CNS	Central Nervous System
CVD	Cardio Vascular Disease
DDH_2O	Deionized Distilled Water
DKD	Diabetic Kidney Disease
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleoside Triphosphates
EDTA	Ethylene Diamine Tetra Acetic Acid
Elisa	Enzyme Linked Immunosorbent Assay
FPG	Fasting Plasma Glucose
G	Guanine
GAD	Glutamic Acid Decarboxylase
GDM	Gestational Diabetes Mellitus
GOD	Glucose Oxidase
GWAS	Genome Wide Association Studies
HbA1c	Glycated Hemoglobin
HDL	High Density Lipoprotein
HOMA-IR	Homeostasis Model Assessment-Insulin Resistance
HRP	Horseradish Peroxidase
ICV	Intracerebroventricular
IDDM	Insulin-Dependent Diabetes Mellitus

IDF	International Diabetes Federation
IGT	Impaired Glucose Tolerance
IR	Insulin Resistance
LADA	Latent Autoimmune Diabetes In Adults
LDL	Low Density Lipoprotein
LPL	Lipoprotein Lipase
METS	Metabolic Equivalents
MODY	Maturity Onset Diabetes Of The Young
mRNA	Messenger Ribose Nucleotide Acid
NAFLD	Non-Alcoholic Fatty Liver Disease
NCBI	National Centre For Biotechnology Information
NCDs	Non-Communicable Diseases
NES1	Nesfatin-1
nT2DM	Newly Diagnosed Type 2 Diabetes Mellitus
NUCB2	Nuclubendin-2
OD	Optical Density
OGTT	Octional Glucose Tolerance Test
OR	Odd Ratio
PASA	PCR Amplification Of Specific Alleles
PC	Prohormone Convertase Enzymes
PCR	Polymerase Chain Reaction
PEPCK	Phosphoenolpyruvate Carboxykinase
PERM	Progressive Encephalomyelitis With Rigidity And Myoclonus
POD	Peroxidase
POMC	Proopiomelanocortin
PPARY	Peroxisome Proliferators' Activator Receptor Y
PVN	Paraventricular Nucleus
RBS	Random Blood Sugar
ROC	Receiver Operating Curve
ROS	Reactive Oxygen Species
rpm	Rotation Per Minute
S	Standard
SD	Standard Deviation
SNPs	Single Nucleotide Polymorphisms
SPS	Stiff Person Syndrome
T1DM	Type One Diabetes Mellitus

T2DM	Type Two Diabetes Mellitus
TBE	Tris Borate Edta
TC	Total Cholesterol
TG	Triglyceride
TMB	Tetramethylbenzidine
TTAB	Tetradecyl Tri Methyl Ammonium Bromide
UV	Ultra Violate
VLDL	Very Low Density Lipoprotein
vWAT	Visceral White Adipose Tissue
W.H.R	Waist-To-Hip Ratio
WAT	White Adipose Tissue

Summary

Type 2 diabetes mellitus is one of metabolic disorders; it is caused by a combination of two basic factors: insufficient insulin secretion by pancreatic β-cells or a failure of insulin-sensitive tissues to respond adequately to insulin. Nesfatin-1 is secreted from the hypothalamic nuclei, it is responsible for controlling appetite and affects many functions such as regulatory effects on energy metabolism through suppression of food intake. In addition, it regulates cardiac functions, decreases blood glucose levels, acts as a neuroendocrine regulator, and causes weight loss along with reduction in energy intake. Nesfatin-1, originating from its precursor protein called nucleobindin- 2 (NUCB2), plays an important role in glucose metabolism and diabetes. This study explored the correlation between NUCB2 genetic variants and type 2 diabetes mellitus (T2DM). The aim of the presented word was to assess the diagnostic accuracy of serum Nesfatin-1 in type 2 diabetes mellitus and its relationship with C-peptide level in obese and non-obese type-2 diabetic women of Iraqi population.

A case-control study was performed on 50 type 2 diabetic patients admitted in Al-Hussein Teaching Hospital and Al-Hassan center of diabetes and endocrinology unit / Kerbala health directorate – Iraq and another 50 control individuals, during the period from April, 2022 – Jan. 2023). The T2DM groups were divided into two groups 25 obese and 25 non-obese; also the control group was divided into 25 obese and 25 non-obese as apparently healthy groups. The ELISA Kit was used to measure serum Nesfatin-1 and C-peptide, and random serum glucose was measured by enzymatic colorimetric method, and lipid profile test were measured through spectrophotometric technique, while HbA1c% was determined using HPLC method.

The results observed indicated that Nesfatin-1 levels show a non-significant decrease in all of type 2 diabetic groups as compared with apparently healthy control group, while the C-peptide was significantly decreased in type 2 diabetic patients when compared with apparently control group. In addition, the random blood glucose

and HbA1c% show significant elevation in type 2 diabetic patients as compared with apparently healthy control groups. The observed data indicated that Nesfatin-1 and C-peptide levels when comparing between type 2 diabetic patients and control in obese groups shown a risk factors depending upon the odd ratio observed (OR = 1.064 (1.011-1.119), 1.0200 (0.992-1.08)) respectively, but only Nesfatin-1 was shown to be significant. According to BMI the levels of Nesfatin-1 and C-peptide, as shown the Nesfatin-1 was significant in obese groups, while the C-peptide as shown significant in normal weight groups. The optimal diagnostic points for Nesfatin-1 were (sensitivity = 98%, specificity = 90%) at a level (Cut-off points) = 39.13, while C-Peptide levels: (sensitivity = 98%, specificity = 94%) at a level (Cut-off points) = 15.99. Both markers have p-values of the AUC were <0.001 and statistically significant. And the result of genotype, the results observed indicated that Nesfatin-1 levels shown a significant in all of type 2 diabetic groups in polymerase gene (rs757081), while the C-peptide and lipid profile, RBS, HbA1C and W.H.R were non-significantly in type 2 diabetic patients in polymerases gene (rs757081). In addition, when compared Nesfatin.1 with polymerase gene in patients showed increased frequencies of CG + GG genotypes in (rs757081) for CG (OR = 1.119 (1.002-1.248), p = 0.045; GG (OR = 1.102 (0.992-1.223), p = 0.05, respectively). Just significantly differences in Nasfatine-1

Accordingly, it was concluded that a significant relationship between circulating Nesfatin-1 levels and type 2 diabetes. Nesfatin-1 appears to be able to contribute to the treatment of obesity and diabetes because of its anorexigenic and antihyperglycemic effects. In addition, C-peptide is a known biomarker of insulin resistance and beta-cell function. High specificity and sensitivity analyzed results were obtained by ROC analysis for both markers in T2DM.

Chapter One

Introduction
&
literature review

1. Introduction

Diabetes mellitus is a condition of chronic hyperglycemia that causes physical damage, physiologic dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, blood vessels, and the brain. The specific glucose level definition of diabetes is an 8-hour post fasting blood glucose >126 mg/dL or a 2-hour postprandial blood glucose >200 mg/dl **(Hansen *et al.,* 2020)**. Modern sedentary lifestyle and excessive calorie intake have increased the chance of developing metabolic diseases such as obesity and type 2 diabetes mellitus T2DM **(Hamilton *et al.,* 2014; Boden *et al.,* 2015)**. Obesity is defined as an excessive amount of body fat. Overweight and obese individuals are defined by measures of weight and height that provide an index of one's mass, referred to as a body mass index (BMI). A BMI of 30 kg/m^2 or greater is the definition of obesity **(Tan *et al.,* 2000).** Excessive weight, until recently associated mostly with T2DM, is also more and more commonly described in the context of type one diabetes T1DM **(DuBose *et al.,* 2015).**

Obesity is the most important risk factor for T2DM and has been postulated to be the major contributor to the current epidemics of T2DM. Moreover; the presence of obesity among type 2 diabetes patients increases the risk of mortality from cardiovascular disease (CVD) **(Han *et al.,* 2018)**. Insulin resistance is commonly linked with obesity; which is a pathophysiologic factor of type 2 diabetes mellitus T2DM **(Khalil *et al.,* 2019)**. Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease characterized by hyperglycemia secondary to insulin resistance and pancreatic β-cell failure **(Di Masi *et al.,* 2015).** In addition, increased expression of mRNA and protein contents of nesfatin-1 and nucleobindin- 2 (NUCB2) have been shown in muscle and adipose tissues of patients with type 2 diabetes mellitus **(Guo *et al.,* 2013)**.

Diabetes is characterized by the chronic hyperglycemia-induced triad of symptoms (polydipsia, polyuria, and polyphagia) caused by elevated blood glucose level and metabolic dysregulation. Untreated diabetes leads to multi organ and systemic injury, including to the heart, kidneys, nerves, and blood vessels, which impair the quality of life and increase the death rate caused by diabetes complications **(Z. Liu *et al.*, 2010).** Some of these complications play a well-defined role in increasing the mortality of people with diabetes. For instance, people with diabetes have a twofold increased risk for cardiovascular mortality **(Hirakawa *et al.*, 2017).** Several studies have demonstrated that in patients with type 2 diabetes and obesity, more extreme dietary energy restriction with very low-calorie diets can reduce A1C to <6.5% (48 mmol/mol) and fasting glucose to <126 mg/dL (7.0mmol/L) in the absence of pharmacologic therapy or ongoing procedures **(Jackness *et al.*, 2013) and (Steven *et al.*, 2016)**.

1.1. Diabetes Mellitus

Diabetes mellitus (DM) is a metabolic disorder where in human body does not produce or properly uses insulin, a hormone that is required to convert sugar, starches and other food into energy. Absence or reduced insulin in turn leads to persistent abnormally high blood sugar and glucose in tolerance. It is probably an oldest disease known to man **(Deepthi *et al.*, 2017)**.It is linked with developing of various serious diseases like micro vascular (nephropathy , retinopathy, neuropathy) and macro vascular (peripheral vascular disease and coronary heart diseases) **(Rao *et al.*, 2010)**. The epidemic of diabetes mellitus and its complications poses a major global health threat. The International Diabetes Federation (IDF) estimated that 1 in 11 adults aged 20–79 years (415 million adults) had diabetes mellitus globally in 2015. This estimate is projected to rise to 642 million by 2040, and the largest increases will come from the regions experiencing economy transitions from low-income to middle-income

levels **(Atlas, 2015)**. Diabetes and its complications are complex, multifactorial conditions with both major environmental and genetic components. When early studies identified differences in diabetic complication susceptibility in patients who seemed otherwise equal with regard to their diabetes glucose control, clinical features and management **(A. D. A. J. D. care, 2018).** Two common forms of diabetes are type 1, which occurs when endogenous insulin production stops, and type 2 where insulin production continues but the tissue response to insulin is reduced. T2DM is commonly associated with overweight and obese individuals. Thus, the belief that obesity causes T2DM. The basic mechanism for obesity is excess glucose and insulin **(Herman *et al.*, 2017).**

Diabetes Mellitus is one of the four major non-communicable diseases (NCDs) comprising - cardiovascular diseases, cancers and chronic respiratory diseases jointly contributing to 63% of NCD deaths worldwide **(Lancet, 2015)**. If the current diabetes trends continue unchanged, both the number of people living with diabetes and the deaths from diabetes are expected to increase. Low income countries are expected to experience the highest increase in diabetes prevalence (92%) followed by lower-middle income countries (57%), upper- middle income countries (46%) and higher income countries (25%) **(Hazaveh *et al.*, 2018).**

Successful treatment of diabetes is a complex and challenging task requiring multiple contemporaneous strategies including lifestyle optimization, adequate antidiabetic medication and individual education. Weight loss and physical exercise represent the fundamental basis of obesity prevention, and its regular implementation is recommended in every international treatment guideline for patients with diabetes **(Chester *et al.*, 2018)**. Finally, more recent studies suggest that high birthweight is also associated with increased risk of obesity and type 2 diabetes **(Harder *et al.*, 2007).**

1.1.1. Epidemiology of Diabetes Mellitus

Type 1 diabetes is the most common type of the diabetes in people with lower age groups. The prevalence of type 1 diabetes is increasing in both prosperous and poverty countries. 85%-95% of type 2 diabetes is predominant in developing countries **(Reuveni *et al.*, 2016)**. Type 2 diabetes (T2D) is a major, rapidly increasing global public health challenge. The number of adults with diabetes is estimated to have increased from 108 to 422 million between 1980 and 2014 making it one of the fastest growing diseases worldwide **(Rawshani *et al.*, 2017)**. The corresponding age-standardized diabetes prevalence increased from 4.3% to 9.0% in men and from 5.0% to 7.9% in women between1980 and 2014, respectively. It has been estimated that if current trends continue, there will be over 700 million people with diabetes before 2030. This increase is due to an increasing prevalence of the disease, an increasing global population size as well as longer life expectancy **(Rawshani *et al.*, 2018).**

1.1.2. General Symptoms of Diabetes Mellitus

Symptoms of diabetes include the following:

- Increased thirst and urination
- Increased hunger, Fatigue
- Blurred vision
- Numbness or tingling in the feet or hands
- Sores that do not heal
- Unexplained weight loss
- Frequent infections, such as gums or skin infections and vaginal infections
- Male sexual dysfunction **(Ozougwu *et al.*, 2013).**

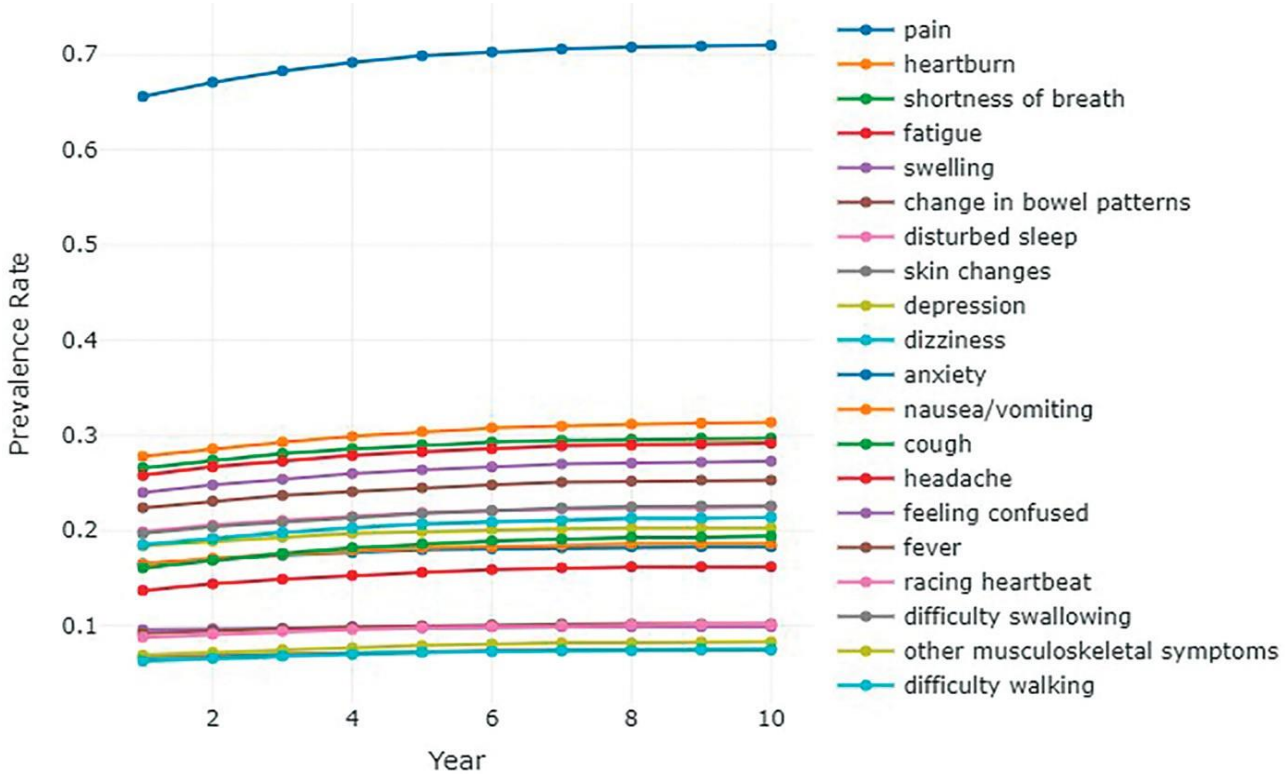


Fig. 1-1: Temporal trajectory of prevalence rates for top 20 most frequent symptoms(Brady *et al.*, 2022).

1.1.2.1. Symptoms of Type 2 Diabetes Mellitus

People with type 2 diabetes experience multiple symptoms, including symptoms of hyperglycemia such as change in appetite and polyuria, symptoms of hypoglycemia such as dizziness, fatigue, pain, sleep disturbance, sensory symptoms, cognitive impairments, depression, anxiety, and changes in vision as a result of their disease or its treatments**(Lin *et al.*, 2019),(K. Harding *et al.*, 2019)**. Clinical and pre- clinical complications of diabetes occur in up to 50% of people newly diagnosed with type 2 diabetes and may manifest with a unique set of symptoms **(Bonora *et al.*, 2022).** The temporal trajectory of prevalence rates of type 2 diabetes symptoms slowly increased after type 2 diabetes onset**(Gregg *et al.*, 2014).** shown in figure 1.1. The most common

co-occurring symptoms were pain and heartburn, pain and swelling, and pain and shortness of breath **(DeVon *et al.*, 2017) .**

1.1.3. Causes of Diabetes

General causes of diabetes mellitus include:

a. Obesity
b. Excess glucocorticoids
c. Excess growth hormone
d. Mutation of insulin receptor
e. Lipodystrophy **(Ozougwu *et al.*, 2013).**

While causes of type 1 diabetes include:

a. Genetic susceptibility to developing type 1 diabetes
b. Certain viruses (e.g. German measles or mumps).
c. Environmental factors.

Type 2 diabetes is the most common form of diabetes mellitus and is highly associated with a family history of diabetes, older age, obesity and lack of exercise **(Baynes, 2015)**. Type 2 diabetes mellitus (T2DM) is considered a multifactorial disease, promoted by both genetic and environmental factors, which is characterized by chronic hyperglycemia and insulin resistance (IR) **(DeFronzo *et al.*, 2015)**.

1.1.4. Diagnosis of Diabetes Mellitus

Diabetes is defined as a state of hyperglycemia in either fasting or postprandial states. The chronic hyperglycemia of diabetes mellitus (DM) is associated with end organ damage, dysfunction, and failure in organs and tissues including the retina, kidney, nerves, heart, and blood vessels **(Fadini *et al.*, 2011)**.The diabetes can be measured by analyzing the blood sugar levels. The blood sugar level in healthy man

on fasting are 80 mg/dl, and in postprandial state after (2 hours) is up to 160 mg/dl **(Singh *et al.*, 2016).**

Diagnostic Criteria of Diabetes Mellitus include measurements of variable venous plasma glucose:

▪ Random plasma glucose value of ≥ 200 mg/dl (≥ 11.1 mmol/l),

▪ Fasting plasma glucose of ≥ 126 mg/dl (7.0 mmol/l) (fasting time 8–12 h),

▪ OGTT 2-h value in venous plasma ≥ 200 mg/dl (≥ 11.1 mmol/l)

▪ HbA1c ≥ 6.5 % (≥ 48mmol/mol Hb) **(Masuch *et al.*, 2019)** .

Diabetes type-1 that is usually diagnosed in children and adults manifest symptoms

and testing for family members, and test is carried out in those with symptoms or without symptoms since it is a familial type. **(Jang, *et al.*, 2020)**.

1.1.4.1. Diagnosis of Type2 Diabetes Mellitus:

A. Fasting plasma glucose test: To perform this test, diabetic patients should be fasting at least 8 hours before doing it. When the level of glucose in plasma more than 110 mg/dl diagnosed as diabetes. Normal value of FBG test (70-110 mg/dl) **(Ketema *et al.*, 2015)**.

B. Random plasma glucose: This test doesn't want for fasting individuals, when the level of glucose in plasma (200mg/dl) or more, the patient consider as diabetes **(Marathe *et al.*, 2017)**.

D. Glucose tolerance test: This test determined the body's ability to handle glucose. In the test a person fasts overnight at least 8 hours but not more than 16 hours then first, the fasting plasma glucose is tested. After this test, the person receives 75 gm of glucose (100 gm of glucose for pregnant women), usually the glucose is in a sweet –

testing liquid that the person drink. Blood sample are taken up to four times to measured blood glucose at 30, 60, 90 and 120 min.

In normal persons, FBG within 70–110 mg/dl, following glucose intake, glucose level rises and reaches at peak within 1 h and then come to normal levels within 1.5–2 h because of normal insulin action and secretion. After 2 h it should be below 120 mg/dl and negative test for glucose in urine.

In diabetic patient, the FBG level is too much higher than normal, glucose level after 1 h of glucose intake also rise and does not reach normal level even after 2 h because of decrease insulin secretion and action. Glucose will appear in urine (glycosuria)**(Wedge *et al.*, 2018)**.

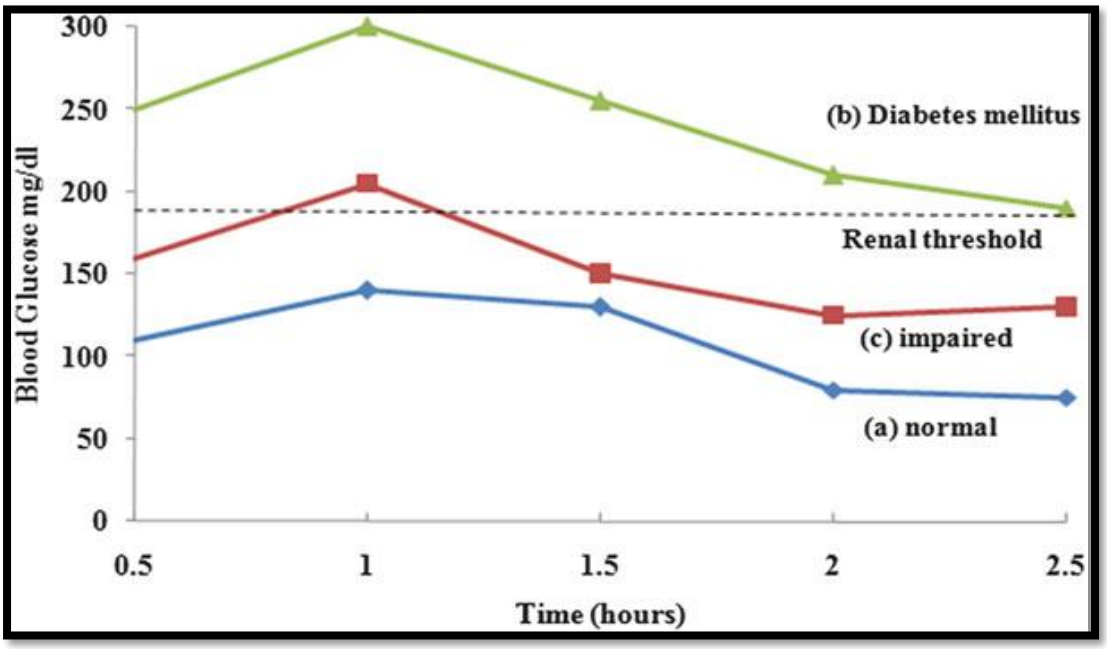


Fig. 1-2: Glucose Tolerance Test (K. Kumar *et al.*, 2018).

E. Hemoglobin A1c (HbA1c): Also called glycated hemoglobin. Glycosylation indicate a modulation or alteration within proteins structure but in the presence of

enzyme, while glycation indicate non–enzymatically attachment between proteins and monosaccharide (usually glucose). HbA1c is glycated hemoglobin in which glucose is attaches to the N-terminal valine residue of the beta-chain (B-chain) of hemoglobin A (HbA). HbA1c test use to diagnosis and monitoring of blood glucose level in diabetic patient over 8-12 weeks based on life span of erythrocytes (120 days), it is more accurate than measurement of blood glucose level because this test does not effect by hormones, food ingestion and exercise. It is expressed as percentage, the normal value of HbA1c about <5.7%, and from 5.7%-6.4% is pre diabetes and from 6.5%-20% and above is diabetes**(Lean *et al.*, 2018) (Welsh, et al., 2 016).**

F. Evaluation of insulin resistance: Measuring of insulin resistance is very important for diabetic patient; the homeostasis model assessment of insulin resistance (HOMA-IR) index is widely used in medicine for clinical studies. HOMA-IR uses fasting measurements of blood glucose and insulin concentrations to calculate indices of both insulin sensitivity and β-cell function. The model assumes that normal-weight subjects aged less than 35 years have an insulin resistance (R) of 1 and 100% β-cell function **(Bakhshalizadeh *et al.*, 2016)**.

G. C-Peptide: C-peptide is a beneficial and widely used procedure of evaluation pancreatic beta cell function. It is produced in equimolar amounts to endogenous insulin but is secreted at a more stable rate over a longer time. Methods of estimation include urinary and serum sampling. After cleavage of proinsulin, insulin and the 31-amino-acid peptide "C-peptide" are generated in equal amounts. The degeneration rate of c-peptide is slower than that of insulin (half-life of 20–30 min, while the half-life of insulin 3–5 min). In healthy individuals the fasting plasma level of c-peptide is 0.3–

0.6 nmol/l, while a postprandial level rise to 1–3 nmol/l. Insulin is metabolized in the liver by first-pass metabolism, whereas c-peptide has small hepatic clearance **(Leighton *et al.,* 2017)**. C-peptide levels interpreted with kidney failure because nearly half of C-peptide produced is extracted by the kidneys, so 5% of total C-peptide produced excreted in the urine. Therefore, blood levels of C-peptide can be elevated with renal impairment **(Elliot *et al.,* 2013)**.

1.1.5. Metabolic Abnormalities in Diabetes Mellitus

The liver is an important organ of glucose metabolism, and it plays an important role in regulating blood glucose concentrations **(Li *et al.,* 2018).** With the decrease of liver reserves and glycogen synthesis, insulin resistance gradually appears, and disorders of glucose metabolism are also aggravated **(Radwan *et al.,* 2019).** Prior studies have suggested that obesity increases the risks of other chronic diseases **(Kahn *et al.,* 2016)** .For instance, excessive lipid accumulation in obese patients suppresses insulin signaling, and results in the occurrence of insulin resistance and type 2 diabetes (T2D) **(Franks *et al.,* 2016).**

Over the decades, over nutrition coupled with a sedentary lifestyle has led to a striking increase in metabolic diseases, such as type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD). Some organs and tissues (e.g., adipose, liver, muscle, skeleton) secrete specific cytokines for inter-organ communication, and the production and secretion of these cytokines alter during nutritional stress and physical activity. Recent studies have shown that certain factors participate in glucose and lipid metabolism and thus may associate with metabolic disorders **(Liu *et al.,* 2018).**

1.1.6. Complication of Diabetes Mellitus

Vascular complications of both the macrovascular system (cardiovascular disease (CVD)) and microvascular system (diabetic kidney disease (DKD), diabetic retinopathy and neuropathy) are the leading cause of morbidity and mortality in individuals with diabetes **(Morrish *et al.*, 2001).** DM leads to long-lasting complications such as kidney failure, nerve damage, loss of vision, leg amputation, and Cardiovascular Disease (CVD) in chronic uncontrolled conditions **(Girach *et al.*, 2006)** . Such complications as CVD, apnoea, and obstructive sleep seem to be especially related with obesity and diabetes **(Wilding, 2014).** Gaining more weight in T2D increases the risk of cardio metabolic complications, which are the main reason of morbidity in T2D **(Wilding, 2014)**. Recent studies highlight that a lot of young people withT2D have a higher risk of complications early after diagnosis than adults with T2D **(Reinehr, 2013)**. Depending on the cardiovascular event or disease (for example, coronary heart disease (CHD), myocardial infarction, heart failure or stroke) and diabetes subtype, individuals with diabetes have anywhere from a twofold to tenfold increased risk of a cardiovascular event compared with individuals without diabetes **(Mulnier *et al.*, 2006)**. Additional risk factors for CVD among individuals with diabetes include the presence of other microvascular complications, as well as sex, age, BMI, glucose control and HbA1c levels, blood pressure and smoking status **(Klein *et al.*, 2004; Mulnier *et al.*, 2006).** The complication of diabetes mellitus was divided into acute and chronic complications:

A. Acute complications: The acute complications of diabetes mellitus include diabetic ketoacidosis, hyperglycaemic hyperosmolar state, hypoglycemia, diabetic coma **(Pinhas-Hamiel *et al.*, 2007).**

B. Chronic complications: The chronic complications was divided into two categories **(Papatheodorou *et al.*, 2018).**

1. Microvascular complications (retinopathy, nephropathy, and neuropathy)
2. Macrovascular complications (cardiovascular disease, coma and atherosclerosis).

1.1.7. Pathophysiology of Diabetes Mellitus

Since its discovery in 1921, insulin has been reported to exert actions in almost every type of cell and tissue. However, insulin's effects vary depending on cell types, including vascular cells. The vascular system includes arteries, veins, capillaries, and venules, often with addition of the myocardium. Its primary mission is to distribute nutrients, hormones, cytokines, and other signaling molecules among various tissues as well as remove their by-products. Equally important is the vascular system's ability to produce many of these signaling metabolites, hormones, and cytokines, with autocrine, paracrine, and hormonal actions, which have profound importance to many physiological processes **(King *et al.*, 2016).** However, there are many interactions between vascular cells and their substromal cells, including adipose cells, which may even cause progenitor cells in the body **(Knights *et al.*, 2020).**

The endothelium can regulate insulin transport into peripheral organs that possess continuous vascular connections such as the central nervous system (CNS), adipose tissue, and skeletal muscle, in contrast to organs with fenestrated capillaries such as the liver, renal glomeruli, and pituitary **(Yazdani *et al.*, 2019)**. In vivo studies have also shown that physiological hyperinsulinemia can increase total skeletal muscle blood flow and rapidly recruit muscle capillaries (by relaxing resistance and terminal arterioles, respectively) in a time-, dose-, and NO-dependent manner, which precedes insulin’s effects of increasing muscle glucose uptake or activating

downstream kinase pathways **(Eelen *et al.*, 2018)**. Furthermore, in obese individuals, insulin has been reported to increase sympathetic nerve activity under basal conditions, but not during an euglycemic hyperinsulinemic clamp as seen in insulin-sensitive controls **(Kanter *et al.*, 2018)** . Individuals with the metabolic syndrome are much more likely to develop type 2 diabetes mellitus (T2DM), cardiovascular diseases (CVDs), and fatty liver disease **(Daryabor *et al.*, 2019)**. Abnormal immune cell activation and subsequent inflammatory environment has an essential role in the progression of T2DM **(Donath *et al.*, 2011)**. Furthermore, chronic elevated serum levels of free fatty acids, seen in obesity and T2DM, induce lipotoxicity in beta-cells and suppress their insulin secretion ability **(Oh *et al.*, 2018)**.

1.1.8. Types of Diabetes Mellitus

The common types of diabetes are detailed below**:**

1.1.8.1. Type 1 Diabetes Mellitus

Type 1 diabetes is an autoimmune condition characterized by the destruction of pancreatic beta cells and absolute insulin deficiency. Affected individuals have impaired glucose metabolism and are prone to chronic complications from hyperglycaemia and acute complications from hypoglycaemia and ketoacidosis. .The strongest predictor of diabetes complications is glycaemic control and achieving near normal glycated hemoglobin (HbA1c $\leq$ 7.0% or 53 mmol/mol) is considered the primary target in diabetes management **(Feinman *et al.*, 2015).** In type 1 diabetes, blood glucose excursions are a function of the input of glucose from food, mainly carbohydrates (starch and sugars), and insulin from predominantly exogenous sources **(Nielsen *et al.*, 2012).** For several decades, type 1 diabetes mellitus (T1DM) was believed to be a T cell- mediated autoimmune disease **(Bottazzo *et al.*, 1985; B. O. J. D. Roep, 2003)**. This notion still holds, but several observations in the past few

years point to a role of β- cells that goes beyond being a non- provoking victim of an autoimmune attack **(B. O. Roep *et al.*, 2016)**, **(Mallone *et al.*, 2020)**. Patients with type 1 diabetes often present with acute symptoms of diabetes and markedly elevated blood glucose levels, and approximately one-third are diagnosed with life-threatening ketoacidosis **(Dabelea *et al.*, 2014).** There is evidence to suggest that early diagnosis may limit acute complications **(Ziegler *et al.*, 2013)**. Few studies also suggested higher relative risk of mortality in females than in males with T1D **(Collier *et al.*, 2018)**.Type 1 diabetes is one of the most common chronic autoimmune disorders that typically manifests in early childhood and adolescence **(De Ferranti *et al.*, 2014).** At older ages, compared with the general population, mortality rates in individuals with type1 diabetes are even higher, largely because of chronic complications of diabetes, such as cardiovascular disease **(Laing *et al.*, 1999).** Indeed, the cardiovascular mortality rate in elderly people with type 1 diabetes is more than ten-times greater than in elderly people in the general population **(Laing *et al.*, 1999).** Type 1 DM is due to an absolute lack of insulin and has an autoimmune basis. This disorder was previously known as insulin-dependent diabetes mellitus (IDDM) until the reclassification of diabetes mellitus based on etiopathology. An immune mediated destruction of b cells is the hallmark of the disorder, and hyperglycemia only ensues when ~90% of B cells are lost **(De Ferranti *et al.*, 2014)**.

1.1.8.2. Gestational Diabetes Mellitus

Carbohydrate intolerance that begins or is first recognized during pregnancy is known as gestational diabetes. Previously undiagnosed diabetes mellitus (either type 1 or type 2) may manifest itself particularly during initial assessments in pregnancy. However, gestational diabetes is considered a separate entity from type2 DM **(McKinney *et al.*, 2004)**. Pregnant women often develop diabetes. During pregnancy large quantities of hormones are produced, these hormones may reduce insulin action

in the mother's body, causing insulin resistance. Women that develop diabetes mellitus during pregnancy and women with undiagnosed asymptomatic type 2 diabetes mellitus that is discovered during pregnancy are classified with gestational diabetes mellitus **(Baynes, 2015)**. Clinical importance of GDM lies in the fact that it is associated with significant maternal and fetal morbidity**(Siddiqui *et al.,* 2013).** However, the phenomenon of hyperglycaemia that develops during pregnancy (generally detected in the late second trimester (13–26 completed weeks of gestation) or early in the third trimester (27–40 weeks)) and resolves following delivery, was noted some time before **(Hadden, 2008).** In the context of this epidemic of hyperglycaemia outside pregnancy, it is highly likely that many cases diagnosed as GDM actually represent undiagnosed pre- pregnancy hyperglycaemia of varying severity **(Diabetes *et al.,* 2010).**

1.1.8.3. Various Types of Diabetes Mellitus

A. LADA (latent autoimmune diabetes in adults) accounts for 2–12% of all cases of diabetes. Patients are typically diagnosed after 35 years of age and are often misdiagnosed as type 2 DM. Glycemic control is initially achieved with sulfonylureas but patients are generally thinner and require insulin therapy more rapidly than in type 2 DM patients **(Nambam *et al.,* 2010)**. The criteria for the diabetes types LADA is shown in Figure 1-3.

B. Genetic defects in β-cell function are associated with numerous forms of diabetes and with an onset of hyperglycemia usually before the age of 25. This heterogeneous group are referred to as maturity onset diabetes of the young (MODY) **(McKinney *et al.,* 2004)**. The criteria for the diabetes types MODY is shown in Figure 1-4.

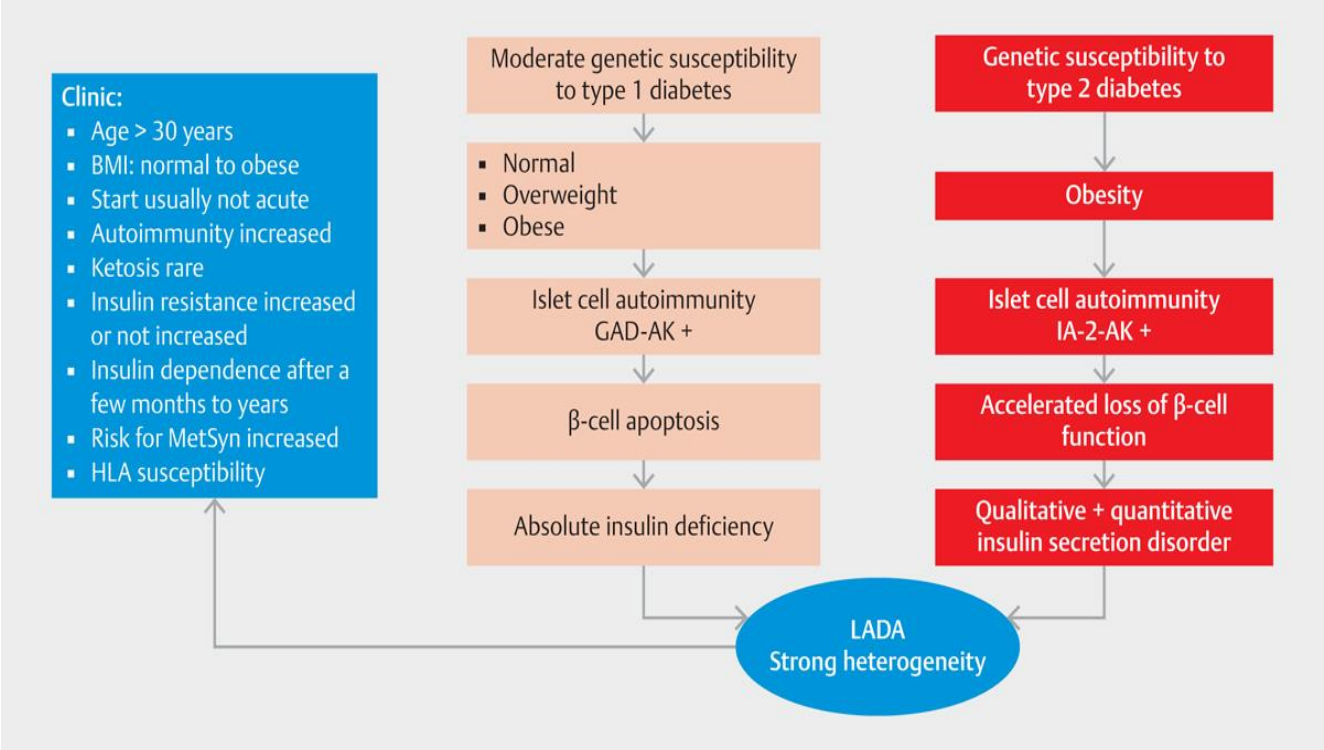


Fig. 1-3: LADA diagnostic criteria (Buzzetti *et al.*, 2017).

C. Autoimmune-mediated diabetes is more common in certain neurologic conditions, particularly those that have a high prevalence of autoantibodies to glutamic acid decarboxylase (GAD Ab). Stiff person syndrome (SPS) and progressive encephalomyelitis with rigidity and myoclonus (PERM) in particular have extremely high titers of GAD Ab. In SPS one-third of individuals go on to develop diabetes **(Walikonis *et al.*, 1998).**

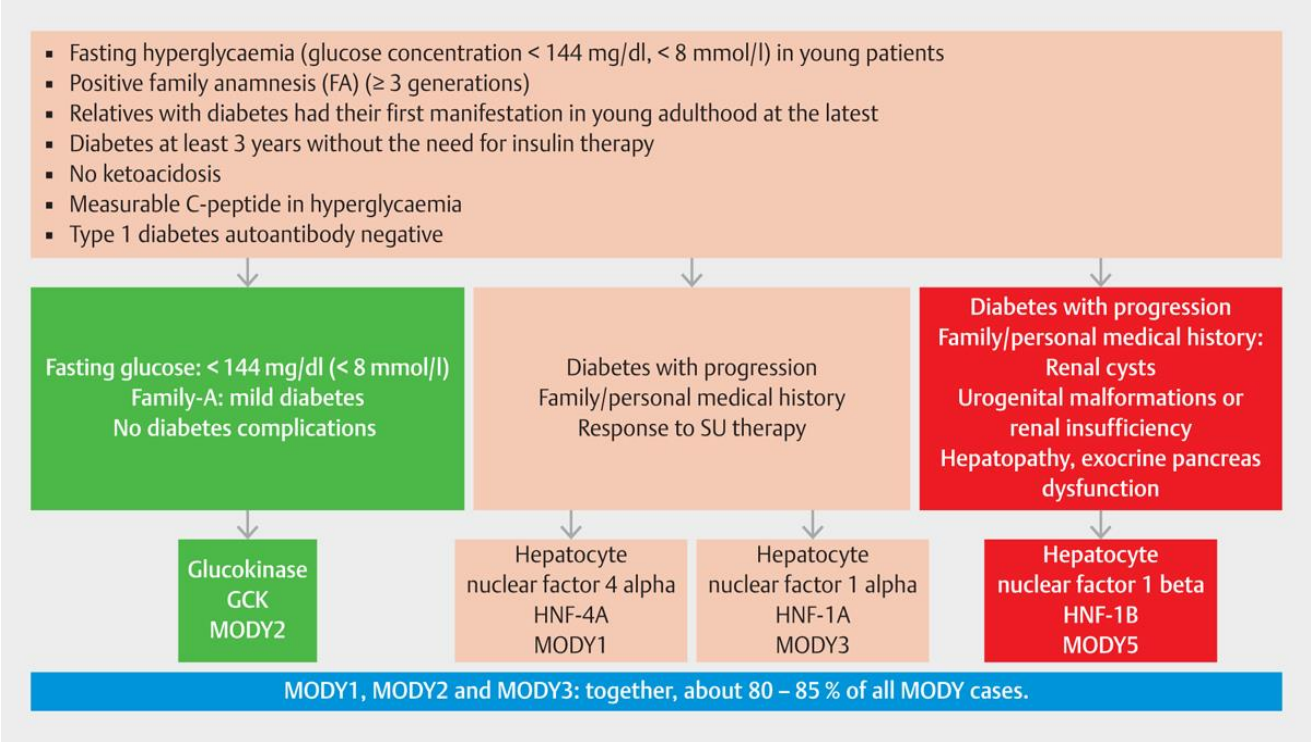


Fig. 1-4: Diagnostic algorithm of the most important MODY forms. According to (Badenhoop, 2017).

1.1.8.4. Type 2 Diabetes Mellitus

Type 2 diabetes mellitus is the commonest form of diabetes and accounts for ~90%–95% of case**s.** It develops secondary to a relative insulin deficiency but the primary defect is insulin resistance **(Care *et al.*, 2010)**.

Type 2 diabetes mellitus T2DM is also known as adult-onset diabetes. The progressive insulin secretary defect on the background of insulin resistance. People with this type of diabetes frequently are resistant to the action of insulin **(Singh *et al.*, 2016).** The most common forms of T2DM are polygenetic, so there are changes in multiple genes, but there are also some rare forms of diabetes that are caused by single gene mutation, known as monogenic diabetes. It is important not be confused with T1DM, so it is crucial to have a correct diagnose in order to receive a proper treatment **(Sharma *et al.*, 2019)**.The increase of adipose tissue is a primary risk factor in

diabetes. In fact, there is a direct correlation between fat percentage and insulin resistant cells, especially if the fat is concentrated in the abdominal area. Around 80% of T2DM patients are obese, which is not, however, a necessary condition to develop diabetes **(Wu *et al.*, 2014).** Obesity and T2DM are two of the most pressing public health concerns worldwide because of their association with life-threatening diseases, including cardiovascular diseases and cancers **(Garg *et al.*, 2014; Scherer *et al.*, 2016).** Obesity, especially pathologic expansion of visceral white adipose tissue (vWAT), increases the risk of developing T2DM. Depending on the race, more than 75%–90% of patients with T2DM are overweight or obese. The strong association of obesity and T2DM is supported by the term "diabesity" **(Farag *et al.*, 2011) (Eckel *et al.*, 2011)**. The findings of human epidemiologic studies indicate that the global prevalence of T2DM is increasing rapidly, and this increase parallels the increase in the prevalence of obesity **(A. S. Kumar *et al.*, 2019)**.

1.1.8.4.1 Causes of Type 2 Diabetes Mellitus

In the last decade, hypothesis-free genome wide association studies (GWAS) have been the single most important contributor to identifying genetic determinants of type 2 diabetes (T2D), leading to the discovery of ~100 associated genomic regions or loci **(Morris *et al.*, 2018)**. This is consistent with the model of heritability of T2D derived from whole-genome sequencing experiments, characterized by a prominent contribution to heritability of common variation, a small contribution of rare variation and evidence of low selective pressure on predisposition alleles **(Meigs, 2019)**. Excess fat is the hallmark of overeating and lack of physical exercise and has been a major focus of genetic research. Large-scale studies of body mass index and related measures have linked genes highly expressed in the central nervous system with general obesity in different ancestries **(Heitkamp *et al.*, 2021).** There is an abundance of epidemiological evidence which indicates that physical inactivity (defined as an

insufficient physical activity level to meet physical activity guidelines) and sedentary behaviour (defined as any waking behaviour characterised by an energy expenditure <1.5 metabolic equivalents (METS), while in a sitting, reclining or lying posture) are major causal factors in the development of obesity, insulin resistance and type 2 diabetes **(Goenka *et al.*, 2017) (Smith *et al.*, 2016; Tremblay *et al.*, 2017)**. T2D is believed to arise due to complex interactions between genetic information, developmental exposures and environmental factors such as diet, physical activity, and pollution **(Nowlin *et al.*, 2012; Ma *et al.*, 2014)**. The reduced sensitivity to insulin is often called insulin resistance and its causes are shown in Table1.1 **(Guyton *et al.*, 2006).**

1.1.8.4.2. Prevalence of Type 2 Diabetes Mellitus

Type-2 diabetes reduces the average lifespan by around ten years **(Saeedi *et al.*, 2019)**, **(Boyle *et al.*, 2010)**. Type 2 diabetes was once considered as a disease of middle-aged and older people, but in recent decades the prevalence of type 2 diabetes in adolescents and young adults has risen considerably around the world, driven largely by increasing obesity prevalence at younger ages. A study from Japan reported that type 2 diabetes increased tenfold in young children (aged 6–12 years) and doubled among adolescents over a 20 year period from 1976 to 1997 **(Kitagawa *et al.*, 1998)**. Similar trends have been found in many countries across the world **(Craig *et al.*, 2007; Dabelea *et al.*, 2009)**. In the past decade, the prevalence of diabetes has grown more rapidly in low and middle-income countries than in high-income countries **(Organization, 2016)**.Increasing aging population, rapid urbanization, overweight and obesity, sedentary life- style, and excessive intake of alcohol and unhealthy diets were identified as significant predictors to the escalation of DM worldwide **(Saeedi *et al.*, 2020)**. Together with the associated changes in lifestyle, urbanization has led to a substantial increase in obesity and diabetes and other non-communicable diseases

(Gong *et al.*, 2012).. In parallel with advances in diabetes care, several recent studies have found a decline over time in the excess risk of death in people with type 2 diabetes **(Harding *et al.*, 2014).**

1.1.8.4.3. Obesity and Type 2 Diabetes Mellitus

Obesity and the progression from obesity to T2DM can partly be explained by changes in adipose tissue (AT) composition and function. The AT is an active endocrine organ secreting several hundreds of bioactive molecules, referred to as adipokines **(Landecho *et al.*, 2019)**. Obesity is characterized by a pathological excess of body fat that results from a persistently positive energy balance **(Hill *et al.*, 2012)**. Diet with low fiber, high fat, and sugar has been linked to obesity **(Cheung *et al.*, 2018)**. which is a most relevant risk factor for T2DM **(Knowler *et al.*, 2002)**. Obesity in patients with T2DM will aggravate insulin resistance IR **(S. E. Kahn *et al.*, 2006)**. Many factors contribute to increasing obesity, including changes in diet, increased affluence, reduced physical activity, and increased urbanization **(Hou *et al.*, 2013)**. In particular, the incidence of T2DM is very high in overweight/obese individuals with visceral adiposity and its linked pathological conditions characterized by interrelated alterations in metabolic and vascular functions such as hyperglycemia, dyslipidemia, insulin resistance, and hypertension **(Barroso *et al.*, 2017)**. Patients with obesity and/or T2DM exhibit significantly reduced circulating adiponectin levels **(Abdella *et al.*, 2018)**. Adiponectin is one of the most abundant adipokines that is highly expressed in white adipose tissues WAT and has anti-obesity, anti-atherogenic, and anti-diabetic effects **(Ohashi *et al.*, 2012)**.

1.2. Nesfatin-1

Nesfatin-1 as a peptide consisting of 82 amino acids. In this study, the protein nucleobindin2 (NUCB2) secreted in the hypothalamic nuclei of mice was analyzed. Nesfatin-1 was discovered by Oh-I and colleagues for the first time in 2006. They showed that nesfatin-1 is secreted from the hypothalamic nuclei, which are responsible for controlling appetite. In the same study, it was reported that nesfatin-1 suppresses food intake, even in obese mice with a knockdown leptin gene **(Oh-I *et al.*, 2006)** . This finding indicates the efficacy of nesfatin-1 as an appetite suppressant, as it works independently of the leptin pathway. Thus, nesfatin-1 has raised interest in the area of treatment of obese individuals with the leptin gene mutation **(Cowley *et al.*, 2006)**.

Nesfatin-1 is secreted by the neurons (hypothalamic paraventricular nucleus, supraoptic nucleus, arcuate nucleus, lateral hypothalamic area, and spinal cord) and peripheral tissues (pancreas, liver, subcutaneous and visceral fat tissues, brown adipose tissue, and skeletal muscles) **(Abaci *et al.*, 2013)**. The secretion is distributed in the body; nesfatin-1 is thought to affect many functions. Previous studies have reported that nesfatin-1 has regulatory effects on energy metabolism through suppression of food intake. In addition, it has been reported that nesfatin1 regulates cardiac functions, decreases blood glucose levels, acts as a neuroendocrine regulator, and causes weight loss along with reduction in energy intake **(S Algul *et al.*, 2016)** .

1.2.1 Chemistry of Nesfatin-1

In 2006, Oh-I *et al* **(Oh-I *et al.*, 2006)**. first described nesfatin-1 as a peptide consisting of 82 amino acids. In this study, the protein nucleobindin2 (NUCB2) secreted in the hypothalamic nuclei of mice was analyzed. This protein consists of 396 amino acids. The NUCB2 prohormone is divided into three components as a result

of proteolytic processes. The N-terminal fragment constitutes nesfatin-1 (1-82), and the C-terminal fragment constitutes nesfatin-2 (85- 163) and nesfatin-3 (166-396). Among the three peptides of NUCB2 prohormone, only nesfatin-1 was found to have an effect on food intake and appetite control **(Andreas Stengel *et al.*, 2011)**. However, the functions of nesfatin-2 and nesfatin-3 are not yet known **(S. J. E. Aydin, 2013)**. The structure of nesfatin-1 consists of three parts. The first part begins from the N-terminal end, and continues to the 23 amino acid, and is called N23. The second part includes amino acids between 23 and 53 and is defined as M30. The third part includes amino acids between 53 and 82 toward the carboxy terminal and is called C29. M30 is reported to be effective in the food intake and appetite control **(S. J. E. Aydin, 2013)**. Figure **1-5** shows the structure of NUCB2 protein and the formation of nesfatin-1 **(Garcia-Galiano *et al.*, 2010)**.

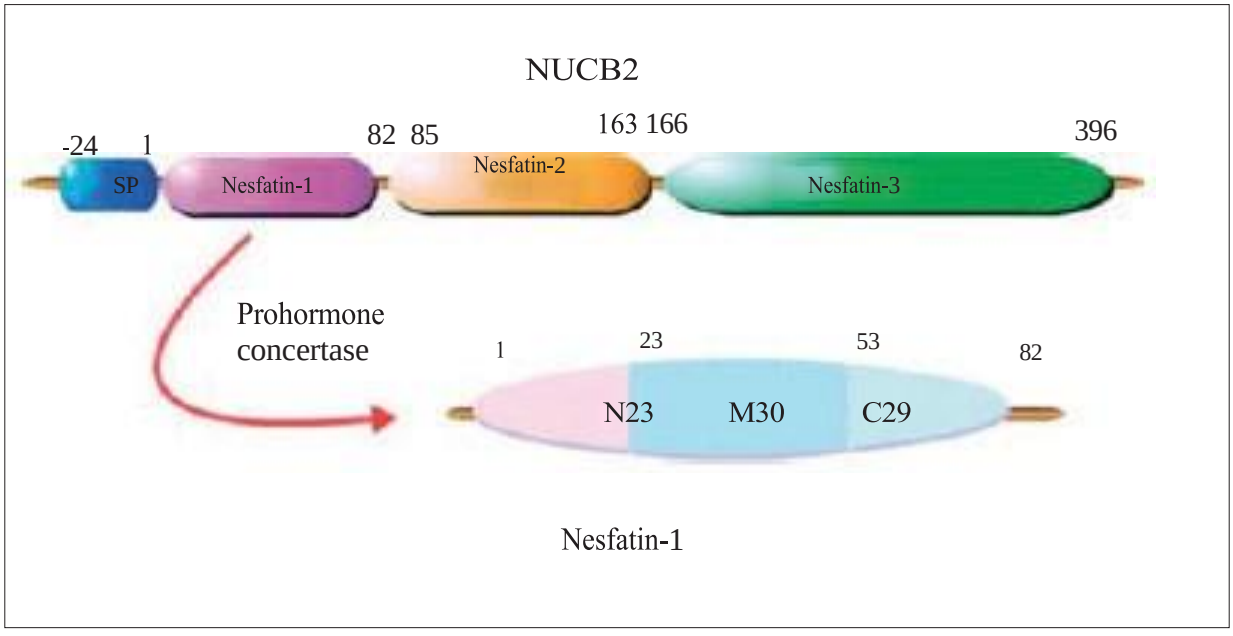


Fig. 1-5: Structure of NUCB2 protein and Nesfatin-1 formation (García-Galiano *et al.*, 2010).

1.2.2. Biochemical Roles of Nesfatin-1

Nesfatin-1 plays a role in satiety regulation and, possibly, energy homeostasis**(Kohno *et al.*, 2008)**. The nesfatin-1 is prominently expressed in several regions of the hypothalamus and spinal cord autonomic nuclei. Also, it has been demonstrated to be present in peripheral tissues including adipocytes, gastric mucosa in human and rat pancreatic beta-cells, indicating the possible involvement of nesfatin-1 in the regulation of insulin secretion from pancreatic beta-cells **(Andreas Stengel *et al.*, 2009) (K. S. Foo *et al.*, 2010)**. As nesfatin-1 may play an important role in the regulation of body weight and insulin secretion, in this study hypothesized that plasma nesfatin-1 levels might be affected by insulin resistance in subjects with newly diagnosed type 2 diabetes mellitus (nT2DM) and impaired glucose tolerance (IGT) **(Rasouli *et al.*, 2008)**.

Besides obesity, some studies have recently suggested a role for nesfatin-1 in the pathophysiology of diabetes mellitus (DM) **(Ding *et al.*, 2015)**and **(Xia *et al.*, 2015)**. Fasting and satiety are regulated by inhibitory (anorexigenic) and stimulatory (orexigenic) factors produced in peripheral tissues or directly in the brain. In the brain, the hypothalamus plays a central role as an area producing factors that regulate food intake. This area combines central and peripheral factors that regulate nutrient uptake and long-term body weight maintenance**(A Stengel *et al.*, 2013)**. The relationship between obesity and nesfatin-1 was investigated because of the effects of nesfatin-1 on food intake and energy consumption. There was a relationship between the polymorphism of the nucleobindin-2 gene and obesity. This may be a risk factor for the development of obesity **(Senin *et al.*, 2015)**. Nesfatin-1 may be a regulator of appetite, energy and glucose homeostasis, and insulin secretion. Thus, it can participate in the etiology of various metabolic diseases including obesity and diabetes**(St-Pierre *et al.*, 2016)**.

1.2.3. Nesfatin-1 and Obesity

Nesfatin-1 has an inhibitory effect on food intake and thus alleviates obesity in a dose- and time-dependent manner upon intracerebroventricular(icv) and intraperitoneal injection, as well as after intranasal administration **(Ozkan *et al.*, 2013)**. The expression of NUCB2 mRNA in peripheral tissues comprising heart, spinal cord, pancreas, islets, stomach, and adipose tissue, muscle have crucial physiological roles in body weight and they also contribute to the pathophysiology of insulin resistance and its associated metabolic problems such as obesity and diabetes **(Khalili *et al.*, 2017)**. Plasma levels of nesfatin-1 can correlate with body mass index **(Senin *et al.*, 2015)**. Nesfatin-1 might will be involved in long-term changes of energy expenditure. Because nesfatin-1 reduces food intake and increases energy expenditure, it induces a negative energy balance, which might be relevant in states of over nutrition but also might reflect conditions of stress **(Schalla *et al.*, 2018)**. insulin resistance, fasting blood glucose, and fasting insulin levels, as well as with body weight and fat mass, suggesting that nesfatin-1, especially fat-derived nesfatin-1, may play an important role in human metabolism and food intake **(Yosten *et al.*, 2013)**.

1.2.4. Nesfatin-1 in Clinical Diagnosis

The structure of the nesfatin-1 molecule is also tripartite; the first section, which begins from the N-terminal and is 23 amino acids long, is called N23; the central fragment from 23 to 53 is named M30, and the section from 53 to 82 near the carboxyl terminus is called C29 **(Aydin, 2013)**. Nesfatin-1 has been identified as the adipokine that is involved in metabolic processes, including glucose homeostasis, and it has a glucose-dependent insulin tropic effect. Nesfatin-1 is expressed in a few regions of the hypothalamus, and it has been proven to affect the feelings of hunger and satiety,

and body weight, and play a key role in regulating food intake**(K. Foo *et al.*, 2008)** . The concentration of nesfatin-1 increases after feeding, and the expression of nesfatin-1 was found in reward-related areas, and this suggests that nesfatin-1 can also play a role in hedonic feeding**(Dore *et al.*, 2017)**. It should be emphasized that nesfatin-1 has also been featured as a regulator of blood glucose concentrations. However, the exact intracellular mechanism of nesfatin-1's effect on decreasing blood glucose concentration is not well recognized. Moreover, nesfatin-1 has been suggested to be involved in type 2 diabetes mellitus (T2DM) pathogenesis by stimulating free acid utilization. A significant decrease in fasting plasma nesfatin-1 levels in T2DM and polycystic ovary syndrome (PCOS) patients has also been confirmed. This could be caused by impaired insulin sensitivity and suggests that nesfatin-1 might be inhibited by insulin resistance, hyperglycemia, and hyperinsulinemia**(Li *et al.*, 2010)**.

Recent studies have found aberrant expressions of nesfatin-1 in the blood of patients with diabetes-related diseases. Although it has been reported that the plasma nesfatin-1 level was significantly decreased in patients with T2DM compared with healthy controls **(F. Liu *et al.*, 2014)**, increasing evidence has indicated a higher level of blood nesfatin-1 in T2DM patients and a close relationship between nesfatin-1 concentration and clinical parameters known to be associated with insulin resistance**(Y. Zhang *et al.*, 2017)**, **(Zhai *et al.*, 2017)**. It has been reported that NES-1 suppresses food intake and reduces weight gain when injected into the third ventricle of rodents or it administered peripherally. So, the major function of NES-1 is inhibition of food intake in time-dependent, dose-dependent, and insulin-dependent manners **(Shimizu *et al.*, 2009)**. 1 Detection of NES-1 localizes with insulin in the pancreatic beta islets indicates the involvement of NES-1 in regulating insulin secretion from pancreatic beta-cells and thus regulation of blood glucose. NES-1 affects glucose metabolism, the suggested mechanism for that is by increasing insulin

sensitivity and decreasing insulin resistance. For measuring insulin resistance, homeostatic model assessment of insulin resistance (HOMA-IR) is used **(Khalili *et al.*, 2017)** .

1.2.5. Nesfatin-1 in Type 2 Diabetes Mellitus

Nesfatin-1 has an effect on the regulation of glucose homeostasis as well as that of food intake **(Serum Algul *et al.*, 2016)**. A study compared the fasting plasma nesfatin-1 levels of healthy individuals and type 2 diabetic individuals. Thus, fasting nesfatin-1 levels were found to be lower in individuals with type 2 diabetes than healthy individuals **(Li *et al.*, 2010)**. Another study found that plasma nesfatin-1 levels were significantly lower in patients with type 2 diabetes compared to healthy subjects **(Serum Algul *et al.*, 2016)**. The production of nesfatin-1 and NUCB2 in pancreatic beta cells indicates their possible effects on the regulation of insulin secretion **(Khalili *et al.*, 2017)**. The effect of nesfatin-1 on insulin secretion is affected by blood glucose concentrations. NUCB2/nesfatin-1 released from the pancreas increased in response to glucose **(Şafak *et al.*, 2017)**. Glucose can increase the production and secretion of nesfatin-1 mRNA **(Kulkarni *et al.*, 2019)**. Nesfatin-1 can increase the synthesis of pre-proinsulin mRNA and stimulate glucose-enhanced insulin release **(Anwar *et al.*, 2014)**. Peripheral nesfatin-1 may have a potential effect on the control of glucose homeostasis **(Khalili *et al.*, 2017)**. Nesfatin-1 is a metabolic controller. Injection of nesfatin-1 can prevent hepatic glucose formation and stimulate glucose uptake **(Şafak *et al.*, 2017) (Prinz *et al.*, 2016)**.

1.3. Nucleobindin-2

1.3.1. Chemistry of Nucleobindin-2

Nucleobindin-2 (NUCB2) was found in the plasma membrane and neuroplasma. Some prohormone convertase enzymes of NUCB2 such as PC3/1 and PC2 converts

NUCB2 to nesfatin-1 (1-82 aa), nesfatin-2 (85-163 aa) and nesfatin-3 (166-396aa) **(Williams *et al.*, 2014)**. In humans, the NUCB2 gene length was 55 kb with 14 exons and 13 introns. The translation region of the NUCB2 gene was described in exon-3. Nesfatin-1 was translated in region between exon-3 and 5 of NUCB2 gene **(Pałasz *et al.*, 2012)**. However, recent studies have shown that NUCB2 was also expressed in other peripheral tissues as the stomach, pancreas, reproductive organs, and adipose tissues **(Suzuki *et al.*, 2012)**.

1.3.2. Biochemical Roles of Nucleobindin-2

NUCB2 is composed of a 24 amino acid signal peptide, followed by a 396 amino acid sequence, which is cleaved into three different peptides: nesfatin-1 (1–82), nesfatin-2 (85–163) and nesfatin-3 (166–396) **(Oh-I *et al.*, 2006)**. Notably, NUCB2 is selectively expressed in pancreatic islet beta cells **(Gonzalez *et al.*, 2009)**.Besides, intracellular NUCB2 mRNA levels and protein synthesis or release in the pancreatic beta cells, are dynamically regulated by glucose levels. In vitro, the expression of human islet NUCB2 mRNA is upregulated under gluco-lipotoxic conditions, and is down-regulated in T2DM patients compared to controls **(Riva *et al.*, 2011)**. A recent study showed that beta-cell-specific NUCB2 knockout mice have elevated blood glucose levels and reduced insulin secretion**(Yang *et al.*, 2019)**. Besides, plasma nesfatin-1 concentrations and islet NUCB2 mRNA are significantly decreased in T2DM patients compared with healthy controls**(F. Liu *et al.*, 2014)**. These previous findings indicate that NUCB2 plays a significant role in the development of T2DM. NUCB2 are associated with an increased risk for T2DM. Thus, dysfunction of expression, secretion, and/or action of NUCB2 might be involved in the development and progression of T2DM.The findings of previous studies report that NUCB2polymorphisms are associated with obesity in adults and children **(Zhai *et al.*, 2017)**.

1.3.3. Nucleobindin-2 as Biomarker used in Pathological Diseases

1.3.3.1 Nucleubindin-2 and Type 2 Diabetes Mellitus

According to the International Diabetes Federation (IDF), one in ten adults in the world currently has diabetes, and this disease is steadily increasing in prevalence worldwide and has acquired the status of a global epidemic**(Yasmin *et al.,* 2022)**.

Nesfatin-1, a recently identified adipokine, is an anorectic hormone derived from nucleobindin-2 (NUCB2) expressed in rat hypothalamus **(Oh-I *et al.,* 2006)**. and contributes to several phenomena, e.g., the control of reproductive and cardiovascular functions, maintenance of glucose homeostasis, and anti-inflammatory and anti-apoptosis effects**(Xu *et al.,* 2020)**, **(Schalla *et al.,* 2018)**. In previous studies, nesfatin-1 has been suggested to be involved in type 2 diabetes mellitus (T2DM) pathogenesis by stimulating free acid utilization. A significant decrease in fasting plasma nesfatin-1 levels in T2DM and polycystic ovary syndrome (PCOS) patients has also been confirmed. This could be caused by impaired insulin sensitivity and suggests that nesfatin-1 might be inhibited by insulin resistance, hyperglycemia, and hyperinsulinemia **(Schalla *et al.,* 2018)** , **(Li *et al.,* 2010)**.

1.3.4 Nucleobindin-2 Gene in Type 2 Diabetes Mellitus

Both genetic and environmental factors contribute to the incidence of T2DM. T2DM is considered to be a complex multi-gene disorder. A number of genes and their polymorphisms have been shown to contribute to the pathogenesis of this disease **(McCarthy *et al.,* 2002)**. A recent study performed in a population of obese subjects indicated an association of three single nucleotide polymorphisms (SNPs) (rs1330, rs214101 and rs757081) in the NUCB2 gene with obesity **(Zegers *et al.,* 2011)**. Chen et al. then reported that one SNP of NUCB2 gene, c.1012C>G (Q338E or rs757081) was correlated with childhood adiposity**(Chen *et al.,* 2013)**. These data indicate that

polymorphisms in the NUCB2 gene could play an important role in the protection against the development of obesity. Therefore, it is hypothesized that c.1012C>G polymorphism of NUCB2 may be associated with the risk of T2DM. **(Riva *et al.*, 2011)**.

1.4 Molecular Studies in Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is associated with several factors including hypertension, chronic hyperglycemia and hyperlipidemia, resulting from insulin resistance or insulin deficiency **(Holman *et al.*, 2015).** These factors have been implicated with overproduction of reactive oxygen species (ROS) in the mitochondrial matrixes that offset cellular redox balance and induce oxidative stress **(Valko *et al.*, 2007).** Excessive ROS-induced oxidative stress exerts significant damage to various cellular biomolecules including lipids, proteins and DNA **(Migdal *et al.*, 2011)**. The resulting dysregulated expression in various genes leads to impaired insulin secretion and impaired insulin signaling. **(Gallagher *et al.*, 2010)**. These risk factors affect the expression of genes involved in insulin secretion in β-cells and insulin sensitivity across peripheral tissues **(Erukainure *et al.*, 2019)**. Despite many in vitro and in vivo studies aimed at elucidating the underlying molecular mechanisms of DM, the precise pathophysiology is not completely understood. In the past decade, hypothesis-free genome-wide association studies (GWAS) have been the most important contributor to identification of genetic determinants of type 2 diabetes, leading to the discovery of more than 100 associated genomic regions or loci**(Morris *et al.*, 2018)**, **(Holman *et al.*, 2015)**. Although most signals are led by common variants with ever smaller effects, new risk alleles include several that are low frequency or rare **(Zhao *et al.*, 2017)**. These findings are consistent with the model of heritability of type 2 diabetes derived from whole-genome sequencing experiments, which is characterized by a prominent contribution to heritability by common

variation, a small contribution of rare variation, and evidence of low selective pressure on predisposition alleles **(Fuchsberger *et al.*, 2016)**.

1.4.1Gene Polymorphism

Molecular markers correspond to a class of genetic markers used to evaluate genetic differences between two or more individuals. They are capable of revealing the existing polymorphism in a set of genetically related individuals. The technology of molecular markers enables the genetic characterization of large numbers of genotypes through relatively simple and inexpensive procedures, and are fundamental in genetic improvement, phylogeography and analysis of similarity or genetic distance, identification of plant accesses, animals, microorganism isolates, and support in taxonomic studies. The ability to reveal polymorphisms is fundamental in selecting molecular markers. If a marker is not able to detect the genetic differences existing in a set of individuals, its usefulness is not effective. In qualitative terms, a marker is considered polymorphic if it has at least two alleles and the most frequent allele has a frequency of up to 99% **(Shete *et al.*, 2000)**. Polymorphism association studies are often comprised of case-control and observational studies that compare the occurrence of a genetic variant in certain individuals suffering from a specific disease to the occurrence in a healthy control population. Furthermore, while several polymorphisms have been identified, most studies often use relatively small homogeneous sample size populations, which severely limits the ability to draw conclusions from general population results and frequently yields conflicting results with mostly non-significant findings. In general, the outcomes of the various analyses are irregular and frequently variable**(Lu *et al.*, 2009)**.

1.4.2 Gene Polymorphism in Type 2 Diabetes Mellitus

T2D.M is associated with chronic hyperglycemia because of resistance to insulin or its inadequate secretion or both**(Boada *et al.*, 2013)**. Beside the environmental and behavioral factors, genetic predisposition has been shown to be a critical risk factor in the pathogenesis of T2D.M and its related complications **(Sanghera *et al.*, 2012)**.However, only a few of the genetic components have been identified so far, despite of the huge number of studies directed towards understanding the genetics of T2D.M **(Sanghera *et al.*, 2012)**. In terms of β-cell activity, the mainstream genes involved play a major part and genetic polymorphisms that affect important proteins involved in glucose metabolism and insulin secretion can also impact susceptibility to T2DM **(Witka *et al.*, 2019)**. Genome-wide association studies (GWASs), the candidate gene approach, and linkage analysis have identified various genes that contribute to T2DM susceptibility**(Mahajan *et al.*, 2018)**. he development of genetic risk scores using combined analysis of loci has significantly contributed to predicting the incidence of T2DM**(Kong *et al.*, 2020)**,**(Zheng *et al.*, 2017)**. T2DM has a strong genetic basis, and individuals with a first-degree family history are at increased risk of developing the disease, and this risk is increased twofold if both parents have diabetes **(Groop, 1996)**. Several risk factors for T2DM have been identified, including obesity and central obesity, ethnicity, family history of diabetes, elevated blood pressure, dyslipidemia, lifestyle factors and dietary intake **(Schwingshackl *et al.*, 2017)**. Some of these risk factors are associated with functional metabolism; therefore, genetic-based diagnoses may provide a more promising diagnostic tool. More than 200 genetic loci have been detected to be associated with T2DM risk**(Mahajan *et al.*, 2018)**.

1.4.3 Nucleobindin-2 Gene Variants

The nucleobindin-2 (NUCB2) is a polypeptide composed of 396 amino acid and is a precursor of nesfatin-1, nesfatin-2 and nesfatin-3, spanning residues 1–82, 85–163 and 85–163, respectively**(Oh-I *et al.*, 2006)**. The peripheral and central administration of NUCB2/nesfatin-1 regulates the glucose and fatty acid metabolism and improves the insulin resistance **(Dong *et al.*, 2013)**. In humans, the NUCB2gene length was 55 kb with 14 exons and 13 introns. The translation region of theNUCB2 gene was described in exon-3. Nesfatin-1 was translated in region between exon-3 and 5 of NUCB2 gene **(Pałasz *et al.*, 2012)**. However, recent studies have shown that NUCB2 was also expressed in other peripheral tissues as the stomach, pancreas, reproductive organs, and adipose tissues**(Suzuki *et al.*, 2012)**. , the microarray and RT-PCR results confirmed that NUCB2 gene expression decreased in the islets of T2DM patients and that expression was enhanced under glucolipotoxic conditions in human pancreatic specimens. This finding suggested that the secretion mechanism of Nesfatin-1 in islets correlated positively with insulin secretory capacity. Recently, Zhang et al**(Z. Zhang *et al.*, 2012)**.

1.4.4 Nucleobindin-2 Gene in Pathological Diseases

NUCB2/Nesfatin-1 has antihyperglycemic effects **(Ayada *et al.*, 2015)**. Blood NUCB2/Nesfatin-1 levels in type 2 diabetes mellitus (DM) and gestational DM patients also seem to be lower **(Deniz *et al.*, 2012)**. This finding - the effects of NUCB2/Nesfatin-1 on energy balance, obesity, glucose metabolism and this being related to delayed puberty of lower NUCB2/Nesfatin-1 - showed that NUCB2/Nesfatin-1 in normal and pathological conditions may be important in the functioning of the ovaries and in reproduction**(Alp *et al.*, 2015)**.

1.5 Aims of the Study

1. Estimation the percentage of obesity among type 2 diabetic women of Kerbala province: Iraq.
2. Determination of Nesfatin-1 and various biochemical markers levels in obese and non-obese type 2 diabetic women and compared with control group.
3. Study the correlation between Nesfatin-1 levels and other biomarkers in obese and non-obese type 2 diabetic women.
4. To study the genetic variants of Gene in obese and non-obese type 2 diabetic women.
5. To determine the correlation between Nesfatin-1 and the resultant data of molecular variants of Gene with the pathogenesis of type 2 diabetes mellitus in obese women as compared with control group.

CHAPTER Two

Subjects, Materials and Methods

2. Subjects, Materials and Methods

2.1 Subjects

The present work included case control study an overall of 100 sample (females) were studied, 25 of them diabetic patients with obesity, 25 of them with type 2 diabetic patients without obesity (T2DM) and another 50 apparently healthy individuals as a control group. The study protocol was approved by the ethical research committee, College of Medicine, University of Kerbala and Kerbala Health Directorate. All blood specimens (patients and control) were collected from individuals admitted in Al-Hussein Teaching Hospital and Al-Hassan center of diabetes and endocrinology unit, Kerbala Health Directorates/ Kerbala – Iraq. The study was managed over the period from April, 2022 – Jan. 2023.The age of all subjects is ranged from 30 to 67 years.

2.1.1. Patient Group

Type II diabetic patients with obesity and type II diabetic patients without obesity were randomly selected from Al-Hassan center for diabetes and endocrinology unit in Karbala.

2.1.1.1 Inclusion Criteria of Patient Group

a. Patient with type 2 diabetes mellitus (obese and non-obese)

b. Age of patients was 30-67 years old.

2.1.1.2 The exclusion criteria of Patient and control Groups include:

- Kidney disease
- Liver disease
- Cancers
- Strokes
- Any acute or chronic inflammatory disease

- Cerebrovascular accidents
- Alcoholics
- Rheumatoid arthritis
- Autoimmune disease
- Patients with type1 diabetes mellitus.
- Epilepsy.
- Anxiety

2.1.2. Control group

Subjects were apparently healthy and selected from the general population when they went to the hospital for check-up and from attendants of patients.

2.1.2.1. Inclusion Criteria of Control Group

a. FBS is less than 110 (mg/dl).

b. HbA1c less than 5.7 %.

c. Patients with no previous medical history of diabetes mellitus complications.

d. No family history of diabetes mellitus.

e. Parallel to patients with respect to age, sex, and geographical distribution.

f. Age at examination >30 years.

g. Normal body mass index (BMI).

2.1.3 Approval of the ethical committee

The Ethical Committees approved this study protocol are: Committee of College of Medicine, University of Kerbala and Department of Medicine and committee of Al-Hussein Teaching Hospital and Al-Hassan canter of diabetes and endocrinology unit, Kerbala Health Directorates/ Kerbala – Iraq.

2.1.4 Blood Sampling

Five ml of blood was drawn from the vein of all subjects by using a disposable syringe then divided into two parts:

The first part (3ml) was place in gel tube and left at room temperature for about (30 min) for clotting, then putt in the centrifuge at 4000 x g to obtain serum which used for the determination of biomarker levels including blood glucose using enzymatic colorimetric method, lipid profile using spectrophotometric technique, and nesfatin-1 level using ELISA kit, and C-peptide level using ELISA kit.

The remaining part of blood (4 ml) was put in two "EDTA containing tubes", the first EDTA tube containing (2ml) of blood used to determine HbA1c% level and the second EDTA tube was stored by freezing at -20˚C until using for DNA extraction and molecular analysis.

2.2. Materials

2.2.1. Chemicals and Kits

All chemicals and kits that were used in this study are listed in Table 2.1.

Table 2-1: Chemicals and Kits

No.	Kits and Chemicals	Source (country)
1.	Genomic DNA Extraction kit	Intron (Korea)
2	Agarose Analytical Grade	Bio Basic / Korea
3	Ethidium Bromide	Sigma / USA
4	Master Mix Kit	Bonier/ KOREA
5	Nuclease Free Water	Promega (U.S.A)
6	Primers	Bonier/ KOREA

7	Tris Borate EDTA (TBE) Buffer X10	Bio Basic / Korea
8	PCR Premix	Bonier/ KOREA
9	DNA leader	Bonier/ KOREA
10	COBAS HbA1c kit	Roche Cobas (Germany)
11	Ethanol 95%	Bio Basic / Korea
12	Human Nesfatin-1 ELISA kit	Pars biochem/ China
13	Human C-Peptide ELISA kit	Pars biochem/ China
14	Lipid profile Kits	Bioteck/ China
15	COBAS Blood glucose kit	Roche Cobas (Germany)

2.2.2. Instruments and Apparatus

The Instruments and apparatus and tools used in this study were shown in table (2-2).

Table 2-2: Instruments and apparatus

No.	Instruments and apparatus	Manufacture
1	PCR - thermocycler	Biometra (Germany)
2	Vortex- Mixture	Cyan (Belgium)
3	Deep freeze	Hitachi / Japan
4	Bench centrifuge	Hettich / Germany
5	Sensitive Balance	Sartorius (Germany)
6	UV source	USA
7	Oven	Binder / USA
8	Minispin centrifuge	Hettich / Germany

10	Nano drop	Bio drop (England)
12	Incubator	Germany
13	Electrophoresis apparatus	Biometra / Germany
14	Photo documentation	UVP (UK)
15	ELISA reader	ELX800-U.S.A
16	ELISAwasher	ELX800-U.S. A
17	Water bath	LabTech / Korea
18	Pipette(100-1000µl)	DRAGON MED/ USA
19	Micropipette(10-100 µl)	DRAGON LAB/ USA
20	Micropipettes	DRAGON LAB/ USA
21	Gilson Tips,1000µl (blue)	China
22	Gilson Micro-tips, 10µl	China
23	Gilson Micro-tips, 100µl	China
24	Eppendorf Tubes	China
25	Gel tubes	China
26	EDTA tubes	China
27	Gloves	China
28	Syringe	China

2.3. Methods

2.3.1. Determination of Body Mass Index and Waist Hip Retio

- The body mass index (BMI) was estimated by below equation:

BMI = Wight in kg/ (Height in meter)^2

For both patients and control groups, weight was classified according to their BMI as shown below (WOH, 2016).

a. Underweight < 18.5 kg/m^2
b. Normal weight 18.5-24.9 kg/m2
c. Over weight 25.0 – 29.9 kg/m2
d. Obese ≥ 30.0

- The waist hip ratio (WHR) was estimated by below equation:
- Calculate WHR by dividing waist circumference by hip circumference.

WHR=Waist in cm/ Hip in cm

According to the study in 2021 by World Health Organization (WHO) a moderate WHR is:

- 0.9 or less in men
- 0.85 or less in women.

Waist-to-hip ratio chart

Health risk	**Women**
low	0.80 or lower
moderate	0.81-0.85
high	0.86 or higher

2.3.2 Measurement of Nesfatin-1 levels and C-Peptide levels in Human serum. By using Sandwich-ELISA Technique:

Principle of Nesfatin-1 and C-Peptide levels assay:

The kit assay Human Nesfatin-1 level and C-Peptide level in the sample use Purified Human Nesfatin-1 antibody to coat micro titer plate wells, make solid-phase antibody, then were add Nesfatin-1 and C-Peptide to wells. Combined Nesfatin-1 and C-Peptide antibody which with HRP labeled become antibody-antigen- enzyme-antibody complex. After washing completely, were add TMB substrate solution, TMB substrate becomes blue color at HRP enzyme-catalyzed, reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of Nesfatin-1and C-Peptide in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Table 2.3: Materials provided with the kits (Nesfatin-1 and C-Peptide levels)

Materials provided with the kit	48determinations	96determinations	Storage
User manual	1	1	---------
Closure plate membrane	2	2	---------
Sealed bags	1	1	---------
MicroELISA strip plate	1	1	2-8℃
Standard：135pg/ml	0.5ml×1 bottle	0.5ml×1 bottle	2-8℃
Standard diluent	1.5ml×1 bottle	1.5ml×1 bottle	2-8℃
HRP-Conjugate reagent	3ml×1 bottle	6ml×1 bottle	2-8℃
Sample diluent	3ml×1 bottle	6ml×1 bottle	2-8℃
Chromogen Solution A	3ml×1 bottle	6ml×1 bottle	2-8℃
Chromogen Solution B	3ml×1 bottle	6ml×1 bottle	2-8℃
Stop Solution	3ml×1 bottle	6ml×1 bottle	2-8℃
wash solution	(20ml×20 fol)×1bottle	(20ml×30 fold)×1bottle	2-8℃

Preparation of reagents:

Stock solutions were prepared according to the procedure of the kit. All reagents were prepared freshly at room temperature before using

Dilution of Standards:

Dilute the standard by small tubes first, then pipette the volume of 50ul from each tube to microplate well, each tube uses two wells, total ten wells.

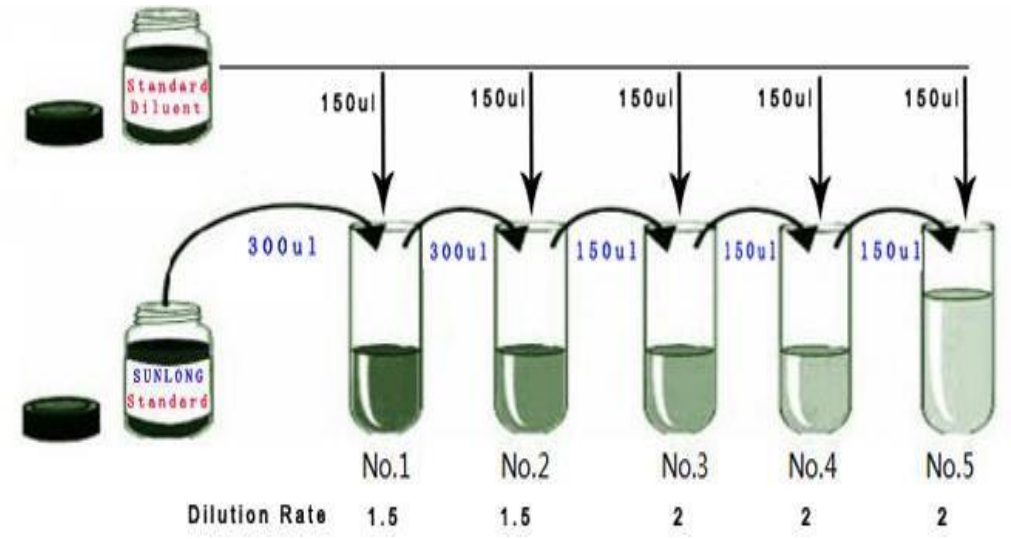


Table 2.4: Dilution of Standards

60 U/ml	Standard No.1	300µl Original Standard + 150µl Standard diluents
40 U/ml	Standard No.2	300µl Standard No.1 + 150µl Standard diluents
20 U/ml	Standard No.3	150µl Standard No.2 + 150µl Standard diluent
10 U/ml	Standard No.4	150µl Standard No.3 + 150µl Standard diluent
5 U/ml	Standard No.5	150µl Standard No.4 + 150µl Standard diluent

Procedures: For both Nesfatin-1 and C-Peptide levels:

1. Dilutes and add sample to Standard: set 10 Standard wells on the ELISA plates coated, were add Standard 100µl to the first and the second well, then add Standard dilution 50µl to the first and the second well, mix; take out 100µl form the first and the second well then add it to the third and the forth well separately. Then add Standard dilution 50µl to the third and the forth well ,mix ; then takeout 50µl from the third and the forth well discard, add 50µl to the fifth and the sixth well ,then add Standard dilution 50µl to the fifth and the sixth well, mix ; take out 50µl from the fifth and the sixth well and add to the seventh and the eighth well, then add Standard dilution 50µl to the seventh and the eighth well ,mix ; take out 50µl from the seventh and the eighth well and add to the ninth and the tenth well, then were add Standard dilution 50µl to the ninth and the tenth well, mix , then were takeout 50µl from the ninth and the tenth well discard(add Sample 50µl to each well after Diluting ,(density: 90 pg/ml ,60 pg/ml ,30 pg/ml ,15 pg/ml ,7.5 pg./ml).
2. Add sample： Set blank wells separately (blank comparison wells don't add sample and HRP-Conjugate reagent; other each step operation is same). testing sample well. add Sample dilution 40µl to testing sample well, then add testing sample 10µl (sample final dilution is 5-fold), then were add sample to wells, don't touch the well wall as far as possible, and Gently mix.
3. Incubate: After closing plate with Closure plate membrane, incubate for 30 min at 37℃.
4. Configuration liquid: 30-fold wash solution diluted 30-fold with distilled water and reserve.
5. Washing Uncover Closure plate membrane, discard Liquid, dry by swing, add washing buffer to every well, still for 30s then drain, repeat 5 times, dry by pat.

6. And then add enzyme, were add HRP-Conjugate reagent 50µl to each well, except blank well.
7. Incubate Operation with 3.
8. Washing Operation with 5.
9. Colour add Chromogen Solution A 50ul and Chromogen Solution B to each well, evade the light preservation for 15 min at 37℃
10. Stop the reaction Add Stop Solution50µl to each well, Stop the reaction (the blue colour change to yellow color).
11. Assay take blank well as zero, read absorbance at 450 nm after Adding Stop Solution and within 15min.

Calculation of results: for both (Nesfatin-1 and C-Peptide levels)

Take the standard density as the horizontal, the OD value for the vertical, draw the standard curve on graph paper, and then were find out the corresponding density according to the sample OD value by the Sample curve, multiplied by the dilution multiple, or calculate the straight line regression equation of the standard curve with the standard density and the OD value, with the sample OD value in the equation, were calculate the sample density, multiplied by the dilution factor, the result is the sample actual density.

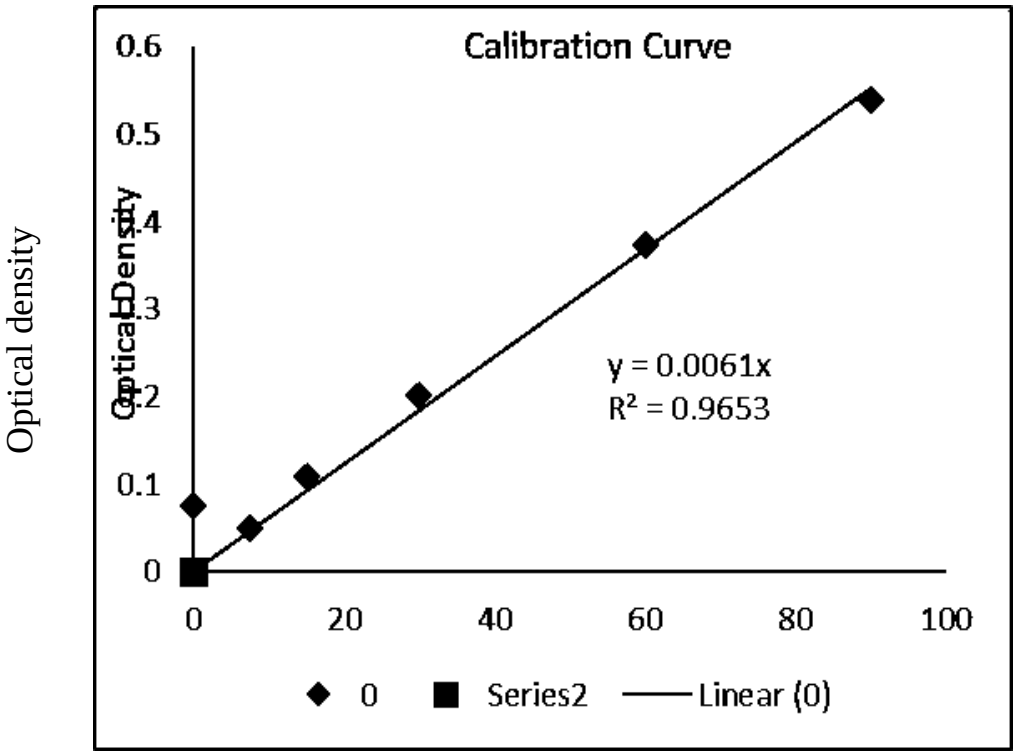


Figure 2.1 Calibration of Nesfatin-1 levels (Calculation of results from ELISA)

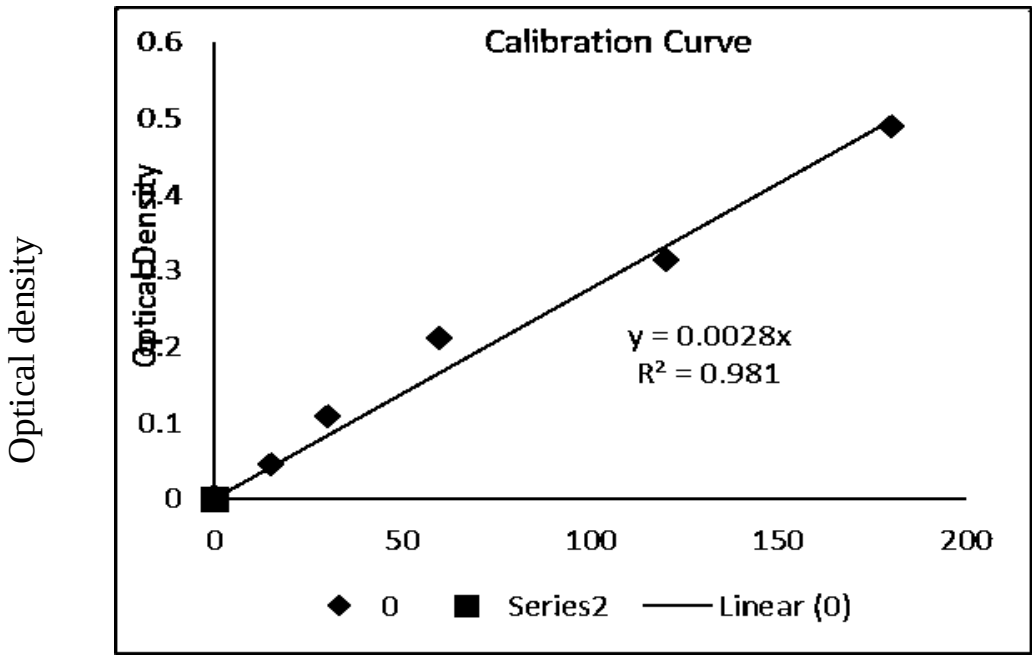


Figure 2.2 Calibration of C-Peptide levels (Calculation of results from ELISA)

2.3.3. Measurement of lipid profile

2.3.3.1 Measurement of Cholesterol levels in Human serum.

By using Spectrophotometric Technique:

Principle:

Esterified cholesterol is hydrolyzed into free cholesterol and fatty acid by cholesterol esterase (CHE). Cholesterol oxidase (CHOD) oxidizes the free cholesterol into cholestene-3-one with formation of hydrogen peroxide. In presence of peroxidase (POD), hydrogen peroxide reacts with a derivative of phenol and 4-aminoantipyrine to produce a colored complex whose color intensity is directly proportional to the total cholesterol concentration in the sample.

$$\text{Cholesterol esters} + H_2O \xrightarrow{CE} \text{cholesterol} + RCOOH$$

$$\text{cholesterol} + O2 \xrightarrow{CHOD} \text{cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4-AAP} + \text{phenol} \xrightarrow{POD} \text{Quinone-imine dye} + 4H_2O$$

Kit components:

Reagent (A) Volume = 50/100/250/1000 ml
Standard Volume = 5 ml

Procedure:

pipette	blank	Sample	standard
Reagent (A)	1000 µl	1000 µl	**1000 µl**
Water	10 µl		
Sample		10 µl	
Standard			**10 µl**

It was mixed and left for 5 min at 37°C after which the absorbance at 500 nm was recorded against a blank reagent the absorbance of the sample (A) and the standard (S).

Calculation: Cholesterol mg/dl = A/S x 200 (standard value).

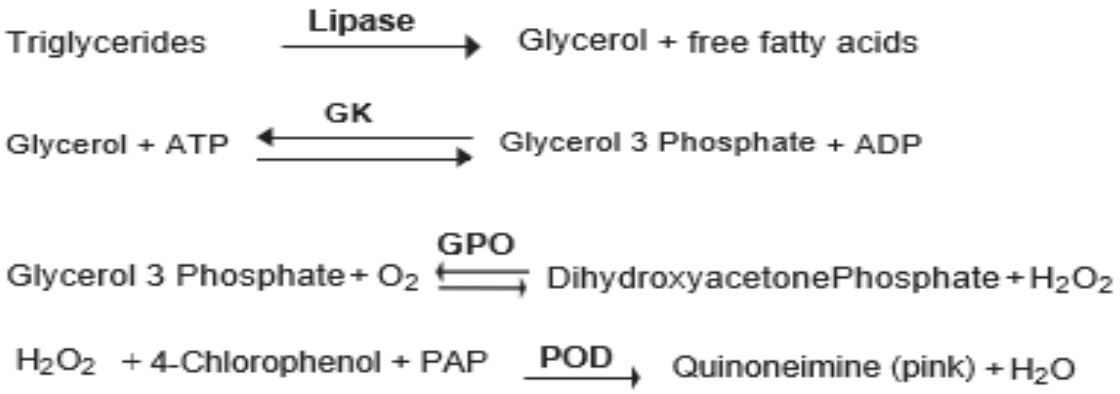
- Absorbance of the sample (A)
- Standard (S).

2.3.3.2 Measurement of Triglycerides levels in Human serum.

By using Spectrophotometric Technique:

Principle:

Reaction scheme is as follows:



The absorbance of the colored complex (quinoneimine), proportional to the amount of triglycerides in the specimen, is measured at 500 nm.

Procedure: reagents and supernatants were placed at room temperature.

	Manual procedure
Reagent	1000 µL
Standard, Controls, Specimen	10 µL

It was mixed and left for 10 min at room temperature after which the absorbance at 505 nm was recorded against a blank reagent.

Calculation: The result was calculated as follows:

Result = Abs (Assay)/ Abs (Standard) x Standard concentration

Reference Values:

- Normal: 150 mg/dL (1.70 mmol/L)
- Borderline/high: 150-199 mg/dL (1.70-2.25 mmol/L)
- High: 200-499 mg/dL (2.26-5.63 mmol/L)

2.3.3.3 Measurement of HDL-Cholesterol levels in Human serum.

By using Spectrophotometric Technique:

Principle: Low density lipoproteins (LDL-Chol), very low density (VLDL) and chylomicrons from specimens are precipitated by phosphotungstic acid (PTA) and Magnesium chloride. HDL-Cholesterol was obtained in supernatant after centrifugation is then measured with Total Cholesterol reagent.

Kit components:

Vial R1 PRECIPITANT	Vial R2 STANDARD
Phosphotungstic aci (PTA)13.9 mmol/L	Cholesterol 100 mg/dL (2.58 mmol/L)
Magnesium chloride 570 mmol/L	_____

Procedure: reagents and supernatants were placed at room temperature.

Pipette in well identified test tubes:	**Blank**	**Standard**	**Assay**
Reagent	1 mL	1 mL	**1 mL**
Demineralised water	25 µL		
Standard 100 mg/dL		25 µL	
Supernatant (*)			**25 µL**

It was mixed and left for 10 min at room temperature after which the absorbance at 500 nm was recorded against a blank reagent.

Calculation: The result was calculated as follows:

Result of cholesterol concentration (mg/dl) = Abs (Assay)/ Abs (Standard) x Standard concentration x 1.1

Expected range: 40 - 60 mg/dL.

2.3.3.4 Estimation of Low Density Lipoprotein-Cholesterol

The low density lipoprotein – cholesterol (LDL-Chol) was estimated by using indirect method based on Friedewald equation **(Friedewald *et al.*, 1972)** as shown below:

LDL-Cholesterol (mg / dl) = Total Cholesterol – HDL + (TG / 5)

Note that TG / 5 = VLDL
Reference Values: less than 180 mg / dl

2.3.4 Estimation of Blood Glucose Level:

Principle:

Estimation of blood glucose level in serum based on using of " Enzymatic colorimetric method " involve the oxidation of glucose to D-gluconate (gluconic acid) by the glucose oxidase (GOD) with the production of hydrogen peroxide, after that and through the activity of peroxidase (POD), a mixture of phenol and 4-aminoantipyrine (4-AA) is oxidized by hydrogen peroxide, to form a red color complex the absorbance of it (measured at 500 nm) is proportional to the concentration of glucose in the serum **(Green *et al.*, 1987),(Trinder, 1969)**.

$$\text{D-Glucose} + H_2O + O_2 \xrightarrow{\textbf{Glucose Oxidase}} \text{D-Gluconate} + H_2O_2$$

$$\text{4-Aminoantuipyrine} + 4\text{Phenol} + 2H_2O \xrightarrow{\textbf{Peroxidase}} \text{Quinoneimine} + 4H_2O$$

Reagents

Table 2-5: Reagents for determination of Glucose Concentration

	Content	Concentration
Reagent	Phosphate buffer	100 mmol/L, pH 7.5
	glucose oxidase (GOD)	10 KU/L
	Peroxidase (POD)	2 KU/L
	Aminoantipyrine (4-AA)	0.5 mmol/L
	phenol	5 mmol/L

Reference Values:

- Normal fasting blood sugar level is less than 105 (mg/dl).
- A fasting blood sugar level (110 -125 mg/dl) is considered pre diabetes.
- A fasting blood sugar more than 126 (mg/dl) is considered diabetes (Ketema et al., 2015).

Calculation:

Automatic calculation.

2.3.5 Determination of HbA1c%

Estimation of glycated hemoglobin (HbA1c) by "COBAS INTEGRA© 400 plus "with "COBAS HbA1c kit", the normal level less than 7% but risk level equal or more than 7%.

Principle:

This method based on using " Immuno turbidimetric assay " which involve firstly addition of Tetradecyl tri methyl ammonium bromide (TTAB) on the whole blood as the hemolyzing reagent to eliminate interference from leukocytes (TTAB does not lyse leukocytes) , the second step involve addition of R1 (Antibody reagent) ,

glycol hemoglobin (HbA1c) which glycated at N-terminal of B-chain in the sample react with (anti-HbA1c AB) and cause agglutination .The agglutination rate is photometrically determined at 550 nm and related to the HbA1c concentration. The final result is calculated automatically expressed as a percentage of HbA1c (Metus et al., 1999).

2.4. Molecular Analysis

DNA was extracted from blood using DNA isolation kit (Intron/ Korea). Genotyping was carried out by allele-specific (Amplification-Refractory Mutation System) ARMS -PCR for – 1012C>G SNP of NUCB2 gene polymorphism (rs757081). Primers and a green master mix kit (Bonier/ KOREA) were used, PCR products were separated on a 1.5% agarose gel.

2.4.1 DNA Extraction

DNA was extracted from whole blood that collected from patient and control groups by using "G- spin TM Total DNA Extraction Kit" (Intron).

Principle

This kit of DNA extraction involves using “proteinase K" enzyme in the first step and other reagents to lyse cells and degrade protein, promote binding of DNA to the silica gel fiber matrix of the spin column. The next step involves removing of Contaminants using a Wash Buffer and ultimately purified genomic DNA is eluted by elution buffer. The purified DNA (approximately 20-30 kb) is suitable for use in PCR or other enzymatic reactions (Vogelstein et al., 1979).

Procedure.

1. Added 20 µl of Proteinase K solution (20 mg/ml) to a 1.5 ml micro-centrifuge tube.

2. Then transferred 200 µl of sample to the 1.5 ml micro-centrifuge tube with Proteinase K solution: If the sample volume is less than 200 µl, we were added the appropriate volume of PBS.

3. Then was added the 20 µl of RNase A Solution (10 mg/ml).

4. Added 200 µl of Binding Solution to the sample tube, and then was mix well by pulse-vortexing for 15 sec.

5. Incubated at 56℃ for 10 min: Longer incubation times have no effect on yield or quality of the purified DNA.

6. Added 200 µl of absolute ethanol and then was mix well by pulse-vortexing for 15 sec: After this step, briefly spin down to get the drops clinging under the lid.

7. Carefully was transferred the lysate into the upper reservoir of the spin column with 2.0ml collection tube without wetting the rim.

8. Centrifuge at 13,000 rpm for 1 min: Pour off the flow-through and assemble the spin column with the 2.0 ml collection tube.

9. Added 500 µl of Washing 1 Solution to the spin column with collection tube and centrifuge at 13,000 rpm for 1 min: Pour off the flow through and assemble the spin column with the 2.0 ml collection tube.

10. And then added 500 µl of Washing 2 Solution to the spin column with collection tube and centrifuge at 13,000 rpm for 1 min: Pour off the flow through and assemble the spin column with the 2.0 ml collection tube.

11. Dried the spin column by additional centrifugation at 13,000 rpm for 1 min to remove the residual ethanol in spin column.

12. Then transferred the spin column to the new 1.5 ml micro-centrifuge tube.

13. Added 100 µl of Elution Solution to the spin column with micro-centrifuge tube, and let stand for at least 1 min.

14. Eluted the genomic DNA by centrifugation at 13,000 rpm for 1 min.

2.4.2. Determination the Concentration and Purity of the Extracted DNA.

Spectrophotometric methods were used to estimate the concentration and purity of extracted DNA **(Cory *et al.*, 2003).**

2.4.2.1 Spectrophotometric Methods

Principle:

The purity and concentration of DNA were measured by absorbance method using the Nano drop instrument. The absorbance readings were done at 260 nm and at 280 nm. At 260 nm the DNA strongly absorbs light while at 280 nm the protein absorbs light most strongly. DNA purity was measured by theA260/A280 ratio. The A260/A280 ratio 1.8-2.0 is commonly accepted **(Tataurov *et al.*, 2008).**

Procedure: Initially 1µL of nuclease free water was smeared on the highly sensitive micro detector of Nano-drop as blank. The micro detector was cleaned up from blank. Then 1µL of DNA sample was applied on the micro detector of Nano-drop. The concentration and A260/A280 ratio of DNA were documented from the instrument **(Bharathi *et al.*, 2008).**

2.4.3. Primers for PCR

A primer is a short single strand of DNA fragments consisting of (18-22) bases known as oligonucleotides that have a sequence that is complementary to the target DNA region. Without the use of primers, the amplification process cannot begin on a single DNA molecule. Thus, it should first be annealed to the single strands that result from the denaturation of the double stranded DNA(Chaitanya et al., 2013). Polymerase chain reaction was performed using a specific primer pairs designed for NUCB2 gene. Based on national center for biotechnology information (NCBI) database, all gene information and SNPs detail, were collected using Genius software designed.

Preparation of the primers in the following steps: -

Materials: Lypholized primers, sterile distal water dH2O.

1. The tube was spin down before opening the cap.

2. Prepare Master Stock, pmoles/µl, the desired amount of sterile dH20 was added according to the manufacturer to obtain a 100 pmoles/µl (Master Stock).

3. The tube was mixed properly to re-suspend the primers equally.

4. Preparing Working Stock, 10 pmoles/µl, ten microliters of the master stock were transferred to a 0.5 ml Eppendorf tube that contains 90µl of sterile dH20 to obtain a 10 pmoles/µl (Working Stock).

5. The master stock was stored at -20 C^{o}.

The sequence of primers used for PCR amplification of NUCB2 gene (rs757081) was illustrated in Tables (2-6).

Table (2-6). Specific primers of NUCB2 gene (rs757081).

Primer	Sequence (5´ –3´)	Allele	Size(bp)	Company
FC	5-TTCCTCTGTGAAGAACTGTTGCTC-3	C	400	BIONEER / Korea
FG	5-TTCCTCTGTGAAGAACTGTTGCTG-3	G	400	BIONEER / Korea
R	5-CACCTGATGTTTGCTTCTTGTGC-3	—	400	BIONEER / Korea

2.4.3.1 Amplification of DNA

Allele-specific PCR reaction protocol was used for SNP detection gene of NUCB2 gene (rs757081) [C/G]. The allele specific -PCR reactions were performed in 23 µl volumes in PCR tubes under sterile conditions, all the volume of the reaction mixture was completed to 23 µl with using deionized distal water(DDH2O) and the master mix which contained optimum concentrations of reaction requirements (MgCl2 1.5 mM, Taq polymerase 1 U, each Deoxynucleoside triphosphates (dNTPs) 200 µM) has been used, table (2-7).

Table 2-7. Components of master mix for detection of NUCB2 gene.

No.	Material	Volume(µl)
1	Master Mix	10
2	Forward	1.5
3	Reverse	1.5
4	Template DNA	3
5	DDH_2O	7
Total		**23µl**

2.4.3.2 Polymerase Chain Reaction

The polymerase chain reaction, also known as PCR, has rapidly emerged as one of the most essential methods in modern biological and medical research(Kang et al., 2017). It amplifies a specific region of a DNA strand to generate thousands to millions of copies of a particular DNA sequence (Guo et al., 2011).

A PCR basically requires the following:

1) DNA template containing the target DNA region.
2) Two primers to initiate DNA synthesis.
3) A thermostable DNA polymerase to catalyze DNA synthesis.
4) Deoxynucleoside triphosphates (dNTPs, the building blocks of new DNA strand).
5) Buffer including bivalent cations, usually Mg^{2+}.

There are three steps of a PCR that are cycled about 25-35 times **(Jones *et al.*, 1993)** this steps including the following:

- Denaturation: This step includes separation of the double DNA strands into two single strands are accomplished by heating for about 94°C.
- Annealing: At lower temperature (54°C), DNA primers (which are short single strand DNA fragments) attach to the ends of each strands of the DNA and initiates the reaction.
- Extension: This step occurs at 72°C, where each primer binding to the DNA template will be extended complementary to the template DNA. This process is carried out in the presence of the Tag DNA polymerase, because of its ability to operate efficiently at high temperatures **(Venkatesan *et al.*, 2015).**

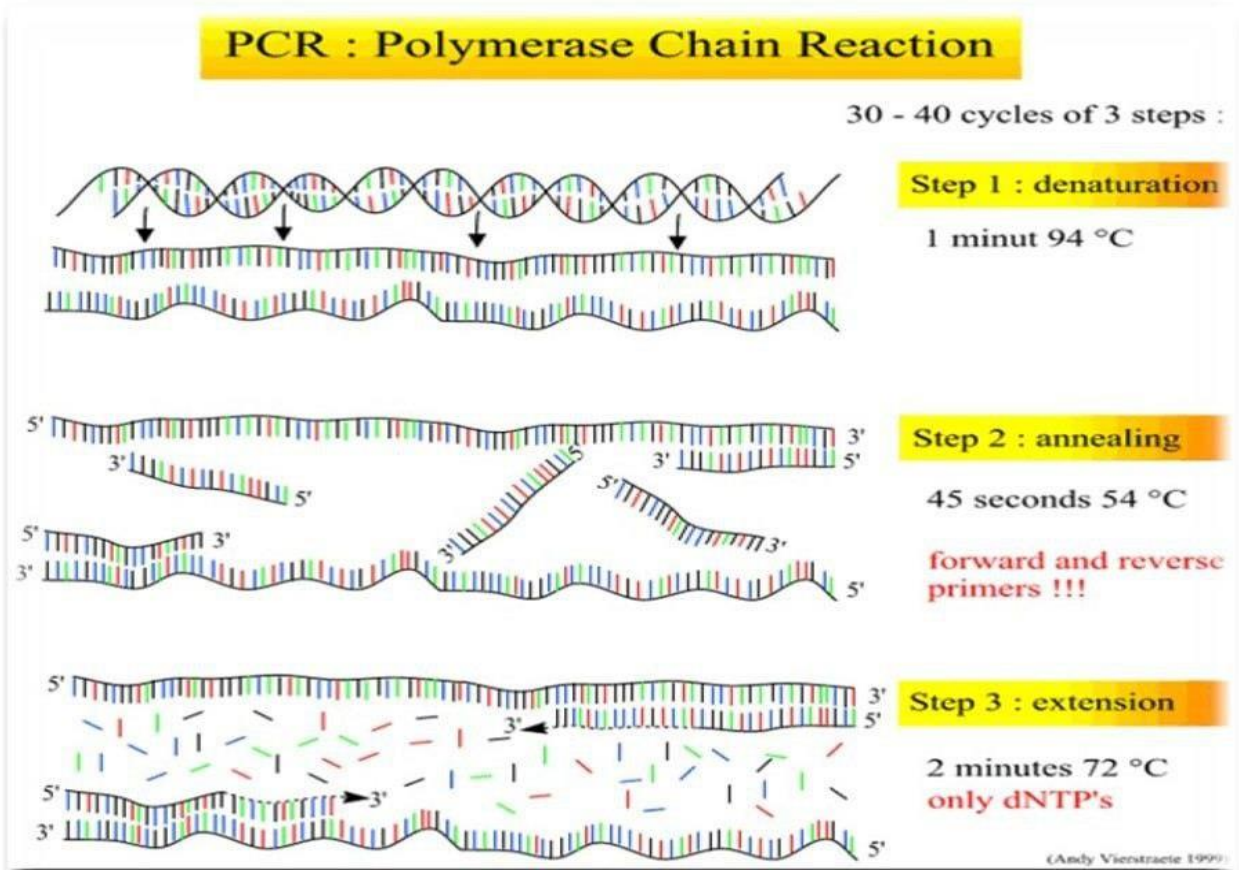


Figure 2.3 Steps of PCR Cycling (Mridha, 2015)

2.4.3.3 Thermo cycler Program for DNA Amplification

The PCR reaction program procedures for SNP (-1012 C / G) in NUCB-2 is represented in table (2.8).

Table 2.8: PCR reaction program

Type of Cycle	Temperature ^{o}C	Time	No. of Cycle
Initial denaturation	95	5 min	1 cycle
Denaturation	94	30 sec	35 cycle
Annealing	54	30 sec	35 cycle
Extension	72	60 sec	35 cycle
Final Extension	72	5 min	1 cycle
Total Time : 1 hour and 35 min			

2.4.3.4 Amplification Refractory Mutation System

The amplification-refractory mutation system (ARMS), which also termed as "allele-specific polymerase chain reaction (ASPCR) or PCR amplification of specific alleles", consider a simple, fast, and reliable technique used for detecting of any mutation include single base changes. ARMS are based on the use of sequence-specific PCR primers that promote amplification of test DNA only when the target allele is included within the specimen and will not amplify the non-target allele. Following an ARMS reaction, the existence or absence of a PCR product is detection for the existence or absence of the target allele. For the mutant-specific primer (M), the 3′ terminal base of the ARMS primer should be complementary to the mutation sequence; for the normal-specific primer (N), the 3′ terminal base should be complementary to the corresponding normal sequence.

2.4.4. Agarose Gel Electrophoresis:

2.4.4.1 Procedure

50 ml of 1% agarose solution was prepared according to the following steps **(M. R. Green *et al.*, 2019).**

A. Preparation of Solution

1X TBE buffer (tris borate EDTA) was prepared by diluting 10X TBE buffer with de ionized water (10 ml of 10X TBE buffer with 90 ml of de ionized water: 1:10 dilution).

B. Preparation of Agarose Gel

1. Firstly 0.5 gm of agarose were weighted and placed into a conical flask, and then 50 ml of 1X (TBE) buffer was added and mixed gently.
2. The solution was stirred on a hot plate until the agarose is dissolved and the solution was clear and then solution was leaved to cool.
3. 3 µl of safe stain was added to the solution.
4. Gel chamber ends were closed with rubber gasket.
5. A combs were pushed in the gel chamber about 1 inch from one ending of tray.
6. A gel solution was poured into the chamber and permitted to be hardened for approximately 30 minutes at room temperature.
7. The combs was removed, and then samples and DNA ladder were loaded (5 µl) on each well with extreme cautions to avoid damages of the wells and cross contamination of neighboring wells.
8. The chamber is placed in a horizontal electrophoresis system and covered with the same TBE buffer that used to prepare the gel.
9. The cathode (black) was connected to the wells side of the unit and the anode (red) to the other side.
10. Electrophoresis is attach to direct current power source until dye markers migrated to the suitable distance, according to the size of DNA fragment that recognized.

2.4.4.2 DNA Electrophoresis

About 5µl of PCR product were loaded to each well with great precaution to prevent damages of the wells and cross contamination of neighboring wells. An electric field (50V for 35 min) was established to the system causing the negatively charged nucleic acids to travel across the gel to the positive electrode (anode).

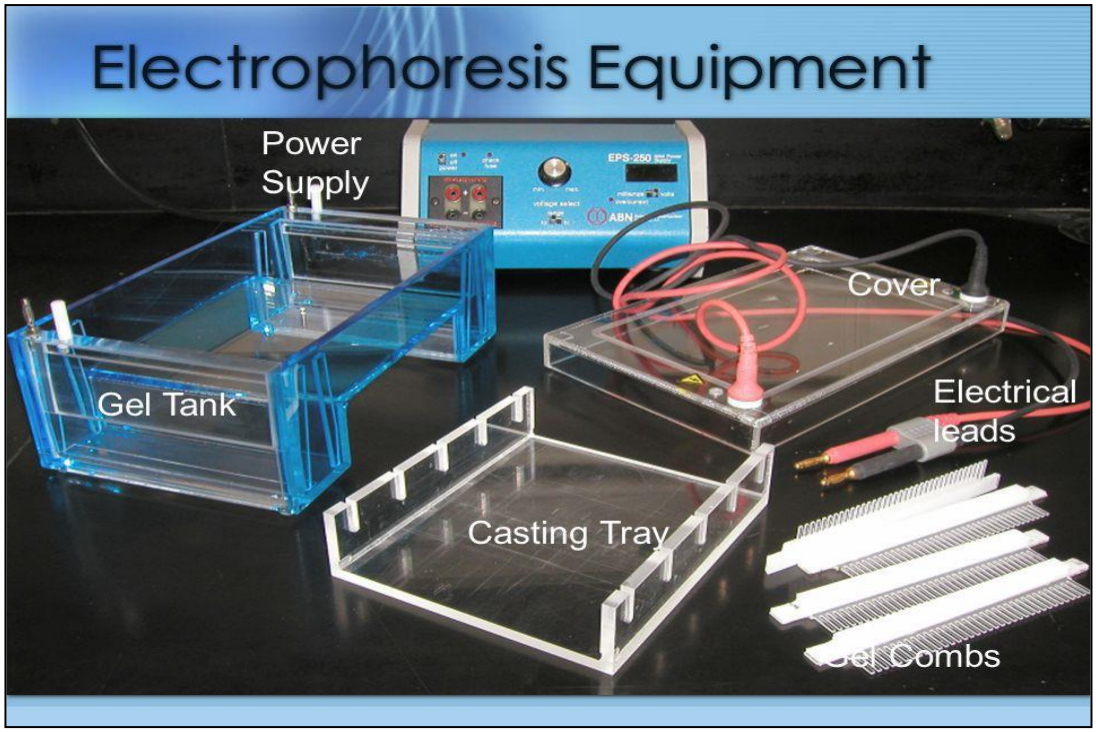


Figure 2-4: Agarose Gel Electrophoresis

2.4.4.3 DNA Ladder

In current study, 5µL of DNA ladder (1500 bp, Intron) was used as standard and band size ladder was 100- 1500 bp.

2.4.4.4 Gel - Band Visualization

To visualize the DNA bands, the agarose gel was placed in the UV trans illuminator device and exposed to UV light and the photos were captured by digital camera linked to PC.

2.5 Statistical analysis:

Information from the questionnaire of all participants were entered a data sheet and were assigned a serial identifier number. Multiple entry was used to avoid errors. The data analysis for this work was generated using The Statistical Package for the Social Sciences software, version 28.0 (IBM, SPSS, Chicago, Illinois, USA) and the Real Statistics Resource Pack software for Mac (Release 7.2) of the resource pack for Excel 2016. Copyright (2013 – 2020). Descriptive statistics was performed on the participants' data of each group. Values were illustrated by n (%) for categorical. The distribution of the data was checked using Shapiro-Wilk test as numerical means of assessing normality.

The association between the analysed factors and presence of diabetes mellitus disease was estimated using odds ratios (ORs) and 95% Confidence Interval Range which calculated by a non-conditional logistic regression.

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

Chapter Three

Results

3 Results of Biochemical markers

3.1 Demographic and clinical characteristics

The clinical demographic characteristics of patients group were summarized in Figure (3.1) . The range age of participants was within the age range of (30-67) years old, (38%) patient for groups (30- 42), (26%) patient for groups (43-54) , and (36%) for groups (55-67). The percentage of participants were nonsmokers (98%) and about half of them (42%) were reported to have hypertension. Furthermore, 50% of the patient group were within normal weight and 50% were obese.

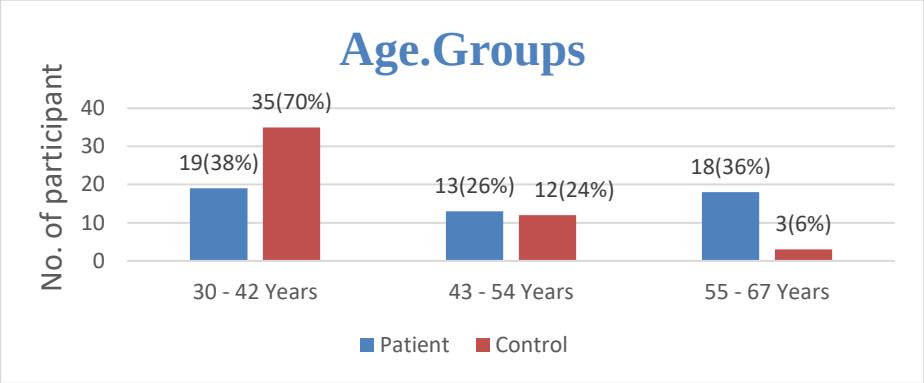


Figure 3.1: Baseline characteristics and Demographic Descriptive of the study population in patients compared to control group, the number of participant (n= 100) for distribution of Age

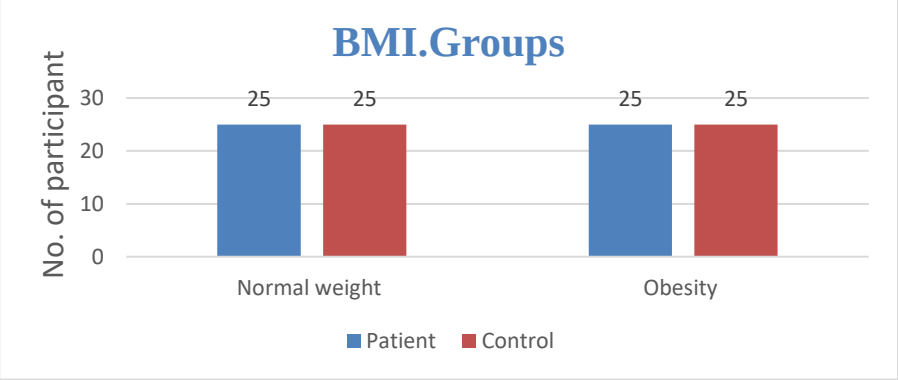


Figure 3.2: Baseline characteristics and Demographic Descriptive of the study population in patients compared to control group, the number of participant (n= 100) for distribution of BMI

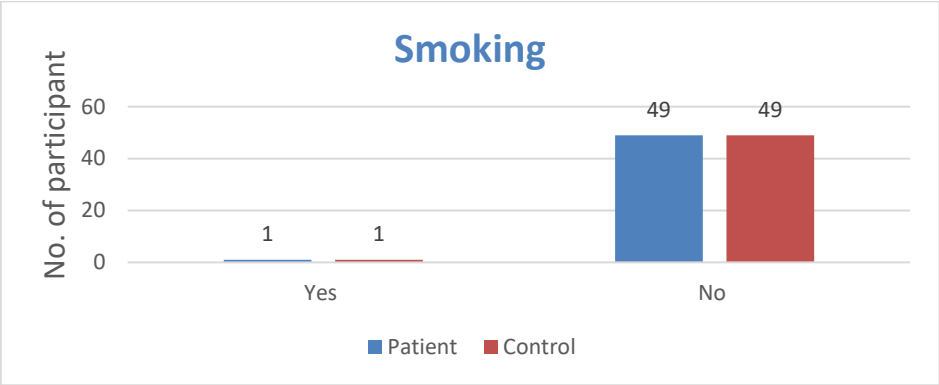


Figure 3.3: Baseline characteristics and Demographic Descriptive of the study population in patients compared to control group, the number of participant (n= 100) for distribution of Smoking

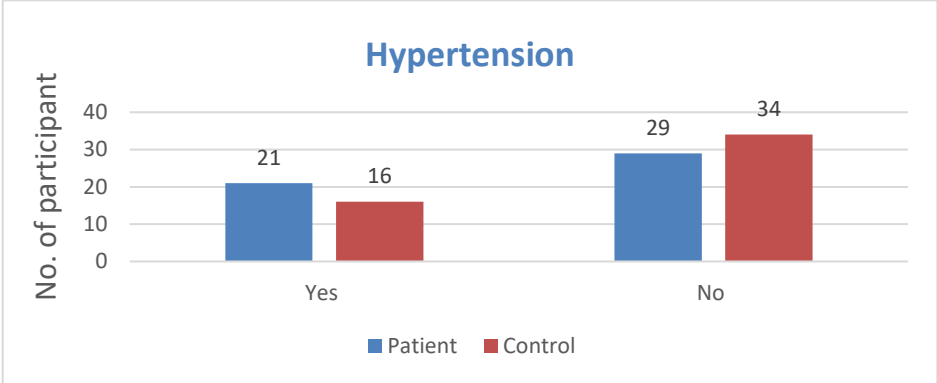


Figure 3.4: Baseline characteristics and Demographic Descriptive of the study population in patients compared to control group, the number of participant (n= 100) for distribution of Hypertension

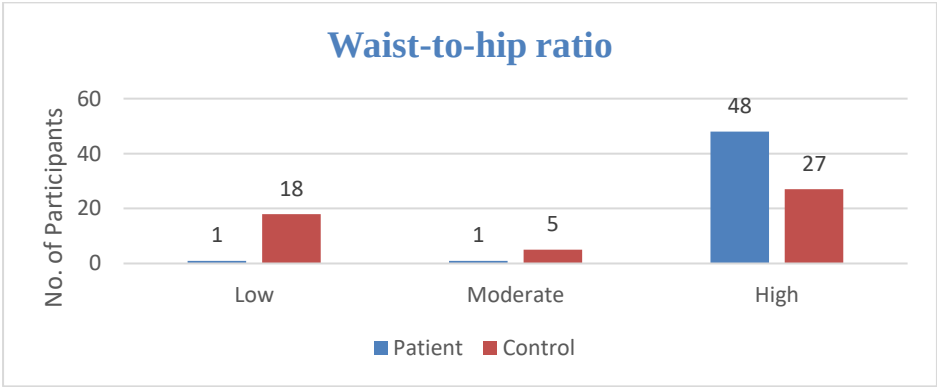


Figure 3.5: Baseline characteristics and Demographic Descriptive of the study population in patients compared to control group, the number of participant (n= 100) for distribution of HWR

Table 1: Illustreated that effect of the biochemical parameters in the Patients and control groups according to the Age groups. The level of Nesfatin.1 was not showing any significant differences in patients group with Age range (30-42) year compared to control.

The level of C.peptide was decreased significantly in patients group with Age range (30-42) year compared to control, the mean level were 113.49±27.5, 159.2±101.4 respectively. While the level of RBS and HbA1c were increased markedly in patients group with same Age range compared to control. The mean level of RBS and HbA1c in patients were (258.58±41.4, 9.36±1.89) while in control were (98.1±12.53 and 4.33±0.71). While, in the lipid profile only level of cholesterol was significantly association compared to control.

Table 3.1:The effect of Age range (30-42) year on the biochemical parameters in Patients and control groups.

Biomarker	Age range (30-42) year		
	Patients Mean±SD N=19	**Control Mean±SD N=35**	**P value**
Nesfatin.1 ng/dl	56.68±18.8	84.95±80.5	0.13[NS]
C. peptide nmol/l	113.49±27.5	159.2±101.4	**0.01[S]**
RBS mg/dl	258.58±41.4	98.1±12.53	**<0.001[S]**
HbA1c%	9.36±1.89	4.33±0.71	**<0.001[S]**
TG mg/dl	171.74±34.8	157.23±86.0	0.38[NS]
Chol mg/dl	196.74±36.6	173.80±33.9	**0.02[S]**

HDL mg/dl	45.09±5.31	46.17±5.47	0.48[NS]
LDL mg/dl	157.53±34.3	171.70±39.8	0.19[NS]
T-test was *: significant at p ≤ 0.05 N: number of cases; SD: standard deviation; S: significant; NS= Non-significant.			

In Age group (**43-54**), results were indicated that , there was a no-statistically difference in the levels of Nesfatin.1 and C.Peptide , there was a statistically difference in the levels of RBC , HbA1c and W.H.R , the mean±SD level were increased in patients group compared to control, while only cholesterol level was increased in same age range of patients compared to control as shown in Table (2)

Table 3.2: The effect of Age range (43-54) year on the biochemical parameters in Patients and control groups.

Biomarker	**Age range (43-54) year**		
	Patients Mean±SD N=13	**Control Mean±SD N=12**	**P value**
Nesfatin.1 ng/dl	61.97±35.61	72.17±16.79	0.37[NS]
C. Peptide nmol/l	151.59±112.1	164.47±61.92	0.72[NS]
RBS mg/dl	290.77±77.94	101.83±11.15	**<0.001[S]**
HbA1c%	9.99±2.08	4.57±0.74	**<0.001[S]**
TG mg/dl	180.23±34.38	170.17±109.18	0.75[NS]
Chol mg/dl	192.46±32.85	163.33±33.28	**0.038[S]**
HDL mg/dl	45.16±4.16	45.83±3.71	0.67[NS]
LDL mg/dl	174.86±29.27	161.43±22.13	0.21[NS]
T-test was *: significant at p ≤ 0.05 N: number of cases; SD: standard deviation; S: significant; NS= Non significant.			

While in the in-age group (55-76), C.Peptide marker was shown a significant difference among patient compared to control. Level of C.Peptide was decreased significantly in patient Age range group (55 –76) years compared to control, as shown in Table (3.3). RBS and HbA1c were incresed significantly in patients group compared to health control. The mean levels in patients were 29.34±7.11, 267.83±77.75 and 9.32±2.17, while in control 19.52±7.11, 101.67±22.50 and 3.83±0.51 respectively, P value <0.05.

While, in the lipid profile only level of triglyceride was significantly association compared to control.

Table 3.3: The effect of Age range (55 – 76) years on the biochemical parameters in Patients and control groups.

Biomarker	Age range (55 – 76)years		
	Patients Mean±SD N=18	**Control Mean±SD N=3**	**P value**
Nesfatin.1ng/dl	59.59±26.60	78.70±19.33	0.25[NS]
C. Peptide nmol/l	107.55±18.25	147.10±47.13	0.013[S]
RBS mg/dl	267.83±77.75	101.67±22.50	**0.002[S]**
HbA1c%	9.32±2.17	3.83±0.51	**<0.001[S]**
TG mg/dl	179.67±28.57	107.00±36.43	**<0.001[S]**
Chol mg/dl	194.94±22.05	172.00±12.17	0.09[NS]
HDL mg/dl	45.49±4.10	44.86±4.12	0.81[NS]
LDL mg/dl	187.70±43.5	165.93±54.5	0.44[NS]
T-test was *: significant at p ≤ 0.05 N: number of cases; SD: standard deviation; S: significant; NS= Non significant.			

Table 3.4 illustrated that effect of type 2 diabetes non-obese women (N= 25) on some biochemical parameters studied in patients and apparently health control groups (N= 25). The mean ± SD observed in non-obese type 2 diabetic women include Nesfatin-1 levels which was non-significantly decreased in type 2 diabetic patients as compared with apparently non-obese healthy control, while the level of C-peptide was significantly decreased in non-obese type 2 diabetic patients as compared with apparently non-obese healthy control. The level of random blood glucose and and HbA1c% were significantly increased markedly in non-obese type 2 diabetic patients group as compared with apparently healthy control group respectively.

The mean ± SD of lipid profile levels shown in the same table including total cholesterol (TC), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C) and triglycerides (TG) which were studies in each type 2 diabetic patients group and healthy control. The data observed indicated that each of total cholesterol and triglycerides were significantly increased as compared with apparently non-obese healthy control group respectively ($P < 0.05$), while the HDL-C and LDL-C were non-significantly changes, $p = 0.73$ and $p = 0.94$ respectively.

Table 3.4: The biochemical parameters studied in non-obese T2DM and control groups.

Variables	(Non-obese) Samples, N = 50		
	T2DM Patients Mean ± SD N = 25	Control Mean ± SD N = 25	P value
Nesfatin-1, ng/dl	67.42 ± 33.49	87.54 ± 68.09	0.19[NS]
C- Peptide, nmol/l	107.82 ± 21.23	176.44 ± 111.53	**0.004[S]**
RBG, mg/dl	279.76 ± 59.33	98.04 ± 13.74	**<0.001[S]**
HbA1c%	9.72 ± 2.20	4.13 ± 0.62	**<0.001[S]**
TG, mg/dl	185.28 ± 37.99	109.36 ± 20.78	**<0.001[S]**
TC, mg/dl	184.44 ± 19.66	164.36 ± 29.60	**0.007[S]**
HDL-C, mg/dl	45.02±3.42	44.71 ± 2.93	0.73[NS]
LDL-C, mg/dl	191.54±33.84	176.38 ± 28.76	0.94[NS]
T-test was *: significant at p ≤ 0.05 N: number of cases; SD: standard deviation; S: significant; NS= Non-significant.			

RBG, Random blood glucose; HbA1c, Glycated hemoglobin; TG, Triglyceride; TC, Total cholesterol; HDL-C, High density lipoprotein-cholesterol; LDL-C, Low density lipoprotein-cholesterol

Table (3.5) illustrated that effect of obese type 2 diabetes women (N = 25) on the same biochemical parameters studied and compared with control groups (N = 25). The mean ± SD results obtained indicated that Nesfatin-1 levels was significantly decreased in obese type 2 diabetic patients as compared with apparently obese healthy control ,and the level of C-peptide was non-significantly decreased in type 2 diabetic patients as compared with apparently obese healthy control. While the level of random blood glucose and HbA1c% were significantly increased in obese type 2 diabetic patients group as compared with apparently obese health control groups respectively.

The mean ± SD of lipid profile levels shown in the same table including also total cholesterol (TC), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C) and triglycerides (TG) which were studies in each

type 2 diabetic patients group and healthy control. The data observed indicated that each of total cholesterol was significantly increased, as compared with apparently obese healthy control group, while the mean ± SD of triglyceride was non-significantly decreased as compared to apparently obese health, while HDL-C and LDL-C were non-significantly decreased as compared with obese control group respectively.

Table 3.5: The biochemical parameters studied in obese T2DM and control groups.

Variables	(Obese) Samples, N = 50		
	T2DM Patients Mean ± SD N = 25	**Control Mean ± SD N = 25**	**P value**
Nesfatin-1, ng/dl	50.79 ± 12.08	75.48 ± 68.53	**0.007[S]**
C- Peptide, nmol/l	134.69 ± 83.39	143.11 ± 59.49	0.68[NS]
RBG, mg/dl	260.80 ± 72.38	100.48 ± 11.64	**<0.001[S]**
HbA1c%	9.30 ± 1.85	4.58 ± 0.75	**<0.001[S]**
TG, mg/dl	168.32 ± 22.83	205.28 ± 105.97	0.10[NS]
TC, mg/dl	205.52 ± 35.74	178.00±34.95	**0.008[S]**
HDL-C, mg/dl	45.48 ± 5.47	47.31 ± 6.18	0.27[NS]
LDL-C, mg/dl	154.25 ± 33.76	161.40 ± 42.62	0.51[NS]
T-test was *: significant at p ≤ 0.05 N: number of cases; SD: standard deviation; S: significant; NS= Non-significant.			

RBG, Random blood glucose; HbA1c, Glycated hemoglobin; TG, Triglyceride; TC, Total cholesterol; HDL-C, High density lipoprotein-cholesterol; LDL-C, Low density lipoprotein cholesterol

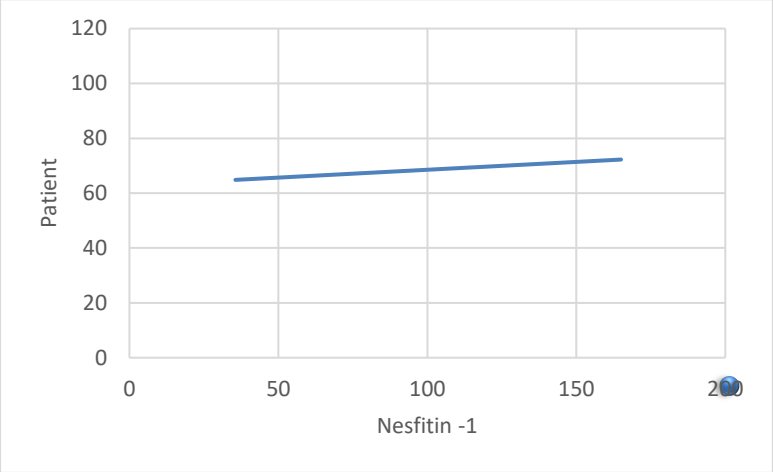


Figure 3.6: Simple linear regression of Nesfatin.1 between control and patient groups

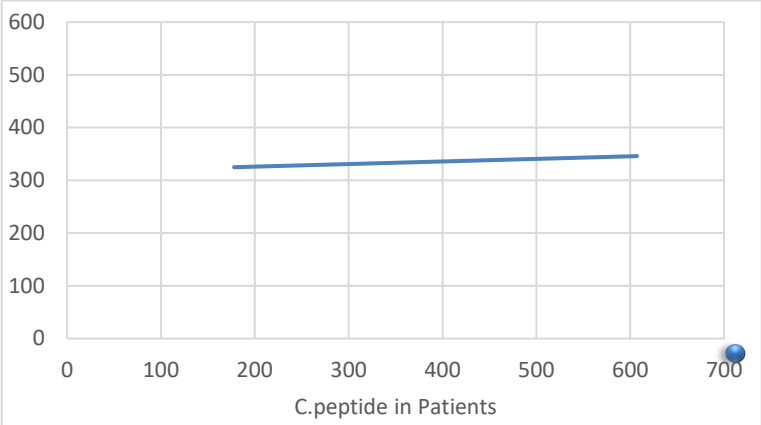


Figure 3.7: Simple linear regression of C. Peptide 1 between control and patient groups

Binary logistic regression was performed and forward logistic regression was adopted to analyze the results. It was found that Nesfatin-1 in non-obese type 2 diabetic patient has a protective factor (OR: 0.987; 95% CI: (0.960-1.015) respectively, while in obese type 2diabetic women patients groups were independent risk factors (OR: 1.064; 95% CI: (1.011-1.119).

In addition, C-Peptide was shown to be a risk factor for T2DM in normal and obese cases (OR: 1.058 and 1.02, 95% CI: (1.021-1.097), 0.992-1.08) as shown in Table 3.6.

Table 3.6: Estimation the Associated of analyzed factors in Patients Compared to control group

Variables	Normal weight		Obese	
	T2DM	Control	T2DM	Control
	OR (Lower–Upper)	P value	OR (Lower–Upper)	P value
Nesfatin-1, ng/dl	0.987(0.960-1.015)	0.37[NS]	1.064(1.011-1.119)	**0.01[S]**
C- Peptide, nmol/l	1.058(1.021-1.097)	0.002[S]	1.0200(0.992-1.08)	0.94[NS]
TG, mg/dl	0.891(0.819-0.969)	**0.007[S]**	1.005 (0.996-1.013)	0.29[NS]
TC, mg/dl	0.969(0.897-1.047)	0.42[NS]	0.974 (0.953-0.996)	**0.023[S]**
HDL-C, mg/dl	0.986(0.596-1.630)	0.95[NS]	1.005 (0.987-1.023)	0.57[NS]
LDL-C, mg/dl	0.898(0.807-0.998)	**0.04[S]**	1.066 (0.956-1.189)	0.24[NS]
p<0.05 considered significantly different, [S]= Significant, [NS]= Non significant 1ª : reference category is Control				

The correlation coefficient was used for determining linear relationships between Nesfatin-1 and obese T2DM and non-obese T2DM, with each of BMI, C-Peptide, RBG and HbA1c% in obese T2DM groups as compared with Non-obese T2DM group. The results showed that there was moderated relationship and a significant correlation between Nesfatin-1 and BMI (p = 0.015, r=0.4), C-peptide (p=0.015, r=-0.4), RBG (p=0.007, r=-0.4), and HbA1c% (p=0.004, r=-0.42) as shown in figure (8,9,10,11).

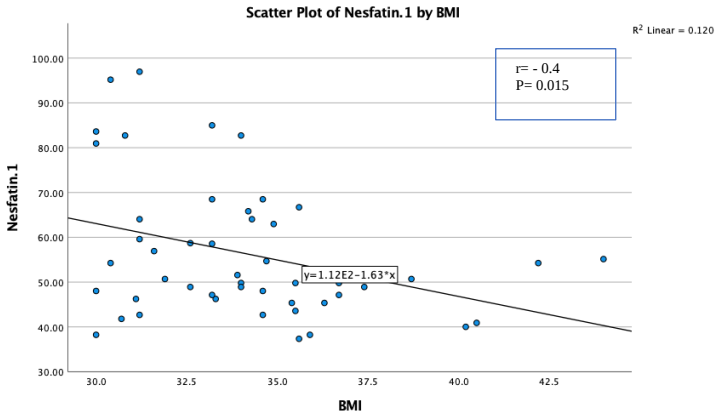


Figure 3.8: Simple linear regression of Nesfatin.1 by BMI between obese (DM & non DM)

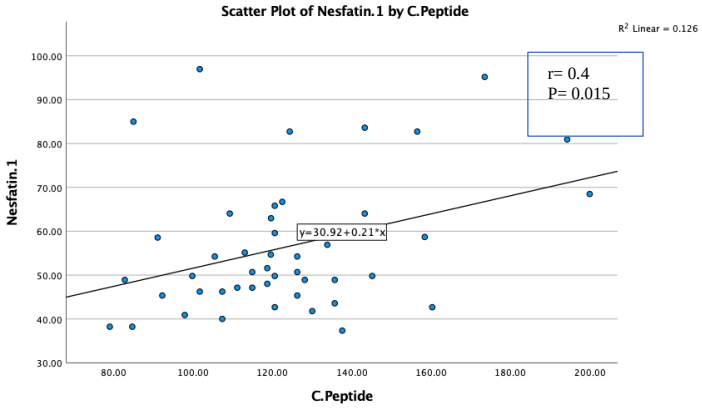


Figure 3.9: Simple linear regression of Nesfatin.1 by C. peptide between obese (DM & non DM)

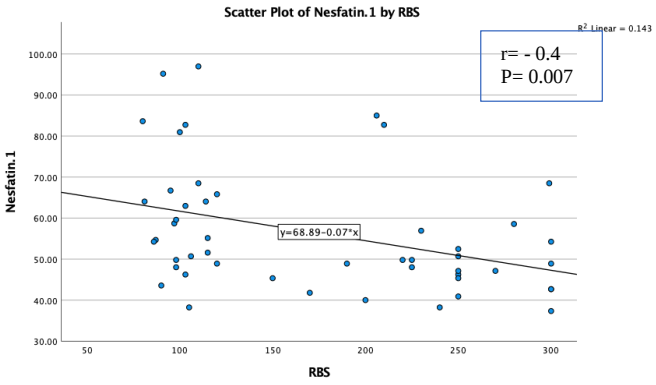


Figure 3.10: Simple linear regression of Nesfatin.1 by RBs between obese (DM & non DM)

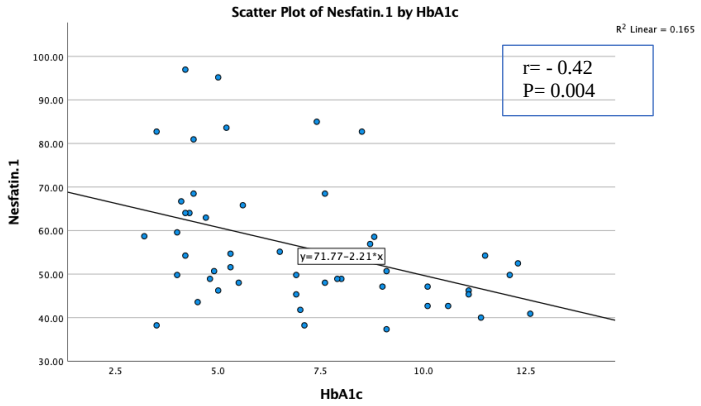


Figure 3.11: Simple linear regression of Nesfatin.1 by HbA1c between obese (DM & non DM)

Serum nesfatin-1 and c-peptide level were measured in the four groups of patients and Healthy control. According to the results, the mean level of serum nesfatin-1 and c-peptide were significantly decreased in obese and non-obese patients group compared to matched groups of control. The mean serum nesfatin-1 and c-peptide levels were differed between the studied groups as shown in Table (3.7) and Figure (8) below, P <0.001).

Table 3.7: Multiple Comparisons of Dependent Variable for Nesfitin-1 & C-peptide level with groups

Biochemical Parameters	(N=100)				P value
	Group 1 Obese Patients N=25	Group 2 Non-Obese Patients N=25	Group 3 Obese control N=25	Group 4 Non-Obese control N=25	
Nesfitin-1 ng/dl	50.79±12.08	67.42±33.49	62.13±15.81	74.93±26.34	**0.005[S]**
C. Peptide nmol/l	118.83±26.3	107.82±21.2	127.66±25.64	135.85±26.86	**0.002[S]**
ANOVA was *: significant at p ≤ 0.05 N: number of cases; SD: standard deviation; S: significant; NS= Non significant					

The results of the receiver operating curve (ROC) and AUC analysis for the Nesfitin-1 and C-peptide (73 %, 71.6%) respectively, as possible diagnostic parameters. Nesfitin-1 was shown a good diagnostic performance for prediction of T2DM patients compared to control group, data are presented in Figure (12) and Table (8). For Nesfitin-1 (sensitivity = 98%, specificity = 90%) at a level = 39.13, while C-peptide levels: (sensitivity = 98%, specificity = 94%) at a level = 15.99, both Nesfitin-1 and C-peptide p values of the AUC for were <0.001 and statistically significant.

Table 3.8: Receiver operating characteristic curve showing sensitivity and specificity of Nesfitin-1 & C-peptide in patients compared to control group

Test Result Variable(s)	Nesfatin-1	C-peptide
AUC	73%	71.6%
Sensitivity %	98%	98%
Specificity %	90%	94%
Youden index	0.369	0.346
Cut-off points	39.13	15.99
CI (95%)	0.624-0.835	0.616-0.816
P value	<0.001	<0.001

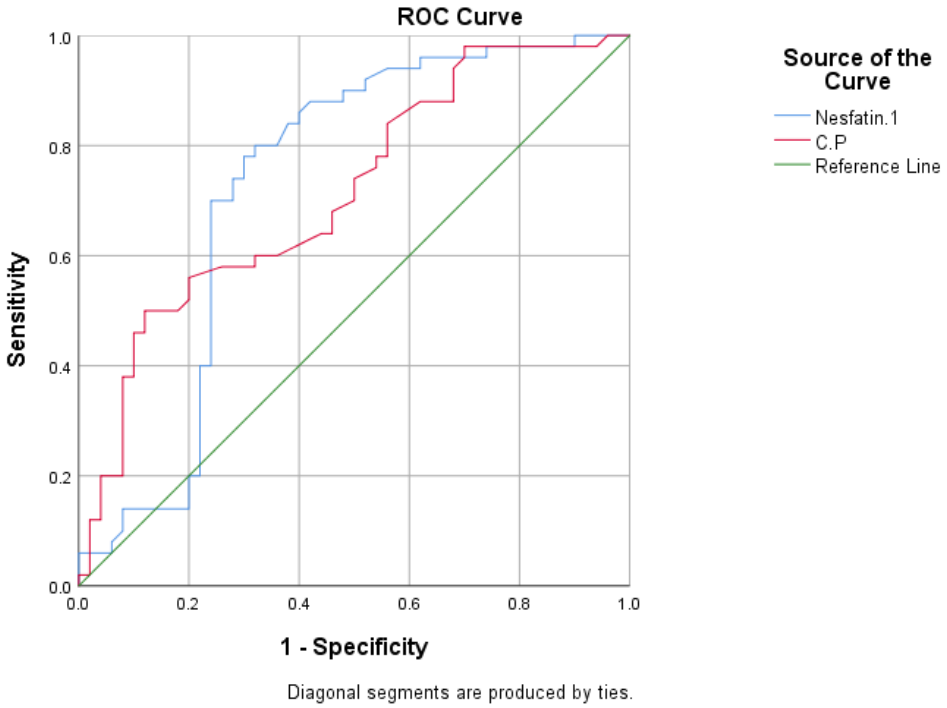


Figure 3.12: ROC curves for Nesfitin-1 & C-peptide in patients to analyses the optimal diagnostic points for predicting of cases compared to control group.

3.2 Results of genetic variants of Nucleobindin-2

The results of variants Genotype (rs 757081) was a clear band with a molecular size 400 bps. (figure 13). The size of amplicon was determined by compare with DNA ladder 100 - 1500 bp. gene polymorphisms

Figure 3.13: The horizontal agarose gel electrophoreses (1.5% w/v) of amplification refractory mutation system-polymerase chain reaction to detect of Nucleobindin-2 rs 757081; C>G SNP. M: 400 bp DNA marker. Lanes 3 and 7 show the wild type (CC), lane 1,4,5 shows the heterozygous mutant type (CG) and lanes 2,6,8 show the homozygous mutant type (GG).

Genotype variants of Nucleobindin-2 of gene (rs 757081) SNP which were classified into three genotypes:

1. The major genotype group (CC) homozygous for the allele C.
2. The minor genotype group (GG) homozygous for the allele G.
3. Heterozygous (CG).

The distribution of genotyping groups of patients shows in table (3.9).

Table 3.9: Distribution of gene variants of Nucleobindin-2 Genotype rs (757081) different genotypes in studied groups

Variable	Group	Frequency	Percentage
Genotype	CC (wild)	8	16
	CG (hetero)	16	32
	GG (homo)	26	52
Data Presented by numbers and percentage			

Result of comparison between observed and anticipated values for SNIP with (rs 757081) in the tested population were shown in Figure (14), and Table (10). The distribution and percentage of individuals having **rs (757081)** differ from those expected under Hardy–Weinberg equilibrium, number of observed vs expected, which were: CC (8, 5.9); GG (26, 22.9); CG (16, 22.2) (goodness-of-fit $\chi 2$ for (rs 757081), 7.637, P= 0.022 and therefore it was statistically significant.

Table 3.10: Hardy–Weinberg equilibrium for (rs 757081**) genotype in studied groups**

Genotypes			Alleles		Hardy–Weinberg equilibrium X^2 test
			C	G	
Genotype N= 100	Frequency	%	0.33	0.67	7.637 P < **0.001** [S]
CC (Wild Type)	8	16			
CG (heterozygous mutant type)	16	32			
GG (homozygous mutant type)	26	52			

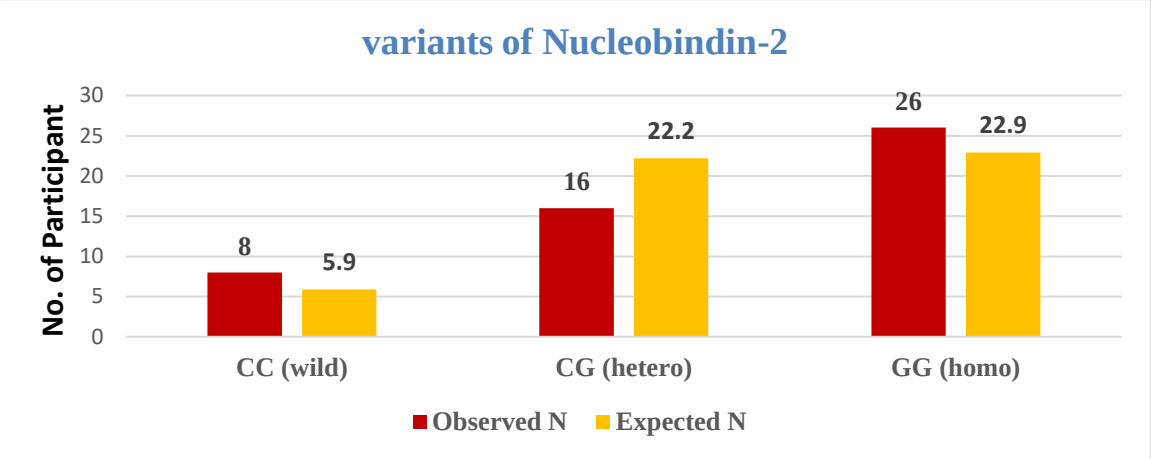


Figure 3.14: variants of Nucleobindin-2 Observed (Obs.) vs expected (Exp.) genotype frequencies % of rs 757081 among individuals' sample

Relationship between demographic characteristics and (rs 757081) SNP

The difference between demographic characteristics and **rs (757081)** SNP (table 11), was performed using one-way ANOVA test to compare the age, BMI and groups of study. No significant difference was found between all groups.

Table 3.11: difference between demographic characteristic in (rs 757081) SNP in studied groups

Demographic parameters		rs 757081 (N= 50)			P-Value
		CC(N=8) Mean±SD	CG(N=16) Mean±SD	GG(N=26) Mean±SD	
Age		46.12±10.69	46.34±11.18	42.96±10.76	0.320 [NS]
BMI		30.04±6.52	27.39±6.09	28.74±6.04	0.340[NS]
Group	Patient	4	7	12	0.903[NS]
	Control	4	9	14	
Study Group	Patient with obese	3	3	7	0.627[NS]
	Patient without obese	1	4	7	
	Control with obese	2	4	7	
	Control without obese	2	5	5	
Results are presented as mean ± SD, or n= number of subjects and percentage, p<0.05 considered significantly different, [S]= Significant, [NS]= non-significant					

Mean Difference of biomarkers and Genotype (rs 757081) SNP in Obese groups:

The difference between biomarkers and **rs (757081)** SNP (table 12) was performed using one-way ANOVA test to compare the mean levels of Nesfatin-1, C. Peptide RBS, HbA1c and Lipid profile panel. Only Nesfatin-1 levels were shown a significant difference among the variants of Nucleobindin-2 Genotype (rs 757081) in obese (Patients & Control) studied groups, p value < 0.05. No further significant difference was found between mean levels of biomarkers and alleles variants (p > 0.05).

Table 3.12: Difference between alleles of variants of Nucleobindin-2 Genotype (rs 757081) with mean levels of biomarkers in obese studied groups

Biomarkers	rs 757081 (N= 50)			P value
	CC(N=8) Mean±SD	CG(N=16) Mean±SD	GG(N=26) Mean±SD	
Nesfatin.1 ng/dl	46.61±8.37	61.77±13.64	57.53±16.19	**0.048** [S]
C. Peptide nmol/l	114.55±16.37	131.41±23.16	122.96±29.71	0.342 [NS]
RBS mg/dl	186.10±79.32	165.83±76.41	170.57±82.49	0.826 [NS]
HbA1c%	7.39±3.05	7.09±2.80	6.71±2.73	0.790 [NS]
W.H.R	1.03±0.03	1.05±0.04	1.04±0.05	0.610 [NS]
TG mg/dl	180.00±68.37	203.17±100.28	182.21±72.55	0.713 [NS]
Chol mg/dl	214.10±50.69	190.00±24.32	184.54±35.13	0.10 [NS]
HDL mg/dl	48.62±6.34	43.32±4.98	46.93±5.68	0.788 [NS]
LDL mg/dl	153.36±54.16	153.68±37.76	161.19±32.64	0.08 [NS]

Chapter Four
Discussion

4. Discussion:

According to figure 1; The findings of this results were similar with other studies conducted in other middle east and even in some western countries. A study in Lebanon found the prevalence of normal weight and obesity were 14% and 17% among the population. In Kuwait the overweight and obesity prevalence were found to be 32% and 8.9% respectively. Whereas in the United States and United Arab Emirates the prevalence of overweight and obesity were 35% among the college students (***Musaiger, 2003***).

Nesfatin-1 is reported to exert an antihyperglycemic effect under impaired glucose metabolism conditions**(Su *et al.,* 2010)**. It may also act in the brain to upregulate Sugar sensitivity**(M. Yang *et al.,* 2012)**.and increase insulin release in beta cells in response to hyperglycemia**(Nakata *et al.,* 2011)** .Nesfatin-1 was also found to inhibit food intake in the central nervous system**(Oh-I *et al.,* 2006)**, but the regulatory mechanism remains unclear. Since nesfatin-1 can cross the brain-blood-barrier**(Pan *et al.,* 2007)**, and hypothalamic nesfatin-1 can significantly inhibit food intake, Li et al. proposed that diabetic polyphagia is caused by decreased circulating nesfatin-1 levels**(Li *et al.,* 2010)**. Studies have also shown that nesfatin-1 can stimulate the lipid metabolism and exhibit anti-inflammatory effects**(Dong *et al.,* 2013)**. Li et al. reported that fasting plasma levels of nesfatin-1 were positively correlated with age **(Li *et al.,* 2010)**.

It has been suggested that low level of serum NES-1 in T2DM patients might be one of the factors causing diabetic because it has been reported that NES-1 has a reducing effect on food intake.

Thus, it acts as an anorexigenic agent, but the regulatory mechanism remains not clear**(Oh-I *et al.,* 2006)**. Other reported that decreased serum nesfatin-1 level among

pre-diabetic and diabetic may be a cause or consequent of IR and hyperinsulinemia. Nesfatin-1 may improve both hepatic and peripheral insulin sensitivity as it enhances glucose uptake by peripheral tissues and inhibits gluconeogenesis via different pathways**(Wu *et al.*, 2014)** .

Moreover, nesfatin-1 mRNA is co-localized almost completely with insulin in β pancreatic islets cells. Also, its processing physiologically occurs in pancreatic islet cell **(Mohan *et al.*, 2016)**. Nesfatin-1 mRNA expressed on pancreatic islet cells from type 2 diabetic patients was lower than that from healthy subjects. This was significantly correlated with insulin secretion capability**(Riva *et al.*, 2011)**.

On the other hand, the role of C-peptide is not well defined in type 2 diabetes, of which insulin resistance and insulin secretion defect both exist. Serum C-peptide had been used in assessing pancreatic function in DM patients for a long time. Recently, several studies started investigating the clinical effect of residual beta cell function in T1DM patients and tried to elucidate the natural course of C-peptide levels in these patients,several recent studies presented a slow decline of serum C-peptide levels and preservation of the insulin secreting capacity **(Luppi *et al.*, 2020)**,**(Grönberg *et al.*, 2020)**. Previously, a preliminary study conducted with a small number of T1DM patients and showed a slight increase in serum C-peptide levels after diagnosis followed by significant decrease 5 years later**(Lee *et al.*, 2013)**.However, the results of this study demonstrate a continuous decline in serum C-peptide levels following diagnosis and an especially significant decline from baseline after 3 years. Several hypotheses are presented to explain the clinical implications of residual C-peptide secretion in T1DM. Some previous studies suggested that C-peptide acts as an endogenous antioxidant, which protects pancreatic beta cells by increasing catalase expression and reducing peroxisomal oxidative stress **(Luppi *et al.*, 2020)**,**(Luppi *et al.*,2017)**,**(Luppi *et al.*,2014*)***.

Additionally, **Thivolet *et al*.** reported an association between residual C-peptide levels and reduction in response to glucagon-like peptide-1 (GLP-1) **(Thivolet *et al.*, 2019)**. Although the mechanism is not clearly elucidated yet, the clinical benefit from preserved C-peptide secretion in DM patients is widely known. Microvascular complications such as diabetic retinopathy and nephropathy were found to be less likely to develop in patients with residual C-peptide production **(Lachin *et al.*, 2014)**, but the results were not always consistent among studies. Also, preserved beta cell function is reported to be related to a decreased risk of hypoglycemia and decreased insulin requirement**(Lam *et al.*, 2021)**. Moreover, with the wide use of continuous glucose monitoring (CGM) systems, recent studies reported on the importance of glycemic variability and its association with C-peptide levels. Patients with residual C-peptide production had a lower mean blood glucose level and higher time in range **(Rickels *et al.*, 2020),(Robertson RP *et al.*,2014).** And fasting C-peptide levels were negatively correlated with glucose coefficient of variation by CGM **(Babaya *et al.*, 2021)** .

Lipid abnormalities are prevalent in DM patients because of IR which affects key enzymes and pathways in lipid metabolism: Apo protein production, regulation of lipoprotein lipase, action of cholesterol ester transfer proteins and hepatic and peripheral actions of insulin **(Gudbjartsson *et al.*, 2019)** .

Hyperglycemia and the high level of IR associated with T2DM has multiple effects on fat metabolism which results in the production of atherogenic dyslipidemia characterized by lipoprotein abnormalities: elevated very low density lipoprotein (VLDL) elevated low density lipoprotein cholesterol (LDL-c), elevated triacylglycerol (TAG) and decreased high density lipoprotein cholesterol (HDL-c) **(Beshara *et al.*, 2016)**. The main cause for lipid abnormalities in T2DM patients is impaired secretion of insulin that affects the liver apolipoprotein production and regulates the enzymatic activity of lipoprotein lipase (LpL) and cholesterol ester

transport protein (CETP). Moreover, its deficiency reduces the activity of hepatic lipase; therefore, several steps involved in the production of biologically active LpL might be altered in T2DM compared to controls**(Rye *et al.,* 2016)**.

Many studies were confirmed the association of Nesfatin-1 levels in obese individuals. It has been reported that the expression of nesfatin-1gene was up-regulated in adipose tissue of a high-fat diet. This suggests a potential role for nesfatin-1 in the lipid accumulation pathway and perhaps diet-induced obesity **(Inhoff T *et al.,* 2010)**, **(Roa *et al.,* 2009).**

They were moderately positively correlated, although not linear. Hence this study suggests that obese patients are hyperinsulinemic. a study which analyzed the insulin secretion in Asian Indians. That study also observed lesser insulin levels in non-obese compared to obese. Also noted by Andrea Tura et al**(Tura *et al.,* 2001)**,in which they measured insulin and C-peptide levels during a three hour oral glucose tolerance test. A positive correlation between BMI and basal serum c-peptide levels was also observed by S.W. Park et al**(Park *et al.,* 1997)**.According to Banerji et al, Asian Indians have an unexpectedly high percentage of body fat relative to BMI and muscle mass; this is associated with a proportionate increase in visceral fat. They are markedly insulin resistant and hyperinsulinemic.

Obesity is caused by excessive intake of nutrients continuously causing fat deposits to become excessive. Deposits of fatty acids in the form of triacylglycerol contained in adiposity cells can protect the body from the toxic effects of fatty acids. Free-form fatty acids can circulate in blood vessels throughout the body and cause oxidative stress which the familiar with lipo-toxicity. The emergence of lipo-toxicity effects caused by several free fatty acids released by the triacylglycerol to compensate for the destruction of excessive fat deposits affects the adipose and non-adipose tissue

and plays a role in the pathophysiology of diseases in various organs such as the liver and pancreas. This release of free fatty acids from excessive triacylglycerol can also inhibit fat synthesis and reduce the clearance of triacylglycerol. This can increase the tendency of hypertriglyceridemia. The release of free fatty acids by endothelial lipoprotein lipase from triglycerides which increases in the increase of lipoprotein β causes lipo-toxicity which also interferes with the function of insulin receptors. The consequence of insulin resistance is hyperglycemia, which is compensated by glucose synthesis from the liver (gluconeogenesis), which contributes to aggravating hyperglycemia. Free fatty acids also contribute to hyperglycemia by reducing glucose use from insulin-stimulated muscles. Lipotoxicity due to excess free fatty acids also decreases insulin secretion from pancreatic β cells, which ultimately β cells will experience fatigue**(H. A. Silitonga *et al.*, 2019)**.

Diabetes is associated with the development of IR. With multiple indices available, examination the validity of the (nesfitin-1) and C-peptide to determine DM by receiver operating characteristic analysis was performed.

Nesfitin-1 was shown a good diagnostic performance for prediction Patients compared to control group. For Nesfitin-1 (sensitivity = 98%, specificity = 90%) at a level = 39.13, while c-peptide levels: (sensitivity = 98%, specificity = 94%) at a level = 0.15.99, both Nesfitin-1 & c-peptide p-values of the AUC were <0.001 and statistically significant.

Since Metabolic Syndrome (MetS) is associated with elevated risk for developing diabetes, many studies were investigating the insulin resistance and many types of Adipokines.

A key component of MetS is the development of insulin resistance (IR). The homeostatic model assessment (HOMA) can determine IR by using insulin or C-

peptide concentrations; however, the efficiency of adipokines (nesfitin-1) and C-peptide to determine Metabolic Syndrome/ or their complication namely diabetes has not been compared. C-peptide is a strong indicator of Metabolic Syndrome. Since C-peptide has recently emerged as a biomolecule with significant importance for inflammatory diseases, monitoring C-peptide levels will aid clinicians in preventing Metabolic Syndrome**(Gonzalez-Mejia *et al.,* 2016)**.Also, Serum nesfatin-1 is possibly associated with weight-related abnormalities in otherwise healthy subjects and diabetes type 2. Obesity and diabetes type 2 may share a common pathologic point in this regard **(Samani *et al.,* 2019)**.

This study was carried out to test the hypothesis that variants in NUCB2 influenced susceptibility to obesity

Generally, Nesfatin-1/NUCB2 plays a key role in the regulation of glucose metabolism. Nesfatin-1 stimulated glucose-induced insulin secretion**(Ademoglu *et al.,* 2017)**. Riva et al. reported that nesfatin-1 also enhanced glucagon secretion. On the other hand, nesfatin-1 caused a small increase in insulin secretion and reduced glucose during intravenous glucose tolerance test in **(Riva *et al.,* 2011)**. Both NUCB2 expression and nesfatin-1 levels were changed during diabetic condition. Recently, the C>G polymorphism of NUCB2 gene was found to be correlated with obesity**(Chen *et al.,* 2013)** . Obesity is a risk factor of developing T2DM. Therefore, a hypothesized that the C>G polymorphism of NUCB2 gene may be also correlated with the risk of developing T2DM. results of our study (Table 8) were not shown any significant effect of the demographic characteristic on the distribution of NUCB2 gene and their phenotypes.

Elucidation of the genetic pathways that influence the risk to T2DM could provide a better understanding of the pathophysiology of the disease and identify possible pharmacologic targets for its treatment.

The distribution and mean level of nesfatin-1 among the polymorphisms of Nucleobindin-2 was significantly different between in obese studied groups. Accumulating evidence has revealed that NUCB2 is not only a novel satiety factor widely expressed in the CNS but is also expressed in peripheral tissues where it regulates glucose and energy metabolism. Recently, it has been reported that infusion of nesfatin-1 into the third cerebral ventricle markedly promotes muscle glucose uptake, inhibits hepatic glucose production, and inhibits hepatic phosphoenolpyruvate carboxykinase (PEPCK) mRNA and protein. This infusion also inhibits enzymatic activity of PEPCK in control and diet-induced obese group**(M. Yang *et al.*, 2012)**. Another study showed that insulin and high glucose levels activate paraventricular nucleus (PVN) nesfatin-1 and proopiomelanocortin (POMC) neurons **(Gantulga *et al.*, 2012)**, suggesting that nesfatin-1 neurons cooperate with melanocortin neurons in the regulation of glucose metabolism. Besides the extensive distribution in the CNS, NUCB2 is also expressed in the periphery, including the stomach, pancreas, testis, and adipose tissues**(J. Kim *et al.*, 2014)**.

Indeed, NUCB2 is co-localized almost exclusively with insulin in the beta-cells of pancreatic islets**(Mohan *et al.*, 2016)** ,**(Riva *et al.*, 2011)**. A recent study showed that beta-cell-specific NUCB2 knockout mice have elevated blood glucose levels and reduced insulin secretion **(Luppi *et al.*, 2020)**,**(Y. Yang *et al.*, 2019)**. Besides, plasma nesfatin-1 concentrations and islet NUCB2 mRNA are significantly decreased in T2DM patients compared with healthy controls**(Liu *et al.*, 2014)**.These previous findings indicate that NUCB2 plays a significant role in the development of T2DM. Similarly,

results indicated that the five tag SNPs of NUCB2 are associated with an increased risk for T2DM. Thus, dysfunction of expression, secretion, and/or action of NUCB2 might be involved in the development and progression of T2DM.

The findings of previous studies report that NUCB2 polymorphisms are associated with obesity in adults and children. It has been shown that SNPs rs757081 are associated with BMI, weight, and fat-free mass in Caucasian males. The C>G polymorphism of the NUCB2 gene (rs757081) is correlated with childhood adiposity**(Chen *et al.*, 2013)**. However, the correlation between NUCB2 polymorphisms and the pathogenesis of T2DM is obscure.

Since obesity is one of the risk factors for DM disease, the variations of Nesfatin-1 gene may play a role in pathogenesis**(Riva *et al.*, 2011)**. The variations that affect the Nesfatin-1 protein functions could be important in affecting lipid levels and development of complications as presented in Table 10. There are many studies regarding the association of Nesfatin-1 gene variations with the obesity, childhood adiposity, metabolic syndrome, type2 diabetes**(Chen *et al.*, 2013)**.Our results indicated that homogenous GG allele and heterogenous CG allele of Nucleobindin-2 Genotype (rs 757081) were significantly have a protective effect on the cholesterol and HDL level among obese patients with DM. Chen Y.Y. et al. has independently demonstrated the association of this allele with adiposity in childhood, providing further support for the role of NUCB2 and its genetic variant in the determination of human adiposity. These results showed that the NUCB2 variant C>G was associated with adiposity. They postulated that the less frequent GG genotype could be viewed as protective against excessive adiposity gain, and factors which predispose to excessive weight gain**(W. Kim *et al.*, 2008)**.

The odds ratios of the detected genotypes of the (rs **757081**) of the patients with levels of biomarkers. The logistic analysis of the (rs **757081**) SNP in Table 11 shows that Nesfatin.1 was a significantly risk factor for both heteogenuos and homogenous Nucleobindin-2 Genotype (rs 757081), that was supported by previous study who confirmed that nesfatin-1 is an insulinotropic peptide and that endogenous pancreatic nesfatin-1 is altered in diabetes and obesity. It found that short-term exposure of nesfatin-1 enhances GSIS from isolated islets and cultured MIN6 cells. These findings provide clear evidence that nesfatin-1 directly acts on the β-cells to stimulate insulin secretion. In addition, nesfatin-1 also stimulated preproinsulin mRNA expression in MIN6 cells, suggesting that this novel insulinotropic peptide could also influence insulin synthesis. Other regulatory peptides, including glucose-dependent insulinotropic peptide, glucagon-like peptide-1, vasoactive intestinal polypeptide, and pituitary adenylate cyclase-activating polypeptide, have been shown to exert glucose-dependent stimulatory effects on insulin release in a meal-responsive manner **(470–512)** Collectively, our results indicate that nesfatin-1 is a glucose-responsive insulinotropic peptide that acts directly on pancreatic islet β-cells. Altered expression of pancreatic nesfatin-1 in both type 1 and type 2 diabetes implicates endogenous nesfatin-1 in the pathophysiology of diabetes and obesity. While the precise mechanisms underlying nesfatin-1 action, particularly within the context of diabetes and obesity, remain elusive, this work illuminates a potential role for nesfatin-1 in regulating glucose homeostasis**(Gonzalez *et al.*, 2011)**.

Chapter Five

Conclusions and Recommendation

5. Conclusions and Recommendations

5.1 Conclusions

1. Nesfatin-1 appears to be able to contribute to the treatment of obesity and diabetes because of its anorexigenic and antihyperglycemic effects. In addition, C-peptide is a known biomarker of insulin resistance and beta-cell function. High specificity and sensitivity analyzed results were obtained by ROC analysis for both markers in T2DM
2. NUCB2 polymorphisms could have a pivotal role in the presence of T2DM. The specific SNP of NUCB2 could account for the differences in clinical features of T2DM in female. Nesfatin-1 has gain attention as a new target to generate, drug for treatment of endocrine nutritional and metabolic disorders like obesity and type 2 diabetes mellitus.

5.2 Recommendations

- In comparison with all other anti-hyperglycemic factors.
- Nesfatin-1 possibly symbolizes a novel agent of insulin contributors. Composed, the anorexigenic and anti-hyperglycemic properties of nesfatin-1 prominently influence both food intake and glucose metabolism, involving its noteworthy parts in the metabolic regulator of the body.

- Human studies have shown that the circulating levels of nesfatin-1 change in several diseases, including epilepsy, diabetes, and anxiety. Therefore, it can be used in the diagnosis of diseases, monitoring of the patient response to treatment, or the progression of the disease both during and after the treatment.

- Guiding parents to their young children through health education recommended by the World Health Organization (WHO), and education is done through schools _
- By following a healthy diet.

Reference

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Appendix

Manuscript Title: **Potentiation of Nucleobindin-2 Variant and Nesfatin-1 serum level as Pathogenic Predictors of Metabolic Disorders and Type 2 Diabetic Mellitus**

Authors: Israa Khalil Ibrahim Al-Yassiri, Maher Abbood Mukheef, Fadhil Jawad altuma

Dear Dr. Prof. Fadhil Jawad altuma

I would like to acknowledge receiving of your manuscript titled "**Potentiation of Nucleobindin-2 Variant and Nesfatin-1 serum level as Pathogenic Predictors of Metabolic Disorders and Type 2 Diabetic Mellitus**". Your manuscript will undergo the review process. You can learn about our review process by visiting APJCP's peer review process page.

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Association between Nesfatin-1 Levels and C-Peptide in Sera of Obese/Non-Obese Type 2 Diabetic Women

Israa Khalil Ibrahim Al-Yassiri[1*], Fadhil Jawad Al-Tu'ma[1], Maher Abbood Mukheef[1], Khosay Baqir Jawad[2]
Baraa Abdul-Kareem Mutar[1]

[1]Department of Chemistry and Biochemistry, College of Medicine, University of Kerbala, Kerbala, Iraq
[2]Al-Hassan Center of Diabetes and Endocrinology Unit, Al-Hussein Medical City, Kerbala Health Directorate, Kerbala, Iraq
*Correspondence to: Israa Khalil Ibrahim Al-Yasiri (E-mail: aalyasiri1@gmail.com)

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Abstract

Objectives: The aim of the presented word was to assess the diagnostic accuracy of serum Nesfatin-1 in type 2 diabetes mellitus and its relationship with C-peptide level in obese and non-obese type-2 diabetic women of Iraqi population.

Methods: (A case-control study was performed on 50 type 2 diabetic patients admitted in Al-Hussein Teaching Hospital and Al-Hassan center of diabetes and endocrinology unit/Kerbala health directorate – Iraq and another 50 control individuals, during the period from April, 2022 – Jan. 2023). The T2DM groups were divided into two groups 25 obese and 25 non-obese; also the control group was divided into 25 obese and 25 non-obese as apparently healthy groups. The ELISA Kit was used to measure serum Nesfatin-1 and C-peptide, and random serum glucose was measured by enzymatic colorimetric method, and lipid profile test were measured through spectrophotometric technique, instead of HbA1c% was determined using HPLC method.

Results: The results observed indicated that Nesfatin-1 levels shown a non-significant decrease in all of type 2 diabetic groups as compared with apparently healthy control group, while the C-peptide were significantly decreased in type 2 diabetic patients when compared with apparently control group. In addition, the random blood glucose and HbA1c% were shown significant elevation in type 2 diabetic patients as compared with apparently healthy control groups. The observed data indicated that Nesfatin-1 and C-peptide levels when comparing between type 2 diabetic patients and control in obese groups shown a risk factors depending upon the odd ratio observed (OR = 1.064 (1.011–1.119), 1.0200 (0.992–1.08)) respectively, but only Nesfatin-1 was shown to be significant. In BMI the levels of Nesfatin-1 and C-peptide, as shown the Nesfatin-1 was significant in obese groups, while the C-peptide as shown significant in normal weight groups. The optimal diagnostic points for Nesfatin-1 were (sensitivity = 98%, specificity = 90%) at a level (Cut-off points) = 39.13, while C-Peptide levels: (sensitivity = 98%, specificity = 94%) at a level (Cut-off points) = 15.99. Both markers have *P*-values of the AUC were <0.001 and statistically significant.

Conclusion: Accordingly, it was concluded that a significant relationship between circulating Nesfatin-1 levels and type 2 diabetes. Nesfatin-1 appears to be able to contribute to the treatment of obesity and diabetes because of its anorexigenic and antihyperglycemic effects. In addition, C-peptide is a known biomarker of insulin resistance and beta-cell function. High specificity and sensitivity analyzed results were obtained by ROC analysis for both markers in T2DM.

Keywords: Nesfatin-1, C-peptide, obese, diabetes mellitus, type 2, body mass index, hypoglycemic agents

Introduction

Diabetes mellitus (DM) is a metabolic disorder where in human body does not produce or properly uses insulin, a hormone that is required to convert sugar, starches and other food into energy. Absence or reduced insulin in turn leads to persistent abnormally high blood sugar and glucose in tolerance. It is probably an oldest disease known to man.[1] Immunohistochemical studies have shown that the precursor of Nesfatin-1, non-esterified fatty acid/nucleobinding 2 (NUCB2), is localized in many places such as the pituitary gland, hypothalamus, brain stem, the forebrain and midbrain nuclei, central amygdaloid nucleus, ventrolateral medulla, and cerebellum. It is linked with developing of various serious diseases like micro vascular (nephropathy, retinopathy, neuropathy) and macro vascular (peripheral vascular disease and coronary heart diseases).[2] Diabetes and its complications are complex, multifactorial conditions with both major environmental and genetic components. When early studies identified differences in diabetic complication susceptibility in patients who seemed otherwise equal with regard to their diabetes glucose control, clinical features and management.[3]

Obesity and T2DM are two of the most pressing public health concerns worldwide because of their association with life-threatening diseases, including cardiovascular diseases and cancers.[4] Obesity, especially pathologic expansion of visceral white adipose tissue (vWAT), increases the risk of developing T2DM. Depending on the race, more than 75–90% of patients with T2DM are overweight or obese. The strong association of obesity and T2DM is supported by the term "disability."[5]

Nesfatin-1 that discovered in 2006 is secreted from the hypothalamic nuclei, which are responsible for controlling appetite. In the same study, it was reported that nesfatin-1 suppresses food intake, even in obese mice with a knockdown leptin gene.[6] The secretion is distributed in the body; nesfatin-1 is thought to affect many functions. Previous studies have reported that nesfatin-1 has regulatory effects on energy metabolism through suppression of food intake. In addition, it has been reported that nesfatin-1 regulates cardiac functions, decreases blood glucose levels, acts as a neuroendocrine regulator, and causes weight loss along with reduction in energy intake.[7]

The relationship between obesity and nesfatin-1 was investigated because of the effects of nesfatin-1 on food intake and energy consumption. There was a relationship between the polymorphism of the nucleobindin-2 gene and obesity. This may be a risk factor for the development of

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Authors: Baraa Abdul-Kareem Mutar, Fadhil Jawad Al-Tu'ma, Thikra Ali Allwsh, Israa Khalil Ibrahim Al-Yassiri, Ammar Ghani Yassin

Dr. Zahra Emam Djomeh
Dean of Aras Intl. Campus (UTAIC)
Conference Scientific Director

Address: End of Qods St., Enghelab Sq., Tehran, Iran.
Cell: 0098 935 438 9168 **Fax:** 00982189776220
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مستشفى النسائية والتوليد التعليمي

شهادة مشاركة

نؤيد مشاركة السيد /...اسراء خليل ابراهيم.....المحترم

بفعاليات اليوم العلمي الثامن لمستشفى النسائية والتوليد التعليمي/ دائرة صحة كربلاء المقدسة

((تحت شعار))

التعليم الطبي والبحث العلمي بين الواقع والطموح

بتاريخ 26-7-2022

8th

د. حيدر صاحب الزاملي

مدير مستشفى النسائية والتوليد التعليمي

University of Kerbala – College of Medicine

Department of Chemistry and Biochemistry

Student Name: Israa Khalil Ibrahim

Supervisors:

Prof. Dr. Fadhil Jawad Al-Tu'ma - College of Medicine – University of Kerbala

Dr. Maher Abbood Mukheef - College of Medicine – University of Kerbala

((Experimental Data))

Nesfatin-1 Levels and Nucleobindin-2 Genetic Variants and their Relations with the Pathogenesis of Obese Type 2 Diabetic Iraqi Women

Sample Number:	Type of study: Case-Control	BMI :
		WHR:
		Age:

Disease Description:

Type 2 diabetes mellitus (Obese and non-obese)

Exclusion criteria:

Kidney diseases, liver disease, cancers, strokes, any acute or chronic inflammatory disease, cerebrovascular accidents, alcoholics, rheumatoid arthritis, autoimmune disease, patients with type 1

Biomarkers

Biomarker	Levels	Unit
Nesfatin-1 Level		
Serum Blood Glucose		
HbA1c%		
Calcium		
Lipid Profiles: Total Serum Cholesterol Serum HDL-Cholesterol Triglyceride LDL-Cholesterol VLDL-Cholesterol		
C-Peptide		

Genetic Variants of Nucleobindin-2:

الملخص

الخلفية: داء السكري من النوع الثاني هو أحد الاضطرابات الأيضية. وهو ناتج عن مزيج من عاملين أساسيين: عدم كفاية إفراز الأنسولين من قبل خلايا البنكرياس أو فشل الأنسجة الحساسة للأنسولين في الاستجابة بشكل كافٍ للأنسولين. يُفرز نسفاتين 1 من نوى الوطاء وهو مسؤول عن التحكم في الشهية ويؤثر على العديد من الوظائف مثل التأثيرات التنظيمية على استقلاب الطاقة من خلال قمع تناول الطعام. بالإضافة إلى ذلك، فهو ينظم وظائف القلب، ويقلل من مستويات الجلوكوز في الدم، ويعمل كمنظم للغدد الصم العصبية، ويسبب فقدان الوزن إلى جانب تقليل استهلاك الطاقة. نيسفاتين-1، الذي نشأ من سلائفه البروتين المسمى ((NUCB2، نيوكليوبيندين-2 الذي يلعب دورًا مهمًا في استقلاب الجلوكوز ومرض السكري. استكشفت هذه الدراسة العلاقة بين المتغيرات الجينية لـ NUCB2 وداء السكري من النوع الثاني .(T2DM)

الهدف: كان الهدف من المقدمة هو تقييم الدقة التشخيصية لمصل نسفاتين -1 في داء السكري من النوع الثاني وعلاقته بمستوى ببتيد- سي في النساء المصابات بالسمنة وغير البدينات من النوع الثاني السكري من السكان العراقيين.

المواد والطرق: تم إجراء دراسة حالة وضبط على 50 مريضاً من مرضى السكري من النوع الثاني في مستشفى الحسين التعليمي ومركز الحسن لأمراض السكر والغدد الصماء / مديرية صحة كربلاء - العراق و50 شخصاً سليمين من مرض السكر خلال الفترة من أبريل 2022 - يناير 2023). تم تقسيم مجموعات السكري النوع الثاني إلى مجموعتين 25 سمنة و25 غير سمنة. كما تم تقسيم المجموعة الضابطة إلى 25 من البدناء و25 من غير البدينين كمجموعات تبدو صحية. تم استخدام ELISA Kit لقياس مصل Nesfatin-1 وC-peptide، وتم قياس نسبة الجلوكوز العشوائي في المصل بالطريقة اللونية الأنزيمية، وتم قياس اختبار ملف تعريف الدهون من خلال تقنية القياس الطيفي، HbA1c ٪ قياس نسبة السكر التراكمي تم تحديدها باستخدام طريقة .HPLC

النتائج: أشارت النتائج التي لوحظت إلى أن مستويات نسفاتين- 1 أظهرت انخفاضًا غير معنوي في جميع مجموعات مرضى السكري من النوع الثاني مقارنةً بمجموعة السيطرة على ما يبدو، بينما انخفض البيتيد -سي بشكل ملحوظ في مرضى السكري من النوع الثاني عند مقارنته مع مجموعة السيطرة الظاهرة. بالإضافة إلى ذلك، أظهر كل من الجلوكوز العشوائي ونسبة HbA1c في الدم ارتفاعًا ملحوظًا في مرضى السكري من النوع الثاني مقارنةً بمجموعات التحكم السليمة ظاهريًا. أشارت البيانات المرصودة إلى أن مستويات Nesfatin-1 وC-peptide عند المقارنة بين مرضى السكري من النوع الثاني والتحكم في مجموعات السمنة أظهرت عوامل خطر اعتمادًا على النسبة الفردية الملاحظة (OR = 1.064 (1.011-1.119)، 1.0200 (0.992-1.08)على التوالي، ولكن تم إظهار نسفاتين- 1 فقط ليكون ذا أهمية. في مؤشر كتلة الجسم، كانت مستويات Nesfatin-1 وC-peptide، كما يتضح من Nesfatin-1 مهماً في المجموعات البدينة، بينما كان البيتيد C مهمًا في مجموعات

الوزن الطبيعي. كانت نقاط التشخيص المثلى لـ Nesfatin-1 هي (الحساسية = 98٪، النوعية = 90٪) عند مستوى (نقاط القطع) = 39.13، بينما مستويات C-Peptide:
(الحساسية = 98٪، النوعية = 94٪) عند مستوى (نقاط القطع) = 15.99. كلتا العلامات لها قيم p لـ AUCالمسافة تحت المنحني، كانت <0.001 وذات دلالة إحصائية. ونتيجة التركيب الجيني، أشارت النتائج التي لوحظت إلى أن مستويات نسفاتين -1 أظهرت معنويًا في جميع مجموعات مرضى السكري من النوع الثاني في جين البوليمير ((rs757081، في حين أن الببتيد C وملف الدهون، وRBSسكر الدم العشوائي، وHbA1C السكر التراكمي، ونسبة الخصر الى الورك W.H.R كانت غير معنوية. في مرضى السكري من النوع الثاني في جين البوليمير.(rs757081) فضلاً عن ذلك،

عند مقارنة نسفاتين 1 بجين البوليمير في المرضى، أظهر زيادة في تواتر الأنماط الجينية CG + GG في ((rs757081ل p = 0.045؛ (OR = 1.102 (0.992-1.223 GG، p = 0.05، على التوالي) فروق ذات دلالة إحصائية في .Nasfatine-1

الخلاصة: بناءً على ذلك، تم التوصل إلى وجود علاقة معنوية بين مستويات نسفاتين- 1 المنتشرة ومرض السكري من النوع 2. يبدو أن نسفاتين-1 قادر على المساهمة في علاج السمنة ومرض السكري بسبب تأثيرات فقدان الشهية ومضادات ارتفاع السكر في الدم. بالإضافة إلى ذلك، يعد الببتيد C علامة حيوية معروفة لمقاومة الأنسولين ووظيفة خلايا بيتا. تم الحصول على نتائج تحليل نوعية وحساسية عالية من خلال تحليل ROC لكلا الواسمتين في السكري من النوع الثاني ونتيجة التركيب الوراثي،

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

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فرع الكيمياء والكيمياء الحياتية

مستويات نسفاتين 1، المتغيرات الجينية نوكليوبيندين- 2 مع أدوارها في احداث داء السكري النوع الثاني في النساء العراقيات البدينات

رسالة ماجستير

إلى مجلس كلية الطب/ فرع الكيمياء والكيمياء الحياتية/ جامعة كربلاء كجزء من متطلبات نيل درجة الماجستير في الكيمياء السريرية

من قبل

اسراء خليل ابراهيم

بكالوريوس تقنية تحليلات مرضية

إشراف

أ. د. فاضل جواد ال طعمة **أ.م.د. ماهر عبود مخيف**

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