SPEXIN AS A NOVEL BIOMARKER AND ITS RELATION WITH CARDIOMETABOLIC PARAMETERS IN METABOLIC SYNDROME

A Thesis submitted to

SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION
AND RESEARCH, TAMAKA, KOLAR, KARNATAKA

For the requirements of the degree

DOCTOR OF PHILOSOPHY
IN
BIOCHEMISTRY

Under Faculty of Medicine

By

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April 2022

DECLARATION BY THE CANDIDATE

I, **Tejaswi Gowdu** hereby declare that this thesis entitled **"Spexin as a biomarker and its relation with cardio-metabolic parameters in metabolic syndrome"** is an original research work carried out by me for the award of **Doctor of Philosophy** in the subject of Biochemistry (Faculty of Medicine) under the supervision of **Dr.C.D.Dayanand** Professor of Biochemistry, Department of Allied Health Sciences, Sri Devaraj Urs Academy of Higher Education and Research, under the Co-supervision of **Dr. Prabhakar.K** Professor of General medicine, Sri Devaraj Urs Medical College and **Dr. Sharath Balakrishna** Associate Professor, Department of Cell Biology andMolecular Genetics, Sri Devaraj Urs Academy of Higher Education and Research.

No part of this thesis has formed the basis for the award of any degree or fellowship previously elsewhere.

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1.	AHA	American Heart Association
2.	Apo-A1	Apolipoprotein A1
3.	Apo-B	Apolipoprotein B
4.	ARC	Arcuate nucleus
5.	AgRP/NPY	Agouti-related protein/neuropeptide Y
6.	ACEI	Angiotensin-converting enzyme inhibitors
7.	ARB	Angiotensin receptor blockers
8.	ASCVD	Atherosclerotic cardiovascular disease
9.	ABCA1	ATP-binding cassette protein A1
10.	BBB	Blood brain barrier
11.	BP	Blood Pressure
12.	CVD	Cardiovascular disease
13.	CRP	C-reactive protein
14.	CETP	Cholesterol ester tranporter protein
15.	CLD	Congenital Leptin deficiency
16.	ESIR	European Group For The Study of Insulin Resistance
17.	ECG	Electrocardiogram
18.	FFA	Free fatty acid
19.	FBG	Fasting Blood Glucose

20.	GalR2	Galanin R-2 receptor
21.	GaIR3	Galanin R-3 receptor
22.	GLUT4	Glucose transporter type 4
23.	HDL-C	High density lipoprotein Cholesterol
24.	$HbA1_C$	Glycosylated Hemoglobin
25.	HOMA-IR	Homeostatic model assessment for Insulin resistance
26.	IDF	International Diabetes Federation
27.	IR	Insulin Resistance
28.	IL-6	Interleukin-6
29.	IKK	IkappaB kinase
30.	IRS1	Insulin receptor substrate-1
31.	JAK2	Janus Kinase 2
32.	JNK	c-Jun N-terminal kinases
33.	KISS	Kisspeptin
34.	LDL-C	Low density lipoprotein Cholesterol
35.	LH	Leutinizing Hormone
36.	LCFA	Long chain fatty acid
37.	Mets	Metabolic syndrome
38.	MI	Myocardial Infarction
39.	MAPK/ERK	Mitogen-activated protein kinases/extracellular signal-regulated kinase
40.	NCEP ATP III	National Cholesterol Education Program-Adult Treatment Panel III

41.	OS	Oxidative stress
42.	PPBS	Post prandial blood sugar
43.	PAI-1	Prothrombin Activator inhibitor-1
44.	P13-K	Phosphoionositol-3-kinase
45.	POMC	Proopiomelanocortin
46.	PTP1B	Protein tyrosine phosphatase 1B
47.	RAAS	Renin-angiotensin-aldosterone system
48.	SPX	Spexin
49.	SNS	Sympathetic Nervous system
50.	SPX1	Mammalian Spexin 1
51.	SPX2	Non- Mammalian Spexin 2
52.	SOCS3	Suppressor of Cytokine signaling 3
53.	STAT3	Signal transducers and activators of transcription 3
54.	T2DM	Type 2 diabetes mellitus
55.	T2DM-HTN	Type 2 diabetes with hypertension
56.	TC	Total Cholesterol
57.	TG	Triglycerides
58.	VLDL	Very low density lipoprotein
59.	WHO	World Health Organization
60.	WAT	White adipose tissue

CHAPTER-1
INTRODUCTION

1.0. BACKGROUND

Metabolic syndrome (MetS) is a growing public-health and serious clinical concern across the globe. Over the next few years, the prevalence of MetS is projected to have dramatic rise by approximately five-fold in diabetes mellitus (DM) and cardiovascular disease (CVD). Furthermore, the MetS is becoming more common not only in the United States and Europe, but also increasing in Asian countries like China, India, and South Korea. In India the highest rate of MetS prevalence found as thirty percent and has been higher risk of cardiovascular morbidity and mortality [1-3].

In 1920, a Swedish Physician, Kylin E demonstrated the MetS as the association of hypertension, hyperglycemia, and hyperuriemia. Whereas the same syndrome in recent time known as a serious public health problem associated with risk of heart disease, diabetes, atherosclerosis and stroke. Excess energy intake, obesity, and sedentary lifestyles are major contributing factors. It is well known as group of interlinked dynamics between physiological, biochemical, clinical, aging and genetic factors that directly contributes to increased risk of DM and cardiovascular complications leads to higher mortality [4].

As per the revised National Cholesterol Education Program Adult Treatment Panel (NCEP/ATP-III), the diagnosis of MetS includes a waist circumference of ≥90 cm in men ≥80 cm in women. The concentration of biochemical parameters like Serum triglycerides ≥150 mg/dl, HDL cholesterol in men < 40mg/dl and < 50mg/dl in women, fasting blood glucose ≥ l00 mg/dL and systolic BP ≥130 mmHg and diastolic BP ≥ 85 mmHg [5,6].

1.1. Pathophysiology underlying in metabolic syndrome

MetS is caused by a cluster of metabolic co-morbidities such as central obesity, physical inactivity, insulin resistance, dyslipidemia and hypertension. Increased visceral adiposity is caused by genetic predisposition, decreased physical activity, and a diet high in saturated fats and low in fiber.

Inflammatory cytokines interleukin-6, interlekin-1, tumour necrosis factor-α and adipocytokines such as interleukin-6, plasminogen-activator inhibitor-1, C-reactive protein, Leptin, ghrelin, resistin, visfatin and retinol binding protein-4 as well as non-esterified fatty acids are produced from visceral fat. These above inflammatory adipokines are key indicators of pathogenesis [7]. Further leads to insulin resistance in skeletal muscle and hepatocytes resulting in hyperinsulinemia, which contributes to atherogenesis and dysfunction of endothelium. The free fatty acids (FFA) also change the profile of hepatic lipid synthesis, making it more atherogenic in terms of having low High Density Cholesterol and elevated triglycerides and Low Density cholesterol. As a whole, the metabolic derangements that make up the MetS are mainly focused on increased visceral adipose tissue lipolysis, incresesed FFAs synthesis and decresed production of apolipoprotein B-100 in the liver leads to fatty liver occurred due to Insulin resistance is a main pathophysiological dearangement.

Eventhough, the actual cause of the MetS is not clear, but also, insulin resistance is believed to be a major contributor in the development and onset of MetS. This has become the primary basis of underlined pathophysiology of MetS[6, 8] as shown in figure 1.

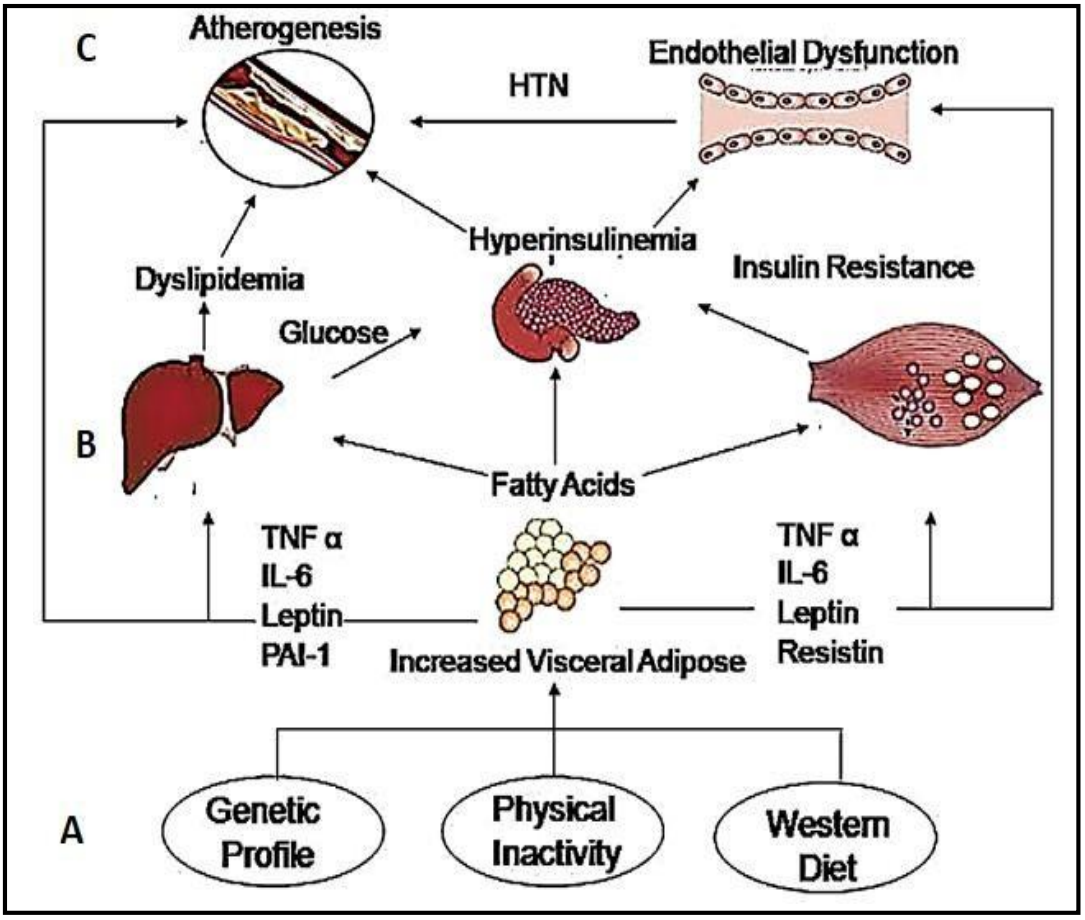


Figure 1: An overview of Pathophysiology of Metabolic Syndrome

A: Due to life style modifications and genetic predisposition, increased Free Fatty Acids (FFA) leads to hypertrophy and hyperplasia in visceral adipose tissue.
B. Increased FFA enters into liver cause's dyslipidemia by accumulation of very low density lipoprotein (VLDL), pancreas and skeletal muscle reduces uptake of glucose and increases insulin levels. Sustained exposure causes B-cell dysfunction, hyperinsulinemia and insulin resistance (Hyperglycemia).
C.Overall increased production of inflammatory cytokines and adipokines, localized low garde-chronic inflammation, macrophage infiltration, oxidative stress, endothelial dysfunction and atherosclerosis.

Source: Potenza MV, Mechanick JI. The metabolic syndrome: definition, global impact, and pathophysiology. Nutr Clin Pract. 2009 Oct-Nov;24 (5):560-77.

1.2. Metabolically triggered Inflammation

The MeS is frequently accompanied by a persistent low-grade inflammatory state, and is related pathophysiological effects [9]. However, because it is not accompanied by infection, there is no substantial tissue destruction, and the inflammatory activation is not large and hence it doesn't follow the standard description of acute or chronic inflammation. As a result, it's commonly referred to as 'low-grade' chronic inflammation, meta-inflammation, which refers to metabolically-triggered inflammation [10] or even para-inflammation, which is a state in between basal and inflammatory states [11].

Low grade chronic inflammation is associated with obesity by over production pro-inflammatory cytokines and adipokines that leads to hypoxia with reduced blood supply that response to necrosis and macrophage infiltration. The role of macrophages is to removal of damaged cells, cellular debris and alters adipokines and inhibits differentiation of adipocytes from stem cells. Hence, large size of macrophages accumulation and delay of removal of debris causes chronic low-grade-inflammation and insulin resistance thereby results in endothelial dysfunction[12]. Few inflammatory and metabolic biomarkers are CRP, leptin is also elevated in MetS[13].

1.3. Management of Metabolic syndrome

American Diabetes Association (ADA) basis for MetS management are primarily by lifestyle adjustments such as food and exercise changes. Furthermore, research reports suggest that diet, exercise, as well as pharmacologic and surgical therapies can help to prevent MetS from progressing to Type 2 diabetes mellitus or CVD [3].

In non-pharmacological management, the weight loss, considerable decreased total cholesterol, increased High density lipoprotein cholesterol, and a shift in the size and volume of low density cholesterol particles are all observed benefits of a "well-formulated" ketogenic diet. Thus, modifiable factors such as nutrition and exercise should be prioritised with metabolic syndrome subjects.

Patients with a Body mass index of 40 kg/m2 or 35 kg/m2 and additional co-morbidities should consider bariatric surgery. Patients with hypertriglyceridemia greater than 150 mg/dL should be investigated with additional testing like comprehensive lipid analysis, thyroid-stimulating hormone level, and liver function tests. Following a thorough clinical examination, patients should be counselled on lifestyle changes such as quit smoking, losing weight, changing their diet and routine exercise.

In pharmacological management, current hypertension therapy follows the standards of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC-7) to attain a target blood pressure (BP) of less than 140/90 mmHg or less than 130/80 mmHg Type 2 Diabetes Mellitus. Angiotensin-converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARBs) are recommended by the American Diabetes Association (ADA) for people with diabetes and hypertension.

According to American college of cardiology, the cholesterol guidelines explains that MetS patients are normally started on a moderate to high-intensity statin medication, however fibrates, niacin, and omega fatty acids can also assist to lower hypertriglyceridemia. Whereas, elevated LDL-C should also be treated in these patients provided the atherosclerotic cardiovascular disease (ASCVD) risk score is

greater than 7.5% that determines a patient's 10-year ASCVD risk. Such patients should receive high-intensity statin therapy with the goal of lowering LDL-C by 50%. [3,6,14] .

when necessary, imaging studies can also be ordered. An ECG is preferable on anyone suspected with atherosclerotic coronary artery disease and symptoms of myocardial ischemia, infarction, or arrhythmias. If advocated, patients should undergo additional cardiac stress testing like ECG stress test, stress echocardiography, stress single-photon emission computed tomography, or myocardial perfusion imaging [15] .

1.4. Diabetes mellitus

Diabetes mellitus (DM) is a chronic non-communicable metabolic disease that has emerged as one of the world's leading health problems with high health-care cost[16]. It affects both adults and children and is strongly linked to morbidity and mortality[17].

According to recent study reports, it is noted that the DM impact is about 382 million (7.7%) in 2013 and is expected to rise to 483 million (8.3%) by 2030. More than half of Patients with DM in developed Nations are over the age of 65 and 8% being under the age of 44 years. In developing Nations about 75% of diabetic patients are 45 years, while 25% of diabetic patients are under 44 years old. As per global data by International Diabetes Federation (IDF), there are about 352 million adults were suffering from hyperglycemia which causes higher risk of contributing to the development of DM by 2045 [18 -20].

DM is a metabolic disorder with hyperglycemia occurred due to impairement in insulin secretion, action or both. The chronic hyperglycemia is associated with long-

term organ dysfunctions & failure especially the eyes, kidneys, nerves, heart, and blood vessels. Symptoms of diabetes mellitus are polyuria, polydipsia, polyphagia, weight loss, sometimes with blurred vision. Long-term complications are retinopathy with potential loss of vision, nephropathy leading to renal failure, peripheral neuropathy with risk of foot ulcers. Autonomic neuropathy causing gastrointestinal, genito urinary and cardiovascular symptoms [6] .

1.5. Classification of diabetes mellitus

Diabetes mellitus categorized in to type 1 diabetes, type 2 diabetes mellitus and Gestational diabetes mellitus. Type 1 occurred by immune response and due to β-cells distruction in pancreas unable to secrete enough insulin. Type 2 Diabetes Mellitus occurred by insulin resistance where cellular receptors unable to responds to insulin action led to obesity. Gestational diabetes charecterised by Pregnancy hyperglycemia without previous history of DM and returns to normal on delivary.

Any disease that causes extensive damage to the pancreas may lead to diabetes exocrine pancreas defect chronic pancreatiis and cystic fibrosis, an autosomal dominant maturity onset Diabetes of the Young, secretion of Insulin antagonistic hormones, glucocorticoids induced Insulin resistance (steroid diabetes) are the other causes. In the study context, we explore the Type 2 diabetes mellitus with hypertension[21].

1.6. Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is common metabolic disease caused by a combination of two basic factors: inadequate insulin production by pancreatic β-cells and the failure of insulin-sensitive tissues to respond to insulin. Insulin release and its action must be precisely to satisfy metabolic demand. As a result, the molecular mechanisms involved in insulin synthesis, secretion as well as insulin response in tissues must be properly regulated. Deficiency in any one of the processes can cause a metabolic imbalance that can lead to pathogenesis of Type 2 diabetes mellitus like peripheral tissue insulin resistance occurs when insulin-receptors or other intermediates in the insulin cell signalling pathways are insensitive.

On acute exposure of environmental and genetic predisposition of adipose tissue causes increased free fatty acids leads to excess fat accumulation (VLDL) in liver through increased gluconeogenesis & lipogenesis. Whereas in muscle, free fatty acids inhibits insulin-mediated glucose uptake leads to insulin in-sensitivity. The consequent increase in diacylglycerol, a secondary messenger causes activation of protein kinase C which inturn activates kappa B kinase (IKK) and c-JUN NH2-terminal kinase (JNK).

Blockage of tyrosine phosphorylation by stimulation of serine-threonine phosphorylation causes dissociation of phosphoinositide-3-kinase (PI3K) from insulin receptor substrate (IRS1) and decreases Akt/protein kinase B (PKB) phosphorylation. Further, inhibition of downstream insulin signaling pathway, reduce expression of glucose transporter type 4 (GLUT4) translocation, decreased glucose uptake, decreased glycogen synthesis and oxidation of fatty acid. Thus prevents GLUT4 translocation to membrane and affect the uptake of glucose which leads to insulin

resistance. Sustained exposure causes pancreatic β-cells dysfunction results in hyperinsulinemia and hyperglycemia [22] as shown in figure 2 & 3.

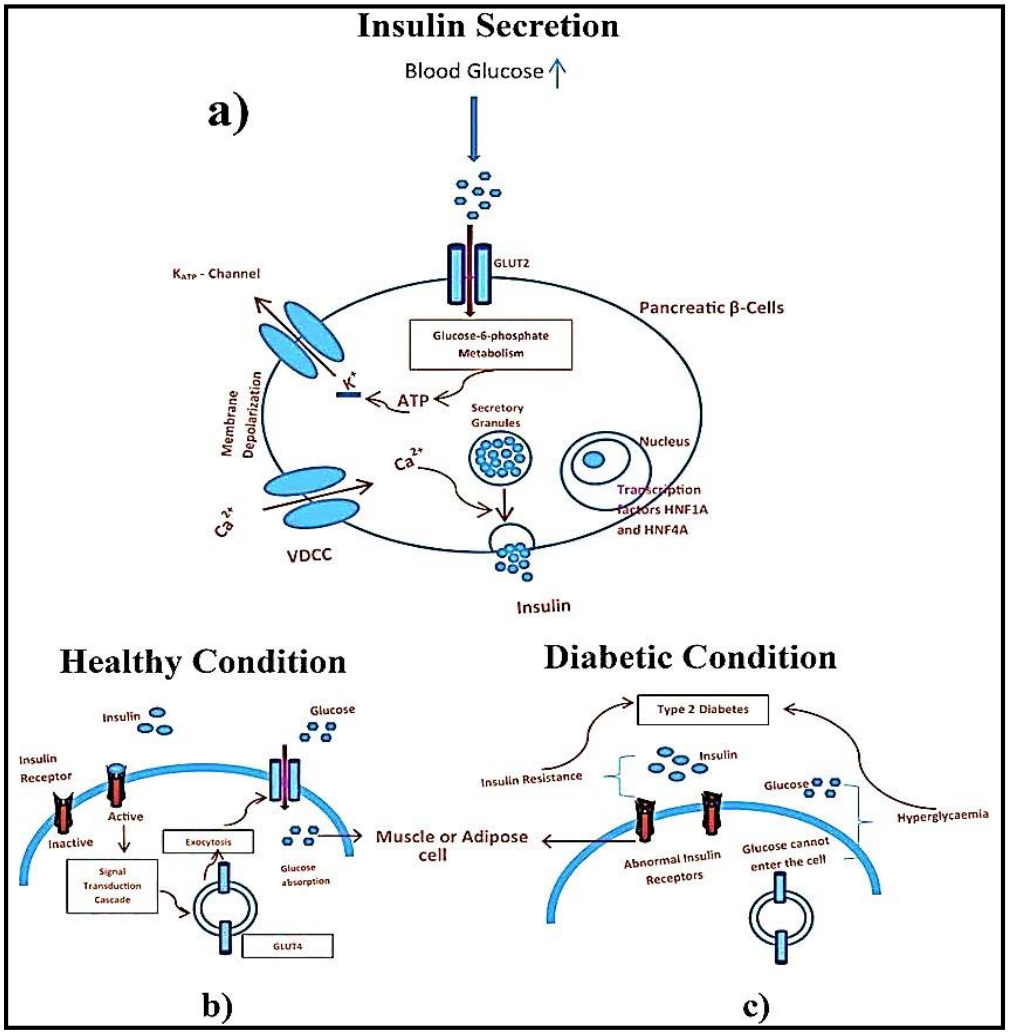


Figure 2: Pathophysiology of Type 2 diabetes mellitus

Source: VSS P, Adapa D, Vana DR, Choudhury A, Jahangir MA, Chatterjee A. et al. Nutritional components relevant to type-2 diabetes: Dietary sources, metabolic functions and glycaemic effects. Journal of Research in Medical and Dental Science. 2018;6(5):52-75.

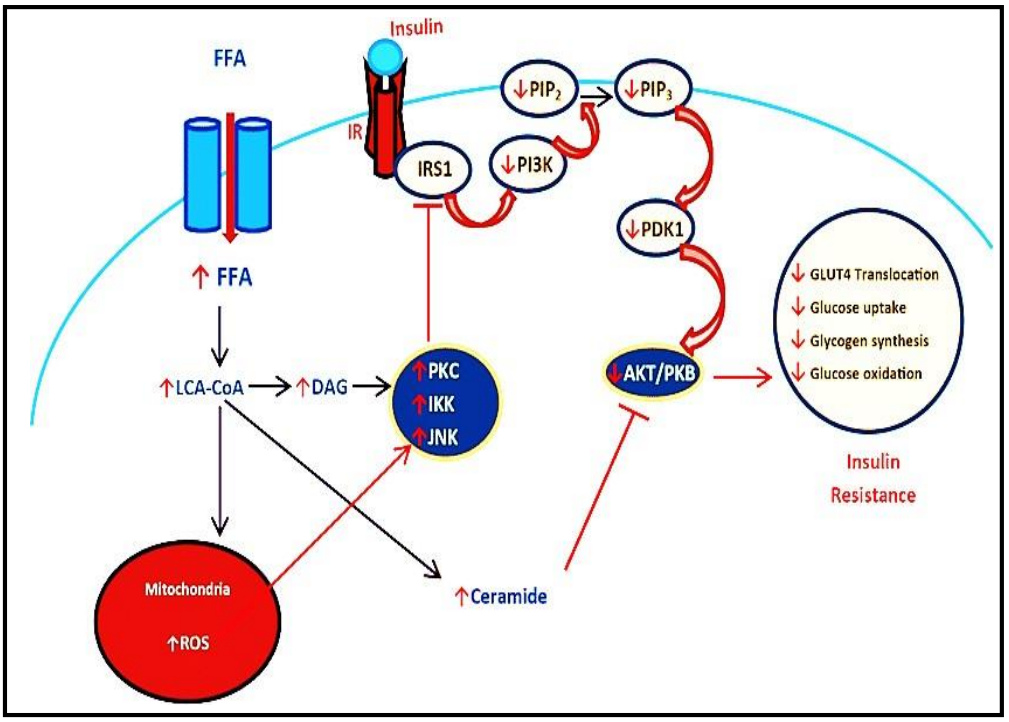


Figure 3: Free Fatty Acids mediated insulin resistance

Source: VSS P, Adapa D, Vana DR, Choudhury A, Jahangir MA, Chatterjee A. Nutritional components relevant to type-2 diabetes: Dietary sources, metabolic functions and glycaemic effects. Journal of Research in Medical and Dental Science. 2018 Aug;6(5):52-75.

1.7. Hyperinsulinemia and Hypertension

Hypertension is caused by a combination of several factors. The state of hyperinsulinemia was first discovered over thirty years ago, which is the most important single cause that contributes to primary hypertension [23]. Insulin resistance results in high glucose levels due to glucose intolerance which leads to hyperinsulinemia. The pancreas tries to compensate dramatically by increasing insulin synthesis, however this results in excessive amounts of serum insulin levels that aren't utilized by the body. Sustained high levels of insulin can also causes overactivation of the sympathetic nervous system, renin-angiotensin-aldosterone system, changes in adipose-derived cytokines causes hyperinsulinemia, structural & functional renal abnormalities and increased salt reabsorption by the kidneys could be one of the consequences of this overproduction which causes rise in extracellular fluid volume, more cardiac output, endothelial dysfunction and vasoconstriction that further causes hypertension [24] as shown in figure 4.

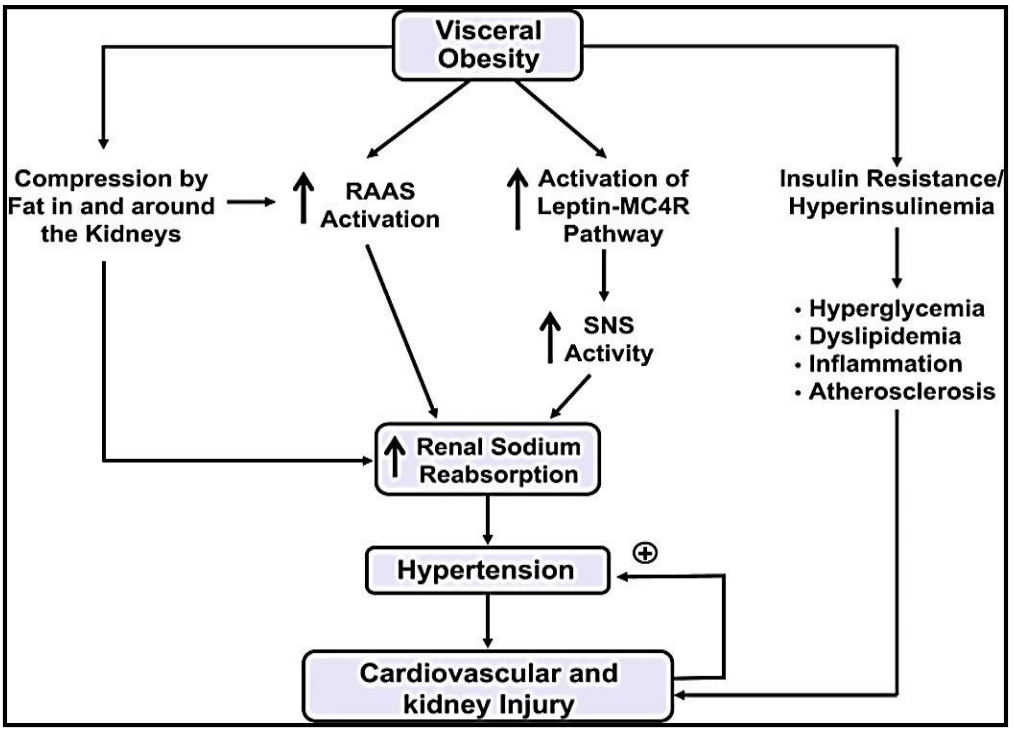


Figure 4: Hypertension in Type 2 diabetes mellitus

Source: Da Silva AA, Carmo JM, Li X, Wang Z, Mouton AJ, Hall JE. Role of hyperinsulinemia and insulin resistance in hypertension: metabolic syndrome revisited. Canadian Journal of Cardiology. 2020;36(5):671-82.

1.8. Complications of Diabetes mellitus

Diabetes mellitus symptoms at intial stages are microvascular complications, acute complications comprising diabetic keto acidisis, hyper osmolar hyperglycemia, but cardiovascular, renal, diabetic foot, vison impairement, and cognitive are long term complications.

Microvascular complications: Complication of Diabetes comprises microvascular or macrovascular in nature. Microvascular complications include neuropathy, nephropathy and retinopathy. Macrovascular complications include cardiovascular disease, peripheral vascular disease and stroke.

Diabetic retinopathy is the most common microvascular complication and is interconnected to long-term hyperglycemia. The most important predictor of visual impairment in persons helps to determined by the severity of the disease. As much as ninty percent of retinopathy-related blindness in adults with diabetes could be avoided if discovered and treated early [25].

Diabetec Neuropathy is the most common cause by neuoronal damage caused by the risk factors such as age, disease duration, smoking, hypertension, increased triglycerides, higher BMI and alcohol [26]. The most common form of diabetic neuropathy is chronic sensory motor distal symmetric polyneuropthy. Sensory loss, muscle weakness, and pain are all symptoms of polyneuropathy. Polyneuropathy usually begins with a slow onset of sensory dysfunction, such as burning and numbness in the feet [25].

Diabetic Nephropathy or kidney disease is by renal tissue damage glomerular lesions and loss of glomerular filtration capacity amounts presence of pathological amount of protein in urine /chronic proteinuria more than 500 mg protein or 300 mg albumin per 24 hours in urine of patients who do not have a urinary tract infection or other conditions that cause proteinuria. One of the primary modifiable risk factors for diabetic nephropathy is metabolic control. Increased blood pressure and hypertension are linked to a higher risk of diabetic renal disease development. Other risk factors are smoking, obesity, anaemia, and hereditary factors [25] .

Macrovascular Complications: In patients with diabetes, cardiovascular disease accounts for up to sixty five percent of all deaths. Ischemic heart disease and stroke account for the majority of diabetes-related morbidity. Hypertension, hypercholesterolemia and smoking are all co-morbidities of CVD just as they are in people without diabetes. However, it appears that having even one of these risk factors leads to poorer results in those with diabetes as compared to those who do not have diabetes.

Type 2 diabetes mellitus is most commonly associated with the MetS which comprises abdominal obesity, hypertension, hyperlipidemia and increased co-agulability. Other risk factors such as low concentrations of the adiponectin levels, increased production of the vascular cell adhesion molecule-1 and subsequent adhesion of T-lymphocytes to the endothelial walls of coronary arteries, higher pro-coagulation with increased expression of plasminogen activator inhibitor-1 (PAI)-1 and increased production of matrix metalloprotease are all factors that may play a role in the progression of macrovascular disease in Type 2 diabetes mellitus [27]. According to Almdal et al 2004 these factors may possibly contribute to the development of

cardiovascular disease [28].

Peripheral vascular disease also referred to as Peripheral arterial disease. The narrowing of blood vessels that supply blood to the arms, legs, stomach, and kidneys causes peripheral vascular disease. The age, duration of diabetes, and presence of neuropathy all raise the risk of peripheral vascular disease in diabetics. Other cardiovascular disease risk variables, such as C-reactive protein and homocysteine levels, have also been linked to risk of Peripheral vascular disease [29] .

1.9. Lipoprotein Metabolism in normal condition

Lipoproteins are conjugated lipids, complex in nature and that have a central hydrophobic core of non-polar lipids cholesteryl esters and triglycerides, this non polar core is surrounded by a hydrophilic membrane consisting of phospholipids, free cholesterol, and apolipoproteins.

Apolipoproteins have four major functions: They serve as structural proteins, act as ligands for lipoprotein receptors, assisting in the production of lipoproteins and serving as inducers or blockers of lipoprotein metabolic enzymes.

The classification of Plasma lipoproteins are categorised into seven classes based on size, lipid composition, and apolipoproteins [30,31]. The categorization of Plasma lipoproteins has shown in table 1.

Table 1: Classification of Lipoprotien

S.No	Lipoprotein	Density (g/ml)	Size (nm)	Major Lipids	Major Apoproteins
1	Chylomicrons	<0.930	75-1200	Triglycerides	Apo B-48, Apo C, Apo E, Apo A-I, A-II, A-IV
2	Chylomicron Remnants	0.930- 1.006	30-80	Triglycerides Cholesterol	Apo B-48, Apo E
3	VLDL	0.930- 1.006	30-80	Triglycerides	Apo B-100, Apo E, Apo C
4	IDL	1.006- 1.019	25-35	Triglycerides Cholesterol	Apo B-100, Apo E, Apo C
5	LDL	1.019- 1.063	18- 25	Cholesterol	Apo B-100
6	HDL	1.063- 1.210	5- 12	Cholesterol Phospholipids	Apo A-I, Apo A-II, Apo C, Apo E
7	Lp (a)	1.055- 1.085	~30	Cholesterol	Apo B-100, Apo (a)

Source: Feingold KR. Introduction to Lipids and Lipoproteins. In: Feingold KR, Anawalt B, Boyce A, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000 [32].

In intestine, the absorption of dietary lipids as chylomicrons begins by exogenous lipoprotein pathway. The triglycerides trapped in chylomicrons are digested by lipoprotein lipase in skeletal muscle and fatty tissue, generating free fatty acids resulting in chylomicron remnants. The liver then absorbs the chylomicron remnants [30,31].

The production of VLDL commences the pathway of lipid transport by endogenously in the hepatocytes. Lipoprotein lipase metabolizes the triglycerides carried by VLDL in skeletal muscle and fatty tissue, producing non-esterified fatty acids and intermediate-density lipoproteins (IDL) and is converted into LDL which is utilised by the LDL receptor in a variety of tissues, including liver which is the primary site of uptake. The liver and stomach produce nascent HDL, which is the initial stage in reverse cholesterol transport. These tiny HDL molecules can then absorb cholesterol and phospholipids from cells, leading in the formation of mature HDL. This progression is regulated by ATP-binding cassette protein A1 [32] shown in figure 5

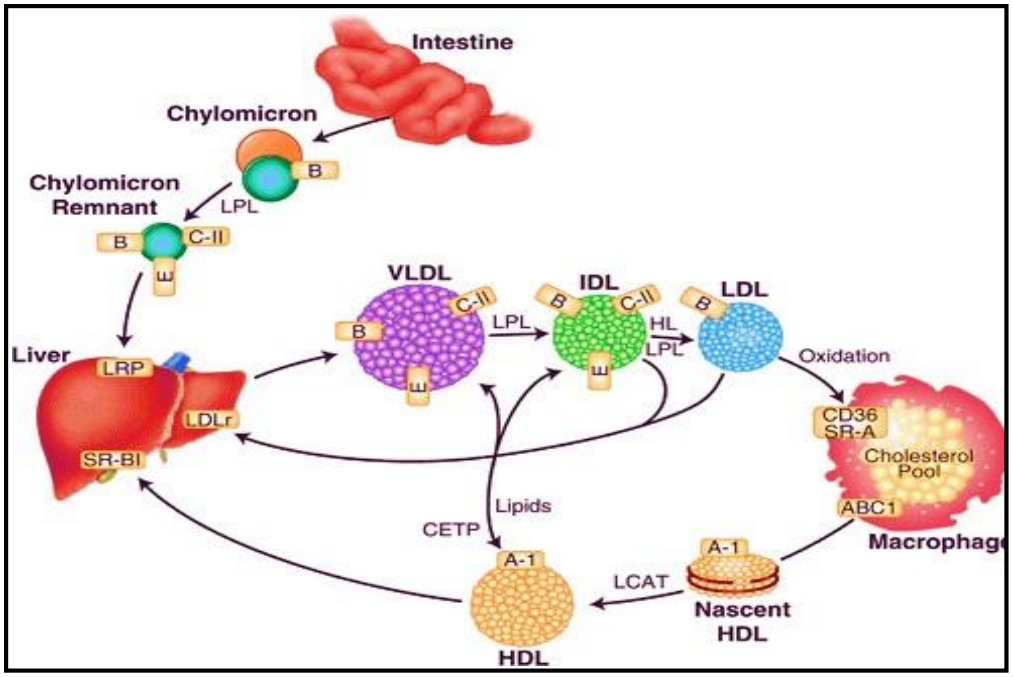


Figure 5: Lipid Metabolism in normal condition
Source: Feingold KR. Introduction to Lipids and Lipoproteins. In: Feingold KR, Anawalt B, Boyce A, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000 **[32] .**

1.10. Dyslipidemia and Hypertension in Diabetes mellitus

Hypertriglyceridemia and low plasma HDL cholesterol are typical lipoprotein abnormalities in type 2 diabetes mellitus. Furthermore, low density lipoprotein is transformed into tiny dense LDL, which may be more atherogenic [33]. Dyslipidemia is frequently identified in prediabetics, or those who have insulin resistance but normal blood glucose levels [34]. As a result, this lipid anomaly is linked to irregularities in insulin function rather than hyperglycemia. Insulin effects on liver apoprotein synthesis, dysregulation of lipoprotein lipase (LpL), inactivities of cholesteryl ester transfer protein (CETP), and peripheral insulin effects on adipose and muscle tissue are all potential contributors to diabetes dyslipidemia [35] as shown in figure 6.

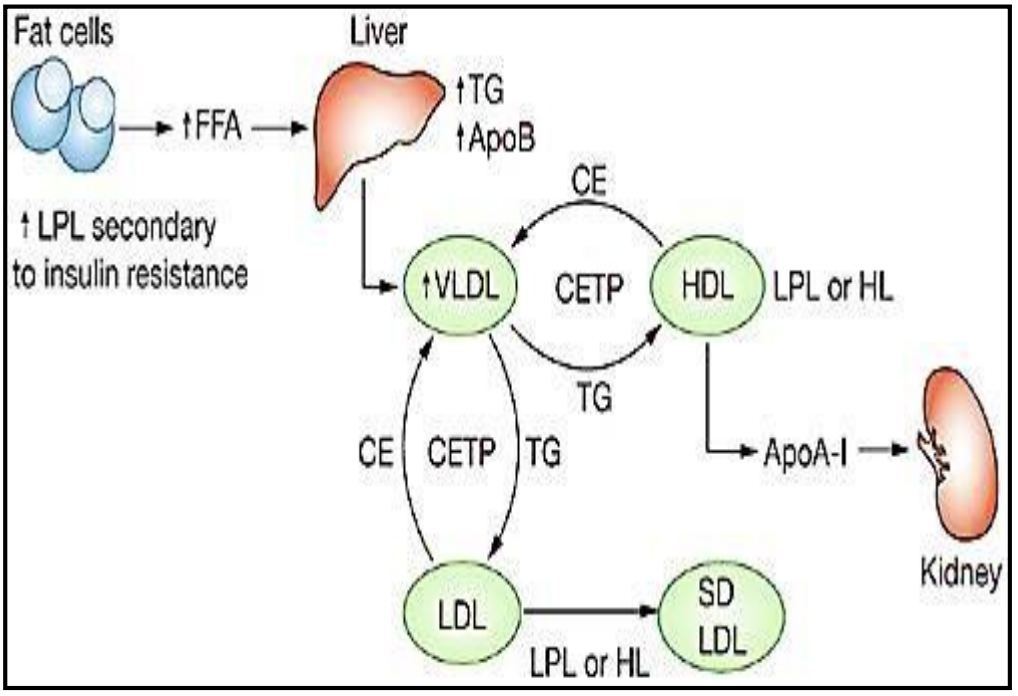


Figure 6: Type 2 diabetes mellitus and Dyslipidemia
Source: Mooradian, A. Dyslipidemia in type 2 diabetes mellitus. Nat Rev Endocrinol 2009;5:50–159 [36]

Research reports suggested that hypertension and cardiovascular disease (CVD) have a similar aetiology. As a result, dyslipidemia which is a powerful predictor of CVD could also predict the occurrence of hypertension. Dyslipidemia is characterised by abnormal lipid levels in the blood (Total Cholesterol >200 mg%, Low Density Lipoprotein >100 mg%, Triglycerides (TGL) >150 mg%, and High Density Lipoprotein (HDL) 40 mg in males and 50 mg in women). According to the NCEP Guidelines-Adult Treatment Panel III (ATP III), hypertension and dyslipidemia are significant components of MetS [37].

The incidence of hypertension and dyslipidemia co-existing is estimated to be 15–31 percent. The interaction of these two risk factors has a greater effect on endothelial dysfunction, which leads to increased atherosclerosis and CVD. Dyslipidemic hypertension is more common in non-familial forms than in familial types. Elevated blood pressure is one symptom for the damage. Endothelial damage and a decrease of vasomotor function are caused by dyslipidemia [37,38].

Atherogenesis is aided by the renin-angiotensin-aldosterone system (RAAS). Angiotensin II (Ag II) stimulates the angiotensin 1 receptor (AT1 receptor) which increases lipid absorption in cells, vasoconstriction and free radical generation promoting both hypertension and atherosclerosis. Hypertension affects the vascular endothelium through altering shear stress and oxidative stress leads to increased collagen and fibronectin synthesis, decreased nitric oxide-dependent vascular relaxation and increased lipoprotein permeability. Upregulation of lipid oxidation enzymes is also linked to hypertension. The vascular endothelium is obviously linked to hypertension (prothrombotic and pro inflammatory).

In the aetiology of atherosclerosis, oxidative stress and vascular inflammation are elevated. The reversal of vascular inflammation occurs when both are reduced [39]. LDL cholesterol is a primary contributor to endothelial dysfunction. Micro-albuminuria is found in hypertension patients, and it is associated to lipid abnormalities such as high LDL and TGL levels, low HDL levels, and elevated LP (a) levels [37,40].

1.12. Leptin

White adipose tissue (WAT) is an important part of the body's energy storage system. Furthermore, different WAT depots act as "endocrine organs" by secreting varied profiles of adipokines, a diverse collection of over fifty cytokines, chemokines, and hormone-like substances that help maintain energy balance. Some locally released adipokines have been demonstrated to alter appetite, satiety, glucose, and lipid metabolism, however they are not solely expressed by white adipose tissue [41].

The Leptin gene (ob) is situated on chromosome 7q31.3 translated to protein Leptin. It is an adipokine peptide hormone secreted by the white adipose tissue, contains 146 amino acid residues with Molecular weight of 16 kDa. Structurally, it has one short loop, 4 antiparallel α-helices fringed by two long crossover links as shown in figure 7.

The physiological function of Leptin are regulation of food intake, body mass index and body fat mass, reproduction, hematopoiesis, angiogenesis, blood pressure, bone mass and lymphoid organ homeostasis [42]. Leptin can binds to six isoforms of receptors such as ObRa, ObRb, ObRc, ObRd, ObRe and ObRf. Amongst, ObRa and ObRc are important in transport of Leptin to blood brain barrier.

The ObRb receptor plays a key role in regulating energy homeostasis and neuroendocrine function in hypothalamus. The ObRb is longest form and has a 302-amino-acid sequence motifs domain towards cytoplasm are known to bind intracellular signaling molecules. It is the only isoform of receptor capable of complete signal transduction [43].

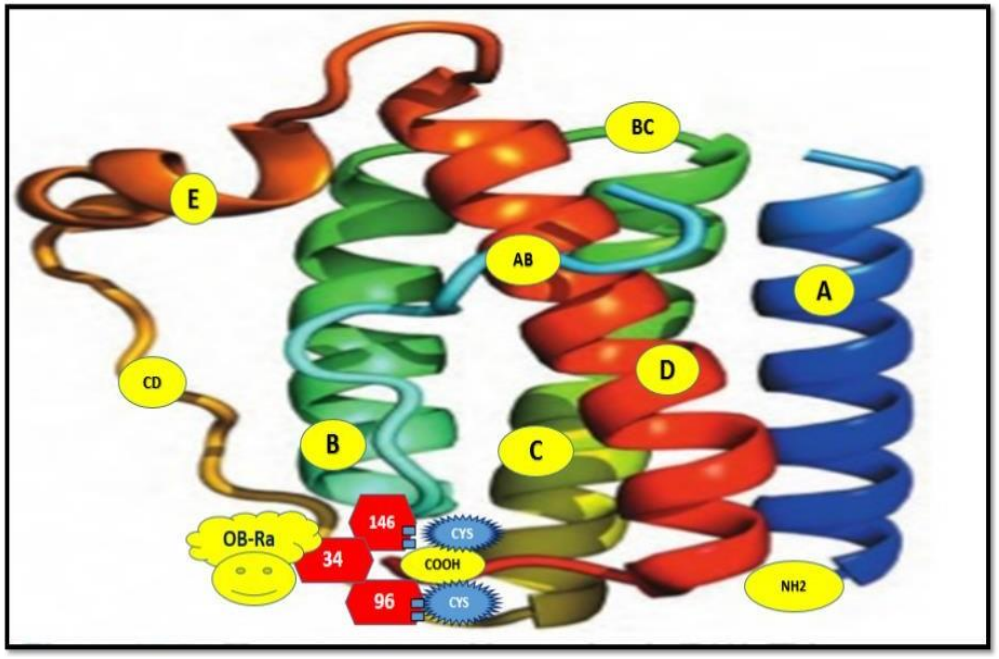


Figure 7: Structure of Leptin

Source: Zhang F, Basinski MB, Beals JM, Briggs SL, Churgay LM, Clawson DK et al. Crystal structure of the obese protein leptin-E100. Nature. 1997; 8:387(6629):206-9 [44].

Leptin receptors (LR) are expressed on the surface of cells such as neuronal, hepatic, pancreatic, cardiac, and perivascular intestine tissue [45]. Some of the well-known hormones like Insulin, steroid hormones, and noradrenaline promotes secretion of Leptin and are known as Secretagogues [46]. Glucocorticoids have the greatest stimulatory effect on Leptin secretion, because they operate directly on adipose tissue [47]. Leptin is also expressed in various tissues like placenta, ovaries, mammary, bone marrow and lymphoid tissues [48]. It has been evidenced that as obesity rises, serum

Leptin levels also rises, potentially leading to BBB transporter resistance, consequently less Leptin will reach the brain and will result in less activation of the body weight regulation signaling system give rise to obesity [49].

Although LEP-ObRb and other cytokine receptors lack kinase activity, they do pair with tyrosine kinases. Leptin binds to Leptin receptor undergoes a conformational change which is critical for Leptin signaling and activation of the associated Janus kinase (JAK2) pathway. JAK2 auto-phosphorylates and simultaneously phosphorylates tyrosine residues permitting binding of Signaling transducer activator transport (STAT) proteins and subsequent translocation to the nucleus.

Other signaling pathways by LEP-ObRb receptor by Phosphoinositol-3 kinase (PI3K) and mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) signaling cascades are also activated when Leptin binds to receptors. The anorexigenic actions of Leptin are aided by the activation of each of these mechanisms involving in suppressing appetite, stimulating weight loss, and increasing thermogenesis.

Hence, increased activity of protein tyrosine phosphatase 1B dephosphorylates JAK2, stops STAT3 activation and thereby inhibits Leptin pathway that leads to Leptin resistance [50] as shown in figure 8.

Subsequently, lack of exogenous Leptin and increased levels of endogenous Leptin together called as Leptin resistance. It further causes insulin resistance, lipid depot in liver inhibits fatty acid oxidation and ultimately leads to obesity and metabolic syndrome [51]. The activities of Leptin on the arcuate (ARC) nucleus of hypothalamus involved in appetite regulation and energy homeostasis. ARC contains anorexigenic pro-opiomelanocortin-containing (POMC) neurons and orexigenic agouti-related

protein/neuropeptide Y-containing (AgRP/NPY) neurons. Leptin stimulates POMC-containing neurons while inhibits AgRP/NPY-containing neurons in the ARC nucleus, resulting in a reduction in hunger [5]. The clinical conditions associated with serum leptin level are Hyperleptinemia and hypoleptinemia [53].

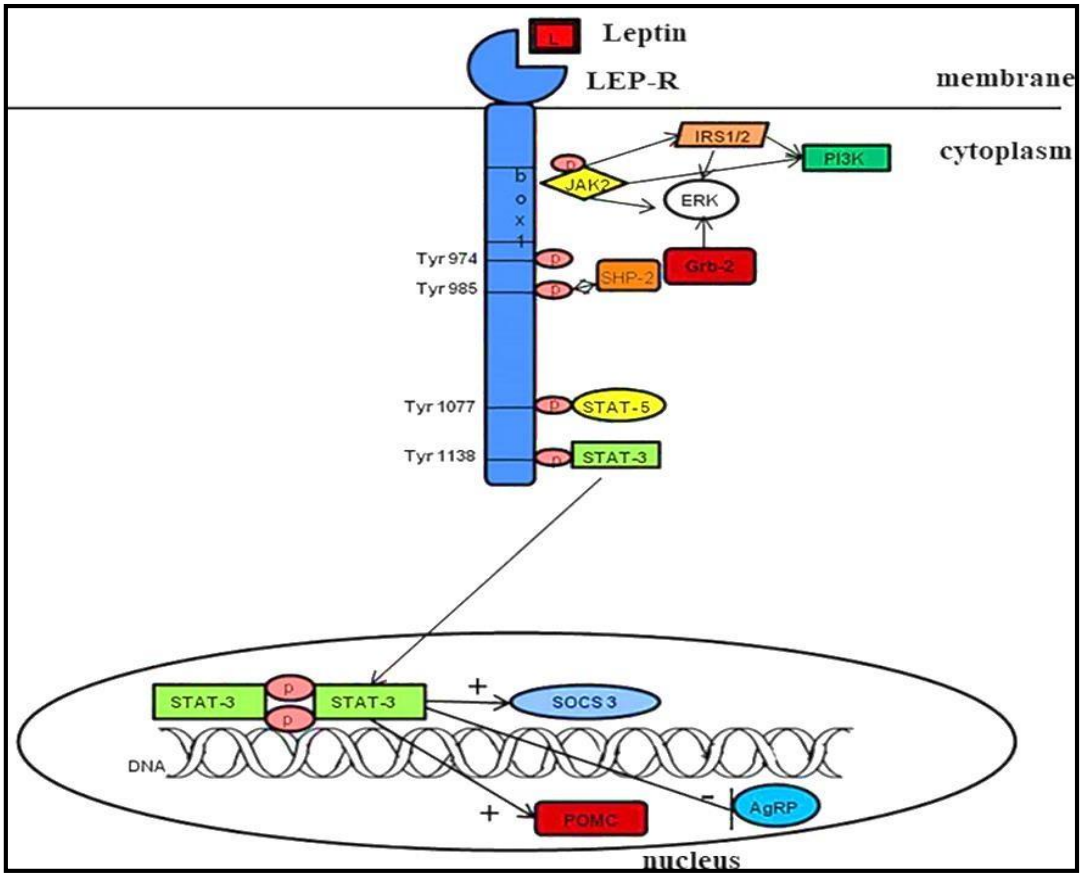


Figure 8: Leptin signaling pathway in food intake

Source: Obradovic M, Sudar-Milovanovic E, Soskic S, Essack M, Arya S, Stewart AJ, Gojobori T, Isenovic ER. Leptin and obesity: role and clinical implication. Frontiers in Endocrinology. 2021;12 [54].

Hypoleptinemia: Severe obesity, decreased satiety, intensive hyperphagia, continual food-seeking behaviour, hyperinsulinemia, dyslipidemia, liver steatosis, recurrent bacterial infections, and hypogonadism are all clinical manifestations of complete leptin deficiency [55]. Congenital hypoleptinemia is caused by mutations in the Leptin or Leptin receptors gene and is referred to as congenital leptin deficiency. Acquired hypo-leptinemias share several of these features and are typically caused by diseases that result in a low body weight. Lipodystrophy syndromes and hypothalamic amenorrhea are examples of acquired disorders.

Hyperleptinemia: Obesity is characterized by hyperleptinemia and leptin resistance. A direct link between blood leptin concentrations and body fat percentage has been found with obese people, having higher leptin serum levels and adipocyte leptin mRNA content than People with normal-weight. With weight loss accompanies decrease blood leptin levels and adipocyte Leptin mRNA content [55].

Inadequacies in leptin transport through the blood-brain barrier or intracellular signalling systems and downstream of the Leptin receptors appear to be connected to Leptin resistance. Non-alcoholic fatty liver disease, neuro-degenerative illnesses, depression and food addiction are all conditions linked to hyperleptinemia [56]. Both hypoleptinemia and hyperleptinemia-related syndromes are being studied using recombinant analogues of leptin. Leptin replacement was originally developed to treat obesity and has only been shown to work in leptin-deficient patients, even with replacement of leptin in obese people found with high leptin levels but having minimal efficacy.

Although certain studies suggest that leptin replacement can reverse some of the anomalies of the syndromes and these illnesses are not yet recognised as a reason to

employ recombinant leptin as a treatment [57].

1.13. Spexin

Neuropeptides and adipokines are secreted from adipose tissues and other organs. Neuropeptide Q (NPQ) is also known as Spexin (SPX) a newly identified neuropeptide hormone encoded by the Ch12orf39 gene, which is located on q short arm of chromosome 12 in humans. The Spexin gene contains six exons and five introns. Spexin is co-evolved with galanin/Kisspeptin family [58]. The biologically active 14 amino acids mature peptide (NWTPQAMLYLKGAQ) is released from a prepropeptide consists of 116 amino acid residues fringed by dibasic cleavage sites [59]. The signal peptide is encoded by the first and second exons, while the mature peptide sequence is encoded by the third exons [60]. The cognitive receptors of Spexin is GalR2 and GalR3 as shown in Figure 9, 10 &11.

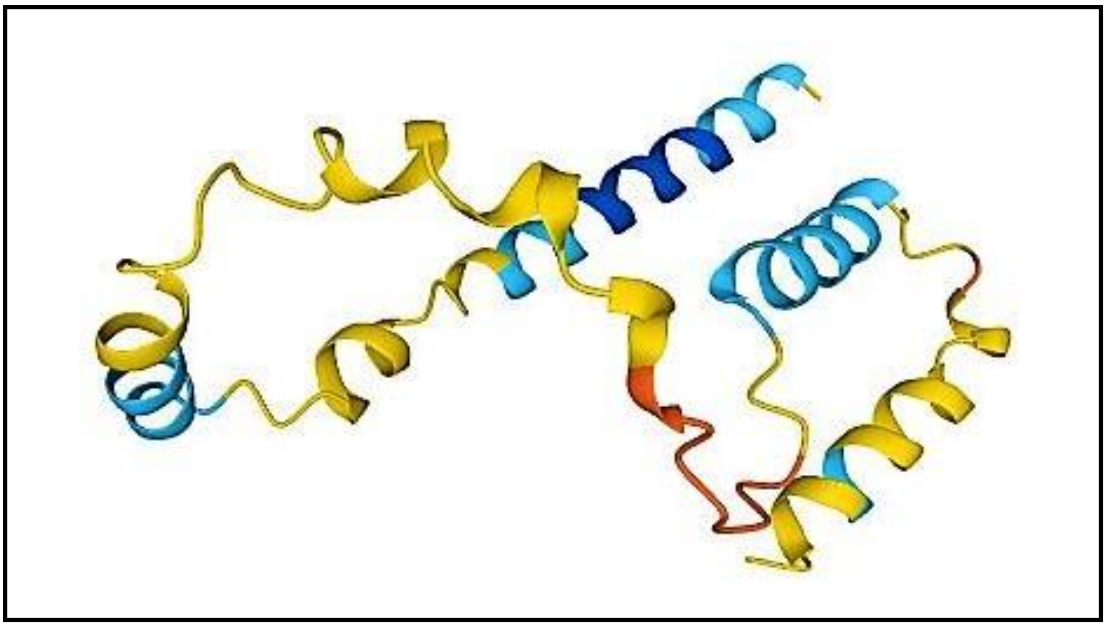


Figure 9: 3D Structure of Spexin
Source: https://www.uniprot.org/uniprot/Q9BT56#structure

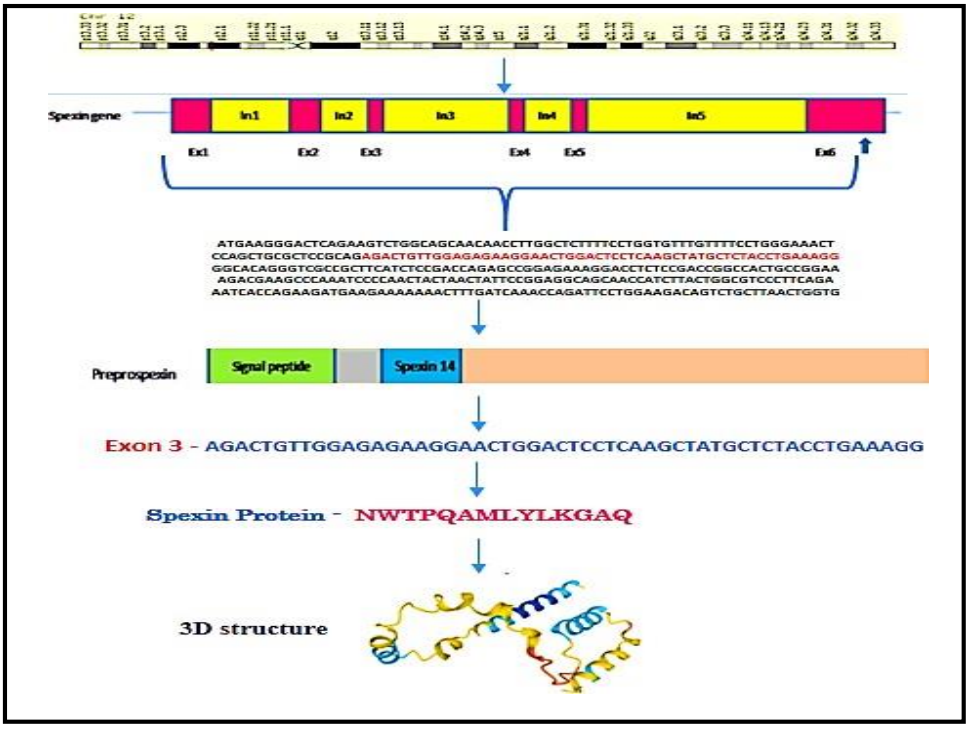


Figure 10: Schematic representation of Spexin gene structure

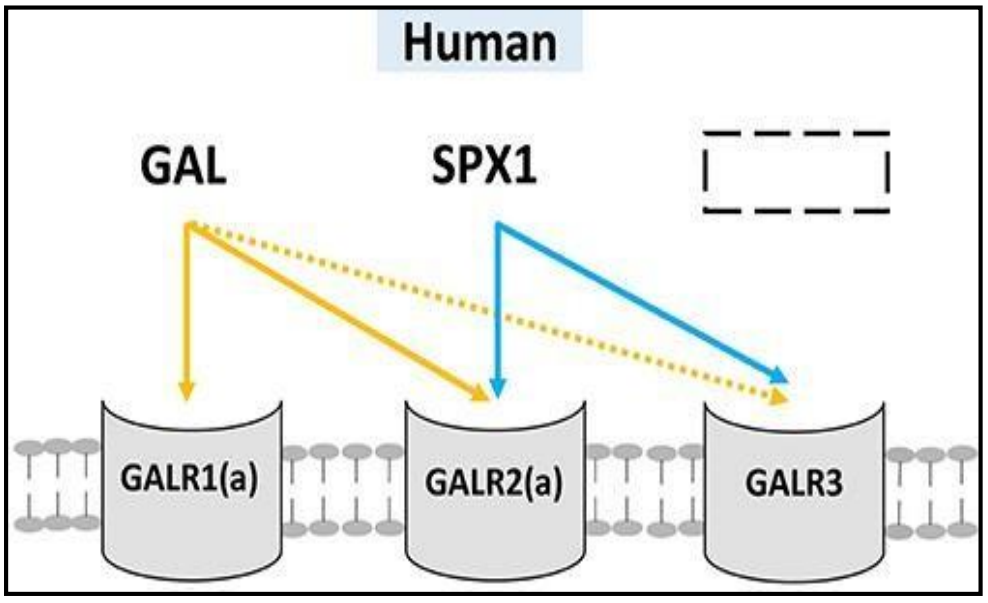


Figure 11: Receptors of Spexin in humans

Source: Lim CH, Lee MYM, Soga T, Parhar I. Evolution of Structural and Functional Diversity of Spexin in Mammalian and Non-mammalian Vertebrate Species. Front Endocrinol (Lausanne). 2019 Jun 19;10: 379 [61].

The mature mammalian Spexin gene sequence found to be conserved, several mRNA transcript variants have been found, phylogenetic and comparative synteny analysis revealed that Spexin gene catergorized into Spexin 1 and Spexin 2. The structure of Spexin 1 & Spexin 2 gene showing number of exons and introns presented in figure 12a & 12b. Whereas Spexin 1 was confined in mammals and Spexin 2 in non-vertebrates [61] as shown in figure 12b.

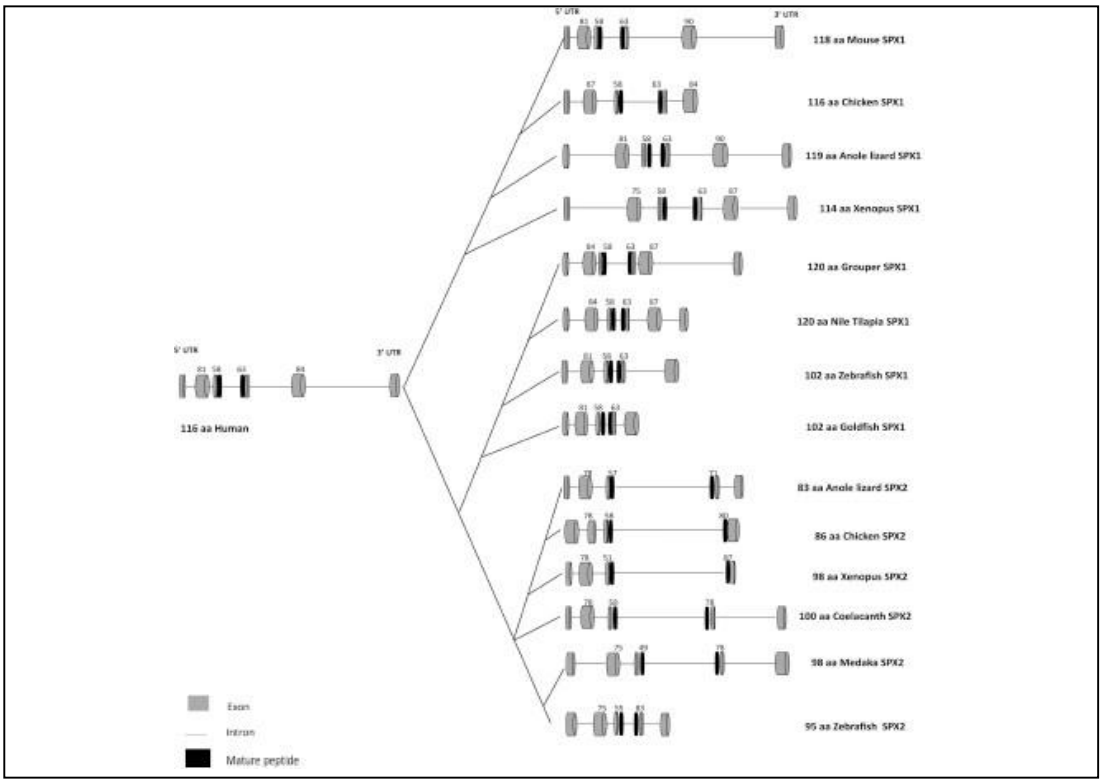


Figure 12a: Spexin exon and intron variation in vertebrates and non-vertebrates
Source: Lim CH, Lee MYM, Soga T, Parhar I. Evolution of Structural and Functional Diversity of Spexin in Mammalian and Non-mammalian Vertebrate Species. Front Endocrinol (Lausanne). 2019 Jun 19;10:379 [61].

Despite, the cleavage site in rat is replaced from Arginine to histidine from Gly-lys-Arg (GKR) instead of Gly-Arg-Arg (GRR) which was found in mammals and variation exists at positions 3, 6, 13, and 14 i,e., 3rd (Thr Vs Gly), 6th (Ala Vs Ser), 13th (Thr Vs Arg or Ala) and 14th (Gln Vs His or Tyr) Kisspeptin & Spexin do not have similar sequence 3rd (Thr Vs Gly), 6th (Ala Vs Ser), 13th (Thr Vs Arg or Ala) and

14th (Gln Vs His or Tyr) suggesting that mature peptide of Spexin 1 and Spexin 2 plays an important physiological role in vertebrates [62].

According to Molecular evolution, the amino acid positions in Spexin 1 is similar with Gal at corresponding positions 2,3,9,10 and 12 which indicates that positions of Gal at Try2, Thr3, Tyr9 are the key determinants for interaction between Spexin and GalR and as well as its binding & activation [63] as shown in figure 12b.

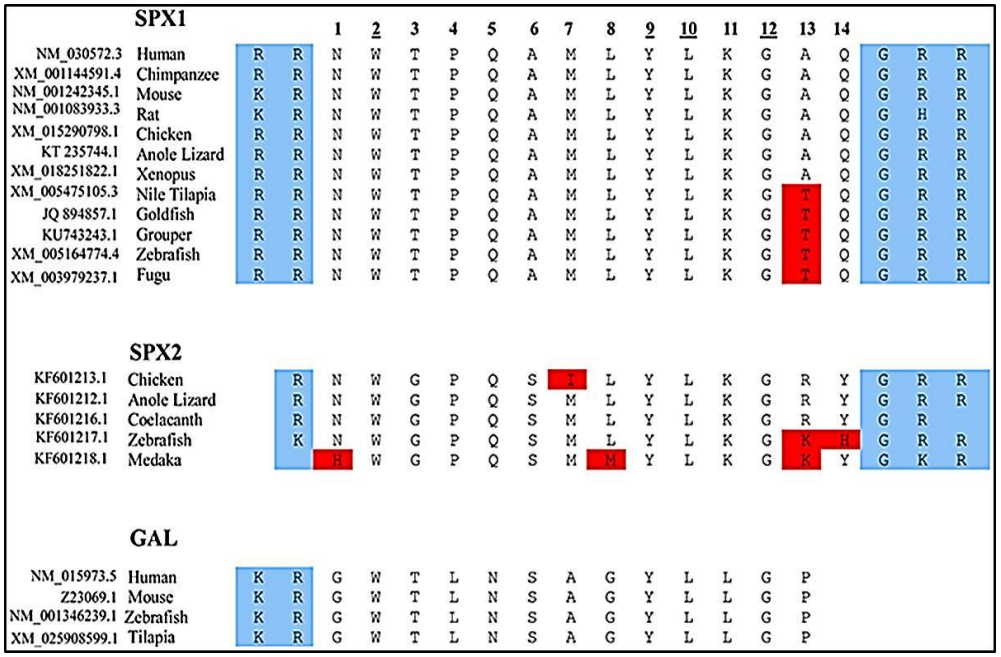


Figure 12b: Spexin 1 mature peptide sequence in humans and non-vertebrates

Source: Lim CH, Lee MYM, Soga T, Parhar I. Evolution of Structural and Functional Diversity of Spexin in Mammalian and Non-mammalian Vertebrate Species. Front Endocrinol (Lausanne). 2019 Jun 19;10:379 [61].

Hence, a ligand-receptor study proved that Spexin 1 is a natural ligand in humans whereas Spexin 2 in non-vertebrate's species activates Galanin receptor 2/3 Spexin undergoes post-translational modification of α-amidation at Carboxy terminal of peptide chains secretes into extracellular space by classical endoplasmic reticulum Golgi dependent pathway via G-protein coupled receptor (GPCR) as mature peptide that has a highly conserved sequence in nature [62].

The biological functions of Galanin are well documented for differential regulation of PI3K/Akt- dependent cascades, MAPK, cAMP/PKA and Calcium through coupling of GalR2/3 receptors with Gq/11 & Gi GPCR subunit [64]. Though the receptors are similar for Spexin and Galanin (i.e) GalR2 and GalR3, the signal transduction pathways which was involved in biological role of Spexin are not illustrated [65].

RNA sequencing of various tissues samples indicated that Spexin peptide is expressed in many tissues but the high tissue specificity/expression found in adipose tissue, decreased trend of expression has been seen in kidney, thyroid, brain, placenta, heart, pancreas, salivary gland, liver and lung [66]. Biochemical analysis revealed that Spexin co-localized with insulin in secretory vesicles of pancreatic β-cell line and suggested that it could be processed and secreted at cellular level by exocytosis. [67].

The protein encoded by the Spexin gene is a hormone brings cardiovascular and kidney function modulations. It has several physiological functions such as inducing stomach contractions, postnatal hypoxia response, nociceptive response, inhibiting adrenal proliferation, inhibit uptake of long chain fatty acid, weight regulation, regulates glucose and lipid metabolism, Cardiovascular and renal modulation [61-62].

Despite, Spexin precursors was predominantly expressed in sub mucosal layer of stomach in rats and mouse oesophagus and causes stimulation of muscle contractions [59]. Research reports shown that decreased circulating Spexin levels are related to blood glucose and lipids in obesity and Type 2 diabetes mellitus, which may represent an adaptation to the increase of glucose and lipid metabolism associated with Type 2 diabetes mellitus [61,66] .

Remarkably, the physiological and pathological functions of Spexin are still at the beginning and the information about the molecular mechanism of the Spexin is

deficient. The signal transduction pathways through Spexin -based galanin receptors are still need to be elucidated. Studies on clinical trials, longitudinal and randomized controlled trails are necessary to explore about Spexin role & its receptors, and Spexin therapies helps to manage obesity and Type 2 diabetes mellitus.

The involvement in human physiological functions needs to be elucidated and established. However, in animal model Spexin known to cause weight loss was evidenced.

1.14. Lacunae of knowledge

Worldwide, 25 percent of people are suffering from MetS [68]. In Indian scenario MetS prevalence refers from 10-40 percent [69]. MetS co-existed with diabetes mellitus and cardiovascular disorders associated with the energy use and storage. As a non-communicable disease, MetS is of great importance due to high risk of cardiovascular problems in diabetes. Central obesity and insulin resistance are two important risk factors mask the body to utilize sugar energy. Life style and nutritional therapy management can helps to prevent the MetS considerably and effectively. Few biomarkers are well known to fix the MetS are larger waist hip ratio, elevated blood glucose, elevated triglycerides, decreased HDL-C and elevated blood pressure [70].

Even though, significant amount of research work carried out in this domain. Yet there is need to look for establishment of any new markers in single or in combination for better understanding or genetic relation for betterment of individual's life. A panel of biomarkers in MetS studied are Leptin, Leptin/adiponectin ratio, Prothrombin activator inhibitor-1, uric acid, IL-6, TNF-α and oxidized HDL-C are generally studied [71].

Various mechanisms proposed to explain the MetS, even though it has become the key challenge of country and attracted the attention of the many scientists. What is clear until now is that to assessing the dynamics of MetS progression that has a vital role in clarifying the pathways of the disorder. Due to the complexity of MetS with the various influences and consequences, it is hard to make a well-defined distinction between the various groups of biomarkers.

Since, there is a growing evidence in research on MetS about Leptin which is an adipokine secreted from white adipose tissue having a role in energy homeostasis. Increased Leptin levels may leads to obesity [49], resistin is an adipokine produced from white adipose tissue play a role in inflammation and lipid metabolism. Increased resistin levels shows an association with insulin insensitivity and obesity [72], Xenin is also an adipokine produced from adipose tissue having a physiological role in food intake. Increased levels of Xenin contributes to development of obesity [73].

Research on prospect of peptides for use in obesity treatment is still not clear. Peptidomics is a new branch of proteomics which is based on the research of endogenous protein fragments that serve an important role of energy metabolism in white and brown adipose tissue. Few peptides as drug with marked achievement for obesity treatment are Glucagon like peptide, adropin, preptin and irisin etc. Hence, there is a further scope for fragments of protein molecules and endogenous peptides produced by adipocyte that have attracted the attension of researchers[74]. Therefore, polypeptide/peptides can prevent insulin resistance and obesity, keeping this in mind the growing research on peptidomics is boon for therapeutic strategy in MetS.

A recent research studies reported that Spexin levels are negatively correlated with blood glucose, HbA1c, TG/LDL/TC in Type 2 Diabetes Mellitus patients and demonstrate that Spexin play a role in glucose and lipid metabolism [66]. It is observed that there is a negative relationship of Spexin with Leptin and increased concentrations of hs-CRP in obesity adolescents, reported low Spexin level and increased Leptin level in the regulation of satiety and cardiovascular risk factors in pediatric obesity [75].

Even though, there is a scope to evaluate Spexin and its genetic polymorphism in Type 2 diabetes mellitus with and without hypertensive subjects.

One of the novel functions of Spexin is regulation of glucose metabolism, recent lilteratures demonstrated that human pancreatic islet is known to have Spexin expression. Spexin immuno-reactivity has been shown to be co-localized with the insulin in secretory vesicles that implies Spexin is co-released with insulin [67]. Since, Spexin levels and its gene expression is markedly down-regulated in obesity and strongly involved in pathogenesis of Type 2 diabetes mellitus, Obesity and Insulin resistance [76].

Our research focused on to find out the possible link between Spexin and Metabolic syndrome and also the underlying possible genetic polymorphism with respect to Spexin in South Indian population having Type 2 diabetes mellitus with and without hypertensive subjects. This research gap has become the need for the study.

CHAPTER-2
REVIEW OF LITERATURE

2.0. REVIEW OF LITERATURE

The following are the recent literature reviewed in relation on Spexin, Leptin and Spexin gene polymorphism

2.1. LEPTIN

In 2022, Peng X and collegues conducted a Cross sectional study in China population to estimated plasma Leptin and resistin concentration in subgroups of Type 2 diabetes mellitus. The subgroups of the study were mild obesity-related diabetes, severe insulin-deficient diabetes, severe insulin-resistant diabetes and mild age related diabetes with total 541 cases. Amongst 285 patients samples were analyzed for Leptin and resistin levels. The study showed increased levels of plasma Leptin in the mild obesity-related diabetes group with relatively decreased levels in the severe insulin-deficient diabetes and severe insulin-resistant diabetes group and resistin showed high in mild age related diabetes. Study concluded that high Leptin levels in mild obesity-related diabetes group associated with BMI and mentioned approach of Leptin therapy for other groups and high resistin levels in severe insulin-resistant diabetes group. Study limitations was done in small sample size and inflammatory markers was not screened. Hence, Study also recommended that longitudinal studies are essential to explore the exact mechanism of Leptin in Type 2 diabetes mellitus [76].

In 2021, Kaze AD and his co-workers conducted a community based cohort study in African American population to evaluate the plasma Leptin and adiponectin, adiponectin-leptin ratio and hsCRP associated with dysglycemia or pre-diabetes/diabetes. The study comprise 3223 participants without diabetes over a period of seven years developed 46.4% glycemic progression. The study concluded increased study parameters associated with development of Type 2 diabetes mellitus [77].

In 2020, Bidulescu A and his collegues carried out similar cohort study among African Americans with 3363 participants without diabetes comprising 1230 males and 2133 females. They evaluated adiponectin and Leptin in Type 2 diabetic patients compared to control. The study finding concluded that adiponectin inversely related to Type 2 diabetes mellitus, whereas Leptin had direct association with Type 2 diabetes mellitus mediated by insulin resistance. This observation was more prominent in men and non-obese participants. Study also indicated to reduce the burden of Type 2 diabetes mellitus by modification of serum Leptin and adiponectin levels is essential [78].

In 2020, Kumar R and his co-workers conducted a Case-control study in Pakistan population to observe the relationship of Leptin in obesity and insulin resistance. The total 92 participants with BMI >25kg/m2 were recruited as cases and BMI <25kg/m2 were enrolled as control group. The study showed significant increased Leptin levels, total cholesterol and insulin resistance in patients with higher BMI. They concluded that increased Leptin levels is linked with high BMI and insulin resistance and mentioned the monitoring of Leptin levels in patients with Type 2 diabetes mellitus has a crucial role in managing obesity. However, the limitations noticed in the study was a leptin levels but it is not associated with Type 2 diabetes mellitus [79].

In 2019, Schnurbein JV and his co-workers conducted a cohort study in Germany population to examine the Leptin substitution by metreleptin in patients with congenital Leptin deficiency. Total 9 patients were recruited in the study. Amongst 6 patients with congenital Leptin deficiency and 3 patients with biallelic disease-causing variants in the Leptin receptor gene. The study observed that short-term substitution of Leptin triggers increased blood pressure and heart rate in 3 patients

and long-term Leptin substitution improves weight loss, decreases resting heart rate in 4 patients. Study concluded that Leptin is not the root cause of obesity-associated hypertension but also has other confounding factors adds to the rise in blood pressure. The study limitations was less samples of pediatric cases [80].

In 2018, in a meta-analysis conducted by Katsiki N and his co-workers on Leptin review accounted that, increased Leptin levels are in relationship with insulin insensitivity and progress of Type 2 diabetes mellitus. Moreover, obesity, hypertension in MetS has endothelial dysfunction with increased Leptin levels. Study concluded that stroke, carotid artery disease, chronic kidney disease, and Type 2 diabetes mellitus all have linked with Leptin levels. Besides, Leptin also involved in promotion of inflammation, thrombosis, arteriosclerosis, angiogenesis, and atherosclerosis. Therefore, statins and antidiabetic medications might influence Leptin concentration [81].

In contrast, Sheikh AL and his collegues conducted a cross sectional study in 2017 in Saudi male population to identify the serum Leptin levels in Diabetic and non-diabetic patients. Amongst them 55 diabetic and 58 healthy subjects. The study results indicated no relationship between Leptin and adiponectin in diabetic and control groups.

The Study findings reported that decreased Leptin and other metabolic parameters as the severity of the disease progress. The study limited only to males hence it cannot be generalized [82].

2.2. SPEXIN

Spexin peptide molecule have attracted insufficient attention research investigations around the world, and information on Spexin levels and their functional significance in obesity and diabetes is limited. Despite the fact that a few papers on Spexin research were described here.

Walewski JL and his collegues in 2014 conducted an experimental study on role of Spexin gene in diet induced obese mice. The study reported that its gene is down-regulated in obesity and play a role in obesity pathogenesis. The Spexin gene was shown to be 14.9 times down-regulated in obese omental and subcutaneous fat. The study also showed that Spexin inhibited fatty acid uptake in DIO mouse adipocytes in vitro. Study conclusion extrapolated that Spexin gene expression is down-regulated in obese people and increases weight reduction in Diet induced obese rats [83].

Kumar S and his collegues in 2016 conducted a cross-sectional study in Florida population to evaluate the effect of Spexin in obese children and its link with cardio-metabolic risk factors in 69 children with age of 12-18yrs. Amongst 69 subjects, 51were obese and 18 were normal weight. Study found that Spexin levels were considerably lower in obese children compared to normal-weight children, but did not correlate with other adipokines, insulin, or glucose levels. Study concluded that obese children have lower amounts of Spexin in the serum. The study limitations was cross-sectional study, lack of longitudinal data and were unable to evaluate the pubertal status, physical activity habits, the duration of obesity regional and/or fat distribution that influenced Spexin levels [84].

Lin CY and co-workers in 2018 conducted a cross-sectional study in China population to investigate the link between Spexin levels, age, BMI, fasting glucose and lipid profile in 68 healthy adult women based on their age groups of 18-65yrs. The study found that serum Spexin levels are linked to age, BMI, fasting glucose, and TG. However, lower Spexin levels were showed to indicate the probability of having a high BMI and high fasting glucose level. Study concluded that circulating Spexin levels decline with age. The limitations of the study were small sample size, limited only in healthy women and not in men, Cut-off value of Spexin could not able to establish in healthy women for the future risk of obesity and Type 2 diabetes mellitus.Therefore, study also recommended that longitudinal studies involving both men and women from young to old will be required to further explore the importance of Spexin in ageing and underlying mechanism that causes circulating Spexin to decrease with age [85].

Kolodziejski PA and his co-workers in 2018 conducted a study in Poland population to investigate the relationship between Spexin or kisspeptin levels and BMI, HOMA-IR, serum levels of insulin, glucagon, leptin, adiponectin, orexin-A, obestatin, ghrelin, and Glucagon-Like-Peptide-2 in obese and non-obese female patients with n=15 per group. The study showed that patients with obese have lower levels of Spexin and kisspeptin than non-obese participants. The study also found negative correlation with HOMA-IR, BMI, insulin and positively with Spexin QUICKI index and adiponectin. Study concluded that Spexin and kisspeptin is having a negative relationship with obesity and insulin resistance. The study limitations include less sample size, immune-histological analysis on physiological role of Spexin in energy metabolism needs to be explored in larger cohort studies [86].

Kumar S and his collegues in 2018 conducted a pilot study in USA population to observe the functional role of leptin, Spexin, and several biomarkers in cardiovascular disease in obesity adolescents with age 15.8 ± 1.7 years in nineteen adolescents with obesity. The study showed inverse relationship between Spexin and Leptin, along the presence of higher hs-CRP concentrations in obese adolescents in the presence of 'low Spexin and high Leptin,' Study concluded that Spexin may serve a central role in the regulation of satiety and some cardiovascular events in obese children. The limited sample size, cross-sectional character of the study, lack of serial measures, and lack of a non-obese control group for comparisons are all limitations of our study. Despite the overwhelming evidence for an negative relation between Spexin and Leptin, the small number of patients in each of the two subgroups ("high Spexin/low leptin" and "low Spexin/high leptin") reduced statistical power to identify significant changes in other outcome variables if any [87].

Daghri AL and his collegues in 2018 conducted a cross sectional study in Saudi Arabia population with sample size included 124 MetS patients (41 men and 83 females) and 136 (21 male and 115 females) healthy individuals and to estimate the relationship between Spexin concentration and MetS components. Study found that lower circulating Spexin levels in MetS subjects. The study concluded that decreased Spexin levels was marginally linked with MetS components and are sex-specific in adults in patients with MetS compared to non-MetS individuals. The study limitations was cross-sectional, the causal relationship between Spexin and MetS could not be established. Furthermore, factors that could influence Spexin, like as food intake and physical activity, were ignored. Furthermore, because of the limited and female-dominated sample size, the findings should be regarded with caution [88].

Karaca A and his co-workers in 2018 conducted a Cross-sectional study in Turkey to estimate a Spexin levels in both type I and Type 2 diabetes mellitus patients and their relevance to glycemic parameters without the presence of obesity or insulin resistance in (n=29) type I and (n=30) Type 2 diabetes mellitus and (n=23) controls. The study found lower Spexin levels in Type I and Type 2 diabetes mellitus patients compared to controls and no correlation between Spexin levels and glycemic parameters in normal weight Type I diabetic patients. Study concluded that lower serum Spexin levels were associated with Type 1 diabetes mellitus and independent of lipid parameters, BMI and glucose [89]. The study limitations, do not explain why Type 2 diabetes mellitus have low Spexin levels, although being slightly greater than Type 1 diabetics. Because the purpose of this study was to compare type 1 diabetic patients to age- and BMI-matched controls, comparing Spexin levels in cohort of Type 2 diabetic patients was not exactly appropriate, which could explain the finding. However, there was no correlation between Spexin levels and glycemic indices in patients with Type 2 diabetes mellitus after adjustment of BMI and age. The study support the idea that glycemic management and serum Spexin levels were unrelated.

Daghri AL and his co-workers in 2019 conducted a Cross-sectional cohort study in Saudi Arabia population to estimate the Spexin levels in 6-month self-monitored lifestyle modification programme from a larger cohort of 294 subjects, 160 comprised Saudi adult males (n=64) and females(n=96 with pre-diabetes were randomly selected. Study found that favorable changes in fasting glucose have an inverse effect on Spexin levels in females with pre-diabetes and concluded that circulating Spexin levels rise over time in people with pre-diabetes, particularly women who responded well to a 6-month lifestyle intervention programme. The study has few limitations like there were more female participants than males and intervention period was

relatively short with small sample size that might have resulted in a non-significant increase in Spexin levels in males. The study rigid for inclusion criteria which limits its application to people with pre-diabetes. Lastly, changes in eating habits, physical activity, and body fat/muscle distribution in study participants were not available and that should be assessed in future studies [90]

Chen. T and his co-workers in 2019 conducted a Cross-sectional study with 40 obese and 32 normal-weight pre-puberty children to determine the potential role of Spexin in obese children and its correlations with obesity-related indicators, insulin sensitivity, and pancreatic cell function. Study showed Spexin levels were considerably lower in obese children compared to non-obese children, and negatively linked with insulin insensitivity and pancreatic cell function markers. Study concluded that Spexin appears to serve a protective role in glucose homeostasis and is linked to cell function in obese children. The study limitations was small sample size, cross sectional study and they would have compare Spexin with leptin a gold standard marker in obese patients [91].

Liu Y and his co-workers in 2020 conducted an expressional study to observe the role of Spexin expression in human and mouse tissues and the impact with and without hypoxia on Spexin levels in primary neonatal rat ventricular myocytes (NRVMs). Study reported that the exposure to hypoxia reduces Spexin levels in NRVMs, whereas exposure to hypoxia with Spexin causes regulation of fatty acid metabolism by enhancing expression of fatty acid translocate/cluster of differentiation 36 (FAT/CD36), Carnitine palmitoyltransferase I (CPT1), peroxisome proliferator-actiavted receptor alpha (PPAR-α) and peroxisome proliferator-actiavted receptor gamma co-activator 1-alpha (PGC1- α), improves glucose uptake, and significantly

prevents down-regulation of mitochondria. Study concluded that Spexin maintains energy and mitochondrial homeostasis under hypoxia in cardiomyocytes implying that Spexin can be useful in the treatment of cardiovascular disorders [92].

Zhang L and his co-workers in 2021 conducted a Cross-sectional study in China with 41 non-alcoholic fatty liver patients to determine the relationship of Adiponectin and Spexin levels in insulin resistance patients with alcoholic steatohepatitis. The study comprised of age with Mean & SD of 35.17±12.29 years and BMI 30.97±2.75 kg/m2, 38 healthy controls with age Mean & SD of 38.47±11.63 years along with BMI 22.83±3.00 kg/m2. They found that significant lower Spexin and Adiponectin levels in non-alcoholic fatty liver disease patients compared to controls and did not correlate with BMI, but they did with HOMA-IR ($r = -0.368$; $P = 0.018$) and Adiponectin ($r=0.378$; $P=0.043$), leading to the conclusion that insulin resistance was strongly connected with Spexin and Adiponectin levels. Limitations of the study is that it was conducted on less sample size [93].

Khadir A and his collegues in 2020 in Kuwait conducted a Cross sectional study on the effects of circulating Spexin concentration in obesity, Type 2 diabetes mellitus and its modulation by physical exercise. The study included 50 healthy subjects, obese with and without Type 2 diabetes mellitus (n=69 and n=66) and subgroup only obese (n=47) participants who underwent a three month exercise programme. Study found decreased plasma Spexin concentration in obese with or without Type 2 diabetes mellitus subjects. Study also reported that Spexin levels significantly increased only in responders to exercise with a concomitant improvement in metabolic profile. Study concluded that more research is needed on the role of physical activity in reducing the effects of metabolic stress linked to obesity and

insulin resistance on Spexin. This study has some limitations, they were unable to identify whether lower Spexin levels related to the development of obesity and diabetes. Furthermore, due to the small number of study participants, unable to generalize study findings. Since, diabetes is linked to ageing, the current study was unable to obtain age-matched healthy normal-weight and obese controls. Furthermore, the investigation was based on a single measurement of fasting Spexin, and dietary consumption was not accounted in the study. Another constraint that prevented from concluding on the status of Spexin was the lack of normal-weight participants in the regular exercise [94].

Gambaro S.E. and his co-workers in 2020 conducted an experimental study in Argentina to evaluate the role of Spexin in improving metabolic and inflammatory profiles in fructose-rich diet obese mice. The study found a positive correlation between body weight before and weight loss after Spexin treatment, as well as improved liver TG, mass, adipocyte hypertrophy also mRNA of leptin in adipose tissue also reduced TNF-α, IL-6, and macrophage M1 (Ly6c-) expression in epidermal adipose tissue. Study concluded that Spexin lowers body weight, improves metabolic profile, and reduces adipocyte hypertrophy, inflammatory marker expression in macrophages [95].

Behrooz M and his co-workers in 2020 conducted a cross-sectional study in Iran with 90 children with metabolic syndrome to observe whether the Spexin levels are related with metabolic syndrome, body composition and dietary intakes in children. The study found that Spexin levels were significantly lower in children with high fat mass and children with Systolic blood pressure (SBP) compared to controls and also showed significant negative associations with dietary fat intake. Study concluded that

there is an association between Spexin adipose tissue and its metabolism. Study indicated a limitations that the causal relationship of Spexin to MetS cannot be determined due to the study's cross-sectional design and limited sample size [96].

Senturk N.G.K. and his co-workers in 2021 conducted a cross sectional case –control study in Turkey with 80 obese patients and 80 healthy controls to assess serum Spexin Levels as well as relationship of Spexin in obese adolescents with MetS Antecedents. The study found that lower Spexin levels in obese patients 50 pg/mL compared to healthy controls 67.0 pg/mL with significant p value =0.035. As well as in obese with MetS subjects had 24.5 pg/mL and in obese without MetS had 69.0 pg/ml. The study also showed significantly negative association with BMI & HOMA-IR with a 75 percent sensitivity and 71 percent specificity, HOMA-IR with a cut-off level of 49.5 pg/mL might predict the occurrence of MetS in obese adolescents with a cut-off level of 49.5 pg/mL. Study concluded that obese adults have reduced Spexin levels, and could be more prone in those with MetS [97].

Amirpour M and his collegues in 2021 conducted a Cross sectional study in Iran to determine a link between serum Spexin levels and MetS components in obese and normal-weight people with or without diabetes. Study found that there was an inverse relationship between Spexin and Waist circumference, TG, FBS in healthy people without diabetes. Study concluded that Spexin may serve as a potential marker for MetS in normal weight people with or without diabetes. The study limits with less sample size, cross sectional study and could not able to compare with other obesity biomarkers like ghrelin, Leptin and adiponectin [98].

Chen Y and his co-workers in 2021 conducted an experimental study on differential regulation by glucose and insulin in glandular stomach and functional implication in feeding Control in mouse and the mice were injected through Intraperitoneal with glucose and insulin respectively to investigate the role of insulin signal caused by glucose uptake in Spexin regulation. The study observed that co-injection of Spexin and glucose in glandular stomach causes Spexin mRNA expression which could be blocked by insulin levels and concluded that Spexin injection can regulate glucose and insulin levels for feeding control. Limitations of the study was in glandular stomach the mechanisms involved for the postprandial changes of Spexin expression are still unknown [99].

Kolodziejski P.A. and his co-workers in 2021 carried out an experimental study on the role of Spexin levels in starvation in Broiler Chickens along with mRNA expression in various chicken tissues. And investigated the link between the serum level of Spexin and other metabolic parameters like insulin, glucagon, glucose, triglycerides, and cholesterol by Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) technique. They found that food restriction increases the expression of Spexin 1, and GalR2/3 in tissues which is linked to glucose and lipid metabolism and hence concluded that Spexin is involved in metabolic control [100].

Beyazit F and his co-workers in 2021 conducted a cross-sectional study on serum Spexin, adiponectin, and concentration of Leptin in polycystic ovarian syndrome in relationship with alpha ketoglutarate dependent dioxygenase gene (FTO + rs9939609) polymorphism and to determine the association between fat mass related adipocytokines and SNPs in the FTO +rs9939609 gene in women with polycystic ovarian syndrome.

They observed that serum Spexin levels were not showing any difference between study groups polycystic ovarian syndrome women (n=91) and healthy control (n =86)and concluded leptin levels and Serum adiponectin may serve as independent indicators for polycystic ovarian syndrome diagnosis and limitations in the study was adipocytokine levels in the blood may not reflect all adipocytokine-related processes. Given that polycystic ovarian syndrome and obesity are linked to long-term low-grade inflammation, it would have been helpful to assess inflammatory markers simultaneously. Second, since the total sample is less, large-scale studies are needed to validate the link between FTO SNPs and polycystic ovarian syndrome susceptibility [101].

Kolodziejski P.A. and his co-workers in 2021 conducted an animal model in Poland on sixty female mice to assess the effects of 30 days of Spexin treatment on serum glucose and lipid levels, insulin sensitivity, and hormonal profile such as insulin, glucagon, adiponectin, leptin, TNF α, IL-6, and IL-1 in mice with induced obesity Type 2 diabetes mellitus. They found that Spexin reduces body weight in control and diet induced obese mice as well as lipid content, IL-6 and TNF-protein levels in diet induced obese and Type 2 diabetes mellitus mice. Study concludes that Spexin treatment for 30 days regulates hormonal and metabolic status that improves insulin sensitivity, improves glucose tolerance, and reduces body weight [102].

Kumar S and his co-workers in 2021 presented a review focused on the role of Spexin as a biomarker in obesity and related cardio-metabolic disease and demonstrated that Spexin is a 14-amino-acid neuropeptide encoded by the Ch12:orf39 gene, it is widely distributed in various body tissues and organs. Spexin also has a key regulatory role in obesity and its co-morbidities. The circulating concentration of Spexin is

significantly lower in obese than in healthy people implying that Spexin has a relationship with cardiovascular disease biomarkers and glucose metabolism, and raising the possibility of Spexin serving as a key biomarker in the early loss of cardiometabolic health and later development of CVD and diabetes [103].

Albeltagy E.S. and his co-workers in 2022 conducted a cross sectional study in Egypt on Spexin levels and their association with obesity, glycemic metabolites and cardiovascular factors in lean individuals compared to physical activity matched obese individuals with the participation of 135 individuals with various BMI. The study found that serum Spexin levels were significantly lower in obese patients than in overweight or normal individuals and in diabetes sub group compared to normal glycemic group. The AUC curve for Spexin with diabetes was 0.912 (95 percent CI 0.085–9.781, P 0.001) when compared to individuals with normal glycemia. Study concluded that Spexin could be a valuable biomarker of metabolic health state.

Few drawbacks are that it was a single-centric trial, authors were unable to determine whether the decreasing Spexin level was causal in the development of Type 2 diabetes mellitus and obesity. Since, research on the physiological and pathological roles of Spexin is limited, and is still in early stages & also information on the Spexin molecular mechanism is lacking. Thereby, future research on receptor activated signal transduction pathways of Spexin as well as clinical trials were demonded [104].

Hala Abdulhade Salih & Khan D et al. in 2020 in a study presented on the Genetic aspects of Iraqi children obesity by determination the role of Spexin gene mutations and its hormone expression. Serum's level of spexin in obese children (n=20) in Healthy children volunteers (n=20) in the age range of 6-12 years were taken which

their BMI ranged (21.34-20.41) kg/m2 and 20 obese group with BMI ranged between (24.22-22.04) kg/m2. They found circulating Spexin levels were significantly lower (1119.4 pg/ml) than children of normal weight (4410.64 pg/ml). And also found 70% of transition and transvertion mutations compared to control group. Study concluded that mutations in the Spexin gene have been documented to affect very low serum Spexin levels and its expression, as well as altering the binding of transcription factors which can disrupt transcription and in turn translation resulting in a reduction in Spexin gene expression [105]. It is the only report available about Spexin gene sequence of the obesity in children group.

Mastsuno S and his co-workers in 2019 conducted a study on genetic polymorphisms using exome sequencing to identify a variant associated with early onset diabetes in the intron of the insulin gene. Study investigated the gene responsible for early onset of diabetes leading to impaired insulin secretion in a family and discovered heterozygous c.18831G>A of intron 2 mutation in the proband – a 43-year-old woman. The polymorphisms found in her two diabetic daughters, but not in her son or her parents. Study concluded that the mutation could be linked to early onset diabetes. However, whole exome sequencing cannot detect relatively large structural variations such as an exon deletion which was one of the study's limitations[106].

Guerini F.R. and his co-workers in 2019 conducted a single nucleotide polymorphisms cohort study on the complex of soluble-N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) to see if genetic variants in the SNARE genes are linked to ischemic heart disease. The total sample size was 100, amongst fifty six patients were with ischemic heart disease, while fourty four were healthy controls. The study found that SNP rs363050 of intron 1 of the SNAP25 gene

has been correlated with SNAP25 protein expression. Study concluded that SNP of SNAP25 gene might involve in the development of Ischemic heart disease in a cohort [107].

CHAPTER-3
AIM AND OBJECTIVE

3.0. AIM AND OBJECTIVES

3.1. Aim

Measurement of Spexin, leptin and cardio-metabolic parameters between Type 2 diabetes mellitus with and without hypertension and healthy subjects with *SPX* gene analysis in whole blood.

3.2. Objectives

1. To estimate and compare Spexin with Type 2 diabetes mellitus and healthy subjects
2. To estimate and compare Spexin in Type 2 diabetes mellitus with hypertension and healthy subjects
3. To determine the association between *SPX* gene polymorphism in Type 2 diabetes mellitus with and without hypertension and healthy subjects

CHAPTER-4
RESEARCH METHODOLOGY

4.0. RESEARCH METHODOLOGY

4.1. MATERIALS

A cross-sectional study design was conducted in the Department of Biochemistry in collaboration with the Department of General Medicine attached to R L Jalappa Hospital and Research Centre, of Sri Devaraj Urs Academy of Higher Education and Research. The ethical approval granted by the Sri Devaraj Urs Medical College institutional Ethics Committee in a vide No. SDUMC/KLR/IEC/28/2019-20. During their visit to the hospital, each participant was educated about the study and written informed consent was obtained and recorded.

4.2. Sample Size Calculation

Based on leptin levels in a study comparing diabetes with non-diabetes mellitus in Saudi males. The study reported an average variance estimate 20.40 ng/ml to detect a difference with an increase is 15.44 ng/ml in leptin levels among diabetes with 80% power and alpha error of 5%. The estimated sample size was total 330 comprising 110 per group [82].

The following formula has been adapted in the study to calculate the sample size of the study groups.

$$n = \frac{2S_P^2 [Z_1\text{-}\alpha/2 + Z_1\text{-}\beta]^2}{\mu_d^2}$$

$$S_P^2 = \frac{S_1^2 + S_2^2}{2}$$

S_1^2 = Standard Deviation in Type 2 diabetic group

S_2^2 = Standard Deviation in Type 2 diabetic with hypertension group

μd^2 = Mean difference between the samples

α = Significance level ($p<0.05$)

$Z_{1-\beta}$ = Power (80%)

$Z_{\alpha/2}$= 95% Confidence Interval (CI)

Based on the above calculation, the sample size arrived for study group is total of 330 subjects in the age group of 40-60years comprising both genders were sub-divided into 3 groups (Group-1, healthy subjects n=110), (Group-2, Type 2 diabetes mellitus n=110) and (Group-3, Type 2 diabetes mellitus with hypertension n=110) subjects. The study participants were recruited according to the inclusion and exclusion criteria on obtaining patients consent.

4.3. Sample Collection

Under Medical supervision, the blood sample was collected in aseptic conditions. In each subject/patient, total 3.0 ml of fasting venous blood samples was drawn from the antecubital vein using vacutainer, samples from all the study participants were transferred to plain & EDTA tubes. The plain tubes were allowed to retract at room temperature for 20 minutes. The plain tubes and EDTA were centrifuged at 3000 rpm for 10mins to obtain a clear serum and plasma which were stored at -80°C until analysis. Whole blood EDTA sample was used for DNA extraction and stored at -80^0C until Spexin gene sequence analysis.

4.4. Inclusion Criteria

Clinically proven cases of Type 2 diabetes mellitus with or without hypertension were included in the study. The criteria for diagnosis of Type 2 diabetes mellitus as per the American Diabetic Association (ADA) 2018 was considered with FBS ≥ 126 mg/dl, PPBS ≥ 200mg/dl, HbAlc ≥ 6.5% and hypertension ≥140/90 [108].

4.5. Exclusion Criteria

The exclusion criteria of the study Participants are Congenital heart diseases, primary myocardial myopathies like cardiomyopathy myocarditis, Cor-pulmonale diseases, pericardial disorders, ischemic heart disease, Angina, myocardial infarction, coronary artery disease, strokes secondary diabetes like any pancreatic diseases, thyroid diseases, pregnancy & lactation, history of surgical removal of gall bladder, CNS disorders, cancer and immune disorders were excluded from the study.

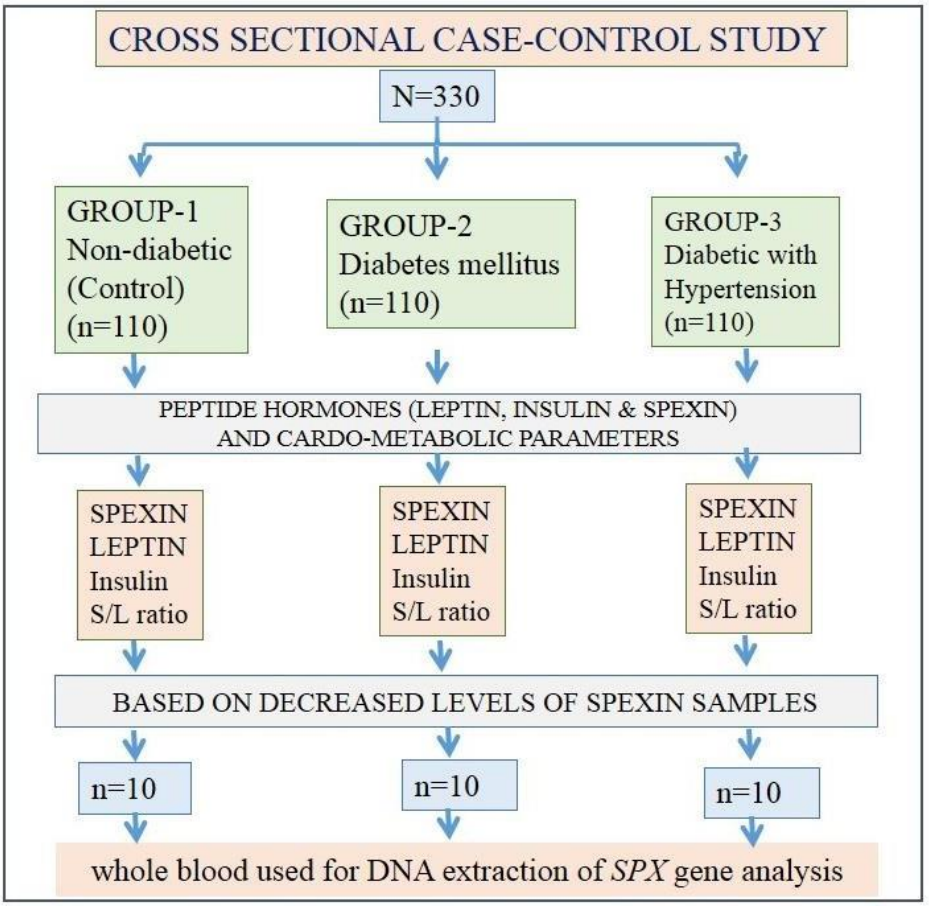


Figure 13: Study Design

4.6. Methods

Table 2: Routine parameters and Methodology

No	PARAMETERS	METHODS	INSTRUMENT
1	Insulin(µIU/ml) [109]	Immunometric immunoassay	Ortho clinical diagnostics-Vitros 5.1, FS
2	Plasma glucose(mg/dl)[109]	Glucose Oxidase Peroxidase	Ortho clinical diagnostics-Vitros 5.1, FS
3	Glycosylated haemoglobin (HbA1c) (%) [109]	High Pressure Liquid Chromatography	Bio-Rad D10
4	Total Cholesterol (TC) (mg/dl)[110]	Cholesterol oxidase method	Ortho clinical diagnostics-Vitros 5.1, FS
5	Triglycerides (TG)(mg/dl)[110]	Lipase hydrolysis colorimetric method	Ortho clinical diagnostics-Vitros 5.1, FS
6	High density lipoprotein (HDL-C)(mg/dl)[110]	Cholesterol ester hydrolase colorimetric method	Ortho clinical diagnostics-Vitros 5.1, FS
7	C-reactive protein (CRP) (mg/dl)[111]	Enzyme heterogenous sandwich immunoassay method	Ortho clinical diagnostics-Vitros 5.1, FS
8	Aspartate aminotransferase (AST) (U/L)[112]	International Federation of Clinical chemistry	Ortho clinical diagnostics-Vitros 5.1, FS
9	Alanine transaminase (ALT) (U/L)[112]	International Federation of Clinical chemistry	Ortho clinical diagnostics-Vitros 5.1, FS
10	Hoemoststic assesement – insulin Resistance (HOMA-IR)(microU/L)x(nmol/L)	Fasting insulin x fasting glucose / 22.5	

CALCULATIONS

LDL cholesterol, VLDL and Non-HDL cholesterol were calculated using the formula given below as per the reference 110.

I. LDL:Friedewald Equation

LDL cholesterol= [Total cholesterol – (HDL cholesterol+Triglyceride/5)]

II. Very Low density Lipoprotein (VLDL)

VLDL= TG/ 5

III. Non- HDL

nHDLc = Total Cholesterol- HDL

The biochemical parameters such as plasma fasting blood sugar, insulin, lipid profile, CRP, Aspartate transaminase, Alanine transaminase were measured in serum bydry chemistry analyzer (Ortho Clinical Diagnostics- Vitros 5.1 FS) and Glycated hemoglobin (HbA1c) was determined by HPLC method on Bio-Rad D10 as shown in table 2.

The study protocol included anthropometric parameters such as body weight, height, waist circumference and hip circumference were measured and recorded. Waist circumference was measured at the lowest point of the costal margins and hip circumference at the widest point of the hip. A baseline anthropometric index such as the body mass index was calculated by dividing the weight (kg) by the square of the height (m^2). The waist and hip ratio (WHR) was determined by means of dividing waist circumference (cm) by hip circumference (cm) [113].

However, study parameters of interest such as serum Spexin and Leptin were measured as per the methodology of manufacturer by using the instruments are as shown in Table 3.

Table 3: Instruments and methods used for the biochemical analysis

NO	Parameters	Method	Make/ Catalog No	Instrument
1.	**Serum Spexin (ng/ml)**	Quantitative Sandwich enzyme Immunoassy Technique	KBH3507 Krishgen Biosystem, India	ELISA-RaytoRT-6100
2.	**Serum Leptin (ng/ml)**	Quantitative Sandwich enzyme Immunoassy Technique	CAN-L-4260, Diagnostics Biochem Cannada, USA.	ELISA-RaytoRT-6100

4.7. ESTIMATION OF SERUM SPEXIN

Method: Spexin C12orf39 is measured by enzyme-linked immunosorbent assay (ELISA) technique as per the protocol given by manufacturers (Krishgen Biosystems, India).

Principle: The assay employs sandwich ELISA technique. Antibodies specific for Spexin are pre-coated onto microwells. Standards and Samples were added into wells. Spexin present in the serum are allowed to bind to pre-coated antibodies. Biotin labeled antibody and Streptavidin-horse radish peroxidase is added and incubated to form a complex. After washing with wash buffer, to remove excess or unbound antibodies, the substrate solution tetramethyl benzidine (TMB) is added to microwells.

On addition of substrate solution, tetramethyl benzidine catalyses conversion of substrate into yellow coloured product measured at 450nm. The intensity of the colour is directly proportionate to the amount of Spexin in the sample. To stop the color development, stop solution is added.

Reagents

1. Standard Diluent
2. Biotinylated C12orf39 Antibody
3. Streptavidin-HRP Conjugate
4. 25X Wash Buffer
5. Tetramethyl benzidine substrate
6. Stop Solution

Procedure:

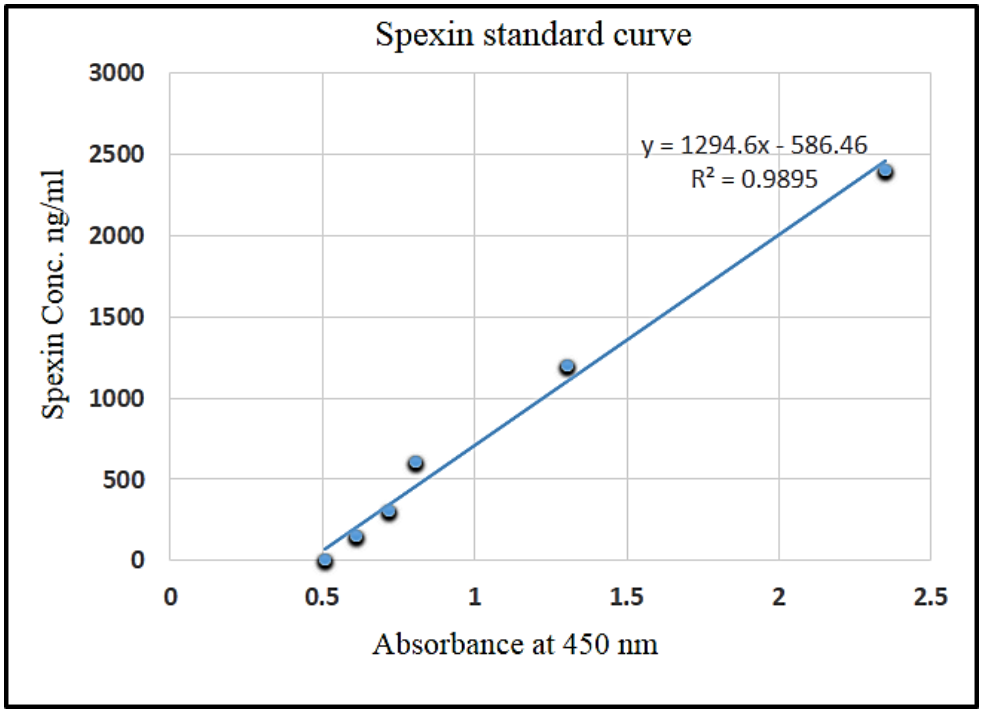
After thawing of all the reagents, buffers and samples, 50 µL of standards of different concentration (S1-S5) were added into the micro titre plate. 40µl of undiluted samples, 10 µl of Biotinylated antibodies, and 50 µl of streptavidin-horseradish peroxidase were added to the respective monoclonal antibody-coated microplate wells and cover the plate with sealer and incubated for 60 minutes at 37 ºC. After incubation, the solution was discarded completely and wells were washed with a 1X washing buffer solution for four times. To the wells, 50µl of substrate A and 50µl of substrate B were added and incubated for 10 minutes at 37 ºC to develop bluish color in positive wells. The reaction was arrested by adding 50µl of stop solution and allowed to turn wells into yellow colour. The optical density of yellow color was measured at 450nm by ELISA reader (Rayto, RT-6100 microplate reader).

Calculation:

Plotting mean absorbance for each standard on the x-axis against concentration on the y-axis yielded the standard curve. The concentration of Spexin in the sample was determined using the sample OD value in the equation (y=1271.2x2– 544.58x +30.93, R2= 0.989). The detection range was 150 to 2400 pg/mL. The sensitivity was 4.95 pg/mL or less. The result was expressed in nanograms per milliliter (ng/ml).

Standard curve for serum Spexin

Standards	**B**	**S-1**	**S-2**	**S-3**	**S-4**	**S-5**
Concentration (ng/ml)	0	150	300	600	1200	2400
Absorbance(450 nm)	0.51	0.615	0.72	0.81	1.30	2.35



4.8. ESTIMATION OF SERUM LEPTIN

Method: Leptin is measured by enzyme-linked immunosorbent assay (ELISA) technique as per the protocol given by manufacturers (Diagnostics Biosystem Cannada, USA).

Principle:

The assay follows sandwich ELISA technique. Antibodies specific for Leptin are pre-coated onto microwells. Standards and Samples were added into wells. Leptin present in the serum are bound to pre-coated antibodies. Biotin labeled antibody and Streptavidin- horse raddish peroxidase is added and incubated to form a complex. After washing with wash buffer, to remove excess or unbound antibodies, the substrate solution tetramethyl benzidine (TMB) is added to microwells. On addition of substrate solution, tetramethyl benzidine catalyses conversion of substrate into yellow coloured product measured at 450nm. To stop the Color development, stop solution is added. The intensity of the colour is directly proportionate to the amount of Leptin in the sample.

Reagents

1. Standard
2. Biotin conjugate
3. Streptavidin-HRP Conjugate
4. Wash Buffer
5. Assay Buffer
6. Tetramethyl benzidine substrate
7. Stop Solution

Procedure:

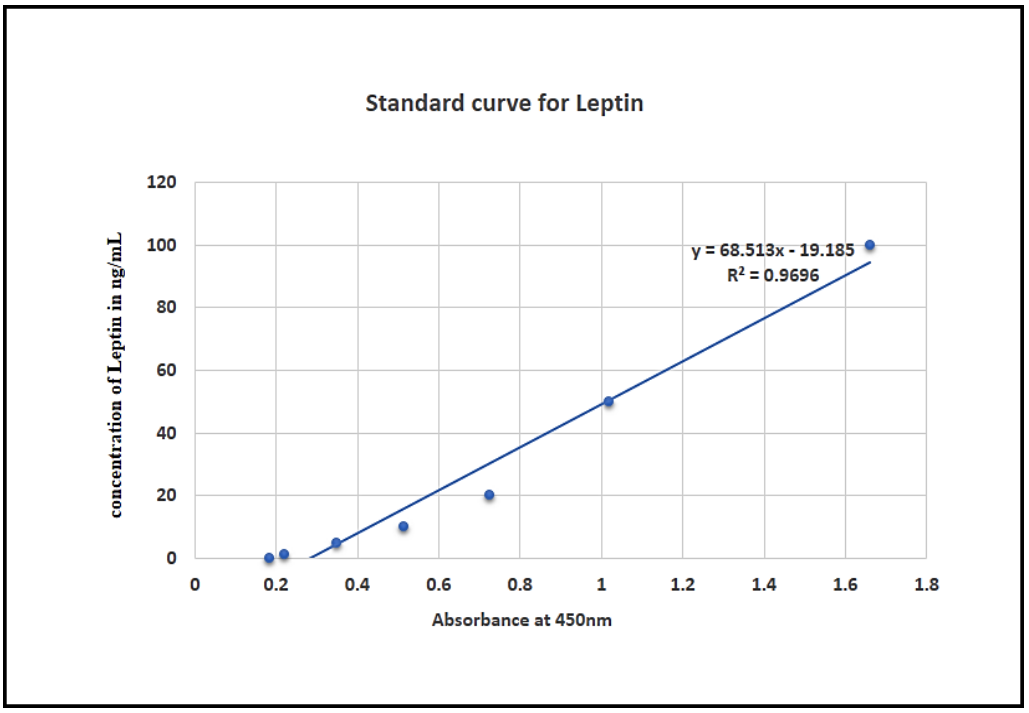
The micro titre plate was loaded with 20µL of standards of different concentration (S1-S7) and samples. Each well (standards and test wells) was added with 80µLof Biotin-antibody. The micro titer plate was sealed with an adhesive strip and incubated at 37°C for 60 minutes. After three rounds of washing, 100µL of streptavidin-horse raddish peroxidase was added to each well, and the plate was incubated at 37°C for 30 minutes. Following the washing step, 100µL of tetramethyl benzidine Substrate was added to each well, and the plate was incubated at 37 ^{0}C for another 10-15 minutes. The OD of the yellow colour developed was measured at 450 nm after addition of 50µL of Stop Solution.

Calculation:

Plotted mean absorbance for each standard on the x-axis against concentration on the y-axis yielded the standard curve. The concentration of Leptin in the sample was determined using the sample OD value in the equation (y=68.51x2– 19.18x +30.93, R2= 0.969). The detection range was 1-100ng/mL. The sensitivity was 0.5 ng/mL or less. The result were expressed in nanograms per milliliter (ng/ml).

Standard curve for serum Leptin

Standards	B	S-1	S-2	S-3	S-4	S-5	S-6
Concentration(ng/ml)	0	1	5	10	20	50	100
Absorbance (450nm)	0.185	0.22	0.35	0.515	0.725	1.02	1.66



4.9. GENETIC ANALYSIS OF SPEXIN GENE:

Spexin (SPX) whole gene sequence Analysis

The Spexin gene located on chromosome 12q.12.1 was analysed using whole blood samples by adapting the following molecular biology techniques. EDTA tube were used for genetic analysis.

Methods used for analysis of Spexin gene

1. Extraction of DNA from whole blood
2. DNA quantification & purification
3. Exons standardization
4. Polymerase chain reaction (PCR) for amplification of DNA
5. Agarose gel electrophoresis
6. Spexin gene Sequencing by Sangers di-deoxy method

I. Extraction of DNA from whole blood

The salting out process was carried out to isolate genomic DNA from whole blood samples[114].

Reagents:

a) **Erythrocyte lysis buffer (ELB) (1L pH 7.4)**: The composition of Erythrocyte lysis buffer are we weighed (155M) 8.26grams of ammonium chloride (NH4Cl), (10mM)1.1g of Potassium bicarbonate (KHCO3) and (0.1mM) 14.6 Ethylene diamine tetra acetic acid (EDTA). The solution was adjusted to pH 7.4 and stored 4^0C.

b) **20 mg/mL Proteinase K:** Dissolve 20mg/mL Proteinase K in 1mL of milli-Q water and transferred in the aliquot of 30 µl to Eppendorf tubes and stored at -20^0C.

c) **20% Sodium Dodecyl Sulphate (SDS):** 20grams of SDS is dissolved in 100mL with milli-Q water, incubated in water bath for 37^0C for 30mins to 2hrs to dissolve completely. The reagent was kept at room temperature to prevent precipitation.

d) **Tris EDTA buffer (1L, 0.5M pH 8.0) 186.2g EDTA and 1M 121.14 Tris-Cl:** Measure 2 mL of (0.5M EDTA) and 1mL of (1M Tris-Cl) Adjusted pH to 8. Dissolved in 1000mL with distilled water and mix thoroughly.

e) **5M Sodium chloride (NaCl):** 146.1 grams of sodium chloride was weighed and made upto 500mL.

f) **80% Ethanol:** (freshly prepared): Add 80mL of absolute ethanol and made upto 100mL with milli-Q water

g) Isopropyl alcohol

Procedure:

EDTA blood was transferred into the Falcon tubes. The blood sample was mixed with erythrocyte lysis buffer and vortexed until foam was formed. Then placed it in the refrigerator for 30 minutes and then kept it at room temperature until it dissolves. Centrifuge the sample for 10 minutes at 3000rpm and the supernatant was discarded. Added 12ml of erythrocyte lysis buffer to it, mixedd well and centrifuged for 6 minutes at 3000rpm till a white colour pellet of heme is obtained. Furthe,r Added 270µl of 20% SDS and 30µl Proteinase K (10mg/ml) mixed for 5 times. Samples were incubated at 37 ° C overnight. Following day, the incubated samples were taken out and added 500µl of 5M sodium chloride and 11ml isopropyl alcohol and gently

mixed until clear DNA is visible. The total precipitated thread like DNA was transferred into sterile micro centrifuge tube followed by added 80% ethanol and centrifuged for 5 minutes at 12000 rpm at 4^{o}C. Supernatant was discarded and the same procedure was repeated 3 times to obtain purified DNA. Allowed to dry for 30 minutes. Further, added 500µl of Tris-EDTA buffer to dissolve DNA and to get a clear solution. The solution was incubated in a water bath at 65^{O}C for 20 minutes. The overnight DNA solution was placed on the rocker following day, isolated DNA was stored at -80°C.

II. DNA Quantification

The purity and quantity of the DNA preparation were determined by spectrophotometry. Measurements were carried out on the UV spectrophotometer (Perkin Elmer model Lambda 35, Waltham, MA, USA) was used to check the concentration and purity of the DNA. 48µL of Tris buffer and 2 µL of DNA was added in cuvette and mixed thoroughly. DNA purity was determined using a spectrophotometer and the ratio of absorbance at 260/280 represent the DNA purity. For PCR reactions, purity of DNA samples with a ratio between 1.7 and 1.9 were considered as pure used for PCR reactions.

III. Exons Standardization

PCR reactions were performed on a gradient thermal cycler (Bio-Rad, California, USA). Spexin gene reference sequence (Accession No: NC-000012.12) was obtained from the NCBI website. The forward and reverse primers set were designed as shown in (Table 4&5) and primers were purchased from (Bioserve Biotechnologies Pvt Ltd, India). Lyophilized primers were dissolved with Tris Buffer made upto final concentration which was followed by the instructions given in the manufacturer kit. And PCR Master Mix was purchased from (VNIR biotechnologies Pvt Ltd, Banglore)

IV. Polymerase chain reaction for amplification of DNA

According to the instructions PCR Mater mix, working stock was prepared by adding 2µl of each genomic DNA (10-100 ng), 500 nM of each primer added to lyophilized VNIR 2X master mix for PCR and the volumes were adjusted to 20 µl with deionized distilled water. PCR programs were: 95°C for 3 minutes (Activation), followed by 30 sec of 95°C for Denaturation, Annealing 72°C for 45 seconds; 72°C for extension in one min, and the final extension was 72°C for 10 minutes as shown in (Table 6).

Reagents for Polymerase chain reaction:

1. Primers ((Bioserve Biotechnologies Pvt Ltd, India).
2. PCR master mix (VNIR biotechnologies Pvt Ltd, Banglore)
3. PCR purification kit (QIAGEN QIAquick)

IV. Agarose gel electrophoresis

The PCR amplified products were electrophoresed on an agarose gel.

Reagents:

a) **1% Agarose gel:** 1g of agarose protein was mixed with 100 mL of 1x TE buffer, allow the solution to cool at room temperature before adding 0.1 mg/ml ethidium bromide.

b) **Tris Acetate EDTA buffer (TAE): 50X stock solution:** Dissolved 242grams of Tris base, 57.1mL glacial acetic acid, 100mL of 500mM EDTA and adjusted to pH 8.0. The volume was made upto 600 mL with distilled water 1X TAE buffer was prepared by diluting 20mL of stock into 980mL of deionised water.

c) **Loading dye:** Prepared 0.042% (W/V) of Bromophenol blue powder, 2.5% of Ficoll and 0.042% (W/V) of Xylene Cyanol.

d) Ethidium bromide

Procedure:

Agarose Gel Electrophoresis was used to determine the amount of DNA present. 1% agarose gel was used to separate the PCR generated products. Poured the agarose gel into the gel-casting tray with the well comb and allowed to harden. In the well comb, added the DNA sampl into a gel tray. The bubbles on the agarose's surface were eliminated. The comb was taken from the gel and placed in an electrophoresis tank with 1X TAE buffer. Each PCR (5µL) product received 1µL of loading dye. 3µL DNA ladder was carefully put into a well on the gel. The electrophoresis tank's lid was covered. The images were obtained in the gel documentation system (Bio Rad

Gel Dock) after the gel was electrophoresed at 2 volts / cm.

DNA Purification

Samples were processed for purification using QIAGEN QIAquick PCR Purification Kit (cat. No. 28104) for the removal of primers, nucleotides, enzymes, mineral oil, salts, agarose, ethidium bromide, and other impurities from DNA samples and other impurities from DNA samples. 100µL of PCR products were added to 500µL of binding buffer and properly mixed. The sample was loaded into a 2.0 ml Collection tube with a QIAquick spin Column and centrifugation was performed for 1 min at 13,000 rpm.

The flow through was discarded, and the QIAquick spin Column was reinserted into the Collection tube. Add 0.75 ml Wash Buffer to the QIAquick spin Column and centrifuged for 1min at 13,000 rpm. Removed the flow through and placed the column back in the Collection tube for 1 minute centrifugation. QIAquick spin Column was placed in sterile 1.5ml vial to elute DNA. Added 50µl of Elution Buffer to the centre of the column and centrifuged for 1 min at 13000 rpm. The eluted DNA was subjected again for centrifugation for 1 minute at 13000 rpm and stored at -20^{o}C.

V. Spexin gene Sequencing by Sangers di-deoxy method

Method: Sanger di-deoxy chain termination method

Sequencing was done on purified samples. The BigDye® Terminator v3.1 Cycle Sequencing Kit was used to sequence all 6 exons of the Spexin gene using an Applied BiosystemsTM MiniAmpTM Plus Thermal Cycler and the Big Dye TM Terminator V3.1 kit according to the manufacturer's instructions. In the Applied Biosystems 3730xL Analyzer, the results of exon sequencing were recorded and subjected to data method to analyze any polymorphisms.

Data analysis

The FASTA sequence was obtained using the Chromas programme. The clustal omega multi-sequencing software was used to compare the mutational analyses to the reference Spexin gene sequence.

1.10. Statistical analysis:

The obtained data was represented as mean±SD. One-way analysis of variance with post hoc Bonferroni's analysis was used to find the difference between more than two groups. Pearson correlation(r) is used to correlate between the parameters. Receiver operating characteristics (ROC) curve analysis was done to assess the diagnostic feasibility of a Spexin parameter in the study. The $p<0.05$ was considered statistically significance. All the statistical analysis was performed using licensed SPSS software, version 20.0 (IBM, USA).

CHAPTER-5
RESULTS AND DISCUSSION

5.0. RESULTS AND DISCUSSION

5.1 Results

A cross-sectional study design was conducted in the Department of Biochemistry in collaboration with the Department of General Medicine attached to R L Jalappa Hospital and Research Centre, of Sri Devaraj Urs Academy of Higher Education and Research. The ethical approval granted by the Sri Devaraj Urs Medical College institutional Ethics Committee in a vide No. SDUMC/KLR/IEC/28/2019-20. During their visit to the hospital, each participant was educated about the study and written informed consent was obtained and recorded.

Total of 330 subjects in the age group of 40-60years comprising both genders were sub-divided into 3 groups (Group-1, healthy subjects n=110), (Group-2, Type 2 diabetes mellitus n=110) and (Group-3, Type 2 diabetes mellitus with hypertension n=110) subjects. The study participants were recruited according to the inclusion and exclusion criteria on obtaining patients consent.

Table 7: Comparison of demographic data in Type 2 diabetes mellitus group

No.	Variables	Controls mean±SD (n=110)	T2DM mean±SD (n=110)	P=value
1	Body mass index (Kg/m^2)	17.6±2.8	20.1±2.7	0.001*
2	Waist/hip ratio	0.9±0.1	0.9±0.1	0.001*
3	Systolic blood pressure (mmHg)	114.6±7.74	136.5±16.9	0.001*
4	Diastolic blood pressure (mmHg)	80.5±1.22	82.6±2.95	0.001*

*P<0.05 considered as statistically significant

Table 8: Comparison of demographic data in Type 2 diabetes mellitus with hypertension group

No	Variables	Controls mean±SD (n=110)	T2DM-HTN mean±SD (n=110)	P=value
1	Body mass index (Kg/m^2)	17.6±2.8	22.1±3.8	0.001*
2	Waist/hip ratio	0.9±0.1	1.0±0.1	0.001*
3	Systolic blood pressure (mmHg)	114.6±7.74	151.1±12.3	0.001*
4	Diastolic blood pressure (mmHg)	80.5±1.22	88.9±7.12	0.001*

*P<0.05 considered as statistically significant

The demographic characteristics of controls, Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension groups were depicted in table7&8.

The table 7&8 depicts about the comparison of demographic & biochemical parameters in control, Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension groups and the results were represented in mean ± SD which are normally distributed. The prevalence of abnormal values for Body mass index, Waist/hip ratio were higher in Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension patients than in the controls. The findings suggested that monitoring for tested patients which directs on the extent of required weight-loss in accordance with the gender, age, blood sugar, and diagnosis of hypertension etc. With regard to systolic blood pressure, there was increased systolic & diastolic blood pressure in Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension when compared to controls. In agreement with the results, the significance p value is 0.001 which was found statistically significant as shown in table 17.

Table 9: Comparison of biochemical parameters in Type 2 diabetes mellitus group

No	Variables	Controls mean±SD (n=110)	T2DM mean±SD (n=110)	P=value
1	Fasting blood sugar (mg/dl)	85.11±9.05	191.7±73.9	0.001*
2	Post prandial blood sugar (mg/dl)	99.5±15.3	233.4±85.1	0.001*
3	Insulin(µIU/L)	9.85±2.94	17.2±2.42	0.002*
4	Glycosylated haemoglobin (%)	5.28±0.57	8.46±6.0	0.001*
5	Homeostatic model assessment-Insulin resistance (uIU/ml)	2.03±1.52	10.65±1.87	0.001*
6	Total cholesterol (mg/dl)	158.2±34.4	239.6±42.0	0.001*
7	Triglycerides (mg/dl)	119.1±30.2	214.9±48.1	0.001*
8	High density lipoprotein (mg/dl)	34.3±3.07	30.41±2.48	0.001*
9	low density lipoprotein (mg/dl)	80.5±9.55	127.3±14.1	0.001*
10	very low density lipoprotein (mg/dl)	23.8±6.0	42.9±9.63	0.001*
11	LDL/HDL ratio	2.3±0.32	4.2±0.55	0.001*
12	Alanine transaminase (U/L)	19.91±7.98	27.58±13.91	0.001*
13	Aspartate transaminase (U/L)	22.44±6.38	27.5±14.2	0.001*

*p<0.05= statistically significant

Table 10: Comparison of biochemical parameters in Type 2 diabetes mellitus with hypertension group

No	Variables	Controls mean±SD (n=110)	T2DM-HTN mean±SD (n=110)	P=value
1	Fasting blood sugar (mg/dl)	85.11±9.05	191.9±61.0	0.001*
2	Post prandial blood sugar (mg/dl)	99.5±15.3	237.9±59.7	0.001*
3	Insulin(µIU/L)	9.85±2.94	16.8±3.21	0.002*
4	Glycosylated haemoglobin (%)	5.28±0.57	8.76±1.90	0.001*
5	Homeostatic model assessment-Insulin resistance (uIU/ml)	2.03±1.52	8.57±2.16	0.001*
6	Total cholesterol (mg/dl)	158.2±34.4	192.8±48.7	0.001*
7	Triglycerides (mg/dl)	119.1±30.2	223.3±43.2	0.001*
8	High density lipoprotein (mg/dl)	34.3±3.07	27.96±2.55	0.001*
9	low density lipoprotein (mg/dl)	80.5±9.55	91.99±8.06	0.001*
10	very low density lipoprotein (mg/dl)	23.8±6.0	44.6±8.65	0.001*
11	LDL/HDL ratio	2.3±0.32	3.31±0.42	0.001*
12	Alanine transaminase (U/L)	19.91±7.98	29.38±15.62	0.001*
13	Aspartate transaminase (U/L)	22.44±6.38	32.8±24.8	0.001*

*p<0.05= statistically significant

Table 9 & 10 depicts about the comparison of biochemical parameters in control, Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension groups and the results were represented in mean ± SD which are normally distributed. All the variables showed significant difference in both Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension when compared to control. Serum levels of fasting blood sugar, post prandial blood sugar, HbA1C, insulin and HOMA-IR is a well-established marker of insulin resistance in Type 2 diabetics individuals, in support of this in the present study, glycemic parameters is estimated and showed increased levels in Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension compared to controls and the p-value is statistically significant (p=0.001) as shown in table 17

Hence, the outcomes of the study clearly reveal that hyperinsulinemia and diabetes is observed in Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension patients. As far as the blood lipid levels are concerned, patients were more dyslipidemic than the healthy controls. According to the results, total cholesterol, triglycerides, VLDL and LDL-C values increased and HDL value decreased in Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension patients than healthy controls and the p-value is statistically significant (p=0.001) as shown in table 17. The LDL-C/HDL-C ratio, which is considered to be a sensitive indicator of hypertension risk, is calculated in control individuals, Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension patients with significant p-value (0.001). The lipid ratios are higher in patients than the controls. Amongst patients with elevated ratios, glycemic parameters and lipid profile were found in Type 2 diabetic patients.

We also examined the associations of serum liver enzymes Alanine aminotransferase and Aspartate aminotransferase in healthy controls, Type 2 diabetes mellitus and for Type 2 diabetes mellitus with hypertension subjects. Our results suggest that these serum liver enzymes were increased in Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension subjects. Amongst, Type 2 diabetes mellitus with hypertension patients showed increased ALT & AST levels and the p-value is statistically significant (p=0.001) indicates increased risk of Type 2 diabetes mellitus & Type 2 diabetes mellitus with hypertension. Results were shown in tables 9, 10 & 17.

Hence, our research findings on demographic and biochemical parameters confirmed that there is a link between deregulation of Cardio-metabolic parameters in Type 2 diabetes mellitus with and without hypertension that contributes to Type 2 Diabetes Mellitus progression.

Table 11: Spexin, leptin and S/L ratio in Type 2 diabetes mellitus group

No	Variables	Controls mean±SD (n=110)	T2DM mean±SD (n=110)	P=value
1	Spexin(ng/ml)	0.94±0.24	0.76±0.19	0.001*
2	Leptin(ng/ml)	22.9±3.01	27.7±2.61	0.001*
3	Spexin/Leptin ratio (ng/mL)	0.042±0.013	0.027±0.007	0.001*
4	C-reactive protein (mg/dl)	6.35±2.98	14.74±11.0	0.001*
5	Fasting blood sugar (mg/dl)	85.11±9.05	191.7±73.9	0.001*
6	Total cholesterol (mg/dl)	158.2±34.4	239.6±42.0	0.001*
7	Triglycerides(mg/dl)	119.1±30.2	214.9±48.1	0.001*
8	Insulin(µIU/L)	9.85±2.94	17.2±2.42	0.002*

* $p<0.05$= statistically significant

Table 12: Spexin, leptin and S/L ratio in Type 2 diabetes mellitus with hypertension group

No	Variables	Controls mean±SD (n=110)	T2DM-HTN mean±SD (n=110)	P=value
1	Spexin(ng/ml)	0.94±0.24	0.61±0.17	0.001*
2	Leptin(ng/ml)	22.9±3.01	35.84±2.49	0.001*
3	Spexin/Leptin ratio (ng/mL)	0.042±0.013	0.017±0.004	0.001*
4	C-reactive protein (mg/dl)	6.35±2.98	26.54±2.70	0.001*
5	Fasting blood sugar (mg/dl)	85.11±9.05	191.9±61.0	0.001*
6	Total cholesterol (mg/dl)	158.2±34.4	192.8±48.7	0.001*
7	Triglycerides(mg/dl)	119.1±30.2	223.3±43.2	0.001*
8	Insulin(µIU/L)	9.85±2.94	16.8±3.21	0.002*

* $p<0.05$= statistically significant

Table 11 & 12 depicts about the comparison of Study parameters in control, Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension groups. All the variables showed significant difference in both the groups and variables like Spexin showed significant decreased levels in both Type 2 Diabetes Mellitus group and remarkable decreased levels was observed in Type 2 diabetes mellitus with hypertension when compared to control.

Leptin showed significant increased levels in Type 2 diabetes mellitus group. Progressively increased levels were observed in Type 2 diabetes mellitus with hypertension when compared to control. Whereas S/L ratio showed significant decreased trend in Type 2 diabetes mellitus and further decreased levels were observed in Type 2 diabetes mellitus with hypertension as shown in figure17. CRP also showed significant increased levels in both the groups compared to control.

Since, Serum Spexin plays crucial role in regulating food intake, weight regulation, regulating glucose and lipid metabolism and inhibits uptake of long chain fatty acids. Hence, sufficient serum Spexin levels are of paramount importance. In the present study, serum Spexin levels were considerably decreased in Type 2 diabetes mellitus compared to controls and significantly decreased in Type 2 diabetes mellitus with hypertension patients and the p-value is statistically significant (p=0.001) as shown in table 17 and figure 17. The overall study results demonstrated that the role of Spexin in regulating glucose and lipid metabolism and also signifies key importance of considering as a new early prediction for the future development of Diabetes and CVD complications.

Another gold standard biomarker is serum leptin also contributes to obesity and diabetes mellitus. Serum Leptin also plays an important role in regulating food intake (Decreases appetite) and weight regulation. Since, serum leptin levels were increased in Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension compared to controls in the present study. Amongst patients, Type 2 diabetes mellitus with hypertension showed significantly increased leptin levels and the p-value is statistically significant (p=0.001) as shown in table 17. Hence, already known fact that increased serum leptin levels is contributing future obesity and diabetes with CVD complications as shown in figure 17 & Table 17.

Decreased trend of Spexin/Leptin ratio was observed in Type 2 diabetes mellitus and significantly 2-fold decreased trend was found in Type 2 diabetes mellitus with hypertension patients. Hence, the results suggested that decreased serum Spexin levels and the trend of decreased Spexin/leptin ratio may indicate future risk of diabetes with CVD complications. However, patients with diabetes and hypertension causes local inflammations in the body and leads to more secretions of inflammatory biomarkers like C-reactive protein which suggests that there is a link between inflammation and diabetes with CVD. Our study findings showed significantly increased CRP levels and the p-value is statistically significant (p=0.001) as shown in table 17.

Taken as a whole, our research findings summarized that Hypo-Spexinemia, hyper-Leptinemia, decreased trend of S/L ratio, hyper-insulinemia, hyper-triglyceridemina and increased inflammatory marker are having a link in energy homeostasis and weight regulation and that contributes to development of Type 2 diabetes mellitus as results were depicted in table 11 & 12.

Table 13: Pearson correlation between Spexin & study variables in Type 2 diabetes mellitus group

No	Variables	Spexin(ng/ml)	
		r	p
1	Body mass index (Kg/m^2)	0.08	0.35
2	Waist/hip ratio	0.05	0.56
3	Leptin (ng/ml)	-0.02	0.82
4	Fasting blood sugar (mg/dl)	0.008	0.39
5	Post prandial blood sugar (mg/dl)	0.004	0.96
6	Insulin (mIU/L)	-0.181	0.05
7	Glycosylated heamoglobin (%)	-0.125	0.19
8	Homeostatic model assessment- Insulin resistance (uIU/ml)	-0.05	0.59
9	Total cholesterol (md/dl)	-0.05	0.59
10	Triglycerides (mg/dl)	-0.08	0.39
11	High density lipoprotein (mg/dl)	0.118	0.22
12	Low density lipoprotein (mg/dl)	-0.02	0.77
13	Very low density lipoprotein (mg/dl)	-0.08	0.39
14	Spexin/Leptin ratio (ng/mL)	0.93	0.001*
15	LDL/HDL ratio	-0.11	0.25
16	Alanine transaminase (U/L)	-0.05	0.58
17	Aspartate transaminase (U/L)	-0.07	0.42
18	C-reactive protein (mg/dl)	-0.01	0.86
19	Systolic blood pressure (mmHg)	0.02	0.82
20	Diastolic blood pressure (mmHg)	-0.08	0.39

* $p<0.05$= statistically significant

Table 14: Pearson correlation between Leptin & study variables in Type 2 diabetes mellitus group

No	Variables	Leptin (ng/ml)	
		r	p
1	Body mass index (Kg/m^2)	0.015	0.87
2	Waist/hip ratio	-0.04	0.65
3	Spexin (ng/ml)	-0.36	0.001*
4	Fasting blood sugar (mg/dl)	0.04	0.66
5	Post prandial blood sugar (mg/dl)	-0.07	0.41
6	Insulin (mIU/L)	-0.03	0.71
7	Glycosylated heamoglobin (%)	0.07	0.42
8	Homeostatic model assessment- Insulin resistance (uIU/ml)	0.04	0.67
9	Total cholesterol (md/dl)	-0.03	0.71
10	Triglycerides (mg/dl)	0.13	0.15
11	High density lipoprotein (mg/dl)	-0.06	0.57
12	Low density lipoprotein (mg/dl)	-0.01	0.89
13	Very low density lipoprotein (mg/dl)	0.07	0.4
	Spexin/Leptin ratio (ng/mL)	-0.17	0.07
14	LDL/HDL ratio	-0.03	0.71
15	Alanine transaminase (U/L)	-0.028	0.77
16	Aspartate transaminase (U/L)	-0.03	0.71
	C-reactive protein (mg/dl)	-0.01	0.87
17	Systolic blood pressure (mmHg)	-0.101	0.29
18	Diastolic blood pressure (mmHg)	0.031	0.74

* $p<0.05$= statistically significant

Table 13 & 14 depicts the correlation between Spexin, leptin and different variables in Type 2 diabetes mellitus patients. Spexin showed weak positive correlation with HDL-C, FBS, PPBS, SBP, BMI and W/H ratio which was not statistically significant but shows strong positive correlation with S/L ratio and found to be statistically significant (p=0.001) as shown in table 13,17 and figure 18 & 19. Spexin showed weak negative non-significant correlation with leptin, insulin, HOMA-IR, CRP, TC, TG, LDL-C, VLDL-C, LDL/HDL ratio, HbA1c, DBP, ALT and AST.

Leptin shows strong negative correlation with Spexin and S/L ratio and was found to be statistically significant but shows weak negative correlation with CRP, TC, TG, HDL-C, LDL-C, LDL/HDL ratio, PPBS, inulin, SBP, W/H ratio, ALT and AST and found to be not statistically significant. Leptin shows positive correlation with TG, VLDL-C, FBS, HbA1c, HOMA-IR, DBP and BMI did not show any statistically significant. Spexin and leptin correlation does not communicate information about whether one variable shows in response to another. In support of the significance values, we can clearly mention that our results are reliable and meaningful. Related outcomes are put on shown in Table 14.

Table 15: Pearson correlation between Spexin & study variables in Type 2 diabetes mellitus with hypertension patients

No	Variables	Spexin(ng/ml) r	p
1	Body mass index (Kg/m^2)	0.01	0.87
2	Waist/hip ratio	-0.06	0.48
3	Leptin (ng/ml)	0.07	0.45
4	Spexin (ng/ml)	-	-
5	Fasting blood sugar (mg/dl)	-0.03	0.73
6	Post prandial blood sugar (mg/dl)	-0.12	0.29
7	Insulin (mIU/L)	0.004	0.96
8	Glycosylated heamoglobin (%)	-0.05	0.55
9	Homeostatic model assessment- Insulin resistance (uIU/ml)	0.008	0.93
10	Total cholesterol (md/dl)	0.007	0.93
11	Triglycerides (mg/dl)	-0.09	0.32
12	High density lipoprotein (mg/dl)	0.02	0.80
13	Low density lipoprotein (mg/dl)	-0.18	0.05
14	Very low density lipoprotein (mg/dl)	-0.09	0.32
15	Spexin/Leptin ratio (ng/mL)	0.96	0.001*
16	LDL/HDL ratio	-0.11	0.24
17	Alanine transaminase (U/L)	0.06	0.47
18	Aspartate transaminase (U/L)	-0.03	0.47
19	C-reactive protein (mg/dl)	-0.11	0.25
20	Systolic blood pressure (mmHg)	0.04	0.67
21	Diastolic blood pressure (mmHg)	-0.01	0.90

*$p<0.05$= statistically significant

Table 16: Pearson correlation between Leptin & study variables in Type 2 diabetes mellitus with hypertension patients

No	Variables	Leptin (ng/ml)	
		r	p
1	Body mass index (Kg/m^2)	-0.08	0.35
2	Waist/hip ratio	0.07	0.42
3	Leptin (ng/ml)	-	-
4	Spexin (ng/ml)	-0.20	0.03*
5	Fasting blood sugar (mg/dl)	-0.06	0.50
6	Post prandial blood sugar (mg/dl)	0.05	0.99
7	Insulin (mIU/L)	-0.008	0.93
8	Glycosylated heamoglobin (%)	0.02	0.76
9	Homeostatic model assessment- Insulin resistance (uIU/ml)	-0.11	0.24
10	Total cholesterol (md/dl)	-0.07	0.41
11	Triglycerides (mg/dl)	0.01	0.91
12	High density lipoprotein (mg/dl)	-0.15	0.10
13	Low density lipoprotein (mg/dl)	-0.05	0.53
14	Very low density lipoprotein (mg/dl)	0.03	0.75
15	Spexin/Leptin ratio (ng/mL)	0.02	0.78
16	LDL/HDL ratio	-0.07	0.41
17	Alanine transaminase (U/L)	-0.04	0.65
18	Aspartate transaminase (U/L)	-0.008	0.93
19	C-reactive protein (mg/dl)	-0.113	0.24
20	Systolic blood pressure (mmHg)	-0.007	0.94
21	Diastolic blood pressure (mmHg)	-0.01	0.85

*$p<0.05$= statistically significant

Table 15 & 16 showed the correlation between Spexin, leptin and different variables in type 2 diabetes mellitus with hypertensive patients. Spexin shows weak positive correlation with Leptin, TC, HDL-C, Insulin, HOMA-IR, SBP, BMI and ALT which was not statiscally significant but shows strong positive correlation with S/L ratio which was found to be statistically significant (p=0.001)as shown in table 15 & 17 and figure 19. Spexin shows weak negative correlation with CRP, TG, LDL-C. VLDL-C, LDL/HDL ratio, FBS, PPBS, HbA1c, DBP, W/H ratio and AST and did not show any statistically significant.

Leptin shows strong non-significant negative correlation with CRP, TC, HDL-C, LDL-C, LDL/HDL ratio, FBS, insulin, HOMA-IR, SBP, DBP, BMI,ALT and AST but strong negatively correlated with Spexin which was found to be statistically significant and positively correlated with S/L ratio, TG, VLDL-C, PPBS, HbA1c and BMI was found to be not statistically significant. Results were shown in table 15.

Table 17: Comparison of biochemical parameters showing significance between groups in the study population

No	Variables	Controls Vs T2DM (N=110)	Control Vs T2DM-HTN (N=110)	T2DM & T2DM-HTN (N=110)
1	Age (Years)	0.001*	0.001*	>0.05
2	Body mass index(Kg/m^2)	0.001*	0.001*	0.001*
3	Waist/hip ratio	0.007*	0.001*	0.001*
4	Insulin(mIU/L)	0.001*	0.001*	>0.05
5	Fasting blood sugar (FBS) (mg/dL)	0.001*	0.001*	>0.05
6	Post prandial blood sugar (PPBS) (mg/dL)	0.001*	0.001*	>0.05
7	Glycosylated heamoglobin (%)	0.001*	0.001*	>0.05
8	Homeostatic model assessment- Insulin resistance (HOMA-IR) (uIU/ml)	0.001*	0.001*	0.001*
9	Lipid profile Total cholesterol (mg/dL)	0.001*	0.001*	0.001*
	Triglycerides (mg/dL)	0.001*	0.001*	>0.05
	High density lipoprotein (mg/dL)	0.001*	0.001*	0.001*
	Low desnity lipoprotein (mg/dL)	0.001*	0.001*	0.001*
	Very low density lipoprotein (mg/dL)	0.001*	0.001*	>0.05
	LDL/HDL ratio (mg/dL)	0.001*	0.001*	0.001*
	TG/HDL ratio (mg/dL)	0.001*	0.001*	0.001*
10	Hypertension Systolic blood pressure (mmHg)	0.001*	0.001*	0.001*
11	Diastolic blood pressure (mmHg)	0.002*	0.001*	0.001*
12	Liver enzymes Alanine aminotransferase (ALT) (U/L)	0.001*	0.001*	>0.05
13	Aspartate aminotransferase (AST) (U/L)	0.007*	0.001*	0.006*
14	Inflammatory protein C-reactive protein (CRP) (mg/dL)	0.001*	0.001*	0.001*
15	Biomarkers Spexin (ng/ml)	0.001*	0.001*	0.001*
	Leptin(ng/ml)	0.001*	0.001*	0.001*
	S/L ratio(ng/mL)	0.001*	0.001*	0.001*

*p<0.05= statistically significant

Table 18: Paired t-test in Type 2 diabetes mellitus with and without hypertension

Variables	Mean ±SD	95% CI		p-value
Pair Spexin with Insulin	16.57±2.46	Lower	upper	0.001*

Table 19: Receiver Operating Characteristic Curve for Spexin and leptin in Type 2 diabetes mellitus group

Type 2 diabetic mellitus group						
Parameter	Cut-off	Sensitivity (%)	Specificity (%)	AUC	95% CI Range	p
Spexin (ng/ml)	0.62	65	62	0.65	0.584-0.728	<0.001
Leptin (ng/ml)	25.1	66	56	0.66	0.597-0.741	<0.001

Table 19: illustrates the sensitivity, specificity and area under curve of the parameters analyzed in Type 2 diabetes mellitus group. The data showed moderate area under curve for Spexin and leptin in Type 2 diabetes mellitus group shown as graph in Figure 17 & 18.

Table 20: Receiver Operating Characteristic Curve for Spexin and leptin in Type 2 diabetes mellitus with hypertension group

Type 2 diabetes mellitus with Hypertension Group						
Parameter	Cut-off	Sensitivity (%)	Specificity (%)	AUC	95% CI Range	p
Spexin (ng/ml)	0.51	81	62	0.83	0.776-0.884	<0.001
Leptin (ng/ml)	27.4	79	65	0.79	0.739-0.855	<0.001

Table 20 illustrates the sensitivity, specificity and area under curve of the parameters analyzed in Type 2 diabetes mellitus with hypertension. The data showed good area under curve for Spexin in Type 2 diabetes mellitus with hypertension shown in graphical representation in Figure 19 & 20.

Furthermore, genetic part of the study deals with genetic variance among 30 subjects consists of 10 healthy controls, 10 Type 2 diabetes mellitus, and 10 Type 2 diabetes mellitus with hypertension subjects. The isolated and purified DNA was amplified by PCR with primers and the sequence information of these primers were gathered from the NCBI Primer BLAST and displayed in Table: 21

Table 21: Spexin gene primers and the sequence information from the NCBI Primer BLAST

Spexin gene C12or39 Exon	Forward Primer (5' – 3')	Reverse Primer (5' – 3')	PCR Amplicon (bp)	Temp ^{0}C
Exon-2	GAAGGTAGAAGGGAAACACCTC	CATTGCAGCTCAGTCCTCTATT	365	63.1
Exon-3	TGTCCACTCTGCTCTTTCTT	TCCTAGGGGTCTTCCATTAT	231	57.3
Exon-4	GGAGGAGCTGAGGTTTAAG	GTGAGGAGACGCTTTCTTT	238	63.1
Exon-5	TGCTGCATGTTAGAATAGGA	CACAGAATCCCGAAAGTAAG	228	59.0
Exon-6	TGTGTCCCAAGCTGAGAAAA	AACAGGACCTGAAGCAATGAAA	453	53.1

Exon-1 was unable to amplify due to six nucleotidesband unable to found difficulty in designing primers set.

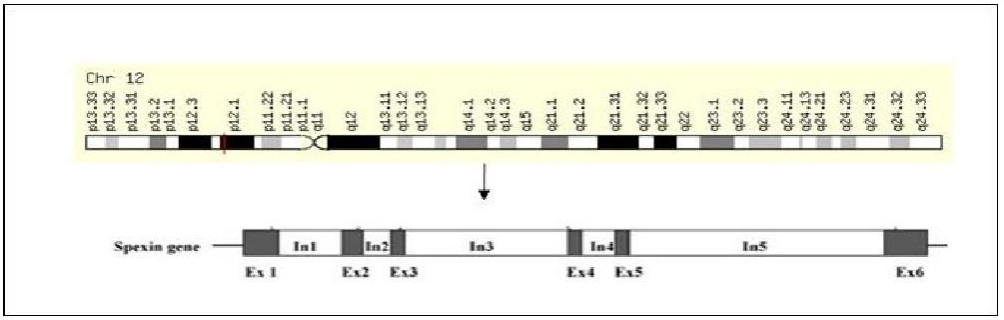
The purified amplicons were separated on 1.5 percent Agarose gel electrophoresis along with ladder DNA markers, and the bands were captured using the Bio-Rad Gel Doc System and recorded. In the present study all the exons were standardised at 63.1^{0}C, 57.3^{0}C, 63.1^{0}C, 59^{0}C and 53.1 ^{0}C. But we were unabled to amplify exon-1 because of six nucleotide which seems to be very small in size to design forward and reverse primers set. The ethidium bromide stained agarose gel photographs were displayed in the figure 14.

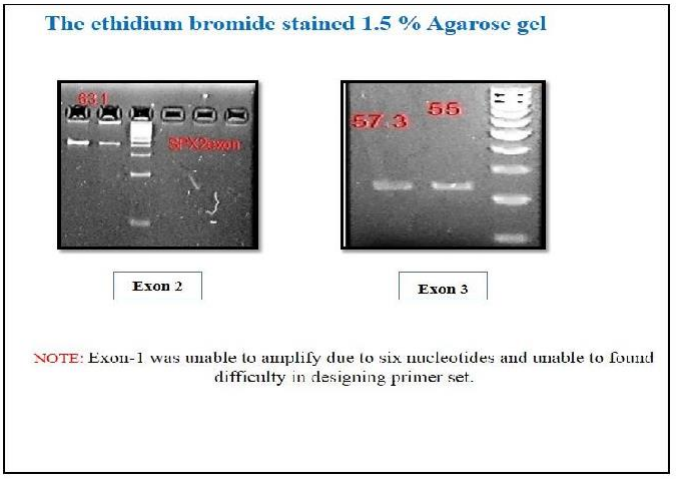
Only a few samples show variation in the sequence. Genetic background may accelerate the contribution of Type 2 diabetes mellitus. The sequencing results of the Spexin gene showed polymorphism in the 6th exon and no polymorphism was detected in the 2nd, 3rd, 4th, and 5th exons. Furthermore, heterogeneous polymorphism were observed in the 2 samples. In these samples stop codon T is mutated to G (T>G), and that codes for glycine residue at 224th and 225th position which results in continuous translation up to 17 amino acids further until a stop codon stops the translation.

Hence, instead of stopping the translation, protein might be translated with atleast 17 extra amino acids which produce long and wrong protein. In the alignment, there seems to be another homozygous polymorphism in exon 6. In the chromatogram, G is mutated to A (G>A) which is point mutation/ missense mutation converted from aspartic acid to aspargine at 212th, -42nd positions. Hence, the present study emphasize that exonic polymorphism (G>A) leads to decreased Spexin levels in serum and might contribute for the future development of diabetes with CVD. Polymorphism were shown in chromatogram figure 15.

A part from exonic mutations, we also found intronic polymorphisms. In intron 5 we found homozygous of -42G>A polymorphism. Rest of the 6 samples showed a heterozygous polymorphism (-42G>A). The significance of this intronic polymorphism was not known but might contribute to early metabolic syndrome. In intron 3, we found deletion polymorphism in the acceptor site of the exon 5'. In this, there was the deletion of one C in the polypyrimidine tract at -21st position from the intron-exon boundary.

Therefore the present study explains that deletion makes any abruption in the splicing, affecting the spliceosome or it leads to alternate splicing. Hence, polymorphism in exon–intron boundaries may alter the binding of transcription factor which may affect transcription, indirectly affected on translation which causes reduction of Spexin gene expression and its protein levels in serum and that contributes to diabetes with CVD complications in MetS dearrangements. Exon and intronic homozygous and heterozygous polymorphism along with fluorescence electrogram was shown in figures 15&16a. The complete sequencing data of Exons in Spexin gene presented in figure 16b.





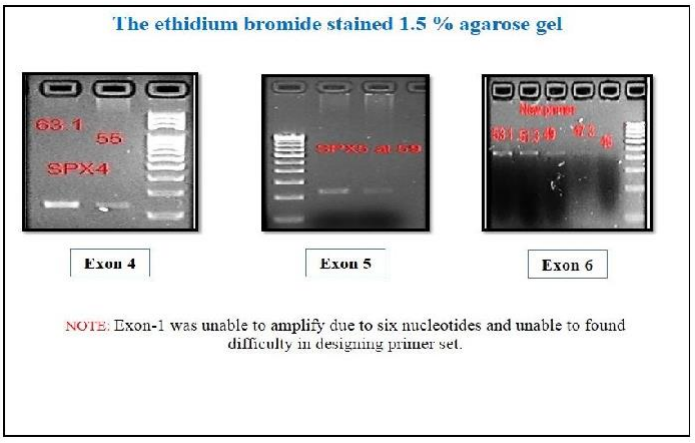


Figure 14: The ethidium bromide stained 1.5% agarose gel of amplified Exons of Spexin gene.

Figure 16b: The complete sequencing data of Exons in Spexin gene

SPEXIN Exon 2

CLUSTAL O(1.2.4) multiple sequence alignment

```
ref         TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample1     TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample2     TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample3     TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample4     TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample5     TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample6     TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample7     TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample8     TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample9     TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample10    TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample11    TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample12    TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample13    TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample14    TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample15    TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample16    TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample17    TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample18    TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample19    TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample20    TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample21    TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample22    TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample23    TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample24    TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
sample25    TGGTGTTTGTTTTCCTGGGAAACTCCAGCTGCGCTCCGCAG   41
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```

Conclusion: In 2nd exon, no SNPs found in all 25 samples

SPEXIN Exon 3

CLUSTAL O(1.2.4) multiple sequence alignment

```
ref        AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample1    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample2    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample3    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample4    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample5    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample6    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample7    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample8    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample9    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample10    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample11    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample12    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample13    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample14    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample15    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample16    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample17    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample18    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample19    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample20    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample21    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample22    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample23    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample24    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
sample25    AGACTGTTGGAGAGAAGGAACTGGACTCCTCAAGCTATGCTCTACCTGAAAGG 53
            ***************************************************
```

Conclusion: In 3rd exon, no SNPs found in all 25 samples

SPEXIN Exon 4

CLUSTAL O(1.2.4) multiple sequence alignment

```
ref       AGGGTCGCCGCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGC  60
sample1   AGGGTCGCCGCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGC  60
sample2   AGGGTCGCCGCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGC  60
sample4   AGGGTCGCCGCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGC  60
sample5   AGGGTCGCCGCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGC  60
sample6   AGGGTCGCCGCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGC  60
sample7   AGGGTCGCCGCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGC  60
sample8   AGGGTCGCCGCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGC  60
sample9   AGGGTCGCCGCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGC  60
sample10  AGGGTCGCCGCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGC  60
sample11  AGGGTCGCCGCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGC  60
sample12  AGGGTCGCCGCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGC  60
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sample14  AGGGTCGCCGCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGC  60
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sample2   CGG  63
sample4   CGG  63
sample5   CGG  63
sample6   CGG  63
sample7   CGG  63
sample8   CGG  63
sample9   CGG  63
sample10  CGG  63
sample11  CGG  63
sample12  CGG  63
sample13  CGG  63
sample14  CGG  63
sample15  CGG  63
sample16  CGG  63
sample17  CGG  63
sample18  CGG  63
sample19  CGG  63
sample20  CGG  63
sample21  CGG  63
sample22  CGG  63
sample23  CGG  63
sample24  CGG  63
sample25  CGG  63
sample3   CGG  59
          ***
```

Conclusion: In 4th exon, no SNPs found in all 25 samples

SPEXIN Exon 5

CLUSTAL O(1.2.4) multiple sequence alignment

```
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sample3    AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample4    AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample5    AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample6    AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample7    AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample8    AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample9    AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample10   AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample11   AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample12   AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample13   AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample14   AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample15   AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample16   AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample17   AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample18   AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample19   AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample20   AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample21   AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample22   AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample23   AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample24   AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
sample25   AAAGACGAAGCCCAAATCCCCAACTACTAACTATTCCGGAGGCAGCAACCATCTTACTGG 60
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ref        CGTCCCTTCAGAAATCACCAGAAG 84
sample1    CGTCCCTTCAGAAATCACCAGAAG 84
sample3    CGTCCCTTCAGAAATCACCAGAAG 84
sample4    CGTCCCTTCAGAAATCACCAGAAG 84
sample5    CGTCCCTTCAGAAATCACCAGAAG 84
sample6    CGTCCCTTCAGAAATCACCAGAAG 84
sample7    CGTCCCTTCAGAAATCACCAGAAG 84
sample8    CGTCCCTTCAGAAATCACCAGAAG 84
sample9    CGTCCCTTCAGAAATCACCAGAAG 84
sample10   CGTCCCTTCAGAAATCACCAGAAG 84
sample11   CGTCCCTTCAGAAATCACCAGAAG 84
sample12   CGTCCCTTCAGAAATCACCAGAAG 84
sample13   CGTCCCTTCAGAAATCACCAGAAG 84
sample14   CGTCCCTTCAGAAATCACCAGAAG 84
sample15   CGTCCCTTCAGAAATCACCAGAAG 84
sample16   CGTCCCTTCAGAAATCACCAGAAG 84
sample17   CGTCCCTTCAGAAATCACCAGAAG 84
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sample20   CGTCCCTTCAGAAATCACCAGAAG 84
sample21   CGTCCCTTCAGAAATCACCAGAAG 84
sample22   CGTCCCTTCAGAAATCACCAGAAG 84
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sample24   CGTCCCTTCAGAAATCACCAGAAG 84
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```
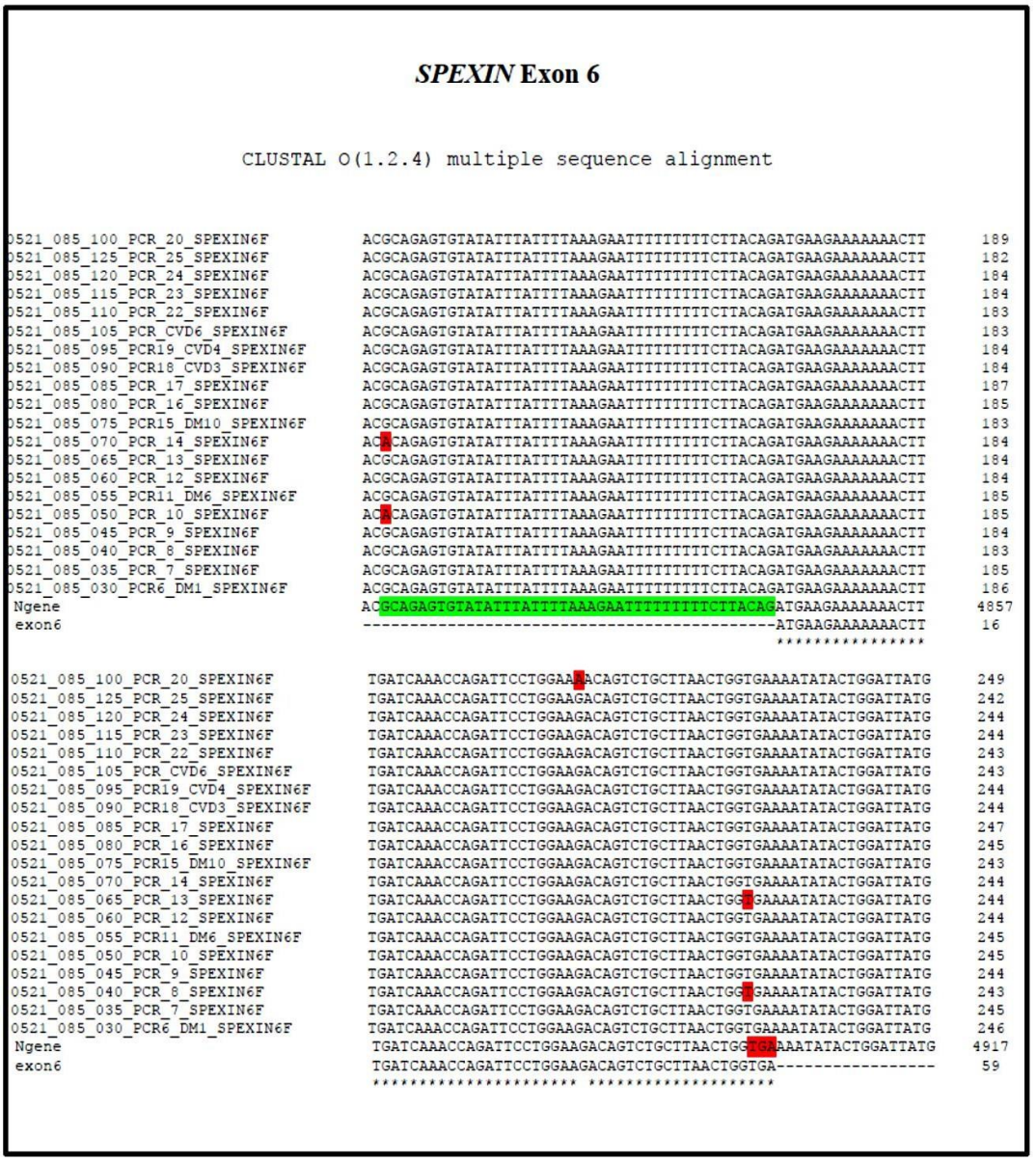
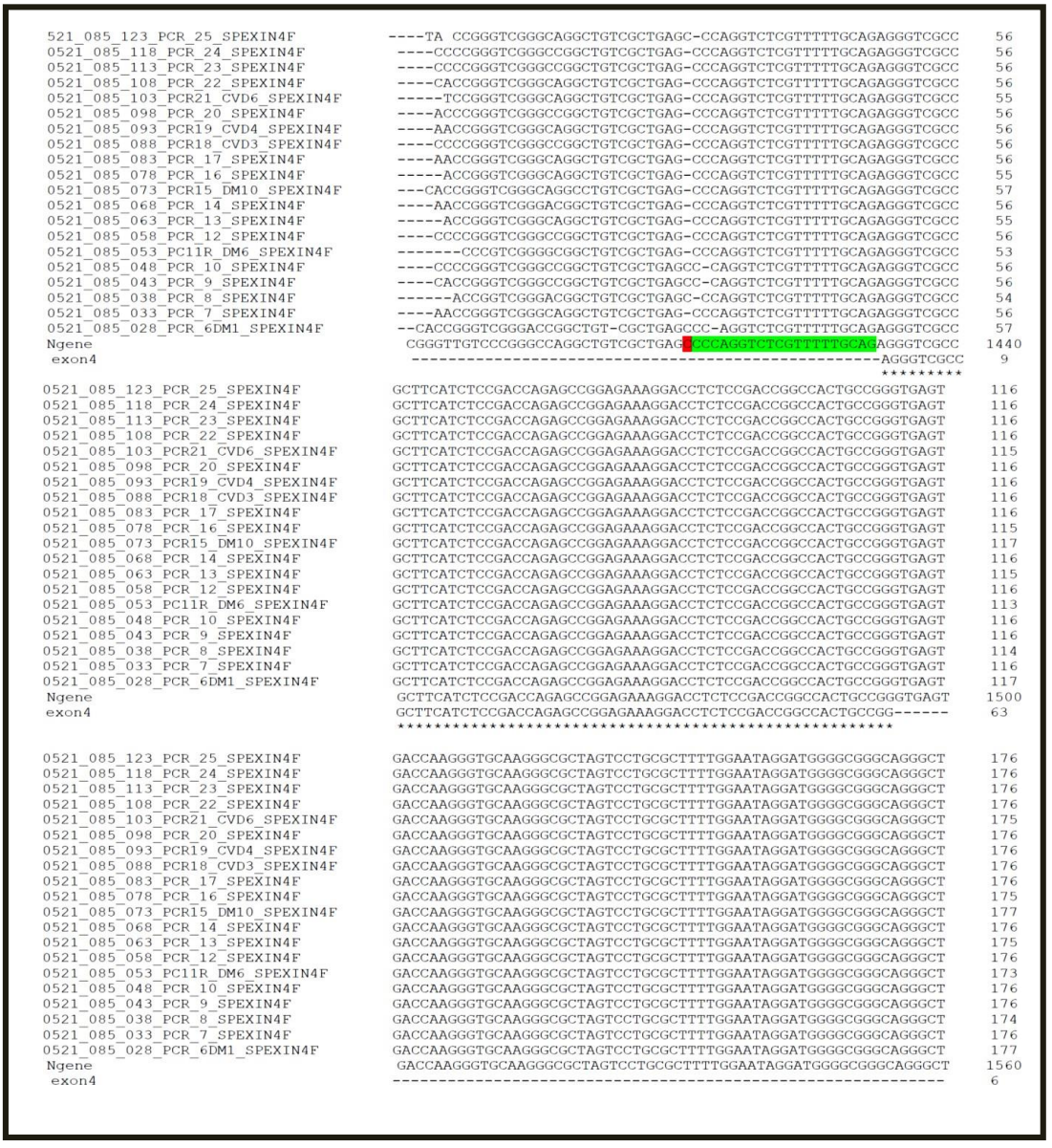

Conclusion: In 5th exon, no SNPs found in all 25 samples

SPEXIN Exon 6

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0521_085_063_PCR_13_SPEXIN4F -----ACCGGGTCGGGCAGGCTGTCGCTGAG-CCCAGGTCTCGTTTTTGCAGAGGGTCGCC 55
0521_085_058_PCR_12_SPEXIN4F ----CCCCGGGTCGGGCCGGCTGTCGCTGAG-CCCAGGTCTCGTTTTTGCAGAGGGTCGCC 56
0521_085_053_PC11R_DM6_SPEXIN4F -------CCCGTCGGGGCGGCTGTCGCTGAG-CCCAGGTCTCGTTTTTGCAGAGGGTCGCC 53
0521_085_048_PCR_10_SPEXIN4F ----CCCCGGGTCGGGCCGGCTGTCGCTGAGCC-CAGGTCTCGTTTTTGCAGAGGGTCGCC 56
0521_085_043_PCR_9_SPEXIN4F ----CACCGGGTCGGGCCGGCTGTCGCTGAGCC-CAGGTCTCGTTTTTGCAGAGGGTCGCC 56
0521_085_038_PCR_8_SPEXIN4F ------ACCGGTCGGGACGGCTGTCGCTGAGC-CCAGGTCTCGTTTTTGCAGAGGGTCGCC 54
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Ngene CGGGTTGTCCCGGGCCAGGCTGTCGCTGAG-CCCAGGTCTCGTTTTTGCAGAGGGTCGCC 1440
exon4 ----------AGGGTCGCC 9
****
0521_085_123_PCR_25_SPEXIN4F GCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGCCGGGTGAGT 116
0521_085_118_PCR_24_SPEXIN4F GCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGCCGGGTGAGT 116
0521_085_113_PCR_23_SPEXIN4F GCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGCCGGGTGAGT 116
0521_085_108_PCR_22_SPEXIN4F GCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGCCGGGTGAGT 116
0521_085_103_PCR21_CVD6_SPEXIN4F GCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGCCGGGTGAGT 115
0521_085_098_PCR_20_SPEXIN4F GCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGCCGGGTGAGT 116
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Ngene GCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGCCGGGTGAGT 1500
exon4 GCTTCATCTCCGACCAGAGCCGGAGAAAGGACCTCTCCGACCGGCCACTGCCGG------ 63
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0521_085_058_PCR_12_SPEXIN4F GACCAAGGGTGCAAGGGCGCTAGTCCTGCGCTTTTGGAATAGGATGGGGCGGGCAGGGCT 176
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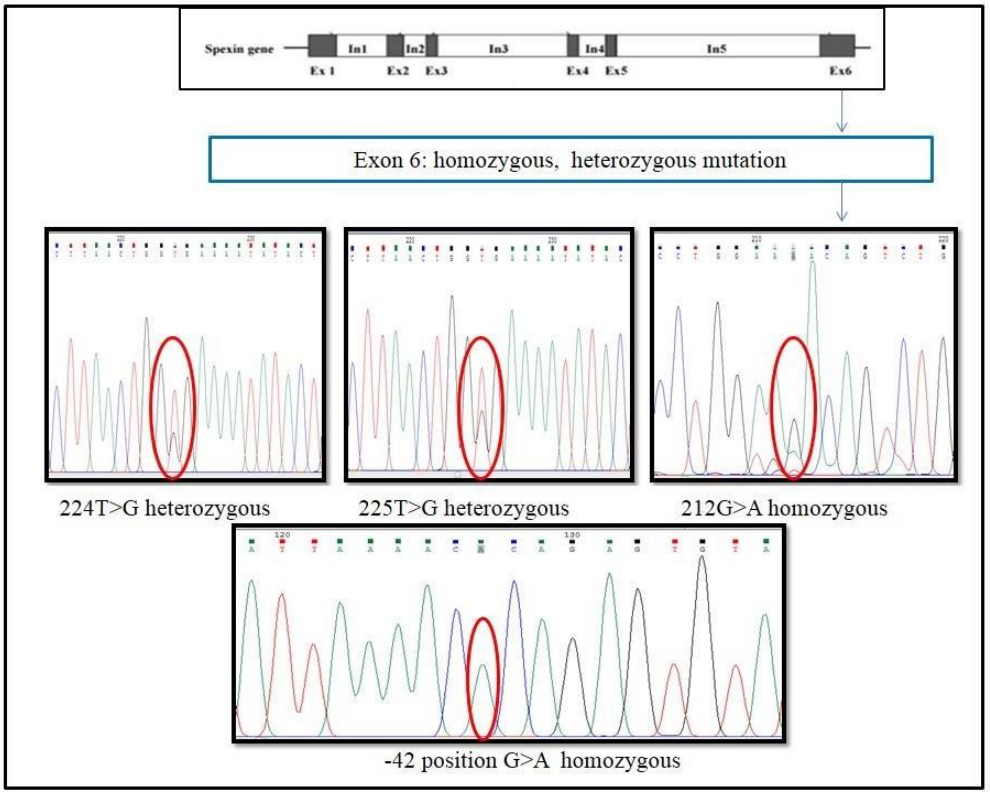


Figure 15: Homozygous & heterozygous polymorphism in 6th Exon

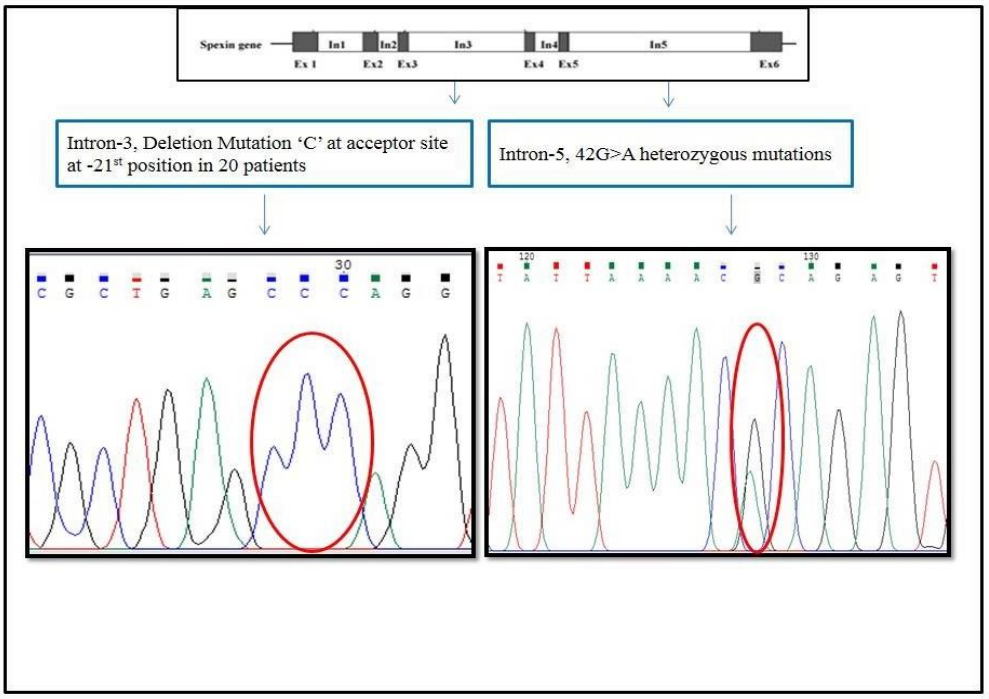


Figure 16a: Polymorphism (heterozygous) of Intron 3 and Intron 5

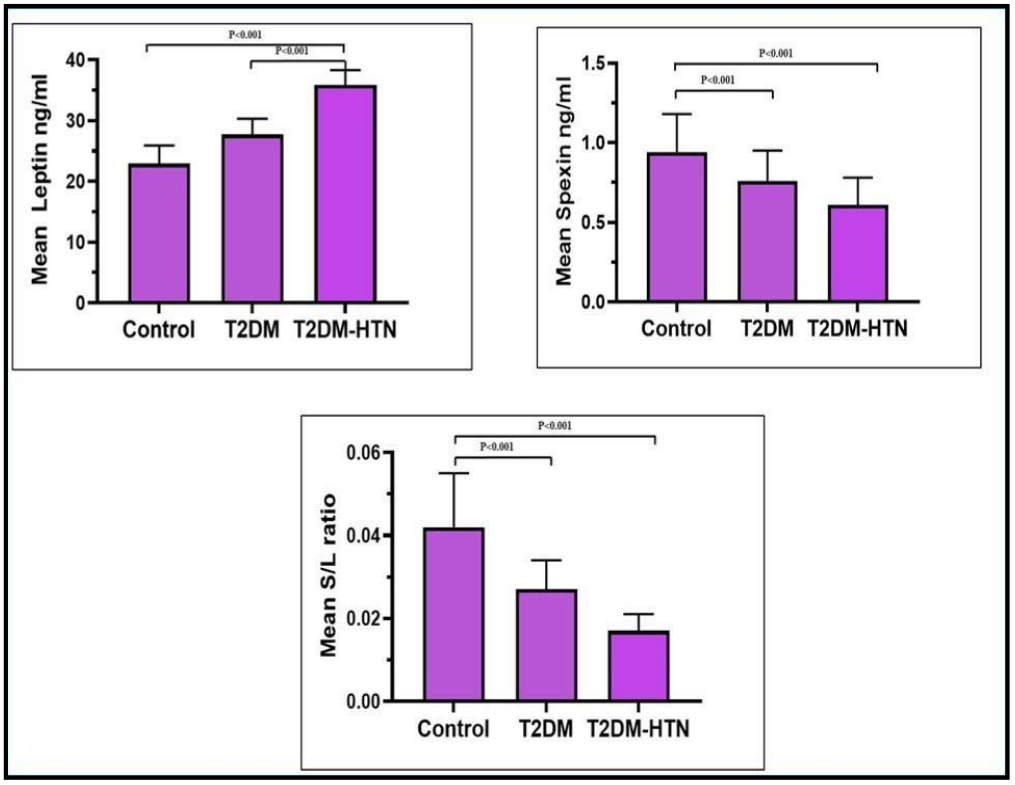


Figure 17: Mean & SD of Spexin, Leptin and S/L ratio concentration in Study groups

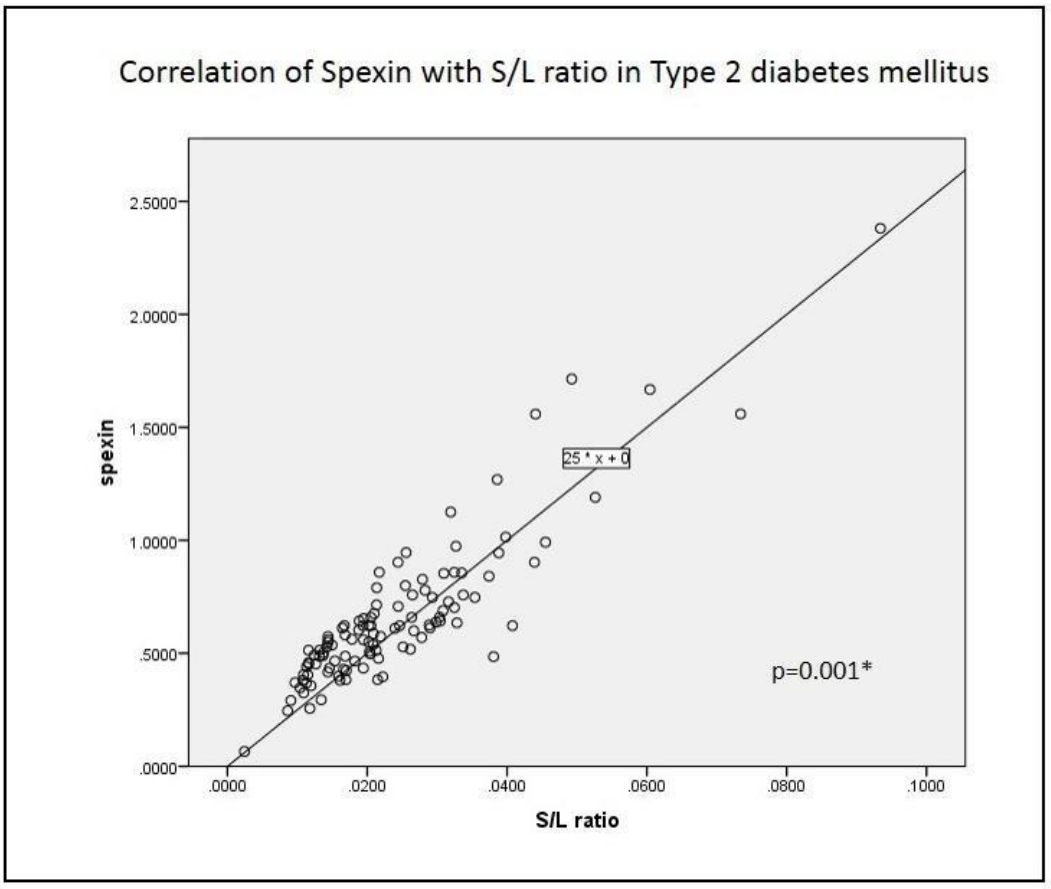


Figure 18: Correlation graph of Spexin with S/L ratio in Type 2 diabetes mellitus

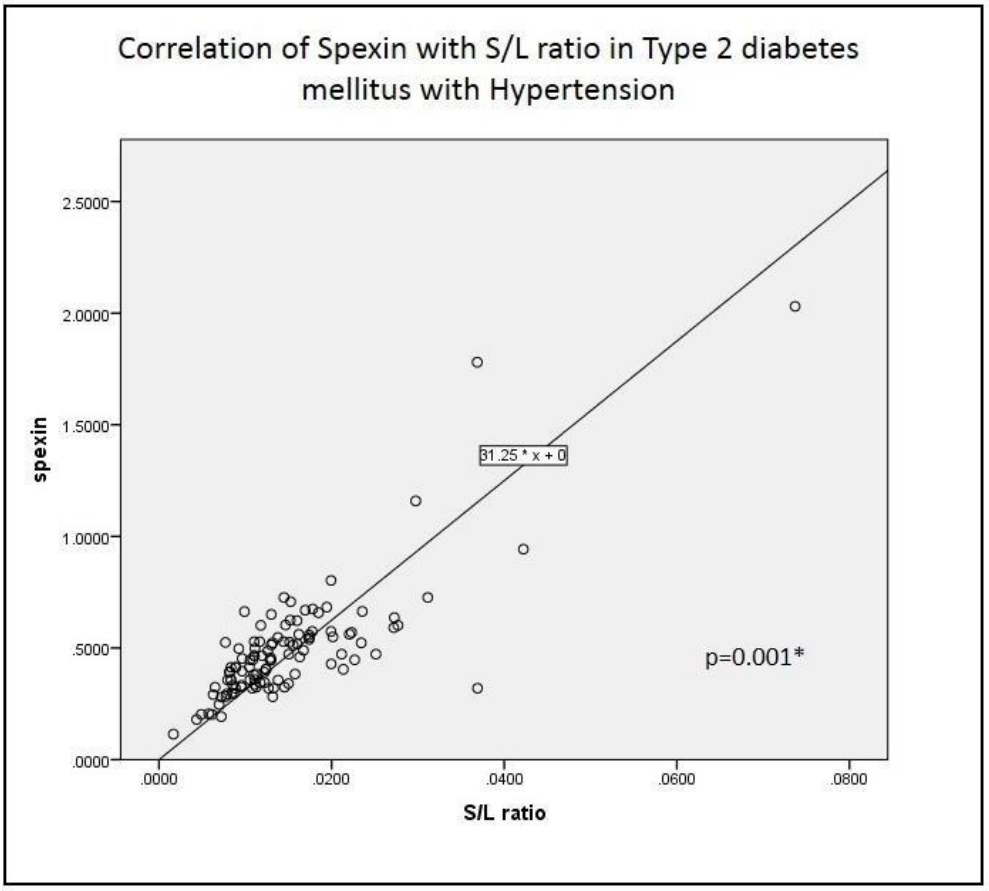


Figure 19: Correlation graph of Spexin with S/L ratio in Type 2 diabetes mellitus with hypertension

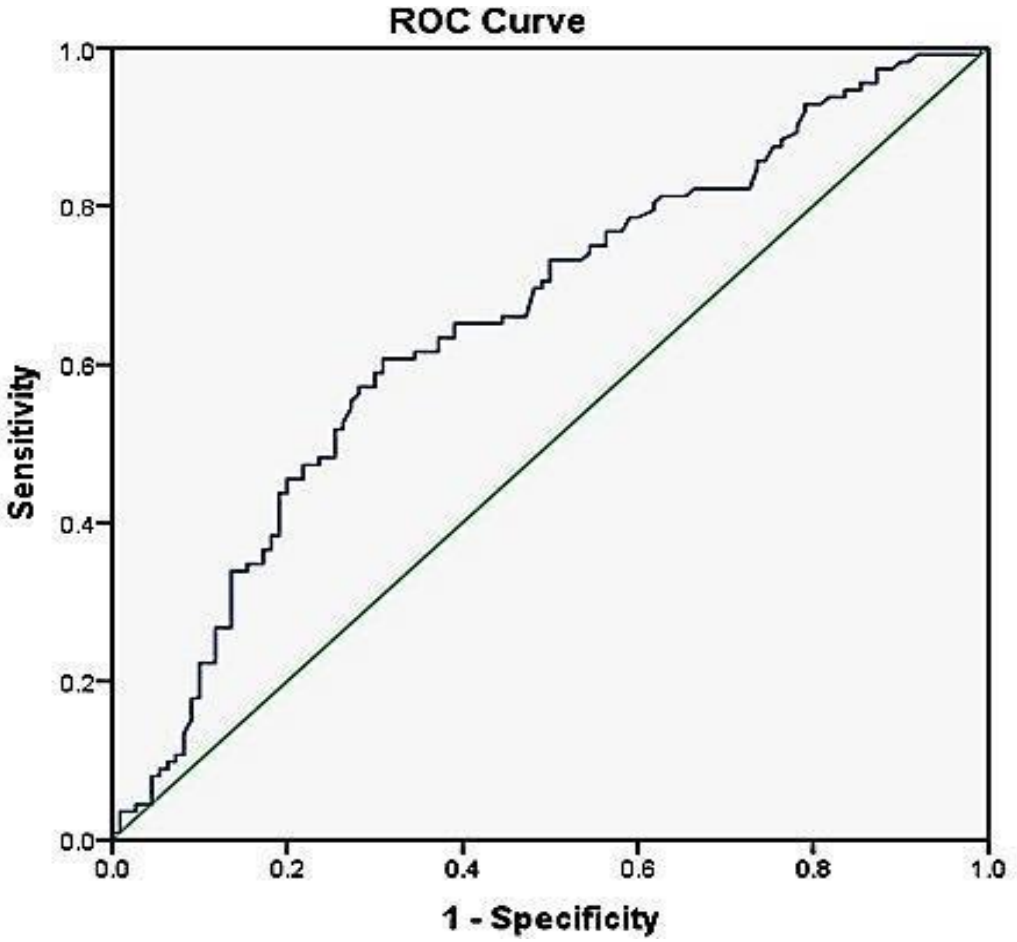


Figure 20: Receiver operating characteristic curve showing the area under curve for Spexin in Type 2 diabetes mellitus

The diagnostic performance of Spexin in Type 2 diabetes mellitus was represented by receiver operating characteristic curve with False Positive Rate (1- specificity) on X - axis and True Positive Rate (sensitivity) on Y-axis. Cut-off of (0.62) showed sensitivity (65%), specificity (62%), and area under curve (AUC) (0.65), 95% confidence interval range (0.584 –0.728) and $p<0.001$. Hence, area under curve shows moderate accuracy for the test parameter in Type 2 diabetes mellitus group.

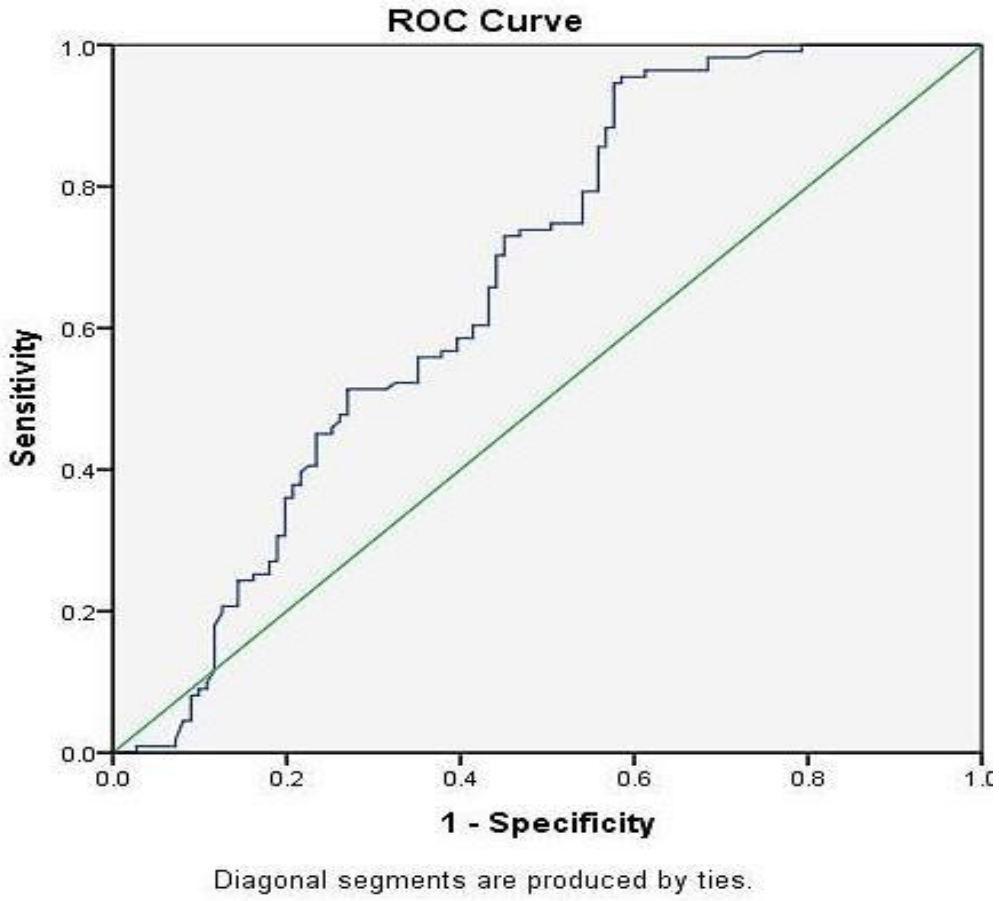


Figure 21: Receiver operating characteristic curve showing the area under curve for Leptin in Type 2 diabetes mellitus

The diagnostic performance of Leptin in Type 2 diabetes mellitus was represented by receiver operating characteristic curve with False Positive Rate (1- specificity) on X-axis and True Positive Rate (sensitivity) on Y-axis. Cut-off of (27.4) showed sensitivity (79%), specificity (65%), and area under curve (AUC) (0.79), 95% confidence interval range (0.739-0.855) and ($p < 0.001$). Hence, area under curve shows moderate accuracy for the test parameter in Type 2 diabetes mellitus group.

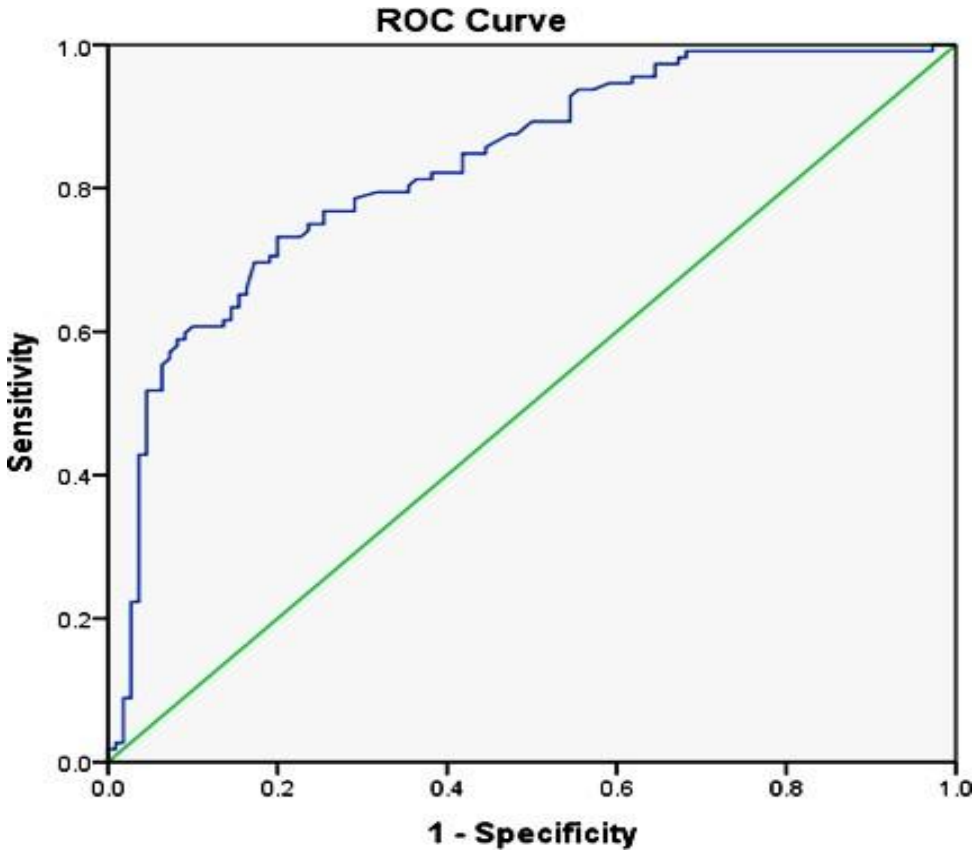


Figure 22: Receiver operating characteristic curve showing the area under curve for Spexin in Type 2 diabetes mellitus with hypertension

The diagnostic performance of Spexin in Type 2 diabetes mellitus with hypertension was represented by receiver operating characteristic curve with False Positive Rate (1-specificity) on X-axis and True Positive Rate (sensitivity) on Y-axis. Cut-off of (0.51) showed sensitivity (81%), specificity (62%), and area under curve (AUC) (0.83), 95% confidence interval range (0.776 –0.884) and ($p < 0.001$). Hence, area under curve shows good accuracy for the test parameter in Type 2 diabetes mellitus with hypertension group.

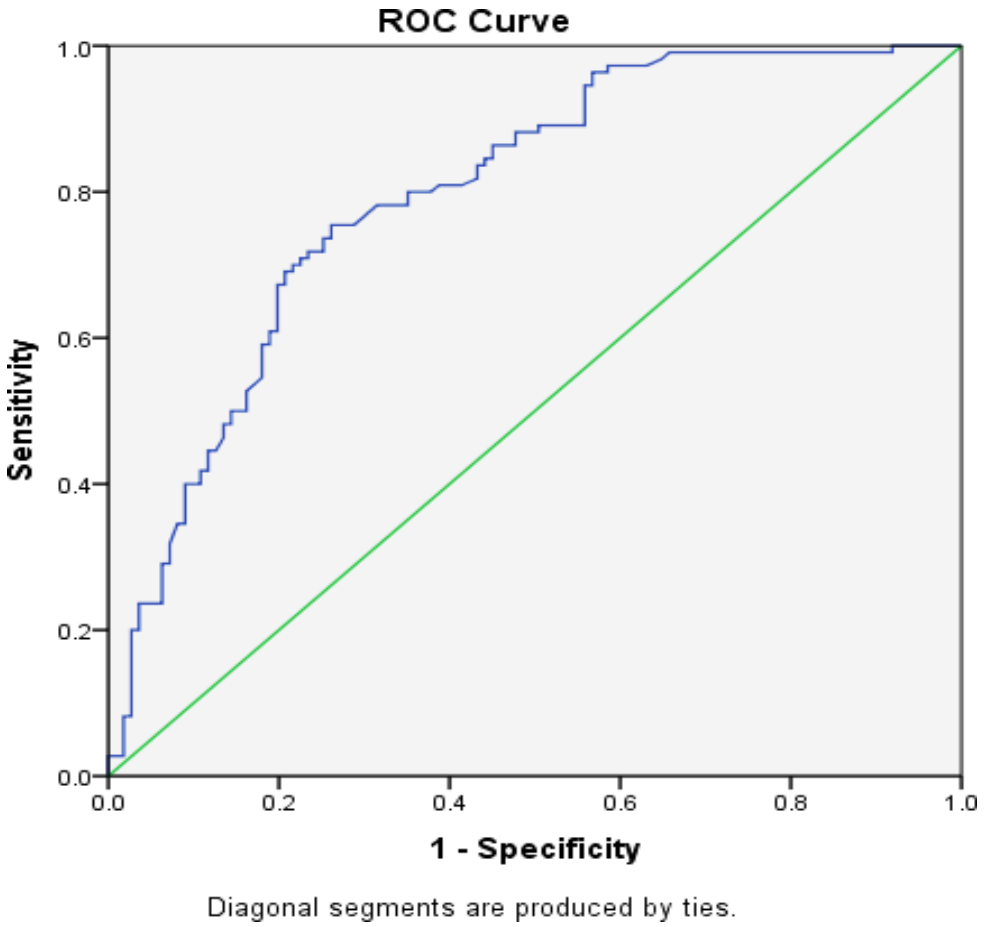
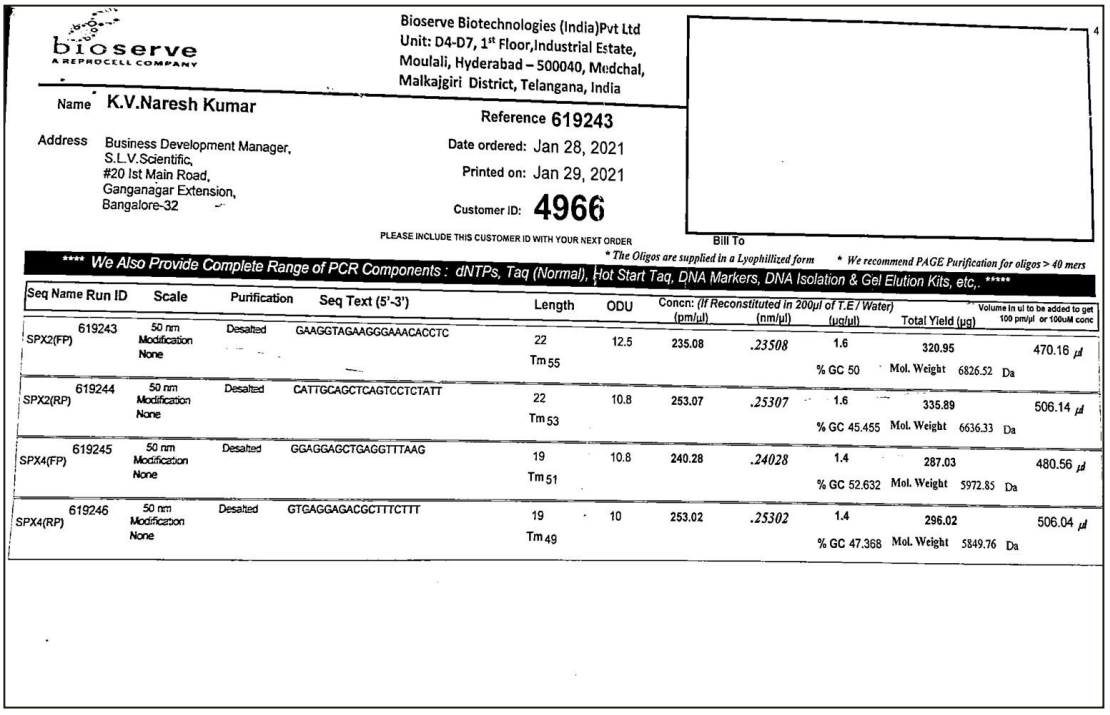


Figure 23: Receiver operating characteristic curve showing the area under curve for leptin in Type 2 diabetes mellitus with hypertension

The diagnostic performance of Leptin in Type 2 diabetes mellitus with hypertension was represented by receiver operating characteristic curve with False Positive Rate (1- specificity) on X -axis and True Positive Rate (sensitivity) on Y-axis. Cut-off of (27.4) showed sensitivity (79%), specificity (65%), and area under curve (AUC) (0.79), 95% confidence interval range (0.739-0.855) and p <0.001. Hence, area under curve shows moderate accuracy for the test parameter in Type 2 diabetes mellitus with hypertension group.



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Address Business Development Manager,
S.L.V.Scientific,
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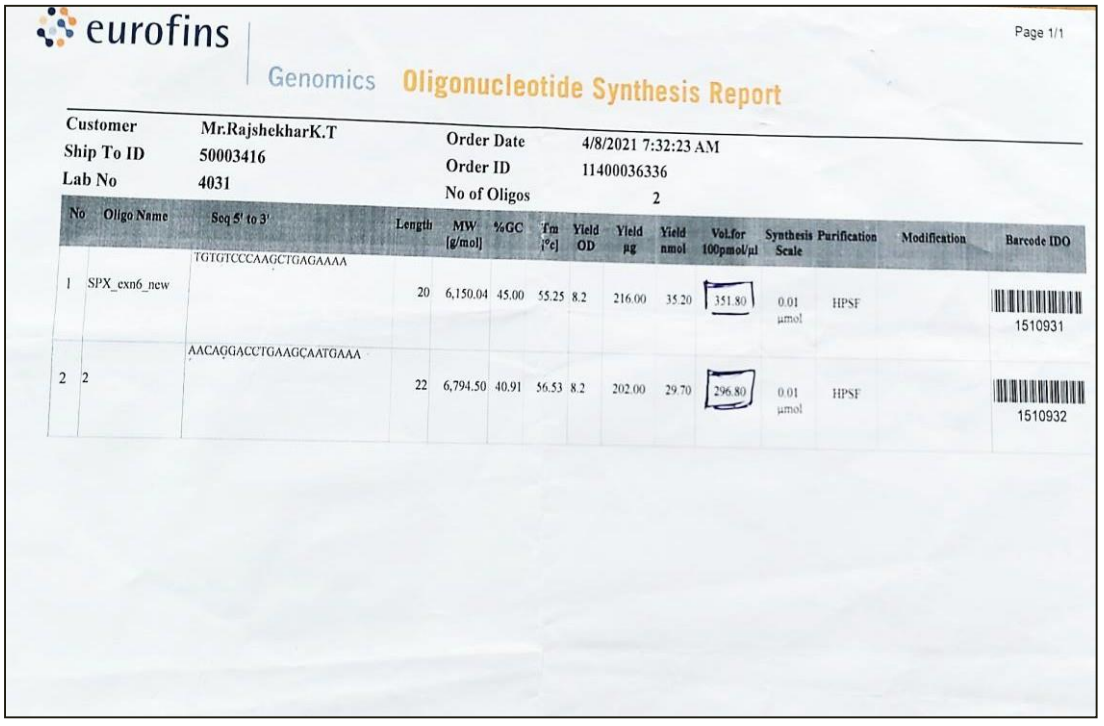
**** We Also Provide Complete Range of PCR Components : dNTPs, Taq (Normal), Hot Start Taq, DNA Markers, DNA Isolation & Gel Elution Kits, etc,. *****

* The Oligos are supplied in a Lyophillized form * We recommend PAGE Purification for oligos > 40 mers

Seq Name	Run ID	Scale	Purification	Seq Text (5'-3')	Length	ODU	Concn: (If Reconstituted in 200µl of T.E / Water) (pm/µl)	(nm/µl)	(µg/µl)	Total Yield (µg)	Volume in ul to be added to get 100 pm/µl or 100uM conc
SPX2(FP)	619243	50 nm Modification None	Desalted	GAAGGTAGAAGGGAAACACCTC	22 Tm 55	12.5	235.08	.23508	1.6 % GC 50	320.95 Mol. Weight 6826.52 Da	470.16 µl
SPX2(RP)	619244	50 nm Modification None	Desalted	CATTGCAGCTCAGTCCTCTATT	22 Tm 53	10.8	253.07	.25307	1.6 % GC 45.455	335.89 Mol. Weight 6636.33 Da	506.14 µl
SPX4(FP)	619245	50 nm Modification None	Desalted	GGAGGAGCTGAGGTTTAAG	19 Tm 51	10.8	240.28	.24028	1.4 % GC 52.632	287.03 Mol. Weight 5972.85 Da	480.56 µl
SPX4(RP)	619246	50 nm Modification None	Desalted	GTGAGGAGACGCTTTCTTT	19 Tm 49	10	253.02	.25302	1.4 % GC 47.368	296.02 Mol. Weight 5849.76 Da	506.04 µl

Table 4: Designed Forward and Reverse Primers on *SPX* Gene



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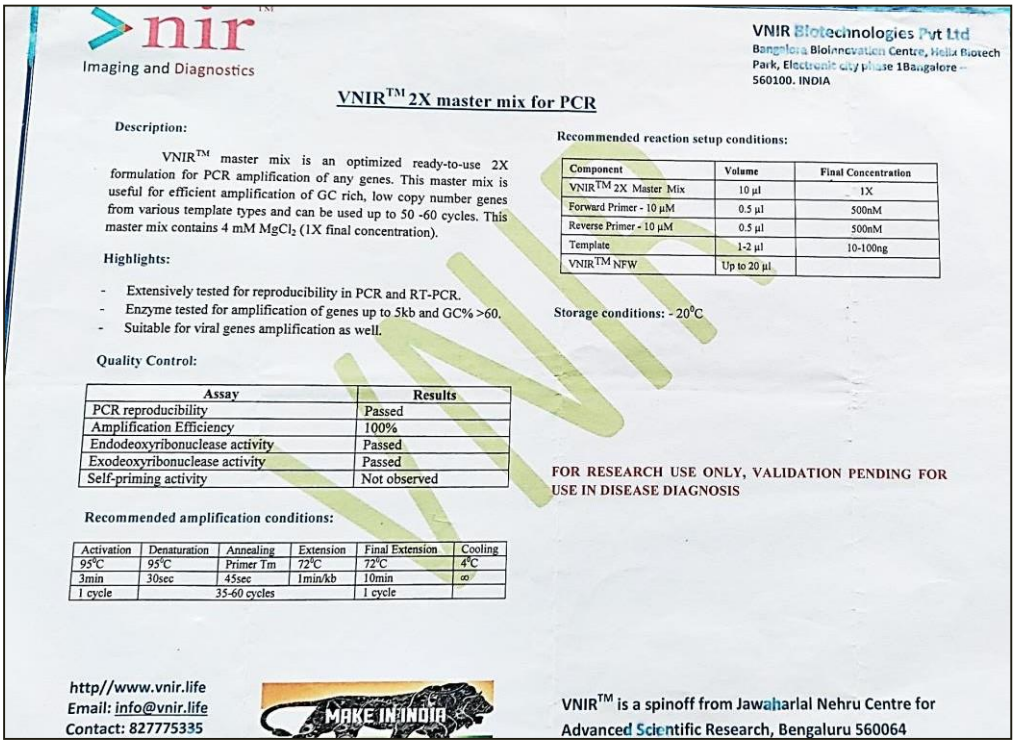
Oligonucleotide Synthesis Report

Customer Mr.RajshekharK.T
Ship To ID 50003416
Lab No 4031
Order Date 4/8/2021 7:32:23 AM
Order ID 11400036336
No of Oligos 2

No	Oligo Name	Seq 5' to 3'	Length	MW [g/mol]	%GC	Tm [°c]	Yield OD	Yield µg	Yield nmol	Vol.for 100pmol/µl	Synthesis Scale	Purification	Modification	Barcode IDO
1	SPX_exn6_new	TGTGTCCCAAGCTGAGAAAA	20	6,150.04	45.00	55.25	8.2	216.00	35.20	351.80	0.01 µmol	HPSF		1510931
2	2	AACAGGACCTGAAGCAATGAAA	22	6,794.50	40.91	56.53	8.2	202.00	29.70	296.80	0.01 µmol	HPSF		1510932

Table 5: Designed Forward and Reverse Primers on *SPX* Gene



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VNIR™ 2X master mix for PCR

Description:

VNIR™ master mix is an optimized ready-to-use 2X formulation for PCR amplification of any genes. This master mix is useful for efficient amplification of GC rich, low copy number genes from various template types and can be used up to 50 -60 cycles. This master mix contains 4 mM $MgCl_2$ (1X final concentration).

Highlights:

- Extensively tested for reproducibility in PCR and RT-PCR.
- Enzyme tested for amplification of genes up to 5kb and GC% >60.
- Suitable for viral genes amplification as well.

Quality Control:

Assay	Results
PCR reproducibility	Passed
Amplification Efficiency	100%
Endodeoxyribonuclease activity	Passed
Exodeoxyribonuclease activity	Passed
Self-priming activity	Not observed

Recommended amplification conditions:

Activation	Denaturation	Annealing	Extension	Final Extension	Cooling
95°C	95°C	Primer Tm	72°C	72°C	4°C
3min	30sec	45sec	1min/kb	10min	∞
1 cycle	35-60 cycles			1 cycle	

Recommended reaction setup conditions:

Component	Volume	Final Concentration
VNIR™ 2X Master Mix	10 µl	1X
Forward Primer - 10 µM	0.5 µl	500nM
Reverse Primer - 10 µM	0.5 µl	500nM
Template	1-2 µl	10-100ng
VNIR™ NFW	Up to 20 µl	

Storage conditions: - 20°C

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Table 6: Details of PCR master mix

5.2. DISCUSSION

Our research findings from the study on demographic analysis clearly give the background information of the study subjects. The increased body mass index and waist/hip ratio along with Systolic and diastolic blood pressure as the disease state progress were found statistically significant between study groups between Type 2 diabetic patients and Type 2 diabetics with hypertension compared to non-diabetic group as control.

We also observed in the study that the routine biochemical parameters indicating glycemic status such as fasting blood glucose/sugar, glycemic homeostasis /control by HbA1c, insulin HOMA-IR, inflammatory status by CRP, and dyslipidemia by lipid profile including Total cholesterol, triglycerides, LDL-C, HDL-C, VLDL, LDL/HDL ratio, and liver enzymes ALT and AST were also found with significant differences in diabetes mellitus and as a whole in Metabolic syndrome.

Few recent research reports also mentioned significant differences of demographic and clinical parameters in diabetes mellitus similar to the current study.

A Cross-sectional study conducted on non-alcoholic fatty liver disease and associated risk factors in Type 2 diabetics by Ali A et al in 2022 showed that increased risk factors such as high BMI, increased HbA1c, lipids and ALT levels in Type 2 diabetic patients. Furthermore, study presented high prevalence of Non-alcoholic fatty liver disease in Type 2 diabetics with associated risk factors like obesity, hyperglycemia, dyslipidemia and liver enzymes [115].

The study recommended early recognition and prevention of obesity by a diet low with carbohydrates and glycemic control, high with omega 3 fatty acids, regular physical activity and life style modifications along with efforts to early recognition

of non-alcoholic fatty liver disease by ultra sonography[115].

A Cohort study about relationship between lipid profiles and glycemic control in Type 2 diabetics conducted by Wang S et al in 2020 showed that fasting plasma glucose significantly associated with TC and HDL-C but not associated with TG and LDL-C. This study suggested that HDL-C and TG should be focused on health management of the progression of Type 2 diabetes mellitus.

Another cross-sectional study on clinical and biological risk factors association with inflammation in Type 2 diabetics conducted by Ellulu M.S et al in 2021 reported about increased CRP levels with high BMI, high fasting blood sugar and low adiponectin levels in Type 2 diabetic patients. Study also reported that an inflammation in Type 2 diabetics linked metabolic abnormalities [117].

A study by Mottaghi T et.al. in 2019 presented an association between BMI and inflammation among diabetic polyneuropathy patients and suggested that BMI has relationship with inflammatory markers [118].

Song Y et.al in 2019 presented an association between CRP and metablolic syndrome suggested that low grade inflammation may increase risk of metabolic syndrome[119].

Yet another study by chan J.C. et.al. in 2018 presented effect of body mass index on the incidence of diabetes and diabetic retiniopathy where suggested that overweight and obesity are risk factors in diabetics [120].

The above studies reports on demographic and biochemical investigations were in agreement and also in support of our study results. In line with the above reported studies, the current study results have also shown a difference of cardio-metabolic parameters such as FBS, HbA1c, increased lipids and CRP in Type 2 diabetic with

and without hypertension patients. And also demographic and biochemical data presented an association between cardio-metabolic parameters like Obesity, insulin resistance, dyslipidemia and hypertension of study groups.

Hence, present study highlights that right clinical management, nutrition, life style modification, physical activity, counseling is essential at the early stage to manage the disorders that contribute to MetS. Therefore, modifiable factors should be prioritised in patients with Type 2 diabetic progression.

The prevalence of MetS in India ranges from 10-41 percent with socio-cultural variations [69], the incidence is generally high with urban population. However, incidence also increasingly high in rural population of developing countries owning to urbanization due to unhealthy diet practice and sedentary life style amounts to MetS [121]. The MetS diagnosis and management requires identification, selection and applications of the suitable markers in the serum or plasma.

The biomarkers studied in assessment of MetS, type 2 diabtes mellitus and risk for CVD was TC, TG, HDL, LDL, Insulin and C-peptide levels. Some of the lipoprotein biomarkers measured to assess are Apolipoprotein A1 (apo-A1) and apo-lipoprotein B (apo-B) for dyslipidemia as predictors for CVD risk. Similarly, some of the peptide hormones like adiponectin, leptin and Ghrelin are emerging biomarkers for insulin resistance [122].

Among the biomarkers, leptin is a well-known marker in metabolic syndrome and insulin resistance. Very few studies have prospectively studied leptin biomarker during progression of MetS to diabetes with CVD [123-125]. Literature on longitudinal measurements of leptin and its role in diabetes is scarce. However, it is known fact that serum marker leptin which is an adipokine have a vital role in prevention and

control of Type 2 diabtes mellitus. According to animal models, by regulation of beta-cell activity and survival improves insulin sensitivity, maintain glucose metabolism and energy homeostasis [126].

Patients with increased serum leptin levels exhibit increased food intake, adipose stores and weight gain. Therefore, we focused on comparision of leptin for identification of Type 2 diabtes mellitus and obesity. We estimated serum leptin levels in the study group's subjects and was found significant difference in Type 2 diabtes mellitus with and without hypertension groups compared with control. Similarly, few recent studies were also reported a significant increased leptin levels in agreement with present study results.

A cross sectional study on plasma Leptin and resistin levels in subgroups of Type 2 Diabetic subjects conducted by Peng et al in 2022. The subgroups of the study were mild obesity-related diabetes, severe insulin-deficient diabetes, severe insulin-resistant diabetes and mild age related diabetes. Samples were estimated for Leptin and resistin levels and showed that increased levels of plasma Leptin in the mild obesity-related diabetes group with relatively decreased levels in the severe insulin-deficient diabetes and severe insulin-resistant diabetes group and resistin showed high in mild age related diabetes. Study concluded that high Leptin levels in mild obesity-related diabetes group associated with BMI and mentioned approach of Leptin therapy for other groups and high resistin levels in severe insulin-resistant diabetes group [76]. Study proposes the need of the longitudinal studies is essential to explore the exact mechanism of Leptin in type 2 diabtes mellitus.

A prospective community based cohort study conducted on plasma Leptin and adiponectin, adiponectin-leptin ratio and hsCRP associated with dysglycemia or pre-diabetes/diabetes by Kaze AD et al in 2021 and his co-workers in African American population. The study comprises 3223 participants without diabetes over a period of seven years developed 46.4% glycemic progression. The study concluded increased study parameters associated with development of type 2 diabtes mellitus [77].

In the similar way, a prospective cohort study was conducted by Bidulescu A and his co-workers in 2020 among African Americans. They evaluated adiponectin and Leptin in Type 2 diabetics patients compared to control. The study results concluded that adiponectin inversely related to Type 2 diabetics patients, whereas Leptin had direct association with Type 2 diabetics mediated by insulin resistance. This observation was more prominent in men. Study also indicated to reduce the burden of Type 2 diabetics modification of serum Leptin and adiponectin levels is essential [78].

In 2020, Kumar R conducted a Case-control study in Pakistan population to observe the relationship of Leptin in obesity and insulin resistance. The study showed significant increased Leptin levels, total cholesterol and insulin resistance in patients with higher BMI. The study concluded that increased Leptin levels is linked with high BMI and insulin resistance and mentioned the monitoring of Leptin levels in patients with Type 2 diabtes mellitus has a crucial role in managing obesity [79].

In contrast, A Cross sectional study conducted on the serum Leptin levels in Diabetic and non-diabetic patients in 2017 in Saudi male population by Sheikh AL and co-workers. The study results indicated no relationship between Leptin and adiponectin in diabetic and control groups. Study concluded that decreased Leptin and other metabolic parameters as the severity of the disease progress [80].

In accordance to the above study results, the present study results showed an increased leptin levels in Type 2 diabtes mellitus and Type 2 diabtes mellitus with hypertension subjects compared to controls with a statistical significance.

The possible explanation on physiological role of leptin in food intake and diabetes was the ObRb receptor of leptin plays a key role in regulating energy homeostasis and neuroendocrine function in hypothalamus. Although LEP-ObRb and other cytokine receptors lack kinase activity, they do pair with tyrosine kinases. Leptin binds to Leptin receptor undergoes a conformational change which is critical for Leptin signaling and activation of the associated Janus kinase (JAK2) pathway. JAK2 auto-phosphorylates and simultaneously phosphorylates tyrosine residues permitting binding of Signaling transducer activator transport (STAT) proteins and subsequent translocation to the nucleus. Other signaling pathways by LEP-ObRb receptor by Phosphoinositol-3 kinase (PI3K) and mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) signaling cascades are also activated when Leptin binds to receptors. The anorexigenic actions of Leptin are aided by the activation of each of these mechanisms involving in suppressing appetite, stimulating weight loss, and increasing thermogenesis.

Hence, increased activity of protein tyrosine phosphatase 1B dephosphorylates JAK2, stops STAT3 activation and thereby inhibits Leptin pathway that leads to Leptin resistance[54].

Several peptides have been extensively studied for the early detection and diagnosis of metabolic syndrome charecterised by obesity, insulin resistance, dyslipidemia and hypertension.

The suggested peptide biomarkers for the early prediction or diagnosis of MetS are Visfatin, resistin, inflammatory markers like C-reactive protein, CD40 ligand, interleukin-6, intercellular adhesion molecule-1, P-selectin, Pentraxin 3 VEGF (vascular endothelial growth factor), adipokines [122].

Due to the complexity of MetS with the various influences and consequences for other diseases, it is hard to make a well-defined distinction between the various groups of biomarkers. Whether changes in concentration levels are causes or consequences of the MetS cannot be defined [122]. Eventhough, still there is a need to study emerging peptides as a biomarker along with existing parameters for the early understanding /prediction of obesity and diabetes with CVD complications.

Therefore, we have choosen Spexin as one of the marker to establish in humans and thus studied in the type 2 diabetes mellitus and type 2 diabetes mellitus with hypertension patients. In the current study scenario, the results evidenced that, decreased Spexin peptide levels in Type 2 diabetes mellitus and significantlow levels in Type 2 diabetes mellitus with hypertension patients. The difference of the variables found to be statistically significance in comparison with the non diabetic control group.

Growing evidence on a new biomarker Spexin having a role in MetS and Type 2 diabetes mellitus is of remarkable importance as one of the novel functions of Spexin is regulation of glucose metabolism in human model for early diagnosis of obesity and Type 2 diabetes mellitus.

To the best of our knowledge, Spexin in and its importance as a marker for early diagnosis of obesity and Type 2 diabetes mellitus in Indian population is seldom. However, few studies conducted in China, Poland and USA population reported measuring Spexin level in Type 2 diabetes mellitus and obesity which has some predictive value for the development of future MetS and Type 2 diabetes mellitus with CVD complications.

A cross-sectional study conducted in Florida population to evaluate the effect of Spexin in obese children and its link with cardio-metabolic risk factors. Study found that Spexin levels were considerably lower in obese children compared to normal-weight children, but did not correlate with other adipokines, insulin, or glucose levels. Study concluded that obese children have lower amounts of Spexin in the serum [84].

Another cross-sectional study conducted by Lin C.Y. et al in China population to investigate the link between Spexin levels, age, BMI, fasting glucose and lipid profile in healthy adult women. The study found that serum Spexin levels are linked to age, BMI, fasting glucose, and TG. However, lower Spexin levels were showed to indicate the probability of having a high BMI and high fasting glucose level. Study concluded that circulating Spexin levels decline with age [85].

Yet another cross sectional case-control study conducted by Kolodziejski P.A. et al. in Poland population to investigate the relationship between Spexin or kisspeptin levels and BMI, HOMA-IR, serum levels of insulin, glucagon, leptin, adiponectin, orexin-A, obestatin, ghrelin, and Glucagon-Like-Peptide-2 in obese and non-obese female patients. The study showed that patients with obese have lower levels of Spexin and kisspeptin than non-obese participants. The study also found negative correlation with HOMA-IR, BMI, insulin and positively with Spexin QUICKI index

and adiponectin. Study concluded that Spexin and kisspeptin is having a negative relationship with obesity and insulin resistance[86].

A study conducted by Daghri A.L. et al. in 2018 conducted a cross sectional study in Saudi Arabia population in healthy individuals to estimate the relationship between Spexin concentration and MetS components. Study found that lower circulating Spexin levels in MetS subjects. The study concluded that decreased Spexin levels was marginally linked with MetS components and are sex-specific in adults in patients with MetS compared to non-MetS individuals[88].

A pilot study conducted in USA population to observe the functional role of leptin, Spexin, and several biomarkers in cardiovascular disease in obesity adolescents. The study showed inverse relationship between Spexin and Leptin, along the presence of higher hs-CRP concentrations in obese adolescents in the presence of 'low Spexin and high Leptin,' Study concluded that Spexin may serve a central role in the regulation of satiety and some cardiovascular events in obese children[87].

Chen. T. et al 2019 conducted a Cross-sectional study to determine the potential role of Spexin in obese children and its correlations with obesity-related indicators, insulin sensitivity, and pancreatic cell function. Study showed Spexin levels were considerably lower in obese children compared to non-obese children, and negatively linked with insulin insensitivity and pancreatic cell function markers. Study concluded that Spexin appears to serve a protective role in glucose homeostasis and is linked to cell function in obese children[91].

Zhang L et al conducted a Cross-sectional study in China to determine the relationship of adiponectin and Spexin levels in insulin resistance patients with alcoholic steatohepatitis. They found that significant lower Spexin and Adiponectin

levels in non-alcoholic fatty liver disease patients compared to controls and did not correlate with BMI, but they did with HOMA-IR and Adiponectin leading to the conclusion that insulin resistance was strongly connected with Spexin and Adiponectin levels[93].

Behrooz M. et al. conducted a cross-sectional study in Iran with children with metabolic syndrome to observe whether the Spexin levels are related with metabolic syndrome, body composition and dietary intakes in children. The study found that Spexin levels were significantly lower in children with high fat mass and children with Systolic blood pressure (SBP) compared to controls and also showed significant negative associations with dietary fat intake. Study concluded that there is an association between Spexin adipose tissue and its metabolism. Study indicated a limitations that the causal relationship of Spexin to MetS cannot be determined due to the study's cross-sectional design and limited sample size [96].

Senturk N.G.K. et al. conducted a cross sectional case –control study in Turkey to assess serum Spexin Levels as well as relationship of Spexin in obese adolescents with MetS Antecedents. The study found that lower Spexin levels in obese patients 50 pg/mL compared to healthy controls 67.0 pg/mL with significant p value =0.035. As well as in obese with MetS subjects had 24.5 pg/mL and in obese without MetS had 69.0 pg/ml. The study also showed significantly negative association with BMI & HOMA-IR with a 75 percent sensitivity and 71 percent specificity, HOMA-IR with a cut-off level of 49.5 pg/mL might predict the occurrence of MetS in obese adolescents with a cut-off level of 49.5 pg/mL. Study concluded that obese adults have reduced Spexin levels, and could be more prone in those with MetS [97].

A cross-sectional study conducted by Amirpour et al. in 2021 in Iran to determine a link between serum Spexin levels and MetS components in obese and normal-weight people with or without diabetes. Study found that there was an inverse relationship between Spexin and Waist circumference, TG, FBS in healthy people without diabetes. Study concluded that Spexin may serve as a potential marker for MetS in normal weight people with or without diabetes[98].

Albeltagy E.S. et al. conducted a cross sectional study in Egypt on Spexin levels and their association with obesity, glycemic metabolites and cardiovascular factors in lean individuals compared to physical activity matched obese individuals with BMI. The study found that serum Spexin levels were significantly lower in obese patients than in overweight or normal individuals and in diabetes sub group compared to normal glycemic group. The AUC curve for Spexin with diabetes was 0.912 when compared to individuals with normal glycemia. Study concluded that Spexin could be a valuable biomarker of a subject with metabolic health state[104].

In contrast to the above, Karaca et al in 2018 conducted a cross-sectional study on decreased Spexin concentration in Type 1, Type 2 diabetes mellitus patients and controls to measure Spexin levels in lean type 1 diabetic patients and their relevance to glycemic parameters without the presence of obesity or insulin resistance and reported that there was no association between Spexin levels and BMI [89].

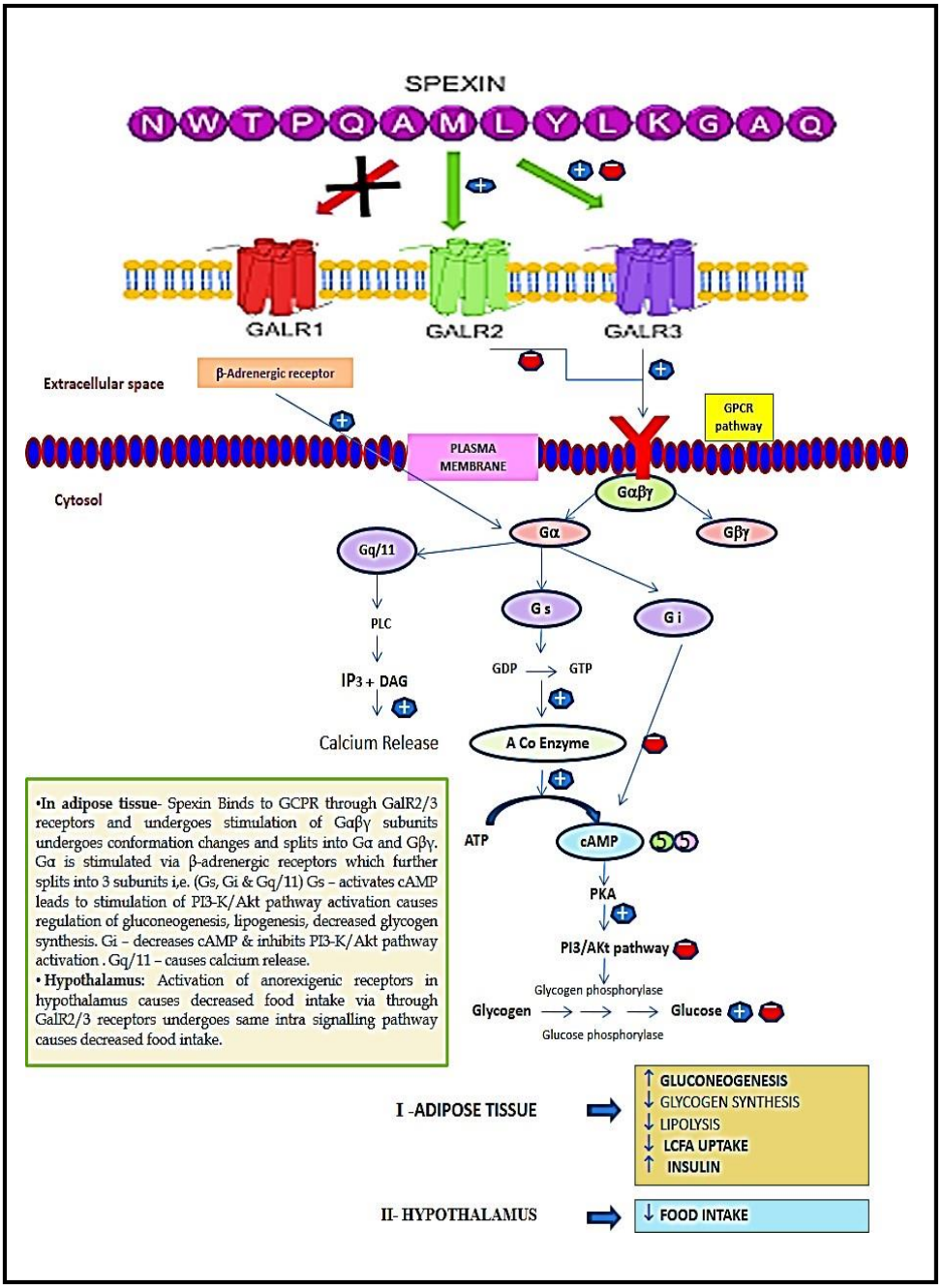
Similarly, Hodges et al 2018 also reported that measuring of Spexin in obesity doesn't show a link between Spexin levels and obesity. Beyazit F et al. in 2021 conducted a cross-sectional study on serum Spexin, adiponectin, and leptin levels in polycystic ovarian syndrome women and found no difference in Spexin levels between the polycystic ovarian syndrome group and control group [127,101].

Few studies reported increased Spexin levels by Daghri A.L. et al. in 2019 conducted a Cross-sectional cohort study in Saudi Arabia population to estimate the Spexin levels in 6-month self-monitored lifestyle modification programme from a larger cohort subjects with pre-diabetes were randomly selected. Study found that favorable changes in fasting glucose have an inverse effect on Spexin levels in females with pre-diabetes and concluded that circulating Spexin levels rise over time in people with pre-diabetes, particularly women who responded well to a 6-month lifestyle intervention programme.

Another study by Khadir A et al. in Kuwait conducted a Cross sectional study on the effects of circulating Spexin concentration in obesity, Type 2 diabetes mellitus and its modulation by physical exercise. Study found decreased plasma Spexin concentration in obese with or without Type 2 diabetes mellitus subjects. Study also reported that Spexin levels significantly increased only in responders to exercise with a concomitant improvement in metabolic profile. Study concluded that more research is needed on the role of physical activity in reducing the effects of metabolic stress linked to obesity and insulin resistance on Spexin. This study has some limitations, they were unable to identify whether lower Spexin levels related to the development of obesity and diabetes[94].

Based on literatures we hypothesized the possible explanation on physiological and pathological role of Spexin was nascent peptide undergoes post-translational modification of α-amidation at Carboxy terminal of peptide chains secretes into extracellular space by classical endoplasmic reticulum golgi dependent pathway as mature peptide that has a highly conserved sequence in nature via G-protein coupled receptor [60], Cognate receptors of Spexin are GalR2/3. The biological functions of Gal

are well documented for differential regulation of PI3K/Akt- dependent cascades, MAPK, cAMP/PKA and Calcium throughcoupling of GalR2 with Gq/11 and GalR3 with Gi receptors and causes increased gluconeogenesis, inhibit uptake of long chain fatty acids, decreased lipolysis and glycogen synthesis, regulate insulin levels, stimulation of anorexigenic receptors and inhibition of orexigenic receptors leads decreased food intake [64].



In obese and Type 2 diabetes mellitus patients the decreased Spexin levels were observed leads to increased uptake of long chain fatty acid, increased gluconeogenesis and increased lipolysis Ma A et al.2018 and Al-Daghri NM et al. 2019 [90,128].

Though the receptors are similar for Spexin and Galanin (i.e) GalR2 and GalR3, the signal transduction pathways which were involved inbiological role of Spexin are not illustrated [65]. Hence, we believed that this might be the reason why Spexin is decreased in obesity and diabetes patients and which might due to lack of Spexin receptors. Hence, the Spexin may also consider as a biomarker in obese and Type 2 diabetes mellitus subjects. However, longitudinal prospective studies are needed to know the Spexin role in obesity and Type 2 diabetes mellitus as biomarker.

Surprisingly, research into the physiological and pathological activities of Spexin is still in its early stages, and knowledge of the Spexin molecular mechanism is limited. As a result, more research into the receptor-activated signal transduction pathways of Spexin as well as clinical trials is required. Furthermore, it is yet unknown if Spexin may traverse the blood-brain barrier.

In line with the reported studies, the current study results shown the decreased Spexin levels in Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension patients indicating that Spexin may have potential role in obesity induced diabetes. Hence, the Spexin may also consider as biomarker in obese and Type 2 diabetes mellitus subjects along with the leptin. However, longitudinal prospective studies are needed to know the Spexin role in obesity and Type 2 diabetes mellitus as biomarker.

The availability of information on the Spexin/leptin ratio and its correlation with diseases state is limited. In the majority, study reports are confined to either independent Spexin or leptin. Kumar S et al. in 2018 reported a negative relation of Spexin with leptin in adult obesity. Hence in the current study, we also attempted to evaluate the Spexin/leptin ratio in Type 2 diabetes mellitus with and without hypertensive patients. We observed that the Spexin/leptin ratio was progressively

decreased in Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertensive groups compared to control. We propose that negative relation of Spexin with leptin in adult obesity. And also the current study results indicating that Spexin alone and Spexin/leptin ratio could also be helpful for diagnosis, prognosis, and intervention monitoring of CVD risks in Type 2 diabetes mellitus patients [74].

Therefore, measurement of Spexin level regarded as a potential biomarker to represent obesity, Type 2 diabetes mellitus and cardio-metabolic complications. Spexin and insulin concentration in the control group is in a balanced state, however, altered levels are observed in disease states. Hence, the results of the study Spexin, leptin and Spexin/leptin ratio associated with atherogenic potency.

Therefore the study results should be interpreted cautiously that Spexin and Spexin /leptin ratio serves as an early predictive biomarker in Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertensive patients.

Further, we were performed the Pearson correlation analysis to find the correlation between the Spexin and other cardiometabolic parameters we found that there was a negative correlation of Spexin with leptin and insulin with significant p=value (p=0.001) and rest of the parameters also showed negative correlation of Spexin with CRP, TC, TG, LDL-C, VLDL-C, LDL/HDL ratio, HbA1c, HOMA-IR, diastolic blood pressure, ALT & AST but not statistically significant and positive significant correlation with S/L ratio (p=0.001) and non-significant positive correlation with BMI, W/H ratio, FBS, PPBS, HDL-C and systolic blood pressure in Type 2 diabetes mellitus

In Type 2 diabetes mellitus with hypertension subjects some parameters showed negative correlation of Spexin with TG, LDL-C, VLDL, LDL/HDL ratio, FBS, PPBS, HbA1c, diastolic blood pressure, W/H ratio and AST but not statistically significant but Spexin showed Significantly negative correlation with insulin (p=0.001) and positive significant correlation with S/L ratio (p=0.001) and positive non-significant with Leptin, BMI, insulin, HOMA-IR, TC, HDL-C, ALT and Systolic blood pressure.

The present context study findings are in agreement with few studies and Chen. T et al in 2019 reported that Spexin was found to be adversely associated with HbA1c, HOMA-IR, adipokines, insulin, and glucose levels. Another study conducted by Zhang L et al. in 2021 in China found that Spexin was negatively correlated with HOMA-IR but not correlate with BMI. Behrooz M et al. in 2020 conducted a cross-sectional study in Iran and observed that significantly negative association with BMI & HOMA-IR.

Albeltagy ES et.al in 2022 conducted a cross-sectional study in Egypt found that serum Spexin levels were negatively correlated with BMI and glycemic parameters in obese individuals [91,93,96,104].

The study also performed receiver operating characteristic curve analysis and showed that the diagnostic performance of Spexin in Type 2 diabetes mellitus with hypertension was represented by receiver operating characteristic curve with False Positive Rate (1- specificity) on X -axis and True Positive Rate (sensitivity) on Y-axis. Cut-off of (0.51) showed sensitivity (81%), specificity (62%), and area under curve (AUC) (0.83), 95% confidence interval range (0.776 –0.884) and p <0.001. Hence, area under curve shows good accuracy for the test parameter in Type 2

diabetes mellitus with hypertension group compared to Type 2 diabetes group.

In contrast to the above studies, Hodges SK et al in 2018 [127] found that Spexin levels did not differ between the groups and it was not showed a significant correlation between the serum Spexin levels and blood biochemical parameters. Karaca et al in 2018 conducted a cross-sectional study and found that though there were decreased Spexin levels in diabetes it is independent of the BMI, glucose, and lipid profile.

Hence, the current study and recent research reports demonstrating that Spexin is having a relation with cardio-metabolic parameters and indicating that Spexin is having a role in causing MetS and diabetes.

Type 2 diabetes mellitus is a multifactorial and multigenetic disease, and its pathogenesis is complex and largely unknown. Several single-nucleotide polymorphisms (SNPs) have been already associated with an increased risk of hypertension and Type 2 diabetes mellitus, while their search is still ongoing. In addition, novel links between these disorders come from epigenetic studies. The involvement of many genes such as IRS (insulin receptor substrate -1), SIRTI gene (sirtuin 1), ADIPOQ (adiponectin), INSR (insulin receptor) contributes to development of Mets and diabetes [129]. Spexin gene is one such gene and its expressional studies were limited in humans.

The low concentration of serum Spexin was reported as a risk marker in development of obesity and diabetes with CVD complications. As per the data, the low levels of Spexin was observed in Type 2 diabetes mellitus and remarkable decreased Spexin levels was noticed in Type 2 diabetes mellitus with hypertension group. The exact reason for decreased Spexin level in Type 2 diabetes mellitus and Type 2 diabetes

mellitus with hypertension is not clearly known.

Our research findings from genetic analysis of Spexin gene and its polymorphism (Accession No: NC_000012.12) subjected to whole exon and intron sequences among healthy controls and Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension patients in the south Indian population.

The sequence analysis of 20 Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension cases revealed polymorphisms in the 6^{th} exon and no polymorphism was detected in the Exon-1, Exon -2, Exon-3, Exon-4 and Exon-5. Furthermore, heterogenous polymorphism were observed in two samples. In these samples stop codon (T>G) mutated, and that codes for glycine residue at 224^{th} and 225^{th} position which results in continuous translation up to 17 amino acids further until a stop codon stops the translation. Hence, instead of stopping the translation, protein might be translated with atleast 17 extra amino acids which produce long and wrong protein.

In the alignment, there seems to be another homozygous polymorphism in exon 6. In the chromatogram, G is mutated to A (G>A) which is point mutation/ missense mutation converted from aspartic acid to aspargine at 212, -42 positions. Hence, the present study emphasize that exon polymorphism (G>A) linked to decreased Spexin levels in serum and might contribute for the future development of diabetes with CVD.

A part from exonic polymorphism, we also found intronic polymorphism. In intron 5 we found homozygous of -42 G>A polymorphism. Rest of the 6 samples showed a heterozygous polymorphism -42G>A. The significance of this intronic polymorphism was not known but might cause an early MetS and in intron 3 we found deletion

polymorphism in the acceptor site of the Exon5'.

In this, there was the deletion of one C in the polypyrimidine tract at -21st position from the intron-exon boundary. Therefore the present study explains that deletion makes any abruption in the splicing, affecting the spliceosome or it leads to alternate splicing. Hence, polymorphism in exon – intron boundaries may alter the binding of transcription factor which may affect transcription, indirectly affected on translation which causes reduction of Spexin gene expression and its protein levels in serum which contributes to DM with CVD complications. Hence, polymorphism in Exon-intron boundaries may affects gene function thus serves as a predisposition for obesity and Type 2 diabetes mellitus.

It is well known that polymorphism in exons leads to disease but polymorphism in introns may also leads to human disease. Some research reports such as Hala Abdulhade Salih et al in 2020 in Iraq conducted a cross sectional study in obese children using whole DNA sequence. They found G>A and T>C polymorphism at 5'UTR and 3'UTR region of Spexin gene. They concluded that polymorphism are responsible for decreased serum Spexin levels and its expression, as well as altering the binding of transcription factors which can disrupt transcription and in turn translation resulting in a reduction in Spexin gene expression [105].

Some other intronic polymorphism studies such as Mastsuno S et al. in 2019[106] in Poland conducted a study on genetic polymorphisms using exome sequencing in the intron of the INS gene (insulin gene) and found G>A SNPs in intron 2 and concluded that G>A was associated with early onset diabetes.

Guerini FR et al in 2019 [107] found soluble-N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) gene and found SNP in intron 1 and suggested that SNP of SNAP25 gene might play a role in MetS, insulin resistance and involve in endothelial dysfunction and ischemic heart disease in a cohort study.

Hence, Exon-intron polymorphism in Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension subjects could prone to development of obesity with diabetes is evident by few studies. Although decreased Spexin levels may contributes to development of diabetes with CVD complications. However, the exact functional role and mechanism of Spexin which directly links to reduce the risk of diabetes still is also not clear.

Since for PCR, chemically synthesized primers of about 20 nucleotides are used for amplification. We are unabled to amplify exon-1 because of six nucleotide which seems to be very small in size to design forward and reverse primers set [130,131]. Sequence analysis results exhibited Spexin (G>A) homozygous and T>G heterozygous polymorphism in 6^{th} exon of coding region. Deletion polymorphism 'C' and -42(G>A) heterozygous polymorphism at 224^{th} 225^{th} 212^{th} -42^{nd} position were found in non-Coding regions and no polymorphism was detected in the 2^{nd}, 3^{rd}, 4^{th}, and 5^{th} exons in South India population. We report this observation for the first time in the South Indian population as per the available data to the best of our awareness.

There is a famine of information on *SPX* gene polymorphism in Type 2 diabetes mellitus in Indian population as a whole. Therefore, there is a scope to evaluate the structure of Spexin expressed from control group and diabetic in order to explore the role of Spexin function at early stage in diabetes and that has create a scope for further research with respective to functionality of Spexin and its receptors.

The limitation of the study is the Type 2 diabetics with and without hypertension subjects at the time of enrollment were on medication for their wellness. Even though, the Spexin level in treatment groups found less significantly. We were able to arrive mean value of Spexin level as cut-off value in the study subjects however it demands to arrive cut-off value in larger populations along with Spexin/leptin ratio for the purpose of diagnostic and prognostic utility.

In the genetic analysis of gene sequencing, promoter sequence of Exon-6 and its expression was not undertaken although identified polymorphism in Exon-6. And also we were not successful in amplification of Exon-1 of Spexin gene due to inadequate base pairs.

The scope of the study is to explore the possibility of therapeutic intervention on anima model and subsequently on human model of longitudinal study design using recombinant Spexin in diabetes mellitus leading to obesity, hypertension and inflammation.

CHAPTER-6
SUMMARY AND CONCLUSION

6.0. SUMMARY AND CONCLUSION

6.1. Summary

All the efforts have been made during the analysis of the research data and our research findings were summarized as below

Decreased Spexin levels and increased Leptin levels in Type 2 diabetes mellitus appraises as marker to metabolic and diabetes in the study population and remarkable decreased Spexin levels and increased Leptin levels were observed in Type 2 diabetes mellitus with hypertension group may also serves as a marker to predict early onset of CVD complications. In Type 2 diabetes mellitus, two-fold decrease in Spexin: Leptin ratio, in Type 2 diabetes mellitus with hypertension, four-fold decrease was observed. Therefore, representation of Spexin:Leptin ratio is used to understand early development of diabetes with CVD problems. Spexin showed good diagnostic utility Area under curve AUC (0.83) in Type 2 diabetes mellitus with hypertension group.

Our research findings satisfied study research question by showing association between Spexin, Leptin and insulin with statistical significant correlation found with respect to Hypo-Spexinemia, Hyper-Leptinemia, Hyper-insulinemia and hyper-lipidemia. Hence, Pepidomics research is vibrant and the use of peptides/polypeptides in therapeutic strategy of control/prevention of obesity has drawn attention. Recent animal models reported that administration of Spexin shown therapeutic benefit and controlling obesity. Monitoring of Spexin, in healthy and disease states is of great advantage to understand early progression and onset of risk in diseases of obesity and metabolic syndrome.

SPX gene sequencing carried out for the first time in the South Indian population as per the available data to the best of our awareness. Sequence analysis of *SPX* gene in cases (n=20) showed a heterozygous & homozygous exon-intron polymorphisms (T>G & G>A) (224,225, 212,-42nd position) of exon- 6.

In Type 2 diabetes mellitus and Type 2 diabetes mellitus with hypertension group showed significant lowered level of Spexin and concomitant polymorphism evidence at exon 6 and intron levels suggested that this might be the one of the relation to lowered Spexin level and progressive disease state to obesity in diabetics.

6.2. Conclusion

Decreased Spexin with increased Leptin levels appraises as marker to diabetes progressiveness in the study population. And progressively decreased trend of S/L ratio in diabetes with hypertension serve to understand early development of diabetes with Cardiac risk problems. Genetic analysis of *SPX* gene sequence in cases showed heterozygous & homozygous exon-intron polymorphism (T>G & G>A) (224,225, 212,-42nd position) of Exon- 6 with concominant low Spexin levels might be associated as one of the reason to find decreased Spexin peptide levels in serum. Hence, Hypo-Spexinemia and Spexin gene analysis has become newer aspects with significant correlation. Therefore, screening of Spexin peptide serves to understand for the development and new therapeutic strategy in prevention of obesity and diabetes with Cardiac risks.

6.3. New Knowledge Generated

Heterozygous & homozygous polymorphism of intron 3,5 and Exon-6 boundaries (T>G & G>A) might be one of the reason for decreased Spexin levels in serum and contributes to development of diabetes with hypertension linked to cardiovascular diseases.

CHAPTER-7
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APPENDIX I

RESEARCH PUBLICATIONS FROM THE Ph.D. TOPIC

SL.NO.	Title	Journal	Indexation
1	Insulin resistance and decreased Spexin in Indian Patients with Type 2 Diabetes Mellitus.	Tejaswi Gowdu CD Dayanand, K Prabhakar	Bioinformation, 17(9): 790-797 (2021)
2	Spexin Gene Polymorphism in Type 2 Diabetes Mellitus Patients of South Indian Population.	Tejaswi Gowdu CD Dayanand, B Sharath K Prabhakar	Romanian Journal of Diabetes, nutrition and metabolic diseases. 2021; 28(3): 01-10
3	Spexin in metabolic syndrome- An overview	Tejaswi Gowdu CD Dayanand	Journal of Scholars Academic and Scientific journal of medicine 2021; 7(1): 15-25

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Research Article

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Discovery at the interface of physical and biological sciences

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Insulin resistance and decreased spexin in Indian Patients with Type 2 Diabetes Mellitus

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Abstract
Spexin is novel biomarker, which plays a potential role in glucose and lipid metabolisms. However, there was paucity of serum spexin levels in obesity and diabetes mellitus subjects. Hence the current study was aimed to find the relationship between the serum spexin levels in type 2 Diabetes mellitus (type 2 DM) with extrapolation of cardiovascular disease (CVD) risk. A cross-sectional study included 330 participants, subdivided as control (n=110), type 2 DM (n=110) and type 2 DM with CVD groups (n=110). HbA1c, insulin, lipid profile, spexin & leptin including blood pressure and body mass index were analyzed from all the participants. The serum spexin levels (ng/ml) were significantly decreased in type 2 DM (mean ± sd: 0.65 ± 0.03) and type 2 DM with CVD (0.48 ± 0.02) groups compared to the control (0.79 ± 0.03) group ($p<0.001$). The decreased spexin levels were observed in type 2 DM, and further more decreased in type 2 DM with CVD patients compared to controls indicating that spexin levels could be served as an early prediction of obesity-induced T2DM with CVD risk.

Keywords: Cardiovascular disease, type 2 Diabetes Mellitus, Spexin

Background:
Obesity manifests metabolically with excess body weight in adults that greatly increases the risk of type 2 diabetes Mellitus (type 2 DM) with cardiovascular disease (CVD), certain cancers, and mortality [1]. High Body Mass Index (BMI) is multi-factorial complex condition linked to genetic, epigenetic, and metabolic deregulation prone towards diabetic and cardiovascular complications [2]. Insulin resistance is recognized as a risk factor in the etiology of diseases state [3, 4]. Spexin is a neuropeptide contains 14 amino acids with molecular weight 1619.9 KDa [5]. Nascent spexin on post-translational modification of h-amidation at Carboxy terminal, secretes into extracellular space is mature peptide having highly conserved sequence [6]. Physiological functions confined are inducing gastric intestinal contractions, postnatal hypoxia response, nociceptive response, inhibiting adrenal proliferation, fatty acid absorption, weight regulation, cardiovascular and renal modulation [7,8,9,10,11,12]. Spexin binding to GalR2/GalR3 receptors belongs to G-protein family functions through the second messenger system [13]. Spexin involves regulating food intake, body weight, and energy homeostasis through neuroendocrine functions and also, it regulates glucose and lipid metabolism [14]. Spexin is also involved in Noonan syndrome- an autosomal dominant genetic disorder characterized by craniofacial abnormalities and Bjornstad syndrome rare disorder with abnormal neural deafness [7,13]. It was reported that downregulation of spexin gene and its concentration in serum is associated with obesity, type 2 DM, hypertension & CVD in metabolic syndrome conditions [15]. Leptin is having 167 amino acid residues with molecular weight of 16 kDa secreted by white adipocytes into the circulation [16]. Circulating leptin binds hypothalamus and activates

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Original Research

Spexin gene polymorphism in type 2 diabetes mellitus patients of South Indian population

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Abstract

Background and aims: The purpose of the study is to assess the concentrations of spexin (SPX) and other specific biochemical parameters, subsequently spexin (SPX) gene polymorphisms among south Indian healthy controls and in T2DM and T2DM +HTN patient's blood samples, which were selected according to certain inclusion and exclusion criteria. **Material and method:** Named and kit methods are used for the evaluation of spexin, biochemical parameters, and for spexin (SPX) gene polymorphism. **Results:** In this prospective study, spexin concentration is a negatively associated biomarker, and elevated levels of glucose, insulin resistance, liver enzymes, and inflammation are independently associated with assessing the diabetic and arterial hypertension disease states. The SPX gene sequencing illustrates exon mutation in the 6th position and no polymorphism is detected in the 2nd, 3rd, 4th, and 5th exons. Whereas intronic mutations are observed in the 3rd and 5th positions. Furthermore, heterogeneous mutations are observed in the 8th and 13th samples. Samples 10 and 14 showed homozygous of -42G>A mutation in exon 6. Samples 11, 12, 18, 19, 21, and 22 showed a heterozygous mutation (-42G>R) in the intron that is present 5 ´ to the exon 6. There is a deletion mutation in the acceptor site of the intron present 5 ´ to the exon 4. **Conclusion:** This is the first study reporting an association between spexin gene polymorphisms and the levels of spexin peptides.

Keywords: diabetes mellitus, exon, hypertension, intron, HOMA-IR, mutation, and spexin gene.

Background and aims

Type 2 diabetes mellitus (T2DM) and hypertension (HTN) are common concomitants. Hypertension is twice as recurrent in patients with diabetes compared with those who do not have diabetes. In addition, patients with hypertension frequently show signs of insulin resistance and are at greater risk of diabetes mounting than in healthy persons [1]. The chief cause of morbidity and mortality in diabetes is cardiovascular disease, which is exacerbated by hypertension. Thus, diabetes and hypertension are intimately interwoven because of alike risk factors, such as endothelial dysfunction, vascular inflammation, arterial remodeling, atherosclerosis, dyslipidemia, and obesity. There is also considerable overlie in the cardiovascular complications of diabetes and hypertension-related primarily to micro and macrovascular disease [2].

Spexin (SPX), namely neuropeptide Q (NPQ), is a newly identified peptide hormone. Spexin was first acknowledged in the human genome via bioinformatic practice. In humans, the gene that encodes SPX is located on chromosome 12, specifically C12orf39. SPX gene encloses 6 exons and 5 introns, encoding a prepropeptide of 116 amino acids. In humans, the precursor of

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Spexin in Metabolic Syndrome-An Overview

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Abstract **Review Article**

Spexin (SPX) is a neuropeptide hormone or adipokine secreted from adipose tissue an endocrine organ and that undergoes post-translational modification of α-amidation at C-terminal. Neuronal signal stimulation causes release of SPX into synapse where it binds to G-protein coupled receptors which involve in signal transduction. SPX co-evolved with galanin/Kisspeptin family. It is 14 amino acids containing mature peptide and its sequence was highly conserved in vertebrates and non-vertebrates. SPX *C12ORF39* gene and mRNA were widely expressed in central nervous system and peripheral nervous system in goldfish, rodents and humans. Cognate receptors of SPX are GalR2/GalR3 involves in regulating food intake, body weight and energy homeostasis through neuroendocrine functions. Current research suggested that SPX also regulate glucose and lipid metabolism. Adipose tissue secretes adipocytokines which plays pivotal role in regulating lipid and glucose metabolism, storage, food intake, energy expenditure. Down regulation of SPX gene and its concentration in serum was associated with obesity which further leads to components of metabolic syndrome such as T2DM, HTN and CVDs. Metabolic syndrome (MetS) is affecting the general population and has been higher risk of developing cardiovascular morbidity and mortality. Severity of this syndrome leads to decreased quality of life in humans. Clear explanation of intracellular signalling pathways and its receptors would help to develop novel therapeutic interventions and drug design for disorders like obesity and T2DM. In this review we focus to provide an updates about SPX and its physiological role in metabolic syndrome.

Keywords: Biomarker, Metabolic syndrome, Spexin.

INTRODUCTION

Adipose tissue is with specialised loose connective tissue composed of adipocytes and endocrine in nature [1]. Two types of adipose tissue are Brown and white adipose tissues, former one present in adult humans, while the latter one is predominantly found in neonates. White adipose tissue mainly as Visceral fat plays a vital role in energy storage which secretes variety of adipokines such as Leptin, adiponectin, resistin, retinol binding protein-4, Vaspin, Tumour necrosis factor-α (TNF-α), Interleukin-6, Visfatin, Apelin, Insulin Growth Factor-1, hormones like estrogen and glucocorticoides [2, 3]. Amongst, adipocytokines, adiponectin, leptin plays a crucial role in inflammation process, metabolism of glucose and lipids [4]. Spexin is a recently identified neuropeptide also termed as Adipokine involved in energy homeostasis [5, 6].

However, human Markov Model-a bioinformatics approach/search tool had been adopted to identify the features of new peptide hormones sequence. Since, spexin is also a short peptide hormone signalling through membrane receptors to exert a crucial role in physiological and pathological conditions. Biochemical analysis revealed that spexin co-localised with insulin in secretory vesicles of pancreatic β-cell line and suggested that it could be processed and secreted at cellular level by exocytosis [7].

Spexin (SPX) has pleotropic functions, it is highly expressed in pancreas and almost all the tissues [8]. SPX acts through cognate receptors with several physiological roles in regulation of glucose and lipid metabolism, GI tract motility, energy balance, nociception/mood disorders, weight loss, acts as satiety inducing peptide, in reproduction, renal and Cardiovascular diseases (CVD) [9, 10]. Despite, SPX precursors was predominantly expressed in sub mucosal layer of stomach in rats and mouse oesophagus and causes stimulation of muscle contractions [7].

POSTER PRESENTATIONS FROM THE Ph.D TOPIC

S.No	TITLE	Conference details	Type of presentation
1.	"Leptin is an independent predictor of CVD"	5th International Diabetes Summit (Virtual)-2021, Pune	Oral Presentation
2.	"Is Aspartate aminotransferase is an independent predictor of CVD risk with type 2 diabetes mellitus"	Genomics Conclave 2021-National e conference (Virtual)-2021, Chettinad.	Oral Presentation

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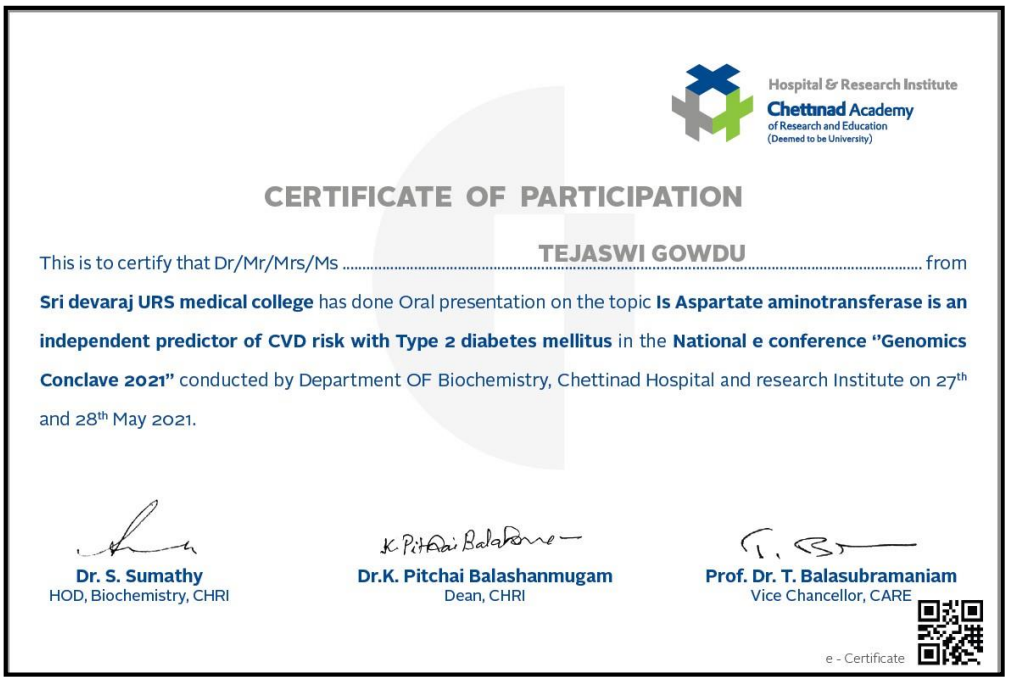

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6.2. Conclusion

Decreased Spexin with increased Leptin levels appraises as marker to diabetes progressiveness in the study population. And progressively decreased trend of S/L ratio in diabetes with hypertension serve to understand early development of diabetes with Cardiac risk problems. Genetic analysis of *SPX* gene sequence in cases showed heterozygous & homozygous exon-intron polymorphism (T>G & G>A) (224,225, 212,-42nd position) of Exon- 6 with concominant low Spexin levels might be associated as one of the reason to find decreased Spexin peptide levels in serum. Hence, Hypo-Spexinemia and Spexin gene analysis has become newer aspects with significant correlation. Therefore, screening of Spexin peptide serves to understand for the development and new therapeutic strategy in prevention of obesity and diabetes with Cardiac risks.

6.3. New Knowledge Generated

Heterozygous & homozygous polymorphism of intron 3,5 and Exon-6 boundaries (T>G & G>A) might be one of the reason for decreased Spexin levels in serum and contributes to development of diabetes with hypertension linked to cardiovascular diseases.