EVALUATION OF EXPRESSION PATTERN OF SELECTED PLASMA MICRORNAS IN TYPE 2 DIABETIC, OBESE AND HEALTHY INDIVIDUALS

MSc. Thesis (2079)

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Recommendation

This is to certify that the research work entitled "**EVALUATION OF EXPRESSION PATTERN OF SELECTED PLASMA MICRORNAS IN TYPE 2 DIABETIC, OBESE AND HEALTHY INDIVIDUAL"** has been carried out by **Mr. Shailesh Adhikari** under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code **BT 621**. The results presented here are from his original findings. I, hereby, recommend this thesis for final evaluation.

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Glossary Acronyms

ADA:	American Diabetes Association
BMI:	Body mass index
dr :	Down-regulated
FPG:	Fasting plasma glucose
GDM:	Gestational diabetes mellitus
HbA1c:	Haemoglobin A1c
IFG :	Impaired fasting glucose
IGT :	Impaired glucose tolerance
LADA :	Late-onset autoimmune diabetes of the adult
LDL:	Low-density lipoprotein
LDLR:	Low-density lipoprotein receptor
MF:	Molecular function
MODY:	Maturity-onset diabetes of the young
NGS:	Next generation sequencing
OGTT:	Oral glucose tolerance test
PCR :	Polymerase chain reaction
qRT-PCR:	real-time quantitative reverse transcription - PCR
T1DM:	Type 1 Diabetes Mellitus
T2DM:	Type 2 Diabetes Mellitus
Ur:	Up-regulated
UPS:	Ubiquitin proteasome system
USD:	US Dollars
WHO:	World Health Organization

Table of contents

List of Tables

LIST OF FIGURES

ABSTRACT

Background

The most common form of diabetes in the world, type 2 diabetes mellitus (T2DM), which is characterized by chronic hyperglycemia, affects almost 95% of all patients with the diabetes. If this disease is caught early on, it may be possible to delay or even prevent its harmful effects. A few drawbacks of standardized diagnostic tests for T2DM are their dependence on blood glucose levels, intrusiveness, and inability to forecast the likelihood that people with normal glucose tolerance may acquire T2DM in the future. As a result, biomarkers that could be employed as a tool for the early and precise detection of T2DM are required.
Small non-coding RNA molecules called microRNAs are important regulators of gene expression and several biological processes. According to studies, dysregulation of microRNAs may cause T2DM and other disorders, and as a result, they may serve as helpful biomarkers for disease diagnosis. Identification of biomarkers, such as microRNAs, as a method for the early and precise identification of T2DM, thus, has enormous potential for diagnostic use.

Methods
In this study, 46 adult subjects aged between 31-60 years were included and classified as healthy Individuals (17), obese (17), and T2DM patients (12). Changes in the plasma expression levels of miR-9, miR-29a, miR-192, and miR-375 were quantified by RT-qPCR. The fold expression of each microRNA was calculated and compared among three study groups and analyzed for their relationship with ANOVA: Two- factor without Replication in EXEL.

Results
All four plasma microRNAs were found to be upregulated in type 2 diabetes and only miR-29a was downregulated (dr) in the obese group but other three were also upregulated. The ANOVA analysis shows the miR-192 was determined to be significantly upregulated *(ur)* in diabetic and obese.

Conclusion
The miR-192 shows significant upregulation in both T2DM and obese patient which can be used as Biomarker.

Keywords: Nepal,Biomarkers, Circulating microRNAs, prediabetes, type 2 diabetes, RT-qPCR

Chapter I

1. Introduction

1.1 Background

Diabetes mellitus is one of a chronic worldwide metabolic disease which is characterised by increased blood glucose levels as a result of defects in production, secretion and signalling of pancreatic hormone insulin due to pancreatic beta cell dysfunction or insulin resistance (Al-Muhtaresh et al 2018, Bhatia et al 2015, Chien et al 2015,Lin and Sun 2010).
There are several types of diabetes, including:
A. Type 1 Diabetes Mellitus (T1DM),
B. Type 2 Diabetes Mellitus (T2DM)
C. Gestational Diabetes Mellitus (GDM)
D. Latent autoimmune diabetes of the adulthood (LADA) and
E. Maturity-onset diabetes of the young (MODY)

Table 1.1: The different form of diabetes with description

TYPE	DESCRIPTION
T1DM	It mainly occurs in children and young adults and is an autoimmune disease and requires daily insulin injections.(American Diabetes Association,2021)
T2DM	It is a metabolic disorder in which the body becomes IR and medication may be necessary. (American Diabetes Association, 2021).
GDM	Due to hormonal changes, it occurs in pregnant women. This type of diabetes occurs during pregnancy in women who never had diabetes before. (American Diabetes Association,2021).
LADA	It is a slow-onset form of type 1 diabetes that occurs in adulthood. (NIDDKD, 2021)
MODY	It is a rare form of diabetes caused by a genetic mutation that affects the way insulin is produced and/or used in the body.(Diabetes UK,2021)

Table 1.1 gives the brief introduction about different types of Diabetes in this thesis we only study about T2DM.

1.1.1 Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by hyperglycemia resulting from insulin resistance and insufficient insulin secretion by pancreatic β-cells (American Diabetes Association, 2022). It is a significant public health concern that affects over 460 million people worldwide (International Diabetes Federation, 2021). T2DM is associated with various complications such as cardiovascular disease, retinopathy, neuropathy, and nephropathy, which can result in a reduced quality of life and increased healthcare costs (Javanbakht et al., 2021). The development and progression of T2DM are influenced by several factors, including genetics, lifestyle factors (e.g., physical

inactivity, unhealthy diet), and environmental factors (e.g., air pollution) (American Diabetes Association, 2022).
Numerous approaches have been employed to manage T2DM, including lifestyle modifications, pharmacotherapy, and bariatric surgery (Javanbakht et al., 2021). Lifestyle modifications such as exercise and a healthy diet are considered the cornerstone of T2DM management (American Diabetes Association, 2022). Pharmacotherapy involves the use of oral antidiabetic medications (e.g., metformin) and injectable therapies such as insulin and glucagon-like peptide-1 (GLP-1) receptor agonists (Javanbakht et al., 2021). Bariatric surgery has been shown to induce significant weight loss and improve glycemic control in obese individuals with T2DM (Schauer et al., 2017). However, the choice of treatment depends on various factors such as disease severity, comorbidities, and patient preferences.

1.1.2 Glucose metabolism

Glucose is an important source of energy for cells in the body. It is the primary fuel used by the brain. The regulation of glucose metabolism is a complex process that involves multiple organs and hormones. After eating, the insulin is released, which stimulates cells to take up glucose from the blood and store it as glycogen in the liver and muscles. When blood glucose levels are low, the pancreas releases glucagon, which stimulates the liver to convert stored glycogen into glucose and release it into the bloodstream. In addition to insulin and glucagon, other hormones such as cortisol, growth hormone, and adrenaline also play a role in glucose regulation. Dysregulation of glucose metabolism can lead to a number of diseases, including diabetes mellitus. (American Diabetes Association, 2021;DeFronzo, 2010,Saltiel & Kahn,2001)
One important organ in glucose regulation is the liver. The liver plays a central role in maintaining blood glucose levels through gluconeogenesis, which is the production of glucose from non-carbohydrate sources. The liver also stores glycogen, which can be rapidly converted to glucose when needed. Another important factor in glucose regulation is the hormone insulin, which is produced by the beta cells of the pancreas. Insulin promotes glucose uptake by cells and the conversion of glucose to glycogen in the liver and muscles. Insulin resistance, which occurs when cells become less receptive to insulin, is a hallmark of type 2 diabetes. (American Diabetes Association, 2021;DeFronzo, 2010,Saltiel & Kahn,2001)
Glucose is maintained within a narrow and well-balanced range and is primary source for energy. Insulin and glucagon hormones mainly drive glucose regulation and homeostasis (Aronoff et al, 2004; Soita, 2009; Triplitt, 2012).

1.1.2.1 Normal glucose regulation

Normal glucose regulation involves interaction of hormones, enzymes etc. The regulation of glucose concentration in the blood is essential for maintaining energy homeostasis in the body. The major hormones involved in glucose regulation are insulin and glucagon. Insulin is produced by pancreatic β-cells and decreases blood glucose levels by increasing uptake of glucose by cells and stimulating storage of glucose in liver and muscle. Glucagon, on the other hand, is produced by pancreatic α-cells and increases blood glucose levels by promoting glycogenlysis in the liver and released into blood. Other hormones involved in glucose regulation include cortisol, growth hormone, and epinephrine. (American Diabetes Association, 2022; McGarry,1992; Saltiel & Kahn, 2001)
The regulation of glucose concentration in the blood is tightly controlled by a negative feedback system. When blood glucose levels rise, insulin is released to bring glucose levels back to normal. Conversely, when blood glucose levels drop, glucagon is released to stimulate the release of glucose from liver. Liver plays a crucial role in glucose regulation as it stores and releases glucose as needed. The enzyme glycogen phosphorylase is responsible for breaking down glycogen into glucose, which can then be released into the bloodstream. Several factors can

disrupt normal glucose regulation, leading to abnormal blood glucose levels.. Other factors that can disrupt glucose regulation include hormonal imbalances, certain medications, and certain diseases such as Cushing's syndrome. Maintaining normal glucose regulation is essential for overall health and well-being. (American Diabetes Association, 2022; McGarry,1992; Saltiel & Kahn, 2001)

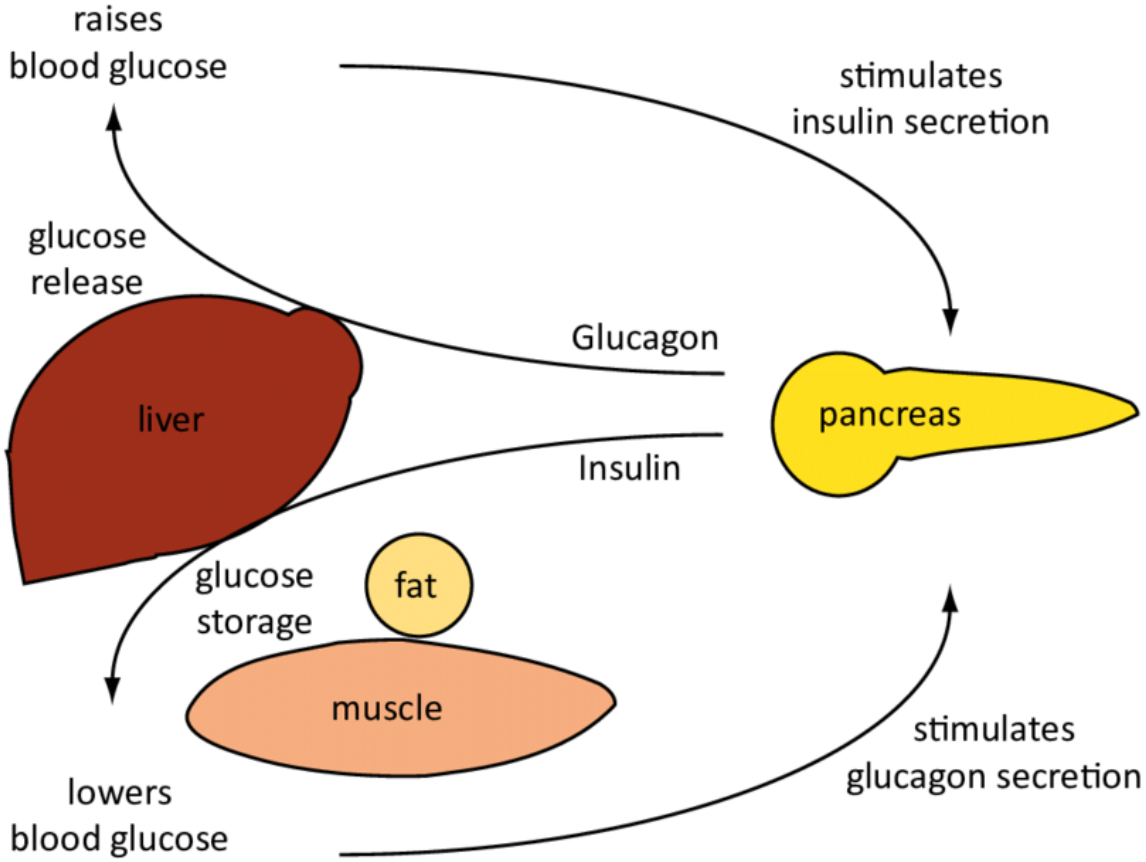


Figure 1.1: The Role of Insulin and Glucagon in Glucose Regulation
(Steinbusch et al, 2011)

1.1.3 Type 2 diabetes mellitus (T2DM) Pathogenesis and pathophysiology

The pathogenesis of T2DM involves a complex interplay of genetic, environmental, and lifestyle factors, leading to impaired glucose homeostasis. Insulin resistance, the reduced ability of insulin to stimulate glucose uptake in peripheral tissues such as muscle, liver, and adipose tissue, is a key pathogenic factor in T2DM. Several mechanisms, including chronic low-grade inflammation, increased fatty acid flux, and mitochondrial dysfunction, contribute to insulin resistance in T2DM (Saltiel & Kahn, 2019).

In addition to insulin resistance, β-cell dysfunction is another critical factor in the pathogenesis of T2DM. β-cell produces insulin response to stimuli, but in T2DM, β-cells fail to balance for IR by rising insulin secretion, leading to hyperglycemia. β-cell dysfunction in T2DM is due to combination of genetic, epigenetic, and environmental factors, including chronic hyperglycemia, glucotoxicity, lipotoxicity, and inflammation (Rhodes & Shoelson, 2017).

The pathophysiology of T2DM involves a range of metabolic and vascular complications that arise from chronic hyperglycemia and insulin resistance. These complications include microvascular and macrovascular disease, neuropathy, retinopathy, nephropathy, and

cardiovascular disease (CVD). Chronic hyperglycemia is the primary driver of these complications, and it exerts its effects through multiple mechanisms, including the AGEs formation, OS and activated of inflammatory pathways (American Diabetes Association, 2021). In addition to hyperglycemia, insulin resistance contributes to the development of cardiovascular disease in T2DM by promoting dyslipidemia, hypertension, and atherosclerosis (Boden et al., 2011).

1.1.3.1. Insulin resistance

Insulin resistance (IR) is a metabolic disorder by which cells become non responsive to the action of insulin for glucose metabolism. This results in high blood sugar levels and increased insulin secretion by the pancreas. IR is a key feature of T2DM, a chronic disease characterized by hyperglycemia, insulin resistance, and impaired insulin secretion. The pathogenesis of insulin resistance involves multiple mechanisms, including genetic, environmental, and lifestyle factors, such as obesity, sedentary behavior, and poor diet quality. Insulin resistance is associated with chronic low-grade inflammation, oxidative stress, and mitochondrial dysfunction, which may contribute to the development of T2DM and its complications. (American Diabetes Association, 2021;Kahn, & Hull,2006;Samuel & Shulman, 2016)

Several biomarkers and imaging techniques have been used to assess insulin resistance and its impact on glucose metabolism. These include the homeostasis model assessment of insulin resistance (HOMA-IR), the oral glucose tolerance test (OGTT), the hyperinsulinemic-euglycemic clamp, and magnetic resonance spectroscopy (MRS) to measure hepatic and muscle lipid content. In clinical practice, the diagnosis of T2DM is based on fasting plasma glucose, HbA1c, or oral glucose tolerance test (OGTT), as recommended by ADA. (American Diabetes Association, 2021;Kahn, & Hull,2006;Samuel & Shulman, 2016)

Treatment strategies for insulin resistance and T2DM include lifestyle modifications, such as weight loss, exercise, and dietary changes, and pharmacological therapies, like metformin, sulfonylureas, insulin, and GLP-1 receptor agonists. The choice of treatment depends on the severity of hyperglycemia, the presence of comorbidities, and individual patient preferences. In conclusion, insulin resistance is a complex metabolic disorder that plays vital role in progression of T2DM. Early detection and management of IR may prevent or delay the onset of T2DM and its complications. (American Diabetes Association, 2021;Kahn, & Hull,2006;Samuel & Shulman, 2016)

1.1.3.2. β-cell dysfunction

Beta cell dysfunction is a key contributor to the pathogenesis of type 2 diabetes mellitus (T2DM), a chronic metabolic disorder characterized by insulin resistance and impaired glucose homeostasis. Beta cells, located in the pancreas, are responsible for producing and releasing insulin, a hormone that regulates glucose uptake and utilization by peripheral tissues. However, in T2DM, beta cells exhibit several functional abnormalities, including reduced insulin secretion, impaired insulin gene expression, and increased apoptosis, leading to inadequate insulin production and secretion, and subsequent hyperglycemia.

One of the major mechanisms underlying beta cell dysfunction in T2DM is oxidative stress(OS). OS lead to impaired insulin secretion and increased beta cell apoptosis. Studies have shown that increased oxidative stress in beta cells is associated with T2DM, and it can disrupt multiple signaling pathways involved in insulin secretion and gene expression, such as the insulin receptor signaling pathway and the peroxisome proliferator-activated receptor gamma (PPARγ) pathway (Evans et al., 2013). Moreover, oxidative stress can also lead to mitochondrial dysfunction in beta cells, which further impairs insulin secretion and promotes beta cell apoptosis (Giacco & Brownlee, 2010).

Another important mechanism contributing to beta cell dysfunction in T2DM is endoplasmic reticulum (ER) stress. ER stress occur and subsequent beta cell dysfunction. Several factors,

such as increased lipotoxicity, glucotoxicity, and inflammation, can induce ER stress in beta cells. ER stress disrupts normal ER function and impairs insulin processing, trafficking, and secretion, leading to reduced insulin secretion and increased beta cell apoptosis. Additionally, ER stress can also impair insulin gene expression and disrupt insulin signaling pathways in beta cells (Cnop et al., 2010).

Furthermore, chronic low-grade inflammation, a hallmark of obesity and insulin resistance, can also contribute to beta cell dysfunction in T2DM. Inflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α, directly impair beta cell function by reducing insulin gene expression and insulin secretion, and by promoting beta cell apoptosis. Inflammatory signaling pathways, such as NF-κB and JNK, activated on beta cells in response to inflammation, leading to impaired insulin secretion and beta cell dysfunction (Donath & Shoelson, 2011).

1.1.4. Complications linked to Type 2 diabetes mellitus

T2DM is complex disorder. The pathophysiology of T2DM involves multiple factors, including genetics, lifestyle, and environmental factors. Despite the availability of various treatment options, complications associated with T2DM remain a significant challenge for healthcare providers. One of the most common complications of T2DM is cardiovascular disease. The prevalence of CVD is higher in individuals with T2DM compared to the general population. Several mechanisms have been proposed to explain the increased risk of CVD in T2DM, including dyslipidemia, hyperglycemia, and inflammation. A recent study showed that individuals with T2DM have a higher risk of heart failure compared to those without T2DM (Alonso et al., 2020).

Another complication associated with T2DM is diabetic nephropathy. Diabetic nephropathy is a progressive kidney disease that affects approximately 40% of individuals with T2DM. The pathogenesis of diabetic nephropathy involves multiple mechanisms, including hyperglycemia, inflammation, and oxidative stress. Early detection and treatment of diabetic nephropathy can significantly improve outcomes (Molitch et al., 2014).

Peripheral neuropathy is another common complication of T2DM. Peripheral neuropathy is characterized by nerve damage that results in numbness, tingling, and burning sensations in the hands and feet. The prevalence of peripheral neuropathy is higher in individuals with T2DM compared to those without T2DM. The pathogenesis of peripheral neuropathy involves multiple mechanisms, including hyperglycemia, inflammation, and oxidative stress (Boulton et al., 2013).

T2DM is a complex metabolic disorder that is associated with multiple complications, including CVD, diabetic nephropathy, and peripheral neuropathy. The pathogenesis of these complications involves multiple mechanisms, and early detection and treatment are crucial for improving outcomes. Healthcare providers should take a comprehensive approach to managing individuals with T2DM to prevent or delay the onset of these complications.

1.1.5 Type 2 Diabetes Mellitus Diagnosis Method

Diagnosing T2DM is crucial for timely initiation of appropriate management strategies to prevent complications. Several diagnostic methods are available, including fasting plasma glucose (FPG), oral glucose tolerance test (OGTT), and glycosylated haemoglobin (HbA1c) levels. (American Diabetes Association, 2021; American Diabetes Association, 2019; World Health Organization, 2016) These methods are widely accepted and recommended by leading healthcare organizations for the diagnosis of T2DM.

1. Fasting Plasma Glucose (FPG):
   FPG is a commonly used method for diagnosing T2DM. It involves measuring blood glucose levels after an overnight fast of at least 8 hours. According to the American Diabetes Association (ADA) guidelines, a fasting plasma glucose level of 126 mg/dL (7.0

mmol/L) or higher on two separate occasions is indicative of T2DM (American Diabetes Association, 2021). FPG is a simple and convenient method that does not require any special preparation, making it a widely used diagnostic tool for T2DM.

2. Oral Glucose Tolerance Test (OGTT):
   OGTT is another diagnostic method for T2DM that involves measuring blood glucose levels after the administration of a standardized oral glucose load. The ADA recommends using a 2-hour plasma glucose level during OGTT to diagnose T2DM. 2-hour plasma glucose level of 200 mg/dL (11.1 mmol/L) or higher is considered diagnostic for T2DM (American Diabetes Association, 2021). OGTT is a more sensitive test compared to FPG as it can detect impaired glucose tolerance and early stages of T2DM, but it requires more time and effort from the patient.
3. Glycosylated Hemoglobin (HbA1c):
   Level HbA1c is a long-term measure of blood glucose control that reflects average blood glucose levels over the past 2-3 months. The ADA recommends using HbA1c levels for the diagnosis of T2DM. An HbA1c level of 6.5% (48 mmol/mol) or higher is considered indicative of T2DM (American Diabetes Association, 2021). HbA1c is a convenient method as it does not require fasting or glucose load, making it more convenient for patients. However, it may not be as sensitive as FPG or OGTT in certain conditions such as anemia and hemoglobinopathies. (World Health Organization,2016)

1.1.6. Obesity

Obesity is complex multifactorial condition in which too much fat accumulate. It causes comorbidities, including T2DM, cardiovascular disease, and some forms of cancer. The etiology of obesity involves various genetic, environmental, and behavioral factors, including diet, physical activity, sleep, and stress (Hruby and Hu, 2015).

The prevalence of obesity has increased dramatically over the past few decades, affecting people of all ages and socioeconomic backgrounds worldwide. Obesity is a major public health concern, and its prevention and treatment require a multidisciplinary approach that involves lifestyle modifications, behavioral therapy, pharmacotherapy, and bariatric surgery (Bray and Kim, 2018).

1.1.6.1 Obesity and Diabetes

Obesity and diabetes are closely interrelated conditions that share common pathophysiological mechanisms. Obesity is a major risk for the development of T2DM, and both conditions has increased dramatically over past few decades worldwide (Kopelman, 2000).

Excess body fat, particularly abdominal adiposity, contributes to insulin resistance and impaired glucose metabolism, develop T2DM. Adipose tissue produces various adipokines, such as leptin, adiponectin, and resistin, which regulate appetite, energy expenditure, and glucose metabolism. Dysregulation of adipokine secretion in obesity may develop insulin resistance and T2DM (Friedman & Halaas, 1998).

1.1.7 MicroRNAs

MicroRNAs (miRNAs) are approximately 18 to 24 nucleotides in length non-coding, short single-stranded RNA molecules, (Saikumar et al, 2012). They are exported to the cytoplasm after transcribed from DNA and processed in the nucleus. At cytoplasm, they regulate gene expression by either degrading the mRNA or inhibiting its translation after binding to target messenger RNA (mRNA) molecules and (Bartel, 2009; Kim et al., 2009).

MiRNAs play important roles in the pathogenesis of various diseases, including cancer, cardiovascular disease, and neurodegenerative disorders. They have also been implicated in a

variety of biological processes, including development, cell differentiation, proliferation, and apoptosis (Bartel, 2009; Kim et al., 2009; O'Brien et al., 2018).
Among thousands of miRNAs identified in various species, some are highly conserved across species (Molitoris and Molitoris, 2011) and others are species-specific (Bartel, 2009). some are also tissue-specific. They have importance in regulating gene expression in a highly specific manner. They can exhibit temporal and spatial expression patterns (Kim et al., 2009).

1.1.6.1. miRNAs biogenesis and their use as biomarkers for diseases

MiRNA biogenesis involves the transcription of primary miRNA (pri-miRNA) by RNA polymerase II, which is processed into precursor miRNA (pre-miRNA) by the RNase III enzyme Drosha and its cofactor DGCR8 in the nucleus. The pre-miRNA is then transported to the cytoplasm by Exportin-5, where it is further processed by the RNase III enzyme Dicer to generate a mature miRNA duplex. One strand of the duplex is incorporated into the RNA-induced silencing complex (RISC), which targets the mRNA for degradation or translational repression.
MiRNAs have been found to be dysregulated in many diseases, including cancer, cardiovascular diseases, and neurological disorders. MiRNAs are stable in body fluids such as blood, urine, and saliva, making them attractive candidates as non-invasive biomarkers for disease diagnosis and prognosis. In addition, miRNAs can distinguish between different disease subtypes and predict treatment response. For example, miR-21 is overexpressed in many types of cancer, including breast, lung, and colon cancer, and is associated with poor prognosis. (Wu et al.,2019). Similarly, miR-126 is downregulated in cardiovascular diseases, and found that circulating miR-126 levels were significantly decreased in patients with acute coronary syndromes, indicating its potential as a diagnostic biomarker. (Zampetaki et al., 2012)
MiRNAs helps in gene regulation. They are involved in pathogenesis of many diseases. Their stable expression in body fluids and tissue-specific expression patterns make them promising biomarkers for disease diagnosis and prognosis. (Wu et al.,2019; Zampetaki et al., 2012)

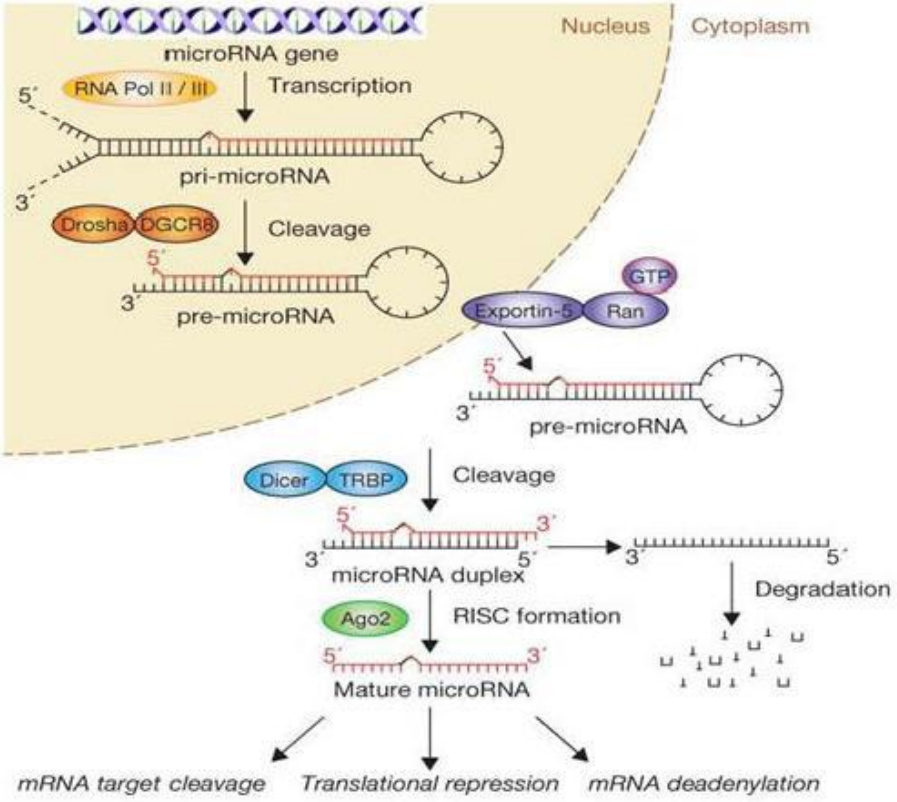


Figure 1.2: miRNAs Biogenesis (Velu et al, 2012)

1.1.6.2. The link between microRNAs and T2DM

MicroRNAs (miRNAs) regulate various biological processes such as insulin secretion, insulin resistance, inflammation, and apoptosis in pancreatic β-cells, adipose tissue, liver, and skeletal muscle. They have been found to play important roles in the development and progression of T2DM (Shen et al., 2015; Ciccacci et al., 2019).

In T2DM specific miRNAs are dysregulated, and some have been identified as potential therapeutic targets. For example, miRNA-29a overexpression increase insulin secretion and glucose homeostasis in mouse models and it has been found to be downregulated in the pancreatic islets of type 2 diabetic patients (Yin et al., 2015). Similarly, miRNA-375 inhibition has been shown to improve β-cell function and glucose tolerance and it has been found to be upregulated in pancreatic β-cells in response to high glucose levels (Poy et al., 2009).

Other miRNAs like miRNA-9, miRNA-146a, miRNA-192, miRNA-375 and miRNA-200 family members have been implicated in T2DM which have been shown to regulate various pathways involved in diabetes, such as inflammation, oxidative stress, and insulin signaling (Shen et al., 2015; Ciccacci et al., 2019).

MiR-9 has been implicated in the regulation of glucose homeostasis through its effects on various target genes in different tissues. Here are some examples of its target tissue, function, and target genes:

In pancreatic beta cells, miR-9 has been shown to regulate insulin secretion by targeting multiple genes involved in the exocytotic machinery, such as Munc18a, Vamp2, and Syt1 (Lovis et al., 2008; Plaisance et al., 2011). MiR-9 overexpression in beta cells leads to impaired insulin secretion and glucose intolerance, whereas its knockdown improves glucose-stimulated insulin secretion (Plaisance et al., 2011).

In adipose tissue, miR-9 has been shown to regulate adipogenesis by targeting the transcription factor Kruppel-like factor 9 (KLF9) (Karbiener et al., 2014). Overexpression of miR-9 in adipose tissue leads to decreased expression of KLF9 and impaired adipocyte differentiation, whereas miR-9 inhibition promotes adipogenesis (Karbiener et al., 2014).

In the liver, miR-9 has been shown to regulate gluconeogenesis by targeting the transcription factor PPARA and its coactivator PGC1A (Yoon et al., 2015). MiR-9 overexpression in the liver leads to increased expression of PPARA and PGC1A and increased gluconeogenesis, whereas its knockdown suppresses gluconeogenesis (Yoon et al., 2015).

MicroRNA 29 (Mir29) regulates gene expression by binding to the 3' untranslated regions (UTRs) of target messenger RNAs (mRNAs). Mir29 has been implicated in various cellular processes, including glucose homeostasis. Mir29 is expressed in several tissues, including the liver, pancreas, and adipose tissue, which play critical roles in glucose homeostasis (Zhang et al., 2020).

Mir29 plays a vital role in regulating glucose homeostasis by targeting various genes involved in insulin signaling, glucose uptake, and glycogen synthesis (Ryu et al., 2014). In particular, Mir29 has been shown to inhibit insulin receptor substrate 1 (IRS1) expression in adipose tissue, which can lead to insulin resistance and glucose intolerance (Liu et al., 2017).

Mir29 has been shown to target several genes involved in glucose homeostasis, including insulin receptor substrate 1 (IRS1), glucose transporter 4 (GLUT4), and glycogen synthase kinase 3 beta (GSK3β) (Ryu et al., 2014; Zhang et al., 2020). Inhibition of IRS1 expression

by Mir29 has been shown to impair insulin signaling and glucose uptake in adipose tissue (Liu et al., 2017). Mir29 also downregulates GLUT4 expression and impairs glucose uptake and utilization in skeletal muscle and adipose tissue (Zhang et al., 2020). Additionally, Mir29 targets GSK3β, a key regulator of glycogen synthesis, leading to reduced glycogen synthesis in the liver and muscle (Ryu et al., 2014).

MiR-192 has been shown to play a role in glucose homeostasis. It is expressed in various tissues including the liver, kidney, and adipose tissue, where it regulates genes involved in glucose metabolism and insulin signaling pathways. One of the target tissues of miR-192 in glucose homeostasis is the liver. miR-192 was found to be upregulated in the liver of diabetic mice, and its overexpression was associated with impaired glucose homeostasis. miR-192 targets several genes involved in glucose metabolism, including Pck1, G6pc, and Fbp1, which are key enzymes in gluconeogenesis. (Rottiers et al, 2013)

Another target tissue of miR-192 in glucose homeostasis is adipose tissue. miR-192 is upregulated in the adipose tissue of obese mice and humans, and its overexpression contributes to insulin resistance. miR-192 targets Pparγ, a transcription factor involved in adipogenesis and insulin sensitivity, and its downregulation improves glucose homeostasis in obese mice. (Chen et al, 2018)

Table 1.2: MiRNAs, their function in regulating glucose homeostasis and their target genes (Lovis et al., 2008; Plaisance et al., 2011;Ryu et al., 2014; Zhang et al., 2020; Rottiers et al, 2013; Yu et al., 2018)

MiRNA	**Target tissue**	**Function**	**Target genes**
miR-29	Muscle, adipose, liver	Glucose transport	IRS1,GLUT4,GSK3β, Insig1, Cav2
miR-192	Kidney	Kidney and diabetic nephropathy development	SIP1, Pck1, G6pc, and Fbp1
miR-375	Adipose, liver, muscle	Insulin secretion, pancreatic islet development, Glucose metabolism	Mtpn, PDK1, PPARγ, SIRT1, HDAC4, Pdk4, Pck1, Igf1r,
miR-103	Liver	Glucose homeostasis	IRS1
miR-9	Pancreas	regulate insulin secretion	Munc18a, Vamp2, and Syt1 Onecut2
miR-107	Liver	Glucose homeostasis	IRS1
miR-143	Adipose tissue	Insulin sensitivity	GLUT4
miR-223	Skeletal muscle	Glucose uptake, insulin signalling	GLUT4, PTEN
miR-124a	Pancreas	islet development	FoxA2

In addition to its role in glucose metabolism, miR-192 has been shown to regulate genes involved in lipid metabolism and inflammation. miR-192 targets SCD1, a key enzyme in fatty acid synthesis, and its downregulation improves hepatic steatosis and insulin sensitivity

in obese mice. (Zhang et al,2020) miR-192 plays an important role in glucose homeostasis by regulating genes involved in glucose and lipid metabolism in various target tissues.

MiR-375 is a microRNA that plays role in glucose regulation by controlling expression of target genes in pancreatic islets and other tissues. In pancreatic islets, miR-375 is primarily expressed in beta cells and has been shown to control insulin and β- cell production (Poy et al., 2009).One of the key target genes of miR-375 in pancreatic beta cells is Myotrophin (Mtpn), which is involved in insulin signaling and beta cell function. Overexpression of miR-375 in beta cells results in downregulation of Mtpn and impaired insulin secretion (Poy et al., 2009). Another target gene of miR-375 in pancreatic islets is PDK1, which is involved in glucose metabolism and insulin signaling (Tong et al., 2019). MiR-375 also plays a role in glucose homeostasis in other tissues such as adipose tissue, liver, and muscle. In adipose tissue, miR-375 regulates adipogenesis and lipid metabolism by targeting PPARγ, SIRT1, and HDAC4 (Li et al., 2016). In liver, miR-375 targets several genes involved in glucose metabolism and insulin signaling, including Pdk4, Pck1, and Igf1r (Zhang et al., 2019). In muscle, miR-375 regulates glucose uptake and metabolism by targeting PDK1 and PDK4 (Yu et al., 2018). Overall, miR-375 plays a crucial role in glucose homeostasis by regulating insulin secretion and glucose metabolism in pancreatic islets and other tissues. Its target genes include Mtpn, PDK1, PPARγ, SIRT1, HDAC4, Pdk4, Pck1, Igf1r.

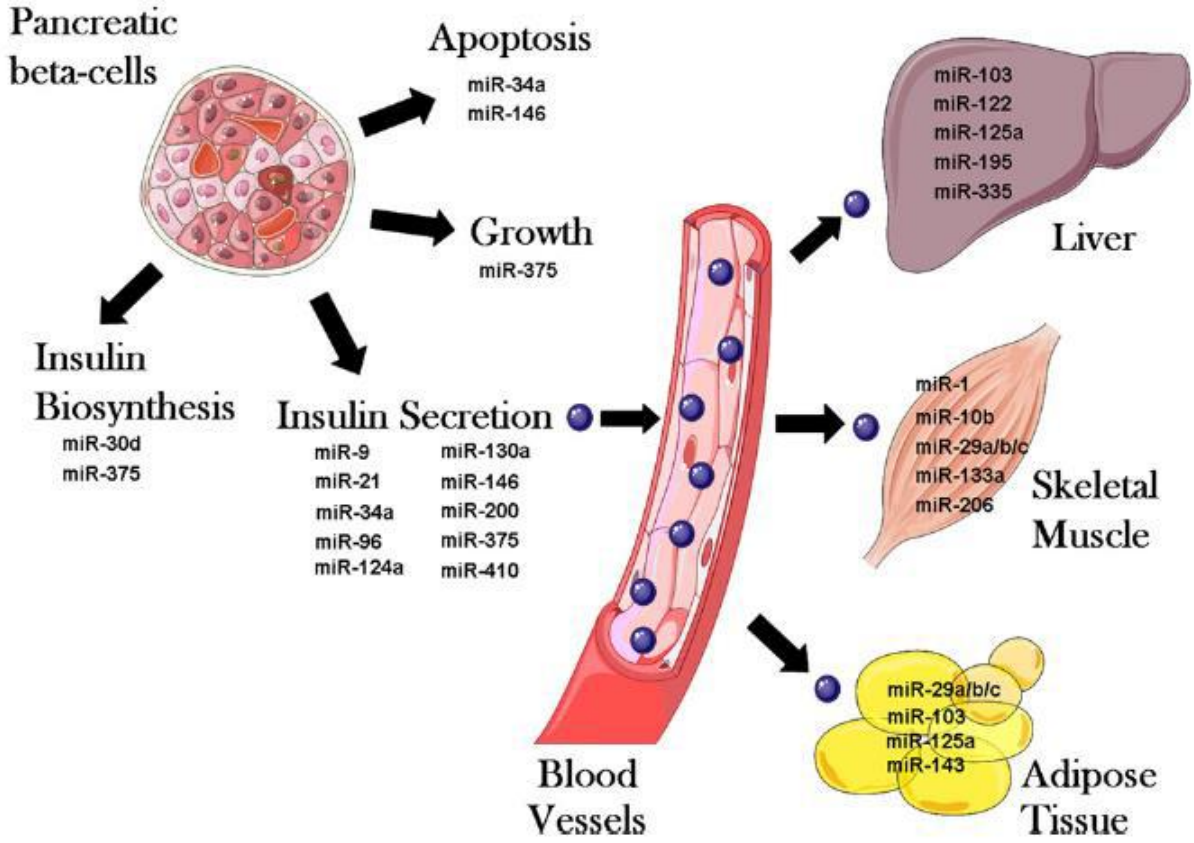


Figure 1.3: The miRNAs implicated in control of insulin target tissues/organs and pancreatic beta-cell activity in T2DM (Guay et al, 2011)

MicroRNAs, small non-coding RNA molecules, control gene by binding to target messenger RNAs and suppressing their translation. Several miRNAs have been identified to contribute

to the development of T2DM. The miR-29a was upregulated in T2DM patients and in high glucose-treated cells. Moreover, miR-29a targeted several genes involved in insulin signaling, including insulin receptor substrate 1 (IRS1) and phosphatidylinositol 3-kinase regulatory subunit alpha (PIK3R1). These findings suggest that miR-29a may contribute to insulin resistance in T2DM. (Wang et al.,2015) . The miR-192 was upregulated in the liver of T2DM patients and in high glucose-treated cells. Moreover, miR-192 targeted several genes involved in glucose and lipid metabolism, including glucose transporter 2 (GLUT2) and peroxisome proliferator-activated receptor alpha (PPARα). These findings suggest that miR-192 may contribute to hepatic insulin resistance and dyslipidemia in T2DM. (Guo et al., 2019)
The miR-27a-3p was downregulated in T2DM patients and in high glucose-treated cells. Moreover, miR-27a-3p targeted several genes involved in insulin signaling, including insulin receptor (INSR) and protein kinase B alpha (AKT1). miR-27a-3p may play a protective role in T2DM by improving insulin sensitivity. (Zhu et al.,2020)
Figure 1.3 shows the different miRNAs involved in T2DM pathogenesis and their targets .As shown in the figure, miR-29a, miR-192, and miR-27a-3p target different organs involved in insulin signaling, glucose and lipid metabolism, and inflammation. Dysregulation of these miRNAs may contribute to the development and progression of T2DM.

1.1.6.3 MicroRNA-9 (miR-9)

MiR-9 is highly conserved across different species and is expressed in various tissues, including the brain, heart, lung, and pancreas. The primary function of miR-9 is to bind to the 3' UTR of messenger RNA (mRNA) molecules, causing either degradation or inhibition. This allows miR-9 to regulate the expression of multiple genes at once, playing a role in a wide range of cellular processes, including development, differentiation, and disease states such as cancer, neurodegeneration, and diabetes (Poy et al., 2004; Sayed et al., 2008; Li et al., 2018).

1.1.6.4 MicroRNA-29 (miR-29)

There are 3 miR-29: miR-29a, miR-29b, and miR-29c. These miRNAs are expressed in various tissues, including the brain, heart, lung, and liver. The primary function of miR-29 is to bind to the 3' UTR of target messenger RNA molecules, causing either degradation or inhibition. This allows miR-29 to regulate the expression of multiple genes at once, playing a role in a wide range of cellular processes, including development, differentiation, and disease states such as cancer, cardiovascular diseases, and fibrosis (van Rooij et al., 2008; Kumarswamy et al., 2011; Li et al., 2018)
MicroRNA-29a belongs to the miR-29 family. It is encoded by the MIR29A gene located on chromosome 7q32.3 in humans. Like other miRNAs, miR-29a plays an important role in post-transcriptional gene regulation by binding to the 3' UTR of target mRNA molecules, leading to either mRNA degradation or translation inhibition. This allows miR-29a to regulate the expression of multiple genes at once and play a role in a variety of biological processes, including apoptosis, differentiation, fibrosis, and tumorigenesis (Duan et al., 2018; Hu et al., 2020; Lu et al., 2020).
miR-29a is involved in the regulation of various diseases, including cancer, cardiovascular disease, and neurological disorders. For example, miR-29a has been shown to play a role in promoting apoptosis and inhibiting proliferation in cancer cells (Liu et al., 2019; Sun et al., 2020). In addition, miR-29a has been shown to regulate cardiac fibrosis by targeting multiple genes involved in extracellular matrix production (Zhou et al., 2018).

1.1.6.5 MicroRNA-192 (miR-192)

MicroRNA-192 (miR-192) is a small non-coding RNA molecule that belongs to the miR-194 family. It is encoded by the MIR192 gene located on chromosome 11q13.1 in humans. miR-192 plays an important role in regulating gene expression by binding to the 3' untranslated region (UTR) of target messenger RNA (mRNA) molecules, leading to either mRNA degradation or translation inhibition. This allows miR-192 to regulate the expression of multiple genes at once and play a role in a variety of biological processes, including liver fibrosis, kidney disease, and cancer (Ji et al., 2019; Meng et al., 2019; Zhang et al., 2020).

Studies have shown that miR-192 is involved in the pathogenesis of liver fibrosis by regulating the expression of multiple genes involved in extracellular matrix production (He et al., 2018; Ji et al., 2019). In addition, miR-192 has been shown to play a role in the progression of kidney disease by regulating the expression of genes involved in apoptosis and inflammation (Meng et al., 2019). Furthermore, miR-192 has been implicated in the development and progression of cancer by regulating the expression of genes involved in tumor growth and metastasis (Zhang et al., 2020).

1.1.6.6 MicroRNA-375 (miR-375)

MicroRNA-375 (miR-375) is a small non-coding RNA molecule that is encoded by the MIR375 gene located on chromosome 2q35 in humans. It is involved in the regulation of gene expression by binding to the 3' untranslated region (UTR) of target messenger RNA (mRNA) molecules, leading to either mRNA degradation or translation inhibition. This allows miR-375 to play a role in a variety of biological processes, including insulin secretion, cell proliferation, and tumorigenesis (Bader et al., 2010; Hara et al., 2013; Li et al., 2018).

miR-375 plays role in regulation of insulin secretion. Specifically, it regulates the expression of multiple genes involved in glucose metabolism and insulin secretion, including PDK1, MYO6, and GK (Bader et al., 2010; Hara et al., 2013). In addition, miR-375 has been shown to play a role in the regulation of cell proliferation and differentiation in multiple tissues, including the lung and liver (Li et al., 2018; Xie et al., 2017). Furthermore, miR-375 has been implicated in the development and progression of multiple types of cancer, including breast cancer and gastric cancer, by regulating the expression of genes involved in tumor growth and metastasis (Li et al., 2018; Tutar & Tutar, 2021).

1.1.6.7Molecular analysis of the miRNAs

The study of miRNAs has gained significant attention due to their potential as biomarkers for various diseases, including cancer. The molecular analysis of miRNAs involves the use of various techniques to identify, quantify, and characterize these molecules.

One of the commonly used methods for miRNA analysis is quantitative reverse transcription PCR (qRT-PCR), which allows for the sensitive and specific detection of miRNAs. The qRT-PCR analyze the expression of miRNAs in extracellular vesicles (EVs) derived from the plasma of patients with pancreatic cancer. The results showed that miR-200a-3p was significantly upregulated in the EVs of patients with pancreatic cancer compared to healthy controls, suggesting its potential as a diagnostic biomarker for this disease. (Gailhouste et al. ,2018)

Another technique for miRNA analysis is miRNA sequencing, which involves the high-throughput sequencing of small RNA libraries. The miRNA sequencing to identify differentially expressed miRNAs in the plasma of patients with hepatocellular carcinoma (HCC) compared to healthy controls revealed that miR-122-5p was downregulated in the plasma of HCC patients, and its levels were negatively correlated with tumor size and alpha-fetoprotein levels, suggesting its potential as a prognostic biomarker for HCC. (Zhang et al.,2021)

In addition to qRT-PCR and miRNA sequencing, various other techniques can be used for miRNA analysis, including microarray analysis and in situ hybridization. The microarray analysis to identify differentially expressed miRNAs in the plasma of patients with non-small cell lung cancer (NSCLC) compared to healthy controls showed that miR-223-3p was significantly upregulated in the plasma of NSCLC patients and could be used as a potential biomarker for this disease. (Wang et al.,2019)
Three techniques are frequently employed to measure miRNA expression levels.: (a) microarrays, (b) NGS technologies, and (c) qRT-PCR. Below is a quick summary of each of these approaches

1.1.6.7.1 Microarrays (Hybridzation-based)
MicroRNA (miRNA) microarrays are an essential tool for molecular analysis that allows the detection of changes in miRNA expression levels between different samples. These arrays are used in various areas of research, including cancer diagnosis, drug discovery, and the study of various diseases. (Kao et al.,2013;Huang et al., 2018, Wang and Wang, 2019)
miRNAs play a important role in post-transcriptional gene regulation. The miRNA microarray technology measures the expression levels of these small RNA molecules, allowing for the identification of miRNAs that are differentially expressed in different conditions. The molecular analysis of miRNA microarrays involves the isolation of total RNA from the samples, labeling of the RNA, hybridization to the microarray, and data analysis. (Kao et al.,2013;Huang et al., 2018, Wang and Wang, 2019)
In the first step of the analysis, the total RNA is extracted from the samples, and the quality and quantity of RNA are assessed. The next step is the labeling of the RNA, which involves the addition of fluorescent dyes to the RNA molecules. This step enables the detection of the miRNA expression levels on the microarray. After labeling, the RNA is hybridized to the microarray, and the microarray is then scanned to detect the fluorescence signal. (Kao et al.,2013;Huang et al., 2018, Wang and Wang, 2019)
The data obtained from the microarray is then analyzed using bioinformatics tools to identify the differentially expressed miRNAs. The data can be analyzed using various statistical methods, such as t-tests, ANOVA, and fold-change analysis. The differentially expressed miRNAs can then be further studied to determine their biological significance and potential roles in disease pathogenesis. (Kao et al.,2013;Huang et al., 2018, Wang and Wang, 2019)
Several studies have utilized miRNA microarrays for the identification of miRNAs involved in various diseases. miRNA microarrays to identify miRNAs involved in ovarian cancer identified several miRNAs that were differentially expressed in ovarian cancer patients, suggesting their potential role as diagnostic markers. Similarly, miRNA microarrays is utilized to identify miRNAs involved in the pathogenesis of diabetic nephropathy. (Kao et al.,2013;Huang et al., 2018, Wang and Wang, 2019)
In conclusion, the molecular analysis of miRNA microarrays is an essential tool for identifying differentially expressed miRNAs and their potential roles in various diseases. The miRNA microarray technology provides a comprehensive view of the miRNA expression profile, which can aid in the development of diagnostic and therapeutic strategies. The application of miRNA microarrays in various fields of research continues to expand, demonstrating the importance and versatility of this technology. (Kao et al., 2013; Huang et al., 2018, Wang and Wang, 2019)

1.1.6.7.2 Next generation sequencing (NGS)
The molecular analysis of miRNAs has become an important area of research in recent years, with next-generation sequencing (NGS) being a powerful tool for miRNA profiling. One of the major advantages of NGS is its ability to generate massive amounts of sequence data in a relatively short amount of time. This allows for the simultaneous profiling of thousands of miRNAs from a single sample, providing a comprehensive analysis of miRNA expression patterns. Additionally, NGS can detect novel miRNAs that may not be represented in current

miRNA databases, allowing for the discovery of new miRNAs and their potential role in gene regulation. (Miao et al, 2018;Li et al., 2018; Han et al., 2019)
NGS-based miRNA profiling has been used in a variety of applications, including cancer research, where miRNA expression patterns have been shown to be useful in cancer diagnosis, prognosis, and treatment. NGS was used to identify a panel of miRNAs that could be used as biomarkers for early detection of lung cancer. (Li et al. 2018) Similarly, used NGS is uesd to identify miRNAs that were differentially expressed in hepatocellular carcinoma, suggesting their potential use as therapeutic targets. (Han et al., 2019)
In conclusion, NGS has emerged as a powerful tool for the molecular analysis of miRNAs. Its ability to generate massive amounts of sequence data allows for the comprehensive analysis of miRNA expression patterns and the discovery of novel miRNAs. With its potential applications in cancer research and other fields, NGS-based miRNA profiling holds great promise for advancing our understanding of gene regulation and disease mechanisms. (Miao et al, 2018;Li et al., 2018; Han et al., 2019)

1.1.6.7.3 qRT-PCR
Quantitative reverse transcription PCR (qRT-PCR) is a widely used technique to analyze miRNA expression levels due to its high sensitivity, specificity, and reproducibility. The procedure of miRNA qRT-PCR involves the reverse transcription of miRNA into cDNA, followed by amplification and detection of the cDNA using specific primers and fluorescent probes. The normalization of miRNA expression is essential to obtain accurate and reliable results, and it is typically achieved by using endogenous control miRNAs such as U6 or RNU48. (Bader et al., 2010; Li et al.,2018; Huang et al., 2020)
Several studies have demonstrated the utility of miRNA qRT-PCR in various research areas, including cancer diagnosis and prognosis, drug discovery, and developmental biology. qRT-PCR is used to identify differentially expressed miRNAs in breast cancer tissues, which could serve as potential diagnostic biomarkers. (Bader et al., 2010) qRT-PCR is employed to evaluate the therapeutic effect of a novel anticancer drug in gastric cancer cell lines. (Li et al.,2018) Moreover, qRT-PCR has been used to investigate the role of miRNAs in embryonic development and organogenesis, on the expression of miRNAs during heart development in zebrafish. (Huang et al., 2020)
In conclusion, miRNA qRT-PCR is a powerful tool for the analysis of miRNA expression levels in various biological contexts. Its high sensitivity and specificity make it an ideal method for the detection of low-abundance miRNAs in complex biological samples. With the increasing availability of miRNA expression data, miRNA qRT-PCR is becoming increasingly important for the identification of miRNAs as potential biomarkers for diseases and the development of miRNA-based therapeutics. (Bader et al., 2010; Li et al.,2018; Huang et al., 2020)

After determining Ct, Delta delta Ct method was used to determine expression. The Ct value is the cycle number at which the fluorescence signal from the amplified product reaches a predefined threshold, and is inversely proportional to the amount of starting material in the reaction.
Delta delta Ct (ΔΔCt) is a widely used method to determine the relative expression of miRNAs in quantitative real-time PCR (qRT-PCR) experiments. This method compares the threshold cycle (Ct) values of a target miRNA to that of a endogenous reference gene, and then calculates the relative expression of the target miRNA using the formula 2^-ΔΔCt. The ΔΔCt is the difference between the Ct value of the target miRNA and the reference miRNA in the experimental group minus the same difference in the control group. (Livak and Schmittgen, 2001)
In summary, ΔΔCt method is a widely used approach to quantify miRNA expression in qRT-PCR experiments. This method allows for the relative quantification of miRNA expression and has been utilized in various biological samples and experimental conditions.

This method allows for the comparison of the relative expression of a target miRNA in different samples or conditions, and is based on the calculation of the difference in the threshold cycle (Ct) values between the target miRNA and a reference gene, followed by normalization to a control sample.

To determine miRNA expression using the ΔΔCt method, the Ct values of the target miRNA and a reference gene are first determined using quantitative PCR (qPCR) analysis. The ΔCt value is then calculated by subtracting the Ct value of the reference gene from the Ct value of the target miRNA. This represents the relative expression of the target miRNA compared to the reference gene. By subtracting the ΔCt value of the control sample from the ΔCt value of each experimental sample, the ΔΔCt value is then calculated. This represents the fold change in expression of the target miRNA in the experimental samples compared to the control sample. (Livak and Schmittgen, 2001)

The ΔΔCt method is a reliable and widely used method for quantifying miRNA expression, with several advantages such as its simplicity, sensitivity, and high reproducibility. However, it is important to carefully select appropriate reference genes for normalization to ensure accurate and reliable results. (Livak and Schmittgen, 2001)

Designing primers for miRNA amplification can be challenging due to the small size of miRNAs and their highly conserved sequences among different species. However, several tools and software programs are available to design miRNA-specific primers that can be used for miRNA detection and quantification. The designed and validated miRNA-specific primers for qRT-PCR using several software programs and compared the performance of these primers to commercially available miRNA-specific primers and found that the newly designed primers performed similarly to the commercial primers and that miRNA expression levels measured using both primer sets were highly correlated. (Mestdagh et al. 2014)

The designed and validated miRNA-specific primers using the miRBase database,the Primer3 and MiRDeep software program when tested these primers on total RNA extracted from various clinical samples and found that the newly designed primers improved miRNA quantification and reduced variability between samples. (Brase et al. 2010, Yang et al. 2011).

In conclusion, designing miRNA-specific primers is essential for accurate detection and quantification of miRNAs using qRT-PCR. Several tools and software programs are available to design these primers, and the studies cited above demonstrate the utility of these methods in designing and validating miRNA-specific primers.

1.1.7 Objectives

1.1.7.1 General Objective

- Comparative evaluation of plasma expression pattern of microRNA in healthy controls, non-diabetic obese and type 2 diabetic patients.

1.1.7.2 Specific Objectives

- Extraction of plasma RNA
- Preparation of cDNA form extracted RNA
- Measurement of concentration of RNA and cDNA
- Measurement of ct values of RNU6B,miRNA 9, miRNA 29a, miRNA 192 and miRNA 375 in T2DM, Healthy and Obese
- Calculation of plasma expression pattern using Pfaffl method

1.1.8 Research Hypothesis

1.1.8.1 Null Hypothesis

Ho: There is no relationship between pattern of plasma circulating microRNA expression and pathogenesis of T2DM

1.1.8.2Alternate Hypothesis

H1: There is relationship between pattern of plasma circulating microRNA expression and pathogenesis of T2DM

Rationale of Study

- Diabetes is worldwide disease and causes 1 death every 5 seconds in 2021.
- The T2DM can be predicted as early as 10 years from different biomarkers like insulin resistance(IR), Oral Glucose Tolerance test(OGTT), microRNA.
- IR, OGTT are hectic, expensive and not much reliable process. So new biomarkers like miRNA is emerging
- miRNAs play a crucial role in the pathogenesis of T2DM
- miRNA is stable and can be determined easily in body fluids like blood, urine etc. so there is worldwide trend to study miRNA as diabetes biomarker
- There is no study of diabetes and miRNA in nepali population so this study is designed

Chapter II

2. Literature Review

2.1Diabetes

Metabolic illnesses, such as diabetes, have attracted the attention of scientists in recent years and have emerged as a serious worldwide health challenge. MiRNAs are essential regulators of metabolically active tissues' growth and physiological status. Changes in their expression can lead to a loss of glucose and lipid homeostasis, which may play a role in metabolic disorders (Chen et al., 2014).

One of the emerging and promising novel biomarkers for T2DM is miRNAs because they are metabolic stable and presence in various fluids. MicroRNAs regulate cellular activities by suppressing gene expression and are a type of noncoding RNA (Dangwal S et al., 2012). They are endogenous and tiny (18‑25 nucleotides). They play a role in a variety of biological processes, like apoptosis, cell growth, proliferation, differentiation, and metabolism (Wang Y et al., 2009).

Diabetes Melitus, in 2022, 537 million adult is already been affected and will rise to 643 million by 2030. Diabetes causes 6.7 million deaths in 2021 i.e. 1 every 5 seconds. (Chien et al,2015;ADA,2021)

Type 2 diabetes mellitus has become a significant public health concern worldwide. It is estimated that the global prevalence of T2DM will increase from 425 million in 2017 to 629 million by 2045. The economic burden of T2DM is enormous, with direct and indirect costs reaching up to $825 billion in 2017. Direct costs of T2DM refer to the medical expenses associated with the diagnosis, treatment, and management of the disease. These costs include hospitalizations, physician visits, medications, and diagnostic tests. The annual direct cost of T2DM in the United States was estimated to be $327 billion in 2017. This figure is expected to increase to $622 billion by 2030 if current trends continue. (ADA,2020) Indirect costs of T2DM refer to the economic burden imposed by the disease on individuals, families, and society as a whole. These costs include lost productivity, absenteeism, disability, and premature mortality. The global indirect cost of T2DM was estimated to be $494 billion in 2019. This figure is expected to increase to $802 billion by 2045. (IDF,2019)Preventive interventions for T2DM aim to reduce the incidence and prevalence of the disease and its associated economic burden. These interventions include lifestyle modifications, such as dietary changes and increased physical activity, and pharmacological interventions, such as metformin and other glucose-lowering medications. A 10% reduction in the incidence of T2DM could result in a savings of $5.5 billion in direct medical costs in the United States. (ADA,2020) T2DM is a significant economic burden worldwide, with direct and indirect costs reaching up to $825 billion in 2017. The economic impact of the disease is expected to increase in the coming years, highlighting the urgent need for preventive interventions. Implementing such interventions could not only improve the health of individuals but also lead to substantial economic benefits. (Khan et al, 2008; Zang, 2020)

Recent research has identified several molecular pathways that are dysregulated in obesity, including those involved in adipogenesis, lipid metabolism, insulin signaling, and

inflammation. Various adipokines, such as leptin, adiponectin, and resistin, have also been implicated in the pathophysiology of obesity and its associated metabolic disorders (Ouchi et al., 2011).

2.2 MicroRNA

In Homo sapiens, up to 2000 miRNAs have been identified with the bulk of genes thought to be regulated directly by them. Furthermore, miRNA can have an impact on multiple levels as it bind and regulate multiple targets at the same time. There appears to be a lot of redundancy among the targets of miRNAs despite the fact that certain circulating miRNAs have low expression. Low-expression miRNAs have a role in the epigenetic regulatory network. In response to pathophysiological stimuli, miRNAs are fine-tuners of gene expression patterns. miRNAs are significantly up-regulated while being expressed at low levels in the absence of pathological stress (Thum T, et al 2015). Many appears in the extracellular space, like blood etc. even though the majority of miRNAs are found intracellularly (Weber JA et al., 2010).

miRNAs can be identified and estimated by northern blotting, qRT-PCR, NGS and microarrays. Each technique have own set of benefits and drawbacks. The Northern blot method was widely adopted because of its great specificity. (Streit S et al., 2009). The use of northern blot analysis measurements reduces due to low efficiency, contamination, and poor sensitivity for probes (Varallyay E and et al., 2008). For reliable and effective identification of miRNAs, NGS, qRT-PCR and microarrays are now the most preferred approaches. Microarray and NGS allows for the testing of hundreds of miRNAs in a single run, while northern blot and qRT-PCR allow for one-by-one examination (Pordzik J and et al. 2018). Furthermore, a newly preferred technique for studying circulating miRNAs is next-generation sequencing (NGS). (Eminaga S et al., 2013). Alternative techniques are the use of nano sensors or nanowires and the enzyme-linked assays (De Rosa S and Indolfi C 2015).

For monitoring the development and progression of diabetes mellitus miRNAs in body fluid appear to be useful as biomarkers. For early detection of type 2 diabetes miR-23a and miR-126 were reliable biomarkers (Liu et al., 2014; Yang et al., 2014).

miRNAs found linked to cancer, and they regulate important processes like cell proliferation, cell adhesion, apoptosis, and angiogenesis, all of which are influenced by dysregulation and play a role in cancer onset, progression, and metastasis. According to numerous studies, breast cancer, leukemia, liver cancer, ovarian cancer, pancreatic cancer, and prostate cancer all have altered miRNA profiles (Iorio et al., 2005). Depending on the cellular context and the genes targeted miRNAs can act as oncogenes or tumor suppressors. miRNA-34a is a major tumor suppressor (Volinia et al., 2006).

The miRNA are18 to 24 nucleotides in lengthed (Saikumar et al, 2012) and in some paper approximately 20-25 nucleotides in length (Dangwal S et al., 2012). They are transcribed from DNA, processed in the nucleus, and exported to the cytoplasm, where they bind to target messenger RNA (mRNA) molecules and regulate gene expression by either degrading the mRNA or inhibiting its translation (Bartel, 2009; Kim et al., 2009).

miRNAs regulate gene in a post-transcriptional manner. They are involved in development, differentiation, proliferation, and apoptosis (Ha & Kim, 2014; O'Brien et al., 2018).

MiRNAs can be classified on the basis of their biogenesis, expression pattern, and function. Some common types of miRNAs include:

1. Canonical miRNAs: These are the most well-known and extensively studied miRNAs. They are transcribed from genomic DNA by RNA polymerase II. Then, processed by the DGCR8 complex in nucleus. Furthermore, processed by Dicer at cytoplasm to form fullyfunctional miRNAs (Ha & Kim, 2014).
2. Non-canonical miRNAs: These miRNAs are generated by alternative processing pathways that bypass one or more of the canonical processing steps. Examples include miRNA-451, which is processed by Ago2 in the absence of Dicer, and miRNA-320, which is processed by Dicer-independent mechanisms (O'Brien et al., 2018).
3. Mirtrons: These are miRNAs that are derived from introns of protein-coding genes. They are processed by splicing and debranching machinery to form pre-miRNA hairpins that are subsequently processed by Dicer (Ha & Kim, 2014).
4. Small nucleolar RNA-derived miRNAs (sdRNAs): These miRNAs are derived from small nucleolar RNAs (snoRNAs) and are generated by the same processing machinery as canonical miRNAs. However, they have different target specificities and functions (O'Brien et al., 2018).
5. Piwi-interacting RNAs (piRNAs): These are a distinct class of small non-coding RNAs that interact with the Piwi subfamily of Argonaute proteins. They are primarily expressed in the germline and are involved in transposon silencing and germ cell development (Ha & Kim, 2014).

2.3 Real-time PCR

Real-time polymerase chain reaction (real-time PCR) is a technique used to amplify and quantify DNA or RNA molecules in real-time as they accumulate during the amplification process. This technique is widely used in various fields, including medical diagnosis, genetic analysis, and environmental monitoring. The principle behind real-time PCR is based on the detection of fluorescence signals generated by fluorophores that are incorporated into the PCR product during amplification. The fluorescence signals are detected by a specialized instrument called a real-time PCR machine, which records the signal intensity in each cycle of the PCR reaction. The signal intensity is proportional to the amount of PCR product that has been synthesized during the reaction. (Bustin, 2000; Mackay et al, 2002; Yang & Qu, 2016)

Real-time PCR has several advantages over traditional PCR methods. Firstly, it allows the detection and quantification of PCR products in real-time, which provides immediate feedback on the success of the reaction. Secondly, it is a more sensitive and specific method compared to traditional PCR because it reduces the risk of false positives due to the detection of non-specific amplification products. Finally, real-time PCR is a more rapid method for detecting and quantifying nucleic acid sequences because it eliminates the need for post-amplification analysis, such as gel electrophoresis. (Bustin, 2000; Mackay et al, 2002; Yang & Qu, 2016)

One of the most common applications of real-time PCR is in medical diagnosis. For example, real-time PCR is used to detect viral infections, such as influenza and HIV, and bacterial infections, such as tuberculosis and streptococcal infections. Real-time PCR is also used in genetic analysis to detect gene mutations and to quantify gene expression levels. Finally, real-time PCR is used in environmental monitoring to detect and quantify the presence of

microorganisms in water and soil samples. (Bustin, 2000; Mackay et al, 2002; Yang & Qu, 2016)

2.4 RNU6B

RNU6B is used to determine the expression pattern of miRNA as it is found to be stable in different studies. RNU6B, also known as U6 snRNA, is a non-coding RNA molecule found in eukaryotic cells that plays an important role in pre-mRNA splicing. In recent years, RNU6B is increasingly used as reference gene for miRNA expression analysis. In the evaluation of the suitability of RNU6B as a reference gene for miRNA quantification in breast cancer tissues, the researchers found that RNU6B was stably expressed across all samples and was therefore a suitable reference gene for miRNA expression analysis in breast cancer tissues (Gómez-Maldonado et al., 2017).

The use of RNU6B as a reference gene for miRNA expression analysis in the context of heart failure, the researchers found that RNU6B was stably expressed across all samples and was therefore a suitable reference gene for miRNA expression analysis in heart failure (Singh et al., 2019).

Furthermore, the use of RNU6B as a reference gene for miRNA expression analysis in various diseases, including cancer and cardiovascular diseases highlighted the importance of using a stable reference gene for miRNA expression analysis and concluded that RNU6B was a suitable reference gene in many cases (Vergara et al., 2020).

In conclusion, RNU6B is a commonly used reference gene for miRNA expression analysis in various diseases, diabetes, including cancer and cardiovascular diseases. Its stability and consistent expression across samples make it a reliable choice for normalizing miRNA expression levels.

2.5 Stem-loop primers

Stem-loop primers are commonly used in the reverse transcription-polymerase chain reaction (RT-PCR) for the detection and quantification of microRNAs (miRNAs). The stem-loop primer is a type of RT primer that consists of a short stem and a loop structure. The stem is usually 3-5 base pairs and forms a stable hairpin structure, while the loop contains the sequence complementary to the target miRNA. The stem-loop structure allows the primer to hybridize specifically to the target miRNA and promotes the efficiency and specificity of reverse transcription. The performance of stem-loop RT-PCR for the detection of miRNAs in serum samples from patients with chronic hepatitis B virus infection was evaluated and found that the stem-loop RT-PCR had high sensitivity and specificity and could detect miRNAs at low concentrations (He et al., 2019).

A stem-loop RT-PCR method for the quantification of miR-21 in breast cancer cell lines found that the stem-loop RT-PCR was more sensitive and specific than a conventional RT-PCR method and could detect miR-21 at concentrations as low as 0.05 femtomoles (Chen et al., 2018).

Overall, stem-loop primers are a useful tool for the detection and quantification of miRNAs in various biological samples. The stem-loop structure of the primer improves the specificity and efficiency of reverse transcription, leading to accurate and sensitive miRNA quantification. Stem loop primers are not used, only forward and reverse primers are used.

2.6 Fluorescent Dye

Intercalating agent like SYBR Green and probe are two commonly used methods for real-time PCR (polymerase chain reaction) detection and quantification of nucleic acids. SYBR Green is a fluorescent dye that binds to double-stranded DNA, while probes are labeled oligonucleotides that hybridize to a specific sequence of nucleotides. The use of SYBR Green and probe for the detection of Human Papillomavirus (HPV) in cervical swab samples evaluated found that both methods had similar sensitivity and specificity, but probe-based detection was more specific for the HPV genotype (Dong et al., 2020).

The use of SYBR Green and probe for the detection of Mycobacterium tuberculosis in sputum samples found that SYBR Green had a higher sensitivity than the probe-based method, but the latter had higher specificity and was less affected by non-specific amplification (Akulich et al., 2019).

Furthermore, there are advantages and disadvantages of using SYBR Green and probe in real-time PCR. The SYBR Green is a cost-effective and versatile method for nucleic acid detection, but it has limitations in terms of specificity and the potential for non-specific amplification. On the other hand, probe-based detection is more specific and has a lower risk of non-specific amplification, but it is more expensive and requires more optimization (Bustin et al., 2016).In conclusion, both SYBR Green and probe are widely used in real-time PCR for nucleic acid detection and quantification, but each method has its advantages and disadvantages.

2.7 Delta-Delta CT method

Several studies have used ΔΔCt method to quantify miRNA expression in various biological samples. (Wang et al. 2019, Wu et al. 2020,) Furthermore, ΔΔCt method has also been used to study the differential expression of miRNAs in response to various treatments. (Huang et al. 2021).

The ΔΔCt method is a widely used approach for determining the relative expression of miRNAs between two or more experimental groups. This method is based on the comparison of Ct values between a control and a treatment group, and normalization to an endogenous reference gene. The method has been extensively validated and is widely used in miRNA expression analysis.

2.8 Primer Selection

The primer used for miRNA expression in this study was obtained from literature review rather than designing as there is more chance of primer failure when designed which is not economical.

The primer design for miRNA-9 amplification was investigated and found many researchers had used a forward primer with the sequence 5’-GCCCGCTCTTTGGTTATCTAG-3’ and a reverse primer with the sequence 5'- CCAGTGCAGGGTCCGAGGT-3' with no cross-reactivity with other miRNAs (Hua et al. 2019, Guo et al. 2020, Xie et al. 2021)

The primer design for RNU6B is a crucial step in the detection and quantification of this small RNA. The use of RNU6B as a reference gene for normalization in qRT-PCR experiments requires the design of specific and efficient primers. The forward primer

CGCTTCGGCAGCACATATAC and reverse primer TTCACGAATTTGCGTGTCAT have been reported in several studies as suitable primers for RNU6B detection and quantification (Chen et al. 2005, Mestdagh et al. 2009, Peltier and Latham 2008).

The primer for miR-29a a forward primer with the sequence CGCGGATCCTGGATTTAAGATTTGGGC and a reverse primer with the sequence CCGGAATTCACATGCAATTCAGGTCAGTG is selected. These primers are designed to amplify the mature miR-29a sequence, which is highly conserved across different species. (Xu et al., 2015, Chen et al, 2017, Jia et al., 2021)

The forward primer AGCCGTTTGTTCGTTCGGCT and reverse primer GTGCAGGGTCCGAGGT for the detection of miRNA-375 is used.(Xu et al 2019, Zhang et al 2017, Yilmaz, M et al 2016)

The primer for miRNA-192 is forward primer (GGGGCTGACCTATGAATTGA) and reverse primer (CAGTGCAGGGTCCGAGGT) for miRNA-192 detection by quantitative PCR (qPCR) method. (Wang et al. 2020, Huang et al. 2019, Wang et al. 2019)

Chapter III

3. Materials and Method

3.1 Sample Collection

The sample was collected Manipal Teaching Hospital, Pokhara, Nepal from before Baisakh 2078. The quantification of the expression level of microRNAs using RT-qPCR was carried out at the Central Department of Biotechnology, Tribhuvan University, Kathmandu, Nepal from Baisakh 2078 to Ashar 2079.

Ethical approval was obtained (MEMG/IRC/241/GA) from the Research and Ethics Committee of the Manipal College of Medical Sciences, Pokhara, Nepal.

A total of 46 adult males and females aged between 31-60 years were included in this study. Of these, 17 were healthy controls, 17 were obese individuals, and 17 were T2DM patients.

3.2 Sample Preparation

The samples were kept in -80 ^{o}C until further processing.

1. The samples were slowly thawed at 4^{o}C.
2. Aliquot 150µL samples in nuclease free eppendorf tubes.
3. Centrifuge the thawed plasma at 4000rpm for 10 min using cooling centrifuge to remove any sedimented debris due to refrigeration
4. The clear 100 µL upper supernatant was used for the experimental work up.

3.3. RNA Extraction

Total RNA was isolated from Plasma by Zymo Direct-zol RNA Microprep Kits (catalog number: R2060) according to manufacturer's instruction.

1. Three times volume TRI Reagent® (300 µl) was added to a sample (plasma100 ul) and mixed thoroughly.
2. Same volume 400 µL of absolute alcohol was added in above mixture and mixed by pipetting thoroughly.
3. The mixture was transfered into a Zymo-Spin™ IC Column in a Collection Tube and centrifuged at 16000Xg for 30 seconds. The column was transfered into a new collection tube and the flowthrough was discarded.
4. 400 µl Direct-zol™ RNA PreWash buffer was added in column and transferred in new collection tube then centrifuge at 16000Xg for 30 seconds then elution was discarded.
5. 80µl of DNase I was added, mixed properly and incubated at room temperature for15minutes.
6. 400µl Direct-zol™ RNA PreWash buffer was added in column and it transferred in new collection tube then centrifuged at 16000Xg for 30 seconds then elution discarded.
7. 700 µl RNA Wash Buffer was added to the column and centrifuged at 16000Xg for 2 minutes to ensure complete removal of the wash buffer. The column was carefully transfered into an RNase-free tube.
8. RNA was eluted by adding 50µl of DNase/RNase-Free Water directly to the column matrix and centrifuged at 16000Xg for 30 seconds.

The concentrations and purity of the RNAs were assessed using a NanoDrop one/one Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).
The RNAs concentrations ranged from 0.035 to 230.44 ng/uL. All RNA samples were stored at -80^{0}C until further processing after making the cDNA.

3.4 Preparation of cDNA:(cDNA synthesis using *iScriptTM* cDNA Synthesis kit)

Reverse transcription (RT) was carried out using iScript cDNA Synthesis Kit BIO-RAD according to manufacturer's instruction.

Table 3.1: Reaction mixture for RT

Components	Volume per reaction
5x iScript reaction mix	4 ul
iScript reverse transcriptase	1 ul
Nuclear- free water	10 ul
RNA template	5 ul
Total	20 ul

Reaction Protocol
The Reverse transcriptase mix was subjected to following PCR conditions in BIO-RAD T100 Thermal Cycler.

Table 3.2: Reaction Protocol for RT

Priming	5min at 25℃
Reverse transcription	20min at 46℃
iScript reverse transcriptase	1 ul
RT inactivation	2min at 95℃
RNA template	5 ul
Optional step	Hold at 4℃

The concentrations and purity of the cDNA were assessed using a NanoDrop one/one Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The concentrations of cDNA ranged from 212.717 to 5424.553 ng/uL. All cDNA samples were stored at -20^{0}C until further processing.

3.5. Quantitative Real-Time PCR

Quantitative real time PCR, Maxima SYBR Green Ref K0221, lot 00615324 and lot 01182733 (thermoscientific; Thermo Fisher Scientific) were used.
Preparation of Real-time PCR mixture:

3.3: Table showing reagents and its volume for RT-PCR

Reagents	Volume (µl)			
SYBR green master mix(2X)	12.5	10	5	2.5
Forward primer	0.5	0.4	0.2	0.1
Reverse primer	0.5	0.4	0.2	0.1
cDNA template	2.5	2	1	.5
NFW	9	7.2	3.6	1.7
Total	25	20	10	5

In a 10µL reaction mix, 1µL of cDNA , 5 µL SYBR green, 1 µL primers and 3 µL NFW were used (most RT-qPCR reactions).
In a 20µL reaction mix, 2µL of cDNA , 10 µL SYBR green, 2 µL gene-specific primers and 6 µL nuclease-free water were used.
cDNA (2.5µL) was used in a 25 µL reaction mix containing 12.5 µL SYBR green, 3 µL gene-specific primers and 7 µL nuclease-free water in some of the reaction. cDNA (.5µL) was used in a 5µL reaction mix containing 2.5 µL SYBR green, .5 µL primers and 1.5 µL NFW in very few reaction.
The primers used are shown below.

Table 3.1: Forward and reverse primers of miRNAs

Target miRNA/gene	**Forward primer (5′3′)**	**Rervese primer (5′3′)**
RNU6B	CGCTTCGGCAGCACATATAC	TTCACGAATTTGCGTGTCAT
mir-9	GCCCGCTCTTTGGTTATCTAG	CCAGTGCAGGGTCCGAGGT
miR-29a	CGCGGATCCTGGATTTAAGATTTGGGC	CCGGAATTCACATGCAATTCAGGTCAGTG
Mir-192	GGGGCTGACCTATGAATTGA	CAGTGCAGGGTCCGAGGT
Mir-375	AGCCGTTTGTTCGTTCGGCT	GTGCAGGGTCCGAGGT

RT-qPCR reactions were run with Lepgen-96 Real-Time PCR system(Lepu Medical, China) for 20 samples , Azure cielo DX Real-Time PCR system (Azure Biosystems, United States) for 31 samples and BIORAD CFX Opus 96 Real-Time PCR System for 2 samples, 95^0C for 10 min, followed by 50 cycles of 95^0C for 15 s and 60^0C for 1 min with melt curve from 50^0C to 95^0C.
For some sample duplicate measurements were performed. The ct values were determined with LEPGEN Full Automatic Medical PCR Analysis System and Bio-Rad CFX Maestro with the automatic setting for assigning baseline. Azure Cielo Manager with threshold setting manually by using Non Template Control was also used.
Ct values were crossed referenced with the melting peak and melting temperature. Only the ct values with bell shaped melting peak around 80^0C were accepted.
The small nuclear RNA, RNU6B, was used for normalization.

3.6: Gel Electrophoresis:

Preparation of Agarose gel electrophoresis:

1. Weigh 0.75gm of agarose and dissolved in 50ml of 1X TAE (Tris base, acetic acid and EDTA) buffer was boiled until the clear solution.
2. Solution was cooled at room temperature and added 2.5µl of EtBr was added in the Solution and poured in the gel tray.
3. Then, allow to solidify and immense the gel tray in TAE buffer in electrophoresis tank.

Loading and visualization:

1. Mixed 1µl loading dye with 3µl DNA sample in loaded into the well.
2. Run the gel at 90V for 30 minutes.
3. Visualized under UV Trans Illuminator/Documentation

3.7. Data Analysis

The analysis was done using EXEL. Data was entered in EXEL sheet and delta-delta-Ct was determined. The Bar-Diagram of expression pattern and ANOVA were also done with EXEL.

Chapter IV

4. Results

4.1. Basic Characteristics

We included 17 obese patients, 12 T2DM patients and 17 non-diabetic healthy individuals (control) in this study.

Table 4.1: Basic characteristics of the study patients

Characteristics	Obese	T2D	Control
Patients numbers	17	12	17
Age	45.78	43.13	43.74
Sex(Female/Male)	9/8	5/7	7/10

4.2. RNA and cDNA concentration

The average concentration of extracted RNA was found to be 13.482 ng/ml and it ranged from 0.6 ng/ml to 222.1 ng/ml. The standard deviation was 37.72.
The average cDNA concentration was found to be 1995.29 ng/ml and it ranged from 287.6 ng/ml to 5424.6 ng/ml. The standard deviation was 1148.16.

4.3. Amplification of micro RNA and cycle Threshold value (Ct)

The Real Time PCR was performed on the cDNA prepared from the extracted RNA using sybr kit. The amplification and melt curve were shown below.

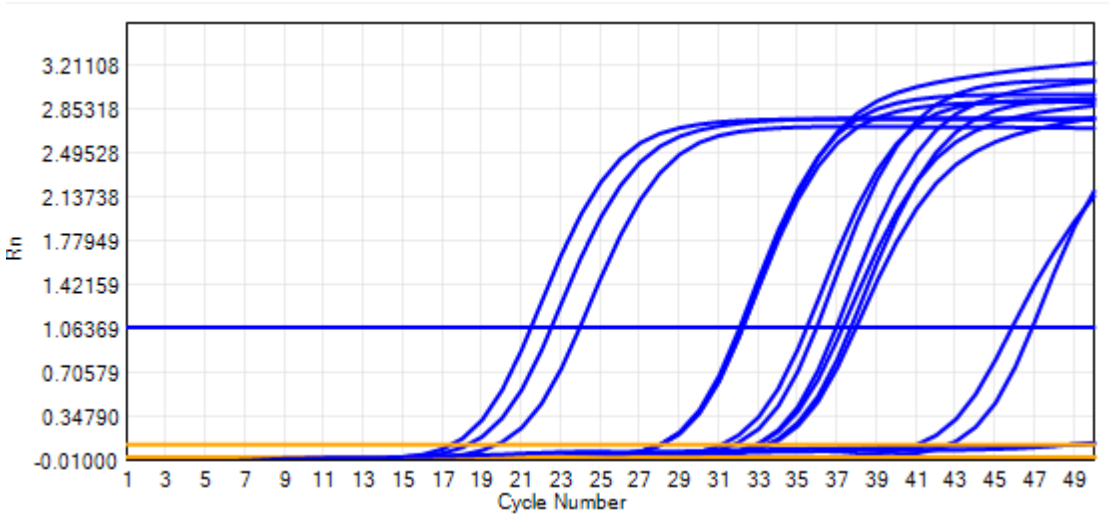


Figure 4.1: Amplification curve of three samples using lepgen

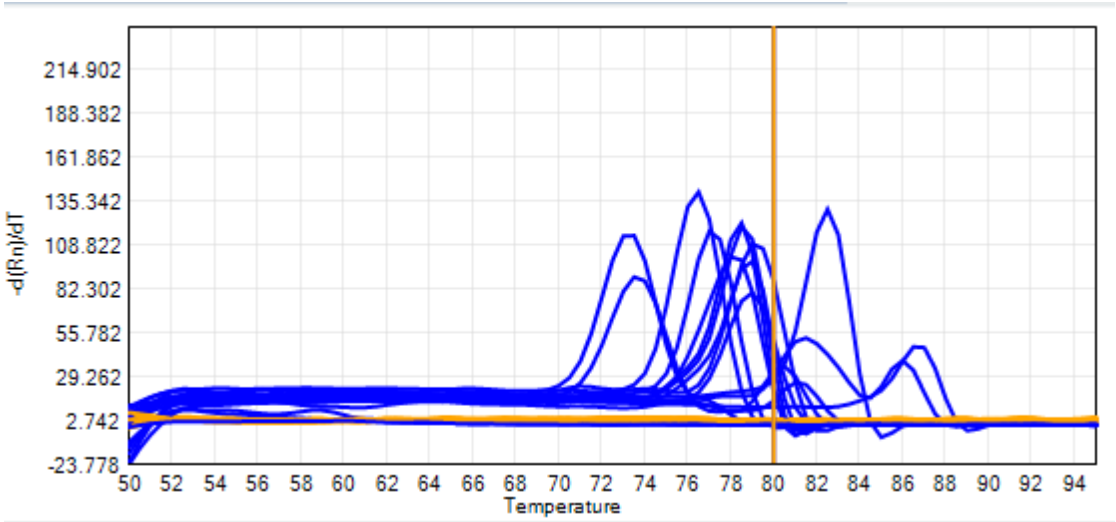


Figure 4.2 Melt peak of three samples using Lepgen

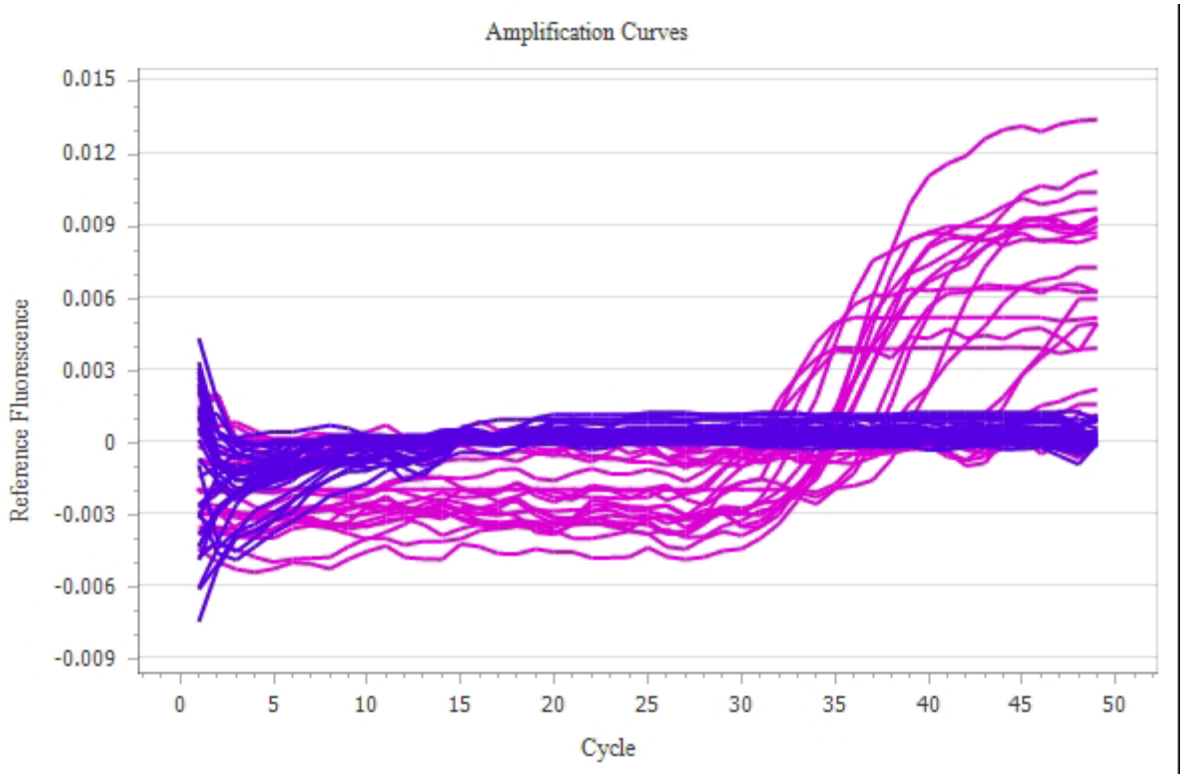


Figure 4.3: Amplification curve using Azure

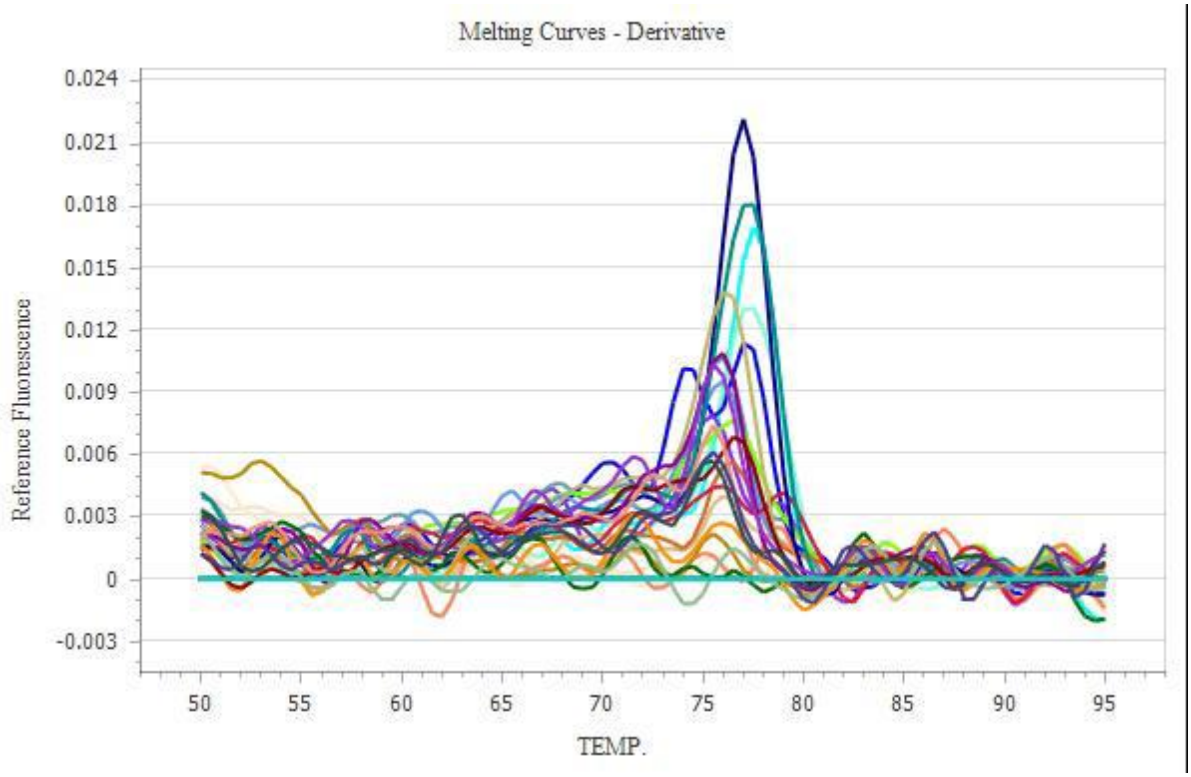


Figure 4.4: Melt curve using azure

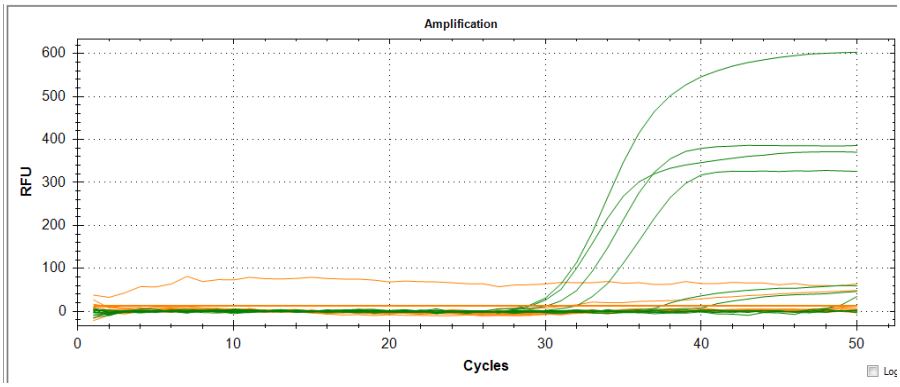


Figure 4.5.Amplification curve using Biorad

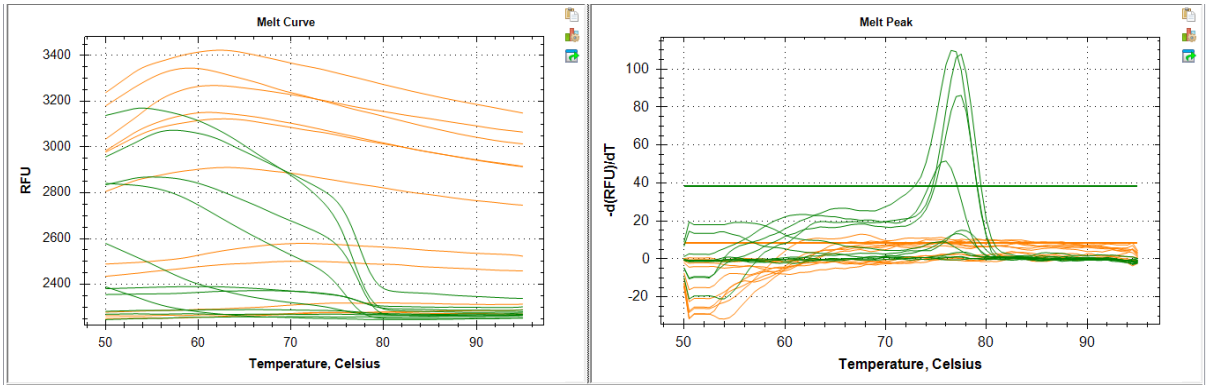


Figure 4.6: Melting Curve using Biorad

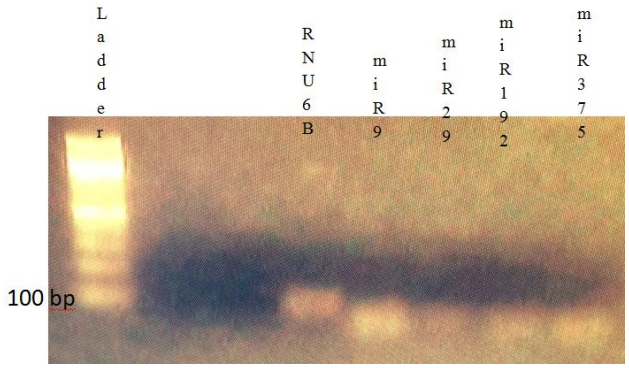
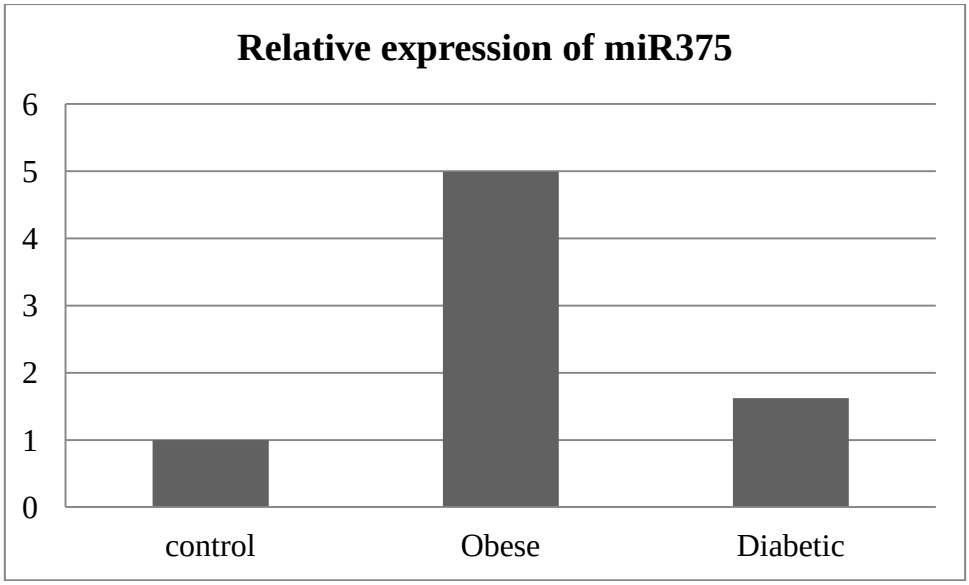


Figure 4.7: Agarose Gel Electrophoresis of RNU6B and miRNA after q-PCR.
The agarose gel electrophoresis was carried out at 1.5% gel using 100bp Solis biodyne ladder 30 minute. The bands of RNU6B was seen near 100 bp and miRNA lower than that.

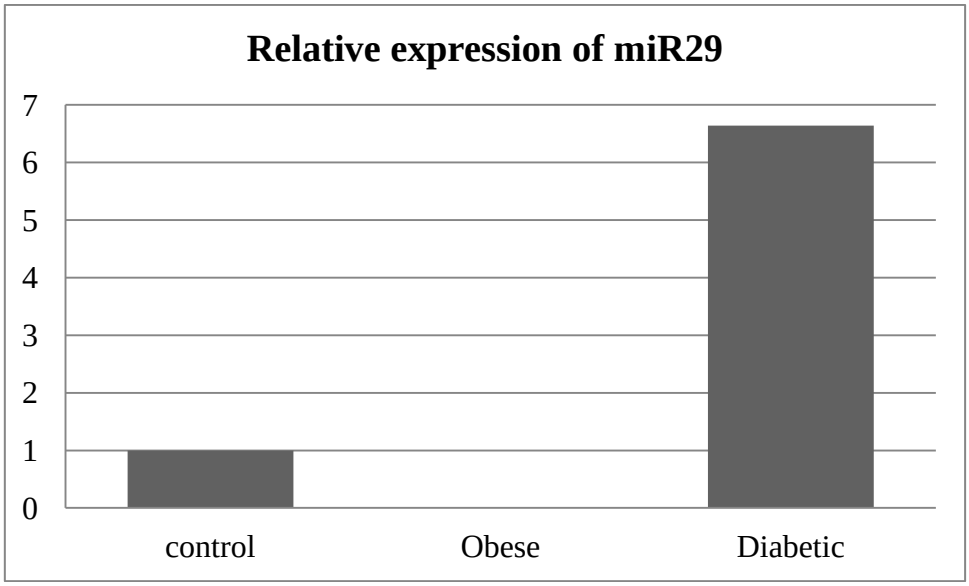
The average RNU6B ct value was 31.39 and all 46 samples gives ct values.
The average miR9 ct value was 35.99 and 44 samples gives ct values.
The average miR192 ct value was 38.21 and 44 samples gives ct values.
The average miR375 ct value was 36.94 and 43 samples gives ct values.
The average miR29 ct value was 40.77 and 18 samples gives ct values.

4.4. Amplification efficiency.
The amplification efficiency of miR9, miR192 and miR375 were found to be 97, 97 and 100 percentage.

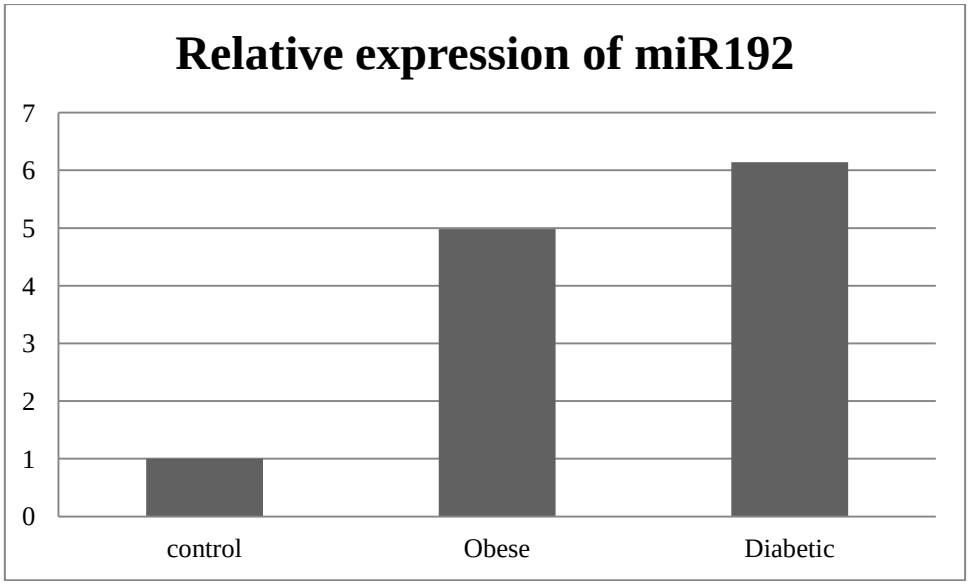
4.5. Relative Expression of miR375, miR192 and miR9 in different Groups
The expression of miR375, miR192 and miR9 were normalized to RNU6B and were determined by SYBR RT-qPCR in plasma of obese, T2DM patients and healthy persons.
Relative expression of miR9 in T2DM patient shows 1.54 for both male and female, 1.20 for female and 1.88 for male, and in Obese patient shows 1.12 for both male and female, 0.84 for female and 1.40 for male.
The relative expression of miR192 in T2DM patient shows 6.14 for both male and female, 2.65 for female and 9.62 for male, and in Obese patient shows 4.98 for both male and female, 4.93 for female and 5.03 for male.
The relative expression of miR375 in T2DM patient shows 1.62 for both male and female, 2.78 for female and 0.45 for male, and in Obese patient shows 4.99 for both male and female, 1.35 for female and 3.65 for male.
The relative expression of miR29 in Obese patient shows 0.006 for both male and female, 0.004 for female and 0.008 for male, and in T2DM patient shows 4.82 for both male and female, 2.95 for female and 6.7 for male.


Relative expression of miR375
6
5
4
3
2
1
0
control
Obese
Diabetic

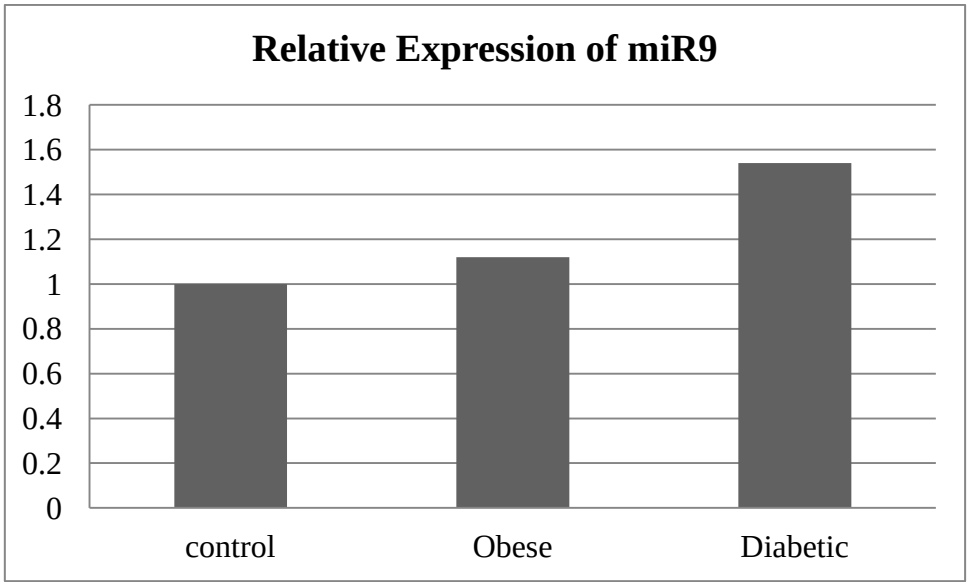
(A)


Relative expression of miR29
7
6
5
4
3
2
1
0
control
Obese
Diabetic

(B)



(C)



(D)

Figure 4.8 . Relative Expression of miR375,miR9, miR129 and miR29 in the Subject Groups.

4.6. ANOVA Analysis

The ANOVA analysis of expression of micro RNA under study in different groups (Diabetic, Obese and Control) shows no significant impact except for miR192 which is p=0.017 between study group and p =0.031 between gender and for miR29 with p=0.0007 for between gender.

Other micro RNA shows no significant impact as p values ranges from 0.11 to 0.99.

Table 4.2: ANOVA Analysis of miR9

Source of Variation	*S.S.*	*D.f.*	*M.S.*	*F*	*P-value*	*F- crit*
Between Study Group	6.71267	8	0.839084	0.913997	0.529535	2.591096
Between Gender	0.015728	2	0.007864	0.008566	0.991475	3.633723
Error	14.68861	16	0.918038			
Total	21.417	26				

Table 4.3: ANOVA Analysis of miR192

Source of Variation	*S.S.*	*D.f.*	*M.S.*	*F*	*P-value*	*F- crit*
Between study group	633.8218	11	57.62016	2.86203	0.017208	2.258518
Between Gender	163.6975	2	81.84877	4.06548	0.031442	3.443357
Error	442.9177	22	20.13262			
Total	1240.437	35				

Table 4.4: ANOVA Analysis of miR375

Source of Variation	*S.S.*	*D.f.*	*M.S.*	*F*	*P-value*	*F- crit*
Between study group	32.13534	12	2.677945	0.647025	0.782091	2.18338
Between Gender	19.37522	2	9.687609	2.340649	0.117855	3.402826
Error	99.33253	24	4.138855			
Total	150.8431	38				

Table 4.5: ANOVA Analysis of miR29

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between study group	0.025103	2	0.012552	1.077194	0.422426	6.944272
Between Gender	1.65514	2	0.82757	71.02255	0.00075	6.944272
Error	0.046609	4	0.011652			
Total	1.726853	8				

Chapter V

Discussion

Type 2 diabetes mellitus (T2DM) is a complex metabolic disorder characterized by insulin resistance and impaired insulin secretion. The development of T2DM involves multiple pathophysiological mechanisms, including dysregulation of gene expression, particularly microRNAs (miRNAs) that play a key role in regulating glucose homeostasis (Shantikumar and Caporali, 2017).

Several miRNAs have been implicated in the development of T2DM, including miRNA-9, miRNA-29a, miRNA-192, and miRNA-375. These miRNAs are involved in the regulation of insulin secretion, insulin sensitivity, and glucose metabolism (Kaviani et al., 2018).

miRNA-9 is expressed in pancreatic β-cells and has been shown to regulate insulin secretion and β-cell proliferation. miRNA-9 may take part in the development of insulin resistance in T2DM by regulating genes taking part in glucose metabolism and insulin signalling (Feng et al., 2016). In our study miR-9 was upregulated in diabetic male, diabetic female and obese male but found downregulated in obese female. This may be due to only 44 samples out of 46 samples for miR-9 gave ct values. Upregulation of Mir9 has been found to be associated with the development and progression of T2DM. Mir9 was upregulated in the pancreas of diabetic mice, and that this upregulation was associated with a decrease in insulin secretion and increased blood glucose levels (Wang et al., 2019). Also, Mir9 was upregulated in the adipose tissue of patients with T2DM, and that this upregulation was associated with insulin resistance (Li et al., 2019). Finally, mir9 was upregulated in the liver of diabetic rats, and that this upregulation was associated with hepatic insulin resistance (Li et al., 2017). miRNA-9 is downregulated in pancreatic β-cells of patients with T2DM and in high-fat diet-induced obese mice, suggesting its role in the pathogenesis of T2DM (Plaisance et al., 2006).

miRNA-29a has been implicated in the regulation of glucose metabolism in the liver and adipose tissue. Studies have shown that miRNA-29a may modulate insulin signaling pathways and contribute to the development of insulin resistance in T2DM (Karolina et al., 2011). In our study miR-29a was found downregulated in Obese but upregulated in T2DM. Only 18 samples out of 46 samples gave Ct values. Mir29a was significantly upregulated in the plasma of T2DM patients compared to healthy controls and the upregulation may be involved in the dysregulation of glucose metabolism and insulin sensitivity seen in T2DM.

(Raitoharju et al. 2015). Mir29a was upregulated in the skeletal muscle of T2DM patients when compared the expression of several microRNAs in skeletal muscle biopsies from T2DM patients and healthy controls, and suggested that Mir29a upregulation may contribute to insulin resistance by inhibiting the expression of genes involved in glucose uptake and metabolism. (Karolina et al. 2011). The expression of several microRNAs in pancreatic islet samples from T2DM patients and healthy controls, found that Mir29a was significantly upregulated in the T2DM group and Mir29a upregulation may contribute to beta-cell dysfunction and impaired insulin secretion, which are key features of T2DM. (Li et al. 2019)

miRNA-29a is also downregulated in T2DM and regulate insulin sensitivity and glucose metabolism in vitro and in vivo (Liang et al., 2014)

The miRNA-192 is predominantly found in the kidney. It has been involved in the control of glucose homeostasis and development of diabetic nephropathy. Studies have suggested that

miRNA-192 may contribute to the pathogenesis of T2DM by regulating the expression of genes involved in glucose metabolism and insulin signaling (Zhang et al., 2017). In our study miR-29a was found upregulated for all groups and 44 samples out of 46 samples gave Ct values. miR-192 was found to be upregulated in the renal tissues of T2DM patients, and its overexpression was associated with the progression of diabetic nephropathy. (Zhang et al. 2016) miR-192 was upregulated in the liver tissues of T2DM mice, and its overexpression was associated with insulin resistance and hepatic steatosis. (Wang et al. 2019) miR-192 was upregulated in the serum of T2DM patients and was positively correlated with fasting blood glucose levels, insulin resistance, and glycated hemoglobin levels. The study also found that miR-192 was a potential diagnostic biomarker for T2DM. (Song et al. 2020)

MiRNA-375 is predominantly expressed in pancreatic islet cells and has been shown to regulate insulin secretion and β-cell proliferation. Studies have suggested that miRNA-375 may be involved in the development of insulin resistance and β-cell dysfunction in T2DM (Wang et al., 2016). In our study miR-375 was found upregulated for all groups and 43 samples out of 46 samples gave Ct values. miRNA-375 is upregulated in pancreatic islets of patients with T2DM and has been shown to regulate insulin secretion and glucose homeostasis (Sharma et al., 2019). miR375 expression was significantly increased in the serum of individuals with T2DM compared to healthy controls, and that this upregulation was correlated with increased insulin resistance and impaired glucose metabolism (Muniyappa et al., 2016). miR375 in vitro led to impaired glucose-stimulated insulin secretion and decreased beta-cell proliferation (Avnit-Sagi et al., 2009) and miR375 expression in mice with T2DM improved insulin sensitivity and glucose tolerance, indicating miR375 may be a potential for the treatment of T2DM (Xu et al., 2021).

miR-192, show significant differential expression in T2DM. Here miR-192 was upregulated (*ur*). According to the results, these miRNA-192 could be employed as T2DM prediction biomarkers.

The statistically insignificant result for other miRNA may be due to many factors. The extraction of RNA was done in this study by using RNA extraction kit may be use of miRNA specific kit would have give better results. MicroRNA (miRNA) extraction kits and RNA extraction kits are two different types of kits used for the isolation of different types of nucleic acids. miRNA extraction kits are designed specifically for the isolation of miRNA from a variety of biological samples, while RNA extraction kits are designed for the isolation of total RNA. The performance of several commercially available miRNA extraction kits found that different kits showed variability in miRNA yield and purity, as well as in the detection and quantification of miRNA by real-time PCR (Mestdagh et al., 2014). RNA extraction kits are designed for the isolation of total RNA, which includes both coding and non-coding RNA, such as miRNA. These kits typically use various lysis methods and column-based purification to isolate high-quality RNA from a variety of biological samples. The performance of several commercially available RNA extraction kits found that different kits showed variability in RNA yield and purity, as well as in the detection and quantification of RNA by real-time PCR (Schmidt et al., 2010). miRNA extraction kits and RNA extraction kits are two different types of kits designed for the isolation of different types of nucleic acids. Researchers should carefully consider the performance of different kits and select the appropriate kit for their specific research question.

Similarly, stem-loop primers were not used in reverse transcription process and also miRNA non specific cDNA preparation kit was used. The use of stem-loop primers and miRNA specific cDNA preparation kit may had given better results. The iScript cDNA Synthesis Kit is a commercially available kit for the preparation of cDNA from RNA samples, including miRNA. The kit uses a combination of oligo(dT) and random primers to ensure efficient cDNA synthesis across a wide range of RNA templates, including miRNA. The iScript cDNA Synthesis Kit has been used in several studies for miRNA cDNA preparation, including for the analysis of miRNA expression in cancer and other diseases (Bader et al., 2010; Zhang et al., 2015).

Furthermore, the samples were processed after three months and were kept in -80^{0}C. It is not recommended to store plasma samples at -80°C for miRNA expression analysis. miRNAs are highly sensitive to degradation, and their expression levels can be affected by storage conditions. Plasma samples should be stored at -20°C or lower to minimize miRNA degradation and ensure reliable miRNA expression analysis. The study found that miRNA levels were stable for up to 1 year when plasma samples were stored at -80°C, but only when the samples were stored in a specialized stabilization buffer. When plasma samples were stored without stabilization buffer, miRNA levels were significantly decreased after 1 week at -80°C. Therefore, if plasma samples are to be used for miRNA expression analysis, it is important to store the samples at -20°C or lower and to use a stabilization buffer if long-term storage at -80°C is necessary. (Pritchard et al., 2012)This may had caused some unwanted effect in our study.

The SYBR green of two different lot had been used in this study which may cause different results than expected. When using SYBR Green for miRNA quantification, it is important to use the same lot of SYBR Green throughout the experiment to minimize variability. Differences in the composition or performance of different lots of SYBR Green can affect the accuracy and precision of miRNA quantification. The performance of two different lots of SYBR Green for miRNA quantification and found that the lots produced comparable results for most miRNAs, but some miRNAs showed significant differences in expression levels between the two lots. Caution should be taken when comparing miRNA expression levels between different SYBR Green lots, and that it may be necessary to validate results using other methods, such as TaqMan probes. (Ko et al., 2013)

Only 46 samples were used in the study. The sample size should be 120 as per 8.5 % prevalence of T2DM at 95% confidence interval with 5% margin of error. Because of the economical constrain the samples cannot be increased. When planning a miRNA study, it is important to consider the statistical power and sample size needed to detect meaningful differences in miRNA expression levels. In general, the sample size needed for miRNA studies depends on several factors, including the expected effect size, the variability of miRNA expression levels, the significance level, and the statistical power. As a general rule, a larger sample size can increase the statistical power and improve the reliability of the results. However, increasing the sample size can also increase the cost and complexity of the study. In the case of having 46 samples available for miRNA research, it is possible to estimate the sample size needed to achieve a certain statistical power based on the expected effect size and the variability of miRNA expression levels.To achieve a higher statistical power or to detect smaller effect sizes, a larger sample size may be needed. As a general

guideline, a sample size of at least 100-200 samples is often recommended for miRNA studies to ensure adequate statistical power and reduce the risk of false-positive or false-negative results. (Cirera & Busk, 2014)

The study was done by using three RT-PCR (Real Time Polymerase Chain Reaction) machine. This may have also affected our results. When performing miRNA research, it is important to maintain consistency and standardization in the experimental procedures, including the use of RT-PCR machines. Using multiple RT-PCR machines can introduce variability in the results, which can affect the accuracy and reproducibility of the miRNA expression data. The effect of using three different RT-PCR machines on the miRNA expression results and found that the use of different machines can result in significant differences in the measured miRNA expression levels. The authors suggested that the variability could be due to differences in the amplification efficiency or detection sensitivity of the different machines. Therefore, it is recommended to use the same RT-PCR machine throughout the experiment to minimize variability and ensure consistency in the results. If it is necessary to use different machines, it is important to perform calibration experiments to assess the variability between the machines and to adjust the data accordingly. In addition to the RT-PCR machines, others factors that can affect the variability of miRNA expression data include the RNA extraction method, the cDNA synthesis protocol, and the normalization strategy. It is important to carefully control these variables and to use appropriate quality control measures to ensure the reliability and accuracy of the miRNA expression data.

Chapter VI

Conclusion

The miRNA-9, miRNA-29a, miRNA-192, and miRNA-375 have been implicated in the regulation of glucose metabolism and insulin signaling pathways in T2DM. The miRNA 192 shows significant upregulation. It can serve as biomarker for T2DM early detection. Similarly, other miRNAs can be used to determine other diseases.

The upregulation of miR-29a has been implicated in the pathogenesis of T2DM. Studies have shown that miR-29a is upregulated in the plasma, skeletal muscle, and pancreatic islets of T2DM patients, and may contribute to the dysregulation of glucose metabolism, insulin resistance, and beta-cell dysfunction seen in the disease. miR-192 upregulation is associated with T2DM pathogenesis and could be a potential diagnostic biomarker for T2DM. Upregulation of Mir375 appears to be involved in the development of T2DM, contributing to insulin resistance and impaired beta-cell function. In order to fully understand the function of miR-29a, miR-192, miR-9 and miR-375 in T2DM and to explore its potential as a therapeutic target more study is required.

Chapter VII

Limitations of the Study

1. Samples were collected only once.
2. Sample size was small and thus findings of this study may not be generalized or extrapolated to other population
3. Total RNA kit was used instead of specific miRNA extraction kit can be used.
3. Stem-loop primers and specific cDNA preparation kit for miRNA can be used.
4. Taqman Probes can be used for specific miRNA.
5. Diagnostic performance of the microRNAs (e.g. sensitivity, specificity, cut off values) could not be determined
6. Being a cross-sectional study, a follow up analysis could not performed to observe the subsequent changes in the plasma expression of microRNA with the progression of the disease.

Recommendation

1. Similar study with large sample size should be carried out to increase the statistical power of the study result
2. Specific microRNA extraction and cDNA synthesis reagent kits should be used to increase the sensitivity and specificity of the experiement
3. A prospective study may be designed and conducted to validate the biomarker potential of the abnormally expressed microRNA
4. Other advanced and specific techniques (e.g. microarray, NGS) may be employed to confirm the finding of this study
5. Further study should be done using other samples like urine.

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APPENDIX

Appendix 1: Table showing ct values with date, volume and machine

Date	Volume	Machine	Sample code	ct value				
				RNU6B	miR9	miR29a	miR197	miR375
21/3/2078	25	Azure	15C	34.338	39.666	39.197	36.201	0
			16D	35.465	0	0	37.881	0
14/7/2021	25	Lepgen	15C	17.43	31.73	31.15	28.05	42.61
	20			18.03				41.05
	25		16D	19.72	32.95	48.94	28.07	0
	25		22O	18.72	32.88	33.4	28.03	33.51
16/7/2021	20	Lepgen	4C	24.81	32.99	35.22	31.22	33.29
			14C	26.29	35.93	39.18	28.34	0
			16C	25.19	35.88	0	29.29	33.53
			22C	23.28	32.13	31.02	29.87	47.36
			41C	26.57	47.96	37.95	28.15	33.3
			42C	25.67	36.65	32.95	30.37	32.18
			4D	25.31	31.61	0	30.14	33.32
			6D	27.11	0	43.86	28.49	33.27
			7D	26.04	39.85	0	28.04	0
			24D	26.41	32.92	0	28.61	0
			26D	26.08	36.59	42.96	28.14	0
			38D	26.26	48.33	0	31.26	32.26
			3O	26.12	33.19	33.48	27.5	32.61
			16O	26.08	0	44.08	28.33	34.29
			22O	25.93	0	33.43	28.02	0
			24O	25.84	0	0	28.25	0
			30O	24.99	0	36.94	28.29	47.11
			47O	26.55	0	32.18	27.55	35.22
			55O	25.73	0	0	27.71	0
22/3/2022	10	Azure	29C	37.93	32.924	0	46.027	35.374
				37.56	33.801	0	47.881	36.036
			34O	38.886	37.405	0	39.488	32.15
				37.99	32.322	0	44.033	31.821
			27D	35.469	35.084	0	0	0
				36.58	35.206	0	0	0
29/3/2022	10	Azure	36C	38.364	37.349	0	47.238	37.832
				39.23	36.615	0	47.881	36.036
			45O	39.388	37.419	0	0	37.374
				38.45	36.786	0	0	0
			35D	36.846	35.079	0	0	36.858
				35.978	34.92	0	0	39.011

31/3/2022	10	Azure	3C	41.807	35.016	33.918	0	34.096
				40.983	34.357	0	37.589	35.044
			19C	39.25	34.966	0	0	35.909
31/3/2022	10	Azure	19C	38.87	35.942	0	36.634	36.762
			40C	34.426	36.326	0	0	37.097
				33.895	35.587	0	35.576	36.417
			1D	35.852	36.674	0	43.898	0
				36.432	36.932	0	41.304	0
			35O	39.308	35.975	0	0	37.893
				38.241	35.306	0	0	0
			36O	0	37.404	0	42.171	34.294
				32.453	35.259	0	0	35.93
			19O	31.321	37.379	0	0	36.728
				30.983	37.084	42.726	0	0
			1D	0	35.264	0	42.18	38.873
				33.345	35.351	0	0	37.904
			43D	35.462	36.439	39.15	0	0
				36.592	35.37	42.349	35.152	37.382
			45O	38.948				34.357
				37.452				37.11
4/4/2022	10	Azure	10C	40.9195	37.999		34.968	39.366
			13C	40.1015	38.983		36.196	40.0735
			12C	38.22	42.6725		38.668	37.2235
			31C	36.56	37.295		34.211	0
			44O	39.39	38.2545		45.875	37.4315
			5O	39.175	36.4725		44.969	36.893
			13O	36.998	38.9325		40.048	36.6075
			6O	37.08	36.2125		37.5475	40.511
			21O	36.718	35.8855		40.511	36.401
9/6/2022	10	Biorad	33C	31.5	32.18	0	0	33.83
			33D	33.06	32.18	0	0	47.93
18/6/2022	10	Biorad	6D	30.58	30.78			30.99
			7D	32.33				31.52
			16D	32.41				35.16
			24D	30.17				32.24
			26D	30.16				31.16
			27D	30.69			34.8	35.77
			4D	30.15	33.5			
			13C	30.75	31.11			32.77

			16C	30.74				48.47
			29C	32.41			40.12	33.35
			10C	31.74	31.05			
18/6/2020	5	Biorad	MC	29.8	27.21	0	30.5	30.3
				29.82				
			DO	28.55	34.37	48.98	32.88	42.22
				30.48				
			PD	29.33	33.27	35.97	42.1	45.83
				30.66				

Appendix 2: Ethical Approval

MANIPAL COLLEGE OF MEDICAL SCIENCES
POKHARA, NEPAL

(INSTITUTIONAL REVIEW COMMITTEE: APPROVED BY NHRC VIDE LETTER REF NO 1296 DATED 16TH FEB 2015)

MEMG/IRC/241/GA

08 May 2019

Dr Daya Ram Pokharel
Professor
Department of Biochemistry
MCOMS/MTH

Subject : **Permission for Conduct of Research Study.**

Dear Dr Daya Ram Pokharel,

1. Reference proposal received under your application dated 03 April 2019.

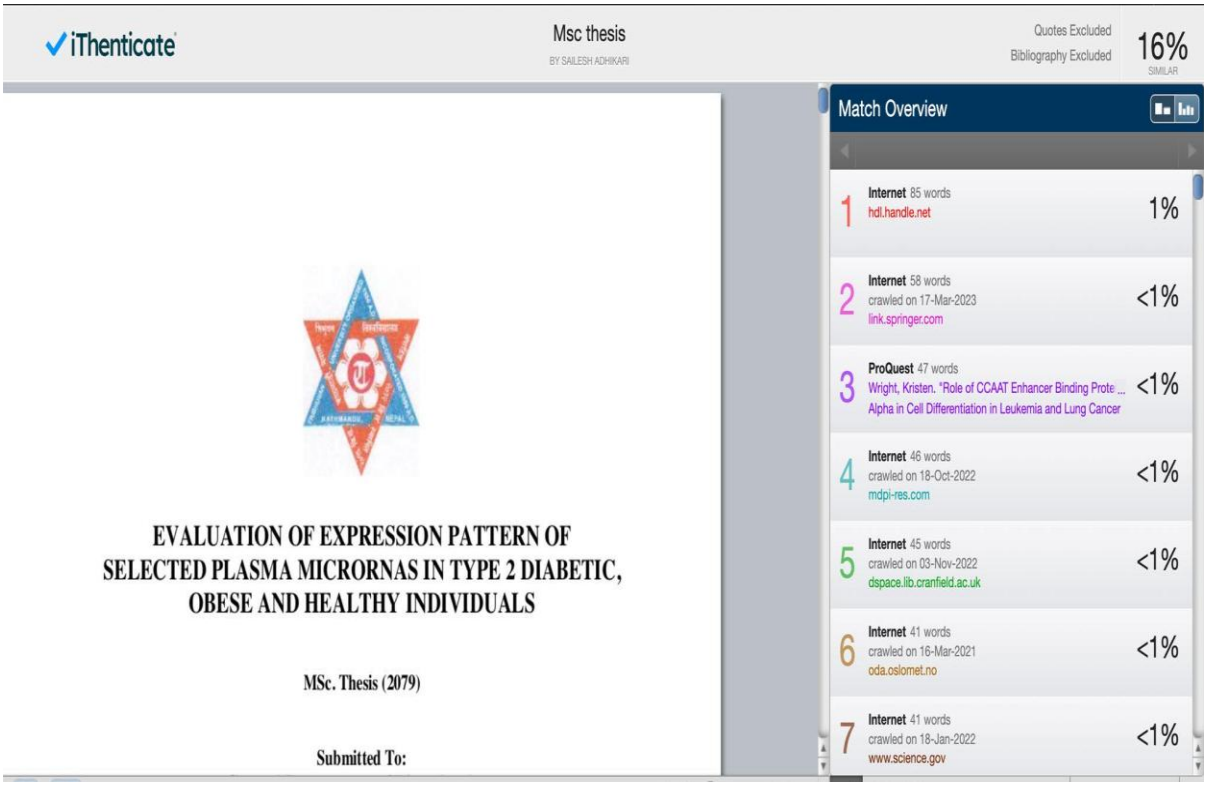
2. Permission is hereby accorded to carry out research study namely, **"Evaluation of Expression Pattern of selected plasma microRNAs as early prediction biomarkers of Type 2 Mellitus : A Pilot Study".**

3. You are required to :-

   (a) Submit progress report on the above study on quarterly basis.

   (b) Submit a copy each of the study on its completion.

Prof Dr VM Alurkar
Chairman,
IRC, MCOMS, Pokhara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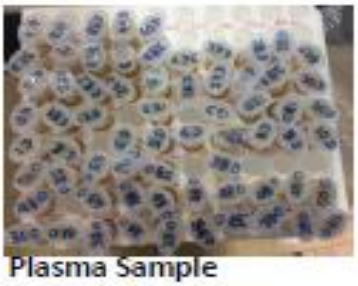


Plasma Sample

RNA Extraction Kit

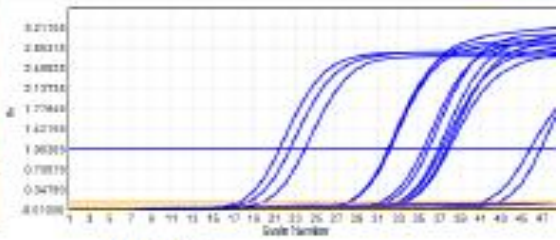
NanoDrop to measure RNA

cDNA preparation Kit

nanoDrop measure cDNA

RT-PCR Kit

RT-PCR machine



Results