Circulating miRNAs as Biomarkers of Type II Diabetes Mellitus Patients in Gaza Strip

جزيئات الحامض النووي الريبوزي الصغيرة كعلامة حيوية لمرضى السكري من النوع الثاني في قطاع غزة

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Declaration

I understand the nature of plagiarism, and I am aware of the University’s policy on this.

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Student's name: Nedal M. Qaddoura
Signature: ___________________________
Date: 1/9/2018
نتيجة الحكم على أطروحة ماجستير

بناءً على موافقة عمادة البحث العلمي والدراسات العليا للجامعة الإسلامية بغزة على تشكيك لجنة الحكم على أطروحة الباحث/ إسلام محمد عوض أبراهيم قيودة لمنطقة درجة الماجستير في كلية العلوم/ برنامج العلوم الطبية تحاليل طبية وموروثية:

جزياتات الحمض النووي الريبوزي الصغيرة كعلامة حيوية لمرض المكري من النوع الثاني في قطاع غزة

Circulating miRNAs as Biomarkers of Type II Diabetes Mellitus Patients in Gaza Strip

وبعد المناقشة التي تمت اليوم السبت 11 محرم 1440هـ الموافق 22/09/2018م الساعات الواحدة ظهرًا، في قاعة اجتماعات الكلية أجمعت لجنة الحكم على الأطروحة والمكونة من:

- صائب حسين البعيري
- محمد عاشي
- كمال عبد الحكوات
- محمد سراج

وبعد المناقشة أوصت اللجنة بمنح الباحث درجة الماجستير في كلية العلوم/ برنامج العلوم الطبية تحاليل طبية.

واللائحة الإذن بإصدار هذه الدرجة فإنما توصى بتقديم الاعتماد والتصريح والطاعة وأن يسخر عمه في خدمة دينه ووطنه.

"ولله ولي التوفيق"

أ. د. مازن إسحاق هنية

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أ. من هي أنجالم نورتون؟

إن أنجالم نورتون هي عالمة في مجال الدراسات الإسلامية. كانت أصلًا تايلاندية ولدت في 1935 وتوفيت في 2012. استقلت مسيرتها الأكاديمية في دولة جزيرة كونكورد في الولايات المتحدة، حيث صدرت كتابها الأول "الإسلام في ظل الاستعمار" في عام 1972. انتقلت إلى إسلامها الديني بعد ذلك. من خلال أعمالها البحثية، تركزت نورتون على دراسة الأفكار الإسلامية في العالم الغربي، وهي عضو في بعض الجمعيات الدراسية الدينية والعلمية.

١٢. اParagraph.

١٢. اParagraph.

١٢. اParagraph.
Abstract

Background and Problem.

Type II Diabetes mellitus (T2DM) is one of the metabolic syndrome and recognized as one of the most serious health problems worldwide. Compared with people without diabetes, individuals with T2DM have a significantly higher risk of cardiovascular morbidity and mortality. Heart attack and stroke are the major causes of early death in people with diabetes. Prognostic biomarkers including Low density lipoprotein (LDL-C), cholesterol (TC), Triglycerides (TG) and high density lipoprotein (HDL-C) and high sensitive C-reactive protein (hsCRP) are widely used to predict cardiovascular diseases (CVDs) in T2DM patients in different population. Recent studies have suggested that microRNAs (miRNAs) play a critical role in the pathogenesis of diabetes and its related cardiovascular complications.

Objectives and aims.

This study is a case-control study aimed to investigate the significance of 8 miRNAs (hsa-miR-29a, hsa-miR-375, hsa-miR-103a-3p, hsa-miR-124-3p, hsa-miR-155-5p and hsa-miR-146a-5p, miR-126 and miR-222) in the prediction of cardiovascular complications among 86 T2DM patients diagnosed with and without cardiovascular diseases (CVDs) and 30 healthy individuals.

Methodology.

Total RNA, including miRNA was isolated from serum samples using the ISOLUTE II Biofluids. hsCRP was measured using immunoturbidimetric technique. TC, TG and HDL-C were measured using colorimetric and kinetic methods and LDL-C was calculated by empirical relationship of Friedewald. Fasting blood sugar (FBS), hemoglobinA1c (HBA1c), Lactate dehydrogenase (LDH), Creatine phosphokinase (CPK), Aspartate aminotransferase (AST), were measured using enzymatic methods.
Results.

The levels of hsCRP, FBS, HbA1c, TC, TG, LDL, LDH, CPK, were significantly increased among T2DM patients compared with the normal control (P≤0.05). Importantly, 68.6%, 87.2% and 48.8% of the cases showed high level of FBS, HbA1c and hsCRP respectively; 35% of cases had high levels of cholesterol > 240 mg/dl while 68% of cases have high level of TG >150mg/dl; 39.6% of cases have high level of LDL-c >130mg/dl and 34.8% of cases have low level of HDL-c <40mg/dl. The expression levels of all tested miRNAs except of miR-375 were significantly decreased in patients groups in comparison with healthy persons. Importantly, among all tested miRNAs, hsa-miR-126-5p showed the highest diagnostic accuracy as revealed by receiver operating characteristic (ROC) curve analysis and can relatively discriminate between patients groups and control.

Conclusions.

Taken together serum miRNAs are differentially expressed between T2DM, T2DM with cardiovascular disorders and healthy persons and could be considered as a candidate’s biomarkers to predict these disease which warrants further evaluation and optimization.

Keywords: Type II diabetes mellitus, miRNA, cardiovascular complications.
المستند

الملخص

خلفية البحث

يعد النوع الثاني من مرض السكري (T2DM) من الأمراض المزمنة والشائعة ويعتبر واحداً من أخطر المشاكل الصحية في العالم، المرضى من هذا النوع لديهم خطر تعرض لأمراض القلب والأوعية الدموية والتي تؤدي إلى زيادة نسبة الوفيات. وتسبب الأطعمة الغفيرة والسكرات الدسمة من أسباب الوفاة المفاجئة لدى مرضى السكري يوجد العديد من المؤشرات والفحوصات التي تستخدم لتتبع بأمراض القلب والشرايين المصاحبة لمرض السكري بشكل مبتكر مثل البروتين الدهني منخفض الكثافة (LDL-C) والكولسترول (TC) في الطائرات الثلاثية، والبروتين الدهني عالي الكثافة (HDL-C) والبروتين الدهني عالي الكثافة (TG) والبروتينات ذات الحساسية العالية (hsCRP)

أهداف البحث

تهدف الدراسة إثبات أهمية 8 أنواع من الميرنا (miRNAs):

( hsa-miR-29a, hsa-miR-375, hsa-miR-103a-3p, hsa-miR-124-3p, hsa-miR-155-5p,
hsa-miR-146a-5p, miR-126 and miR-222 )

في التنبؤ بمضاعفات أمراض القلب في نوع الثاني من مرض السكري الذين تم تشخيصهم أو لم يتم تشخيصهم بأمراض القلب بالإضافة إلى 30 شخص سليم.

منهجية البحث

تم استخلاص الحمض النووي من عينات الدم بواسطة ISOLATE II Biofluids colorimetric and kinetic، وتتم فحص البروتينات ذات الحساسية العالية (hsCRP) باستخدام تقنية IMMUNOTURBIDIMETRIC وTC وTG، وتتم فحص البروتين الدهني منخفض الكثافة (HDL-C)، وتتم أيضاً فحص السكر في الدم للصائم (FBS)، مخزون السكر في الدم (HbA1c)، LDH، CPK، AST باستخدام الطرق الأنزيمية.

النتائج

تبين النتائج أن فحوصات مستويات البروتينات علي الحساسية، السكر في الدم، نسبة مخزون السكر في الدم، البروتين الدهني منخفض الكثافة، والكولسترول، الدهون الثلاثية، البروتين الدهني عالي الكثافة، لها أهمية تشخيصية ويعد معلمها عن العامل الطبيعي في مرضى السكري من النوع الثاني مقارنة بالأفراد الأصحاء (p≤0.05). أظهرت النتائج أيضاً أن 68.6، 87.2، 48.8 % من الحالات شهدت ارتفاع في
على التوالي، بالمقابل 35% من الحالات معدل الكولسترول عالي >240 mg/dl، بينما 68% من الحالات كانت الدهون الثلاثية فيها >150mg/dl. في المقابل، 39.6% من الحالات كان معدل الدهون منخفضة الكثافة <130mg/dl، و 34.8% من الحالات تحتوي على معدل منخفض من الدهون عالية الكثافة <40mg/dl.

تبيّن أنّها منخفضة عند مرضى السكري مقارنة بالأشخاص الأصحاء. مقارنة بجميع miRNA المفحوصة، تبين أن hsa-miR-126-5p له قيمة تشخيصية عالية في منحنى ROC عند مقارنة المرضى بالأشخاص الأصحاء.

الاستنتاجات

فحص miRNA له أهمية فارقة وكبيرة في تشخيص والتفرّق بين مرضى السكري من النوع الثاني والأصحاء. وهذا يضيفة الحاجة إلى مزيداً من الدراسات المستقبلية.

الكلمات المفتاحية

السكر من النوع الثاني، الميرنا، امراض القلب والأوعية الدموية.
Dedication

I would like to dedicate my work to my special, profound and affectionate thanks, love, affectionate gratitude and deep indebtedness are due to my wife, Nahed, who has been struggling with me, hand by hand, to secure and shape brighter future. Her understanding, support, commitment and looking after my children during my study all stand behind my success. At the same time, I would like to express my love and thanks to the beats of my heart, my children, Ahmed, Huda, Mohammed, Abdulrahman and Hadeel, who are the only source of inspiration to me, and it is their love and innocent smiles that have made the hardship of this task bearable.

I wish to express my thanks and gratitude to my parents, Mohammed Awad and Huda, the ones who can never ever be thanked enough, for the overwhelming love and care they bestow upon me. Your prayers for me was what sustained me thus far.

Without the support of my dear sisters Mervat, Basma, Fadwa, Rajeha and Hanadi, and dear brothers Ibrahim, Wesam, Raed and Mohammed, I could not possibly have finished this work.

I do not forget to express my deep sense of gratitude to my uncle Mr. Yacoub Soliman, Dr. Fae’q Naouq, Dr. Mansour Al Yazji and Dr. Ahmed Eid Alnajjar for their unlimited moral support to finish this work.

This work also is dedicated to the martyrs of the Palestinian people.
Acknowledgment

All praise and thanks are due to the Almighty Allah who always guides me to the right path and has helped me to complete this thesis.

There are many people whom I have to acknowledge for their support, help and encouragement during the journey of preparing this thesis. So, I will attempt to give them their due here, and I sincerely apologize for any omissions.

First and foremost, I would like to record my gratitude to my supervisors Dr. Saeb Aliwaini (Assistant Prof. of Molecular Cell Biology- Islamic University of Gaza), and Dr. Basim Ayesh (Associate Prof. of Molecular Biochemistry - Al-Aqsa University - Gaza) for their supervision, advice and guidance from the early stage of this research as well as giving me extraordinary experiences throughout the work. Above all and the most needed, they provided me unflinching encouragement and support in various ways. I am really indebted to them more than they know.

I wish to express my sincere thanks and gratitude to Mr. Rabee Awad and Dr. Essam Dawood for their helping me to handle the statistical analyses of my research.

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<th>Definition</th>
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<tr>
<td>AGEs</td>
<td>advanced glycation end products</td>
</tr>
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<td>AMI</td>
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</tr>
<tr>
<td>AR</td>
<td>acute rejection</td>
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<td>AUC</td>
<td>Area under the curve</td>
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</tr>
<tr>
<td>LDLc</td>
<td>low-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
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<tr>
<td>LPSs</td>
<td>lipopolysaccharides</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCV</td>
<td>mean cell volume</td>
</tr>
<tr>
<td>MDH</td>
<td>malate dehydrogenase</td>
</tr>
<tr>
<td>miRNAs</td>
<td>microRNAs</td>
</tr>
<tr>
<td>MOH</td>
<td>ministry of Health</td>
</tr>
<tr>
<td>NAC</td>
<td>N-Acetylcysteine</td>
</tr>
<tr>
<td>ncRNA</td>
<td>non-coding RNA</td>
</tr>
<tr>
<td>NDR</td>
<td>National Diabetes Register</td>
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<tr>
<td>NGOs</td>
<td>Non-Governmental Organizations</td>
</tr>
<tr>
<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
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<tr>
<td>NOD</td>
<td>nonobese diabetic</td>
</tr>
<tr>
<td>OGTT</td>
<td>oral glucose tolerance test</td>
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<tr>
<td>oxLDL</td>
<td>oxidized low density lipoprotein</td>
</tr>
<tr>
<td>PAI1</td>
<td>Plasminogen activator inhibitor 1</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PDK1</td>
<td>protein 3-phosphoinositide-dependent protein kinase-1</td>
</tr>
<tr>
<td>PER3</td>
<td>Period Circadian Regulator 3</td>
</tr>
<tr>
<td>PHIC</td>
<td>Palestinian Health Information Center</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMMS</td>
<td>Palestinian Military Medical Services</td>
</tr>
<tr>
<td>POD</td>
<td>peroxidase</td>
</tr>
<tr>
<td>PPARG</td>
<td>Peroxisome Proliferator Activated Receptor Gamma</td>
</tr>
<tr>
<td>PPP1R13L</td>
<td>protein phosphatase 1 regulatory subunit 13 like</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PUMA</td>
<td>P53 up-regulated modulator of apoptosis</td>
</tr>
<tr>
<td>RBP4</td>
<td>Retinol-binding protein 4</td>
</tr>
<tr>
<td>RDW</td>
<td>red blood cells distribution width</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNA Pol II</td>
<td>RNA polymerase II</td>
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<tr>
<td>RNA Pol III</td>
<td>RNA polymerase III</td>
</tr>
<tr>
<td>SHIP1</td>
<td>SH-2 containing inositol 5' polyphosphatase 1</td>
</tr>
<tr>
<td>SOCS1</td>
<td>Suppressor Of Cytokine Signaling 1</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type I diabetes mellitus</td>
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<tr>
<td>T2DM</td>
<td>Type II diabetes mellitus</td>
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<tr>
<td>T2DM/CVD</td>
<td>T2DM with cardiovascular disease</td>
</tr>
<tr>
<td>TCF7L2</td>
<td>The transcription factors 7-like 2</td>
</tr>
<tr>
<td>TG</td>
<td>triglycerides</td>
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<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLRs</td>
<td>toll like receptors</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>ROC</td>
<td>receiver operating characteristic</td>
</tr>
<tr>
<td>TOM1</td>
<td>target of Myb protein 1</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor-associated factor 6</td>
</tr>
<tr>
<td>TU</td>
<td>transcriptional unit</td>
</tr>
<tr>
<td>UPR</td>
<td>unfolded protein response</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSDs</td>
<td>ventricular septal defect</td>
</tr>
<tr>
<td>XBP1</td>
<td>X box-binding protein 1</td>
</tr>
<tr>
<td>β-ME</td>
<td>β-Mercaptoethanol</td>
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</table>
Chapter 1
Introduction
Chapter 1

Introduction

1.1 Overview

Type II Diabetes mellitus (T2DM) is a global health problem, individuals of all ages, genders and races may be affected by this disease. In 2012, 9 % of adult persons worldwide were diagnosed with T2DM and 1.5 million died of it (Sharma, Nazareth, & Petersen, 2016).

T2DM is associated with insulin resistance and long term hyperglycemia which result in serious complications that affects different organs and systems. Macrovascular complications (coronary artery disease, peripheral arterial disease and stroke) and microvascular complications (diabetic nephropathy, neuropathy and retinopathy) are the most common causes of the great morbidity in T2DM subjects (Zhang et al., 2010; Kong et al., 2011). Prognostic biomarkers including low density lipoprotein cholesterol (LDL-c), high density lipoprotein cholesterol (HDL-c) are widely used to predict cardiovascular diseases (CVD) in different populations such as T2DM patients. Another group of biomarkers such as hs-CRP, interleukin-6 (IL-6), MCP-1, tumour necrosis factor-α (TNFα) and Vascular endothelial growth factor (VEGF) are applied in diagnosis and prognosis of CVD (Shoamanesh et al., 2015; Leeuw et al., 2016).

Recently, microRNAs (miRNAs) have been shown to be key modulators in the various cardiovascular events through the regulation of cardiac gene expression. Besides their credible involvement in controlling the cellular processes, they are also released into the circulation in disease states where they serve as potential diagnostic biomarkers for the disease. However, their potential role in diabetic heart disease as modulators as well as diagnostic biomarkers still under investigations.

Certain miRNAs in particular level of eight miRNAs, namely has-miR-29a-3p, has-miR-155-5p, has-miR-126-5p, has-miR-103a-3p, has-miR-124-3p, has-miR-146a-5p, has-miR-222-3p and has-miR-375 have been shown to have abnormal expression in T2DM and in certain CVD such as acute myocardial infarction (AMI) (Ai et al., 2010; Long et al., 2012; Leijia, et al., 2013; Peng et al., 2014; Zhu & Leung, 2015; Caporali, Miscianinov, Saif, & Emanueli, 2016; Chien et al., 2016). Altered mir-29
expression has been reported in type 2 diabetes patients which influences glucose and lipid metabolism in skeletal muscle regulate the genesis of myocardial fibrosis (Roncarati et al., 2014; Massart et al., 2017).

However, it's not known whether these miRNAs can be used as biomarkers to predict cardiovascular complications among T2DM. The current study is the first to investigate these miRNAs among Palestinian individuals in Gaza strip.

1.2 **Aim of the study:**
To evaluate the potential role of circulating miRNAs as a new diagnostic biomarkers of T2DM Patients in Gaza Strip.

1.3 **Specific objectives:**
   1. To evaluate the differential expression of specific microRNAs (hsa-miR-29a-3p, hsa-miR-155-5p, hsa-miR-126-5p, hsa-miR-103a-3p, hsa-miR-124-3p, hsa-miR-146a-5p, hsa-miR-222-3p and hsa-miR-375) in Palestinian individuals with T2DM, T2DM with CVD in comparison to healthy individuals.
   2. To investigate the level of insulin, C-peptide, lipid profile, glycated Albumin, HbA1c, fasting blood sugar (FBS), kidney function tests in the blood of the studied cases and control.
   3. To test the correlation between important biochemical and clinical markers for T2DM and T2DM/CVD with the expression of the tested miRNAs.

1.3 **Significance**
T2DM is the most common chronic disease worldwide and in Palestine and most of T2DM related deaths are due to its cardiovascular complications. In spite of the huge efforts spent to predict diabetes mellitus and it complications, this disease still diagnosed late. Therefore, determining confident biomarkers to predict this disease is very significant. In Gaza this project has a special value as it is the first study to identify miRNAs as biomarkers for T2DM and its complications. This project further
provides more information about the traditional biomarkers and the association between these biomarkers and miRNAs.
Chapter 2
Literature Review
Chapter 2
Literature Review

2.1 Diabetes Mellitus

One of the most common public health problems today is diabetes mellitus (DM), which constitutes a series of metabolic dysfunction that is manifested by a chronic increase of glucose level, which is caused by defective insulin secretion, insulin action or both (Kharroubi & Darwish, 2015).

Diabetes consists of two major types: Type one diabetes mellitus (T1DM) constitutes an autoimmune disorder leading to the destruction of pancreatic β-cells and T2DM which is much more common and primarily caused by progressively impaired glucose regulation as result of dysfunctional pancreatic β-cells and insulin resistance (Kerner, Bruckel, & German Diabetes, 2014; Ignatavicius & Workman, 2015).

2.1.1 T1DM

T1DM is a chronic autoimmune disease with both environmental and genetic factors that is characterized by insulin deficiency caused by pancreatic β-cell loss and subsequently leading to hyperglycemia (Katsarou et al., 2017).

According to the International Diabetes Federation (IDF), 8.8% of adults worldwide are diabetic and 10-15% of them are T1DM and most of them are children (Katsarou et al., 2017; Kharroubi & Darwish, 2015). The prevalence of T1DM is growing worldwide with more than 86,000 children (new cases) are detected each year. In 2015 the global prevalence of T1DM in children was 542,000 cases (International Diabetes Federation, IDF, 2015).

2.1.2 T2DM

T2DM is the most prevalent form of diabetes and accounts for at least 85-90% of all diabetic patients (González, Johansson, Wallander, & Rodríguez, 2009). It is characterized by chronic hyperglycemia (elevated blood glucose level) linked to both insulin resistance and pancreatic β-cells dysfunction that is leads to impaired insulin secretion (Petrie, Pearson, & Sutherland, 2011). Patients suffering from this type of diabetes may present normal or only elevated insulin levels especially if β-cells function as normal. However, insulin secretion process is found to be impaired and
incapable of compensating the insulin resistance in this type of diabetes mellitus (World Health Organization, WHO, 2006).

2.1.3 Prevalence of T2DM worldwide

Today diabetes mellitus constitutes a common disease in almost every population in the world (Ogurtsova et al., 2017) and about 0.5 million adult died by diabetes in 2015 only (IDF, 2015). Epidemiological evidence suggest that without effective prevention and control programs, diabetes is likely to continue in the rise. The number of individuals suffering from diabetes is increasing due to population growth, aging, urbanization, and the growing rate of obesity and physical inactivity (Wild, Roglic, Green, Sicree, & King, 2004).

The global prevalence of T2DM in adults has increased over the past decades. In 1964, it was reported that 30 million people worldwide suffered from diabetes (Entmacher & Marks, 1965). According to the World Health Organization (WHO), the global prevalence of T2DM was 135 million in 1995, 171 million in 2000, 220 million in 2004, and this is likely to increase to 422 million by 2014 (WHO, 2006, 2016).

IDF has produced other estimations for the global prevalence of diabetes. It showed that 382 million T2DM patients were diagnosed in 2013 and this number increased to 415 million during 2015 (Ogurtsova et al., 2017). Unfortunately, this number is expected to reach to 642 million by 2040 (IDF, 2015) Figure (2.1). Thus, T2DM has become a major global public health concern (DeFronzo et al., 2015).
Figure (2.1): Prevalence of T2DM: In each box, the top value represents the number of people with T2DM (in millions) in 2013, and the middle number represents the estimated number of people expected to have T2DM in 2035. The bottom value represents the estimated increase from 2013 to 2035 (DeFronzo et al., 2015).

Unfortunately, the Arab world (North Africa, Middle East, and the Gulf region) is expected to record the second highest increase in percentage of people with DM in 2030 compared to other parts of the world (Sweileh, Abu-Hadeed, Al-Jabi, & Sa’ed, 2014).

2.1.4 Prevalence of T2DM in Palestine

Medical services for diabetic patients in Palestine are provided by different sectors including ministry of Health (MOH), United Nations Relief and Works Agency, Non-Governmental Organizations (NGOs), Palestinian Military Medical Services (PMMS), and the private sector. Diabetes prevalence rate were difficult to be defined due to dispersing of large population of refugees throughout the adjacent countries and the discontinued nature of the health system. However, studies estimated that the prevalence rate of diabetes reached between 10% and 20.8% among Palestinians by 2020 (Abu Al-Halaweh et al., 2017).

According to Palestinian Health Information Center (PHIC) the total (new and old) of 59,047 T2DM patients were registered in the Gaza Strip in 2016 with prevalence of 3.1 per 100 people (Ministry of Health, 2016). Females constitute about 58% (3.6 per 100) and males 42% (2.6 per 100) were males.
Importantly, diabetes and its complications are estimated to be responsible for about 5.7% of mortality among Palestinians (El Sharif, Samara, Titi, & Awartani, 2015). The mortality rate of patients with diabetes 0.3 per 1000 patient, the proportion of males accounted for 60.7% of total deaths (Ministry of Health, 2016).

Regarding treatment systems, only 0.3% of the diabetic patients are on diet, 13.8% of patients follow compound treatment (tablets and insulin), 21.2% of patients on insulin treatment, and 64.5 are on oral hypoglycemic drugs (Ministry of Health, 2016).

### 2.2 Risk factors of T2DM

Several studies have investigated into the risk factors related to T2DM. Body mass index (BMI), high levels of lipids, hypertension, smoking, physical inactivity, low education, dietary patterns, family history, and also specific genes have been identified as the most common risk factors for T2DM (Bassuk & Manson, 2008; Chan et al., 2009).

#### 2.2.1 BMI

A number of long-term studies have established that increased BMI constitutes a strong risk factor for T2DM (Almdal, Scharling, Jensen, & Vestergaard, 2008). A strong positive relation between obesity and T2DM has been observed in both genders (Almdal et al., 2008). Obesity seems to increase the risk of developing insulin resistance and T2DM. Adipose tissues of obese people releases higher amounts of non-esterified fatty acids, glycerol, hormones, pro-inflammatory cytokines, and other substances associated with insulin resistance. When insulin resistance is accompanied by dysfunction of the β-cells, the blood glucose level is not controlled, thus leading to T2DM (Kahn, Hull, & Utzschneider, 2006).

#### 2.2.2 Lipids level

Unfavorable blood lipids have also been identified as a risk factor for T2DM. An inverse relationship between HDLc and risk of T2DM have been documented in several studies (Jacobsen, Bønnaa, & Njølstad, 2002). Low HDL cholesterol is found
to constitute a stronger risk factor for T2DM in women than it is in men (Campagna et al., 1997).

A study measuring non-fasting triglycerides (TG) has identified elevated triglyceride levels as an independent risk factor for T2DM (Almdal et al., 2008). High plasma triglycerides and low plasma HDLc levels have been observed in relation with the prediabetic state of the insulin resistance syndrome (Taskinen, 2003). This suggests that nonfasting triglycerides and HDLc levels offer a firm indication of the degree of insulin resistance. It is suggested that the increased circulating levels of free fatty acids are responsible for this development due to the increased insulin levels and increased chylomicron-assembly and secretion in the gut, the latter process is the result of localized insulin resistance in the intestine. Cross-sectional studies have shown that high BMI is associated with a higher level of total cholesterol and unfavorable lipid pattern showing low concentrations of HDL cholesterol and high triglyceride concentrations (Rainwater et al., 2000; Wild & Byrne, 2006).

### 2.2.3 Hypertension

A number of existing prospective and case control studies have established that hypertension progression constitutes an independent predictor of T2DM (Conen, Ridker, Mora, Buring, & Glynn, 2007). Several factors are likely to cause the association between T2DM and hypertension. Endothelial dysfunction may represent a common pathophysiological pathway that explains the strong association between blood pressure and incident T2DM. Studies have indicated that the markers of endothelial dysfunction are associated with new-onset diabetes (Meigs et al., 2006). Endothelial dysfunction is also closely related to blood pressure and hypertension (Gokce et al., 2001). Markers of inflammation such as C-reactive protein have been consistently related to the incidence of T2DM (Hu, Meigs, Li, Rifai, & Manson, 2004) as well as to increased blood pressure levels (Buckley, Fu, Freeman, Rogers, & Helfand, 2009). This suggests that inflammation may constitute another factor explaining the frequently observed association between blood pressure, the metabolic syndrome, and incident T2DM (Ridker, Buring, Cook, & Rifai, 2003).
Finally, although existing studies have shown that blood pressure increases with increasing BMI, the risk of T2DM associated with hypertension is independent of any measured changes in BMI (Joseph, Svartberg, Njølstad, & Schirmer, 2010).

2.2.4 Cigarette smoking
Several prospective studies have established that nicotine consumption constitutes a risk factor for developing T2DM (Hur et al., 2007). A meta-analysis involving 25 prospective studies has established that frequent cigarette smoking is associated with a 44% increased risk of diabetes (Willi, Bodenmann, Ghali, Faris, & Cornuz, 2007). The association between smoking and T2DM is stronger for heavy smokers (those consuming ≥ 20 cigarettes per day) compared to light smokers or former smokers (Willi et al., 2007). An increased risk of developing T2DM seems to persist during the first two to three years after smoking cessation (Hur et al., 2007). Smoking is known to lead to insulin resistance and an inadequate compensatory insulin secretion response. This can be explained by attributing a direct effect of nicotinic or other components of cigarette smoke on the β-cells of the pancreas. Cigarette smoking has been associated with chronic pancreatitis and pancreatic cancer (Talamini et al., 1999). It has also been suggested that heavy smokers with evidence of increased systemic inflammation and have gained substantially in their body weight after quitting the habit are at a considerably high risk of developing T2DM (Duncan, Schmidt, Chambless, Folsom, & Heiss, 2003). As expected, quitting smoking has been firmly associated with a reduction in risk of developing T2DM in the long run (Wannamethee, Shaper, & Perry, 2001).

2.2.5 Physical inactivity
Longitudinal studies have identified the lack of physical exercise or physical inactivity to constitute a strong risk factor for T2DM (Fretts et al., 2009). Such common every day prolonged sessions in front of the TV or the computer have been positively associated with diabetes risk in both men and women (Krishnan, Rosenberg, & Palmer, 2008). In contrast, frequent moderate and vigorous physical activity has been associated with a lower risk of T2DM (Fretts et al., 2009). Evidence from clinical trials which incorporated certain forms of physical activity have
suggested that the onset of T2DM can be prevented or delayed by the subjects increased physical activity (Ramachandran et al., 2006). Physical activity has been identified as playing a key role in delaying or even preventing the development of T2DM. This is achieved directly by improving the insulin sensitivity and reducing the insulin resistance of risk individuals and indirectly by changes in body mass and body composition (Ramachandran et al., 2006).

### 2.2.6 Low education

Previous prospective studies have examined the association between educational attainment and the incidence of diabetes. They have found low education to be a significant predictor of T2DM (Maty, Everson-Rose, Haan, Raghunathan, & Kaplan, 2005). A cross-sectional study documented that individuals who did not complete their high school education were almost twice as likely to have diabetes compared to others who got bachelor degree or more (Tang, Chen, & Krewski, 2003). Another study established that lower educated women are more likely to have diabetic than higher educated women. Furthermore, the association varied according to the individuals’ race, ethnicity and gender as Caucasians, Hispanics and women in general exhibited a stronger association between education and diabetes than African-Americans and men (Borrell, Dallo, & White, 2006). More recent cross-sectional study has documented that the risk of developing T2DM is higher in individuals who are lesser educated, obese and inactive compared to those who are more educated (Dasgupta, Khan, & Ross, 2010). The above mentioned studies suggest that a higher level of education promotes a healthier lifestyle. Individuals who are more aware of health risks are able to reduce those risks and adopt healthier habits (Joseph et al., 2010).

### 2.2.7 Dietary pattern

Another important life style factor associated with the development of T2DM is diet. A positive association has been established between the risk of T2DM and different dietary habits (Sun et al., 2010). A high dietary glycemic index is consistently associated with a high risk of developing T2DM in prospective cohort studies.
According to "On diet and risk of type 2 diabetes: the role of fat and carbohydrate", a review that included 19 studies, a higher intake of polyunsaturated fat and long-chain fatty acid is beneficial, whereas a higher intake of saturated fat and trans-fat adversely affects the consumers’ glucose metabolism and insulin resistance (Hu & Liu, 2001). Another prospective study observed in this respect that a higher consumption of butter, potatoes and whole milk was associated with an increased risk of developing T2DM, whereas a higher consumption of fruits and vegetables was associated with a reduced risk (Montonen et al., 2005). The possible mechanisms suggested are that insoluble fiber intake leads to an improved insulin sensitivity and thus decreases the risk of T2DM (Meyer et al., 2000). Furthermore, large-scale observational studies have documented the association between low vitamin D intake and an increased incidence of T2DM (Knekt et al., 2008). Vitamin D deficiency may contribute to β-cell dysfunction, insulin resistance and inflammation, which in turn may result in developing T2DM. The effect particular dietary habits have on increasing or decreasing the risk of developing T2DM are known to be independent of any BMI change (Joseph et al., 2010).

### 2.2.8 Genetics

Several studies have suggested that genetic components also play an important role in the pathogenesis of T2DM (Amini & Janghorbani, 2007). According to several prospective studies and cross-sectional studies, positive family history among first degree relatives confers an increased risk of developing T2DM. This risk is reportedly greater when both parents are affected (Amini & Janghorbani, 2007; Ma et al., 2008). A study involving twin siblings established that the concordance estimate for T2DM was higher in monozygotic twins compared to dizygotic twins, and the rate increased within the period of subsequent follow ups (Medici, Hawa, Ianari, Pyke, & Leslie, 1999). The prevalence of diabetes varies substantially among different ethnic groups (Das & Elbein, 2006).

Recent studies have identified variants in 11 genes (The transcription factors 7-like 2 (TCF7L2), Peroxisome Proliferator Activated Receptor (PPARG), FTO, KCNJ11, NOTCH2, WFS1, CDKAL1, IGF2BP2, SLC30A8, JAZF1, and HHEX) that are
significantly associated with the risk of T2DM independently of other clinical risk factors. The variants in eight genes were associated with impaired β-cell function (Ahlqvist, Ahluwalia, & Groop, 2011). TCF7L2 represents the locus of the highest risk of T2DM (Lyssenko, 2008).

2.3 Complications of T2DM
Complications of diabetes constitute two major classes, acute complications include hypoglycemia, hyperglycemia, and diabetic ketoacidosis (DKA), the second class is chronic complications such as Macrovascular (hypertension, hyperlipidemia, cardiovascular, cerebral vascular disease, and peripheral vascular disease) and microvascular (nephropathy, neuropathy, and retinopathy) (Benhalima, Standl, & Mathieu, 2011).
Diabetic microvascular complications have been found to be closely linked to the severity and duration of hyperglycaemia (Holman, Paul, Bethel, Matthews, & Neil, 2008). Hyperglycaemia promotes the development of microvascular complications by activating six major pathways, including enhanced polyol pathway flux, increased formation of advanced glycation end products (AGEs), increased AGE receptor expression, activation of Protein kinase C (PKC) isoforms, enhanced hexosamine flux and increased intracellular reactive oxygen species (Brownlee, 2005; Giacco & Brownlee, 2010). Genetic factors play a pivotal role in determining the susceptibility to microvascular complications. T2DM also affects the macrovasculature, and the incidence of myocardial infarction and peripheral vascular disease and stroke is markedly increased. Accelerated atherosclerotic cardiovascular disease is associated with several risk factors such as insulin resistance and hyperinsulinaemia, activation of inflammatory pathways, and the presence of multiple cardiovascular risk factors (hypertriglyceridemia, reduced HDLc, and small dense LDLc particles (Taskinen & Borén, 2015), hypertension, endothelial dysfunction, increased plasminogen activator inhibitor 1 levels, visceral obesity and non-alcoholic steatohepatitis or non-alcoholic fatty liver disease) (DeFronzo, 2010).
Hypertension is two or three times more common in people with T2DM and greatly increases the risk of further macrovascular complications (such as myocardial infarction, stroke, peripheral vascular disease and congestive heart failure),
microvascular complications (including retinopathy and nephropathy) and premature death (Adler et al., 2000). Multiple factors contribute to the increased incidence of hypertension in T2DM, including disturbed blood pressure circadian rhythms (higher nocturnal blood pressure), impaired blood flow autoregulation, stiffening of large arteries, increased intracellular sodium concentration, increased arterial sensitivity to angiotensin II, insulin resistance, endothelial dysfunction, obesity and genetic susceptibility (DeFronzo et al., 2015; Williams, 2015).

2.4 Pathogenesis of T2DM
Under normal physiological conditions, concentrations of glucose are maintained within regulated dynamic interaction between tissue sensitivity to insulin and insulin secretion (Ozougwu, 2013). In T2DM these mechanisms cease to function, resulting in the two main pathological defects which are impaired insulin secretion through dysfunctional pancreatic β-cells and dysfunction of insulin action through insulin resistance (Holt, 2004).

2.5 Pathophysiology of T2DM
T2DM is a multifactorial disease that involves genetic and environmental factors (Scheen, 2016; Chavarria-Avila et al., 2017). The pathophysiology of T2DM is complex, the most important determinant being an altered balance between insulin sensitivity and insulin secretion (Wilmot & Idris, 2014). The known pathophysiological changes include β-cell dysfunction, insulin resistance, and chronic inflammation, all of which increasingly reduce the control of blood glucose levels and cause micro- and macrovascular complications (Scheen, 2016; Chavarria-Avila et al., 2017).

2.5.1 Insulin hormone
The insulin hormone facilitates the transport of blood glucose across the cell membrane. It binds to a receptor on the cell membrane, allowing the entry of glucose into different cells and form subsequently forms fatty acids, glycogen to generate triglycerides and amino acids for protein synthesis (Cosford, 1999). If the binding of insulin to the receptors is disturbed, the insulin activity will be reduced and less
glucose will be able to enter the cells, therefore pancreas detects elevated blood sugar (Cosford, 1999).

2.5.2 Insulin sensitivity
Insulin sensitivity is the capacity of insulin to maintain glucose level within normal limits by signaling the insulin-sensitive tissues or organs to reabsorb glucose. These signals include stimulating utilization of glucose in both muscles and fatty tissue and inhibiting the production of glucose by the liver in order to drop the concentration of plasma glucose. The impaired glucose metabolism is influenced by the insulin sensitivity of cells within the body and by the reserve capacity of the pancreatic cells (Jellinger, 2007).

2.5.3 Insulin resistance
Insulin resistance constitutes a significant manifestation of T2DM that can be observed 10–20 years before the onset of hyperglycemia (Zhao & Townsend, 2009). Insulin resistance is identified by inability of tissues such as adipose, liver, skeletal and cardiac muscle to respond to insulin (Steppan & Lazar, 2002). Thus, hyperinsulinaemia constitutes a common manifestation in T2DM cases (Zhao & Townsend, 2009). Although standard definitions of insulin resistance still focus on the effects of insulin on glucose metabolism, more recent explanations transformed from the traditional glucocentric perspective of diabetes to an increasingly confirmed lipocentric perspective. This hypothesis holds that abnormalities in fatty acid metabolism may result in the disproportional accumulation of fats in liver, muscles, and β-cells. Mover, high free fatty acids concentrations might induce insulin resistance in different tissues (Perry, Samuel, Petersen, & Shulman, 2014).
Obesity and physical inactivity can cause insulin resistance, which together with a genetic predisposition place stress on β-cells to fail and lead to the progressive decline in insulin secretion (DeFronzo et al., 2015). Signs of insulin resistance are present in muscle and in the liver (DeFronzo, 2009), the two tissues responsible for the majority of glucose disposal following carbohydrate ingestion, and also present in adipose (Guilherme, Virbasius, Puri, & Czech, 2008), kidney (Gerich, Meyer, Woerle, & Stumvoll, 2001), gastrointestinal tract (Honka et al., 2013), vasculature
(Meijer et al., 2012), brain tissue (Kleinridders, Ferris, Cai, & Kahn, 2014), and pancreatic β-cells (Oliveira, Rebuffat, Gasa, & Gomis, 2014).

In muscles, a number of abnormalities may contribute to the development of insulin resistance this includes defects in different places as insulin signaling, glucose transport, glucose phosphorylation, glycogen synthesis, pyruvate dehydrogenase complex activity, and mitochondrial oxidative activity (Oliveira et al., 2014).

In the liver, insulin resistance is associated with insulin deficiency, hyper-glucagonaemia, enhanced glucagon sensitivity and increased substrate delivery of fatty acids, lactate, glycerol and amino acids (Samuel et al., 2009). This leads to increased gluconeogenesis which is responsible for the increased basis rate of glucose production and fasting hyperglycemia (DeFronzo, 2009).

Insulin resistance in the kidney and augmented renal gluconeogenesis also contributes to fasting hyperglycaemia (Gerich et al., 2001). Impaired suppression of hepatic glucose production, decreased hepatic glucose uptake, muscle insulin resistance, reduced noninsulin-mediated glucose uptake and excessive renal glucose reabsorption contribute to postprandial hyperglycaemia in T2DM (DeFronzo et al., 2013).

In addition, insulin resistance in the vascular endothelium impairs the vasodilating effects of insulin and thus further reduces not only its own delivery but also that of glucose (Barrett, Wang, Upchurch, & Liu, 2011).

2.5.4 Mechanisms of insulin resistance

Binding of insulin to its receptor activates insulin receptor tyrosine kinase and the phosphorylation of insulin receptor substrates (IRSs), especially IRS1 and IRS2 (Krüger et al., 2008) Figure (2.2). These phosphorylated IRS proteins activate intracellular signaling molecules, the most important enzyme is phosphatidylinositol 3-kinase (PI3K). This kinase promotes glucose transporter type 4 (GLUT4) translocation to the plasma membrane, causes the glucose uptake into skeletal muscle and phosphorylates and inactivates the transcription factor forkhead box protein O1 (FOXO1) and thus alters the transcription of downstream genes including cell cycle arrest and DNA repair genes. Insulin also stimulates the RAS mitogen-activated
protein kinase (MAPK) pathway which is crucial for cell division (DeFronzo et al., 2015).

Insulin resistance in obesity and T2DM is mainly linked to the PI3K pathway (Cusi et al., 2000; Krook et al., 2000) and usually associated with the increased serine phosphorylation (Ser-p) of IRS proteins, which inhibits tyrosine phosphorylation and leads to insulin resistance (Kleinridders et al., 2014). In some cases, serine phosphorylation also increases IRS degradation, thus further increasing the insulin resistance (Hiratani et al., 2005). There exist numerous factors that cause increased serine phosphorylation such as ectopic lipid accumulation, mitochondrial dysfunction, inflammation and endoplasmic reticulum (ER) stress (DeFronzo et al., 2015).

**Figure (2.2): Mechanisms of insulin resistance.** In adipocytes, insulin resistance (also caused by increased insulin receptor substrate (IRS) serine phosphorylation) and inflammation produces and releases free fatty acids (FFAs) and insulin-resistance-provoking pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor (TNF) and insulin-sensitizing adipokines such as adiponectin, conversely, ameliorate insulin resistance. Retinol-binding protein 4 (RBP4) also increases insulin resistance. Plasminogen activator inhibitor 1 (PAI1) does not affect insulin resistance but has been implicated in complications of obesity, including accelerated atherosclerosis and type 2 diabetes. These factors contribute to the accumulation of toxic lipid metabolites (diacylglycerol (DAG), ceramides and acyl-CoAs) in myocytes and hepatocytes, which impair insulin signaling (IRS–phosphatidylinositol 3-kinase (PI3K) pathway) and activate inflammatory pathways (JUN amino-terminal kinase (JNK), IκB kinase (IKK) and mitogen-activated protein kinase (MAPK)), which further impair the insulin signal transduction pathway. FA, fatty acid; mTOR, mammalian target of rapamycin; SOCS, suppressors of cytokine signaling; Toll-like receptor 4 (TLR4); TNFR, TNF receptor; unfolded protein response (UPR); X box-binding protein 1(XBP1) (DeFronzo et al., 2015).
2.5.5 Pancreatic β-cells function

Normally, pancreatic β-cells secrete insulin in response to glucose stimulation through a chain of electrical reactions across the cell membrane. Glucose metabolism in β-cells generates bursts of action potentials and eventually into the intracellular calcium flow along with secretion of insulin. The ATP sensitive potassium channels (K-ATP) typically maintain the β-cell resting membrane potential and prevent entry of calcium. The K-ATP channel is inhibited when the ratio of ATP to ADP is increased within the β-cell cytosol as it occurs during glycolysis. Subsequent polarization of the β-cell membrane activates calcium channels, and the accumulation of intracellular calcium leads to secretion of insulin (MacDonald et al., 2005; Moneva & Dagogo-Jack, 2002).

T2DM occurs when the β-cells are unable to secrete sufficient amounts of insulin and cells lost insulin sensitivity (Kahn, Cooper, & Prato, 2014). Multiple factors contribute to β-cell failure including aging, genetic abnormalities, incretin hormone glucagon-like peptide 1 (GLP1) and gastric inhibitory polypeptide (GIP) resistance and/or deficiency (Madsbad, 2014; Nauck, Vardarli, Deacon, Holst, & Meier, 2011). Other reasons also include lipotoxicity (Ferrannini & Mari, 2014), glucotoxicity (Bensellam, Laybutt, & Jonas, 2012), insulin resistance leading to β-cell stress (DeFronzo, 2010), hypersecretion of islet amyloid polypeptide (IAPP) (Ritzel, Meier, Lin, Veldhuis, & Butler, 2007), reactive oxygen stress (Collins, Pi, & Yehuda-Shnaidman, 2012), and activation of the inflammatory pathway (DeFronzo, 2010).

According to the British Prospective Diabetes Study, extensive studies of newly discovered patients with T2DM, long-term increases in fasting blood sugar levels are accompanied by a progressive drop in β-cell function amounting to 4% yearly. By the time of diagnosis, the mean β-cells function was already below than 50%, and the β-cell function further deteriorated despite the diabetic therapies that were administered. The extrapolation of the β-cell drop rate predict that diminished of β-cell function occurs 12 years prior to onset (Holman, 1998). Progressive decline of β-cell function interpreting why patients need an increasing frequency and dose of oral hypoglycemic agents by time. It also explains why they eventually become refractory to the oral treatments and require insulin (Holt, 2004).
Although the mechanisms underlying β-cell dysfunction remain uncertain and unclear, they are commonly caused by a number of factors such as genetic factors (Pratley & Weyer, 2001), socioeconomic factors (including childhood malnutrition and obesity) and hyperglycaemia and hyperlipidaemia have been found to accelerate the decline in β-cell function (Prentki, Joly, El-Assaad, & Roduit, 2002). Other evidence indicates that hyperglycemia does not occur without β-cell dysfunction (Kahn, 2003; Marchetti, Dotta, Lauro, & Purrello, 2008).

### 2.5.6 Mechanisms of β-cell dysfunction

The initial alterations in β-cell function are likely to reflect intrinsic defects, whereas the accelerated β-cell dysfunction mainly occurs with overt hyperglycemia resulting from glucolipotoxicity (Wajchenberg, 2007). This reflects a genetic predisposition for β-cell defects. The subsequent β-cell dysfunction may be caused by other environmental conditions. The role of cellular dysfunction in the natural history of T2DM is included in Figure (2.3) (Popa & Mot, 2013).

The molecular mechanisms of β-cell dysfunction have not been fully clarified. Loss of β-cell differentiation can be observed in the early stage of T2DM. Decreased gene expression of insulin-sensitive glucose transporter 2 (GLUT-2), glucokinase, pyruvate carboxylase, transcription factors and increased gene expression of lactate dehydrogenase, hexokinase, glucose-6-phosphatase and transcription factor cellular myelocytomatosis (c-myc) have been reported. The loss of β-cell mass is also associated with the deposition of amyloid, which is a product of human islet amyloid polypeptide normally produced in the β-cells and secreted along with insulin. Islet amyloid constitutes a characteristic pathological finding in more than 90% of cases of T2DM. Patients who require insulin treatment present greatly reduced islet mass and the most prominent amyloid deposits. Islet amyloid deposition has been implicated in β-cell cytotoxicity through the expression of apoptosis-related genes, oxidative stress and/or formation of ion channels. The degree of islet amyloid formation correlates with the degree of hyperproinsulinemia. Lowered insulin secretory capacity linked to amyloid-induced cytotoxicity may serve as the focal point for therapies directed toward preserving the islet function in T2DM. Future
therapies may be directed at inhibiting human islet amyloid polypeptide production or inhibiting amyloid fibrillogenesis (Moneva & Dagogo-Jack, 2002)

![Figure 2.3](image)

*Figure (2.3): β-cell dysfunction in natural history of type 2 diabetes (Popa & Mot, 2013)*

### 2.6 Current diagnostic markers for T2DM

The routine diagnosis of T2DM is generally based on the plasma glucose value while the patient is fasting (fasting blood sugar, FBS), and two hours after the oral intake of 75g glucose (oral glucose tolerance test, OGTT) (Stumvoll, Goldstein, & van Haeften, 2005). In 2009, a global expert committee also recommended the use of glycated hemoglobin (HbA1c) levels to diagnose diabetes mellitus and pre-diabetes (International Expert Committee, 2009). In order to administer the fasting blood sugar test (FBS) >126 mg/dl, the subject must not consume any calories for at least eight hours. The oral glucose tolerance test can be given after two hours (OGTT) >200 mg/dl using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water. The test using glycated hemoglobin (HbA1c)>6.5% should be
performed in a clinical laboratory using a technique developed by the National Glycohemoglobin Standardization Program (NGSP) and identical to the Diabetes Control and Complications Trial (DCCT) assay. All of the tests above go along with signs of hyperglycemia suggestive of diabetes mellitus, as shown in Table (2.1) (International Expert Committee, 2009). These classical biomarkers are identified in combination with serum measurements of lipid metabolisms (triglycerides, cholesterol and lipoproteins), small molecule intermediates (2-hydroxybutyrate), and creatinine serve prediction of T2DM with a probability of about 0.65–0.75 (Schulze et al., 2009). Supplemental information about lifestyle factors (including physical inactivity, diet, smoking), physical factors (including BMI, waist-hip ratio, blood pressure) and genetic risk factors further increase the probability values to 0.85–0.90 (Schulze et al., 2009).

Table 2.1: Classical biomarkers for T2DM (International Expert Committee, 2009).

<table>
<thead>
<tr>
<th>Classical biomarkers</th>
<th>Normal</th>
<th>Prediabetic</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting blood sugar test (FBS)</td>
<td>≤100 mg/dl</td>
<td>100–125.99 mg/dl</td>
<td>&gt;126 mg/dl</td>
</tr>
<tr>
<td>Oral glucose tolerance test (OGTT)</td>
<td>≤140 mg/dl</td>
<td>140–199.99 mg/dl</td>
<td>&gt;200 mg/dl</td>
</tr>
<tr>
<td>Glycated haemoglobin (HbA1C)</td>
<td>≤5.7%</td>
<td>5.7–5.99%</td>
<td>At least 6.5%</td>
</tr>
</tbody>
</table>

2.6.1 HbA1c as a diagnostic test for diabetes mellitus

The level of glycated hemoglobin HbA1c indicates any case of chronic hyperglycemia existing postdating a period of six to eight weeks rather than at the time the test is taken (Chen et al., 2015). HbA1c is used to diagnose diabetes as recommended by the International Expert Committee in 2009 (International Expert Committee, 2009), and endorsed by the ADA (American Diabetes Association, ADA, 2014), the Endocrine Society and the WHO (WHO, 2016). The HbA1c test offers a number of advantages over the FBS and OGTT, including greater convenience (fasting not required), greater pre-analytical stability, stronger correlation with microvascular complications, especially that of retinopathy, a marker for glycemic control and protein glycation (Kharroubi & Darwish, 2015), and
fewer perturbations during periods of stress and illness. It is recommended to repeat the HbA1c test in asymptomatic patients within two weeks to reaffirm the diagnostic result (McDonald & Warren, 2014).

However, these known advantages are offset by the lower sensitivity of the HbA1c test at the designated cut point, greater cost, limited availability of HbA1c testing in certain regions of the developing world, and the imperfect correlation between HbA1c and average glucose levels in certain individuals. Most studies with different ethnic groups have endorsed a cutoff value for an HbA1c of ≥6.5% (48 mmol/mol) to diagnose diabetes as has been recommended by the International Expert Committee (International Expert Committee, 2009). In this respect it is important to take age, race or ethnicity, and anemia or hemoglobinopathies into consideration when using the HbA1c to diagnose diabetes (Cowie et al., 2010). The HbA1c cut-off value of ≥6.5% (48 mmol/mol) is being used in many countries with different ethnic populations, even though it is known that ethnicity may affect those values (Hui et al., 2013). The cut-off values of 5.5% (37 mmol/mol) and 6.5% (48 mmol/mol) have been reported in a Japanese study, 6.0% (42 mmol/mol) in the National Health and Nutrition Examination Survey (NHANES) in the United States, 6.2% (44 mmol/mol) in a Pima Indian study, 6.3% (45 mmol/mol) in an Egyptian study as reported by Davidson, and three cut-off values are reported in Chinese studies. Australia has recommended the use of ≤ 5.5% to “rule-out” and ≥ 7.0% to “rule-in” diabetes (Kharroubi, Darwish, Abu Al-Halawe, & Khammash, 2014).

HbA1c is very stable after collection with no change in its concentration in the collection tube. When measuring plasma glucose, the venous blood sample should be spun and the plasma separated within minutes of taking the sample. Red blood cells continue to consume glucose at about 7% per hour in vitro leading to the glucose level being measured as too low.

On the other hand, if the sample is collected in a container using antiglycolytic preservative (fluoride), is also only partially effective (Bruns & Knowler, 2009). Ideally, the sample should be placed in iced water and processed within 30 to 60 minutes, although most laboratories are known not to fulfill these rigorous sample handling requirements. However, HbA1c possesses high pre-analytical stability (one week at 4 °C). Within-subject biological variation of HbA1c is recorded at 3.6%,
compared to 5.7% for fasting blood sugar and 16.7% for the two-hour post-OGTT value (Selvin, Crainiceanu, Brancati, & Coresh, 2007). The analytical precision achieved through HbA1c now approaches the precision achieved for glucose, with intra- and between-laboratory analytic variability in the order of 2.5%. The standardization of HbA1c measurement is also deemed as superior than that of glucose (Florkowski, 2013).

The HbA1c test should be repeated on asymptomatic patients within two weeks in order to confirm the initial diagnostic result (McDonald & Warren, 2014). In terms of medical research, fewer studies used HbA1c testing to diagnose diabetes and preferred FBS and OGTT (ADA, 2016).

### 2.6.2 Novel biomarkers for T2DM

Recent studies also support the use of novel biomarkers as shown in Table (2.2) like combinations of C-reactive protein, adipokines, incretins, and cytokines for predicting T2DM showing similar probabilities as the traditional biomarkers (Blüher & Mantzoros, 2015; Othman et al., 2015). However, these biomarkers are not entirely specific for T2DM and are predictive of other metabolic disorders as well (Guay & Regazzi, 2013). The detection speed of these biomarkers is generally slow and are only effective in individuals who are already displaying metabolic imbalances (Urdea et al., 2009). Therefore, these biomarkers can predict T2DM susceptibility only a few years before the actual manifestation of diabetes. They can’t be used to assess disease susceptibility in the general population (Herder, Kowall, Tabak, & Rathmann, 2014). This calls for exploring new biomarkers that can help identify the individuals who are at risk of developing T2DM long before the metabolic imbalance sets in. Such a step would help better assess the therapeutics and drug targets (Bhatia, Raina, Chugh, & Sharma, 2015).
Table 2.2: Novel biomarkers for Type 2 diabetes mellitus (Bhatia et al., 2015).

<table>
<thead>
<tr>
<th>Novel biomarker</th>
<th>Association</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive protein</td>
<td>Higher expression in T2DM</td>
<td>(Shafizadeh et al., 2011)</td>
</tr>
<tr>
<td>Leptins and adipokines</td>
<td>Lower expression in T2DM</td>
<td>(Vinagre et al., 2014)</td>
</tr>
<tr>
<td>Incretins (such as GLP-1)</td>
<td>Anti-diabetic</td>
<td>(Shafizadeh et al., 2011)</td>
</tr>
<tr>
<td>Ferritin</td>
<td>Higher expression in T2DM</td>
<td>(Shafizadeh et al., 2011)</td>
</tr>
<tr>
<td>Cytokines (such as, IL-6, IL-18, TGF-β1, TNF-α)</td>
<td>Higher expression in T2DM</td>
<td>(Vinagre et al., 2014)</td>
</tr>
<tr>
<td>Chemokines (such as, MCP-1)</td>
<td>Higher expression in T2DM</td>
<td>(Vinagre et al., 2014)</td>
</tr>
<tr>
<td>1-Deoxysphingolipids</td>
<td>Higher expression in T2DM</td>
<td>(Othman et al., 2015)</td>
</tr>
</tbody>
</table>
2.7 MicroRNA

MicroRNAs (miRNAs) are group of specific highly conserved sequences composed of single strand none coding RNA (19–25 nucleotides in length) (Sebastiani et al., 2017). The main function of miRNAs is to regulate gene expression post-transcriptionally by binding to complementary sequences on the 3’-UTR regions of their target messenger RNAs (mRNAs). This leads to translational inhibition and/or mRNA degradation (Bhatia et al., 2015; Parrizas & Novials, 2016; Sebastiani et al., 2017).

According to miRNA data bases, there are mature 2588 miRNA in the human genome [http://www.mirbase.org/]. Due to the presence of similar seed sequences in multiple mRNAs, all of the 2588 miRNAs may actually target and regulate various mRNAs. On the other hand, single mRNAs may be targeted by several miRNAs. Therefore, it is not a surprise that miRNAs are found in the most biological processes and that their subsequent dysfunction may lead to various diseases that include diabetes and other complications (Soifer, Rossi, & Sætrom, 2007). Thus, miRNA can help in the modulation of pathological and physiological pathway in human diseases such as CVDs, diabetes, cancer and others. For example, various miRNAs are engaged in the development of β-cell and regulation of insulin secretion. However, certain miRNAs cause β-cell dysfunction and promote the development of different forms of diabetes mellitus (Guay & Regazzi, 2016).

Alternation of expression of miRNAs impacts the complications of diabetes in kidney, peripheral nerves and retina. Every miRNA has the potential of regulating the multiple genes in the biological processes including cell proliferation, apoptosis and differentiation. This has also confirmed that dysregulation of the miRNAs would affect pathological pathways and diabetic complication (Kantharidis, Wang, Carew, & Lan, 2011).

2.8 Genomic distribution and gene structure

The miRNA genes are present invariably in the human chromosomes with the exception of Y chromosome (Kim & Nam, 2006). About 50% of the recognized miRNAs are present in clustered manner and they are named as polycistronic primary transcripts. It has been seen that in a cluster of genes, the miRNAs are
related and this suggests that gene cluster takes place due to the phenomenon of gene duplication. On the contrary, in a gene cluster, there are also unrelated miRNAs (Kim & Nam, 2006). It was initially thought that most miRNA genes were located in intergenic regions. However, investigation of miRNA gene locations showed that the majority of mammalian miRNA genes are located in defined transcription units (TUs) (Rodriguez, Jones, Ashurst, & Bradley, 2004).

Rodriguez et al. (2004) detailed that the miRNA registry consists of 232 mammalian miRNAs (http://www.sanger.ac.uk/Programming/Rfam/mirna/; Griffiths-Jones 2004). Of these 232 miRNAs, they recognized 117 miRNAs situated in introns of protein-coding genes or long non-coding RNA (ncRNA) transcripts. Roughly, 40% (90 miRNAs) of these 117 miRNAs are found inside introns of protein-coding genes, while 10% (27 miRNAs) are situated inside introns of long ncRNA transcripts. Strikingly, 30 miRNAs of the 232 miRNAs located in exons of ncRNAs. Of the 232, 14 miRNAs are situated in either an exon or an intron (mixed) contingent upon alternative splicing of the host transcript. This perception shows that miRNAs are usually connected with complex transcriptional loci and transcriptionally related to mRNAs or ncRNAs (Rodriguez et al., 2004).

In this way, miRNA genes can be classified in view of their genomic areas: intronic miRNAs in protein-coding transcriptional unit (TU), intronic miRNAs in noncoding TU, and exonic miRNAs in noncoding TU Figure (2.4). 'Mixed' miRNA genes can be relegated to one of the above groups relying on the given joining splicing pattern (Kim & Nam, 2006).

**Figure (2.4): Genomic organization and structure of the miRNA genes.**

(a) The intronic miRNA present in the protein code transcriptional unit (TU). For an example, the miR-10 in the HOX4B gene is seen. Green triangles in the figure show location of the miRNA stem loop and the yellow triangle show the exons. (b) The non-coding transcripts containing intronic miRNAs. The miR-15a~16-1 can be seen that is present in that fourth intron of the earlier determined DLEU2 that is non-coding RNA genes. (c) Structure of the exonic miRNA in the non-coding transcripts, for instance the miR-155. (Kim & Nam, 2006).
2.9 Biogenesis and release of miRNAs

miRNAs biogenesis, function, maturation and secretion are an exceptionally complex molecular mechanism. miRNAs biogenesis begins with miRNA gene transcription by RNA polymerase II (RNA Pol II) as long precursor molecules that contain a distinguished stem-loop structure (Rodriguez et al., 2004). Later, it has been watched that additionally RNA polymerase III (RNA Pol III) is associated with that process (Haurie et al., 2010). Based on transcription, long primary transcript (pri-miRNA) is produced, which is typically poly-adenylated, capped, and with at least one long hairpin structure. These primary transcripts are cleaved by the ribonuclease III enzyme Drosha to create a ~60–70 nucleotide long hairpin structure pre-miRNA. These molecules are then transported by means of an Exportin-5-dependent process into the cytoplasm, where they are additionally cleaved by the endoribonuclease Dicer to create defective duplexes of ~22 nucleotides comprising of a guide strand (miRNA) and a passenger strand (miRNA*). The guide miRNA strand is the most thermodynamically stable and integrated together with Argonaute proteins forming RNA-induced silencing complex (RISC). The passenger miRNA* strand is typically degraded but in some cases it will be loaded into the RISC complex and be functional (Guay & Regazzi, 2013; Sebastiani et al., 2017). Mature miRNAs exert their activity by controlling the RISC complex to complementary sequences inside the 3′-UTR region of target mRNAs, promoting translational suppression and finally transcript degradation. Target recognition is primarily controlled by miRNA complementarity with the alleged seed sequences (bases 2–8 of the miRNA) of target mRNAs. Both translational suppression and mRNA degradation have been proposed as miRNAs mechanisms responsible for the negative regulation of their target mRNAs. Strangely, a single miRNA can possibly bind and regulate the expression of several targets, while a single 3′-UTR region can be targeted by various distinctive miRNAs Figure (2.5) (Chien et al., 2015; Sebastiani et al., 2017). Because of their function as regulators of gene expression, miRNAs are usually thought to be exclusively intracellular. However, recent breakthrough studies exhibited that miRNAs are likewise extracellular, being available in a cell-free circulating form in the circulation system (plasma or serum) and in numerous other
diverse biological fluids, for example, urine, milk, or aqueous humor (Fehlmann, Ludwig, Backes, Meese, & Keller, 2016).

Figure (2.5): Biogenesis and release of miRNAs.
Pre-miRNAs are generated in the nucleus by the ribonuclease III enzyme Drosha after cleaving the pri-miRNAs (1). The pre-miRNAs are then transported into the cytoplasm through a process including Exportin-5 and the GTP-binding protein Ran (2) in the cytoplasm pre-miRNA is further cleaved by Dicer to yield 21–23 nucleotide duplexes (3). One strand of the miRNA duplex can either associate to the RISC complex to guide translational repression of target mRNAs (4) or to be released by the cells. In the latter case, the mature miRNA binds to RNA-binding proteins such as Argonaute-2 (5) or to lipoproteins (6). On the other hand, the miRNAs can be loaded in microvesicles formed by plasma membrane blebbing (7) or in exosomes that are released in the extracellular space upon exocytic fusion of multivesicular bodies with the plasma membrane (8) (Guay & Regazzi, 2013).
2.10 Role of miRNAs in diabetes pathogenesis

According to the recent data, there is a significant role played by the miRNAs in case of secretion of insulin, development and differentiation of the pancreatic β-cell and indirect control of the lipid and glucose metabolism, and along with involving into the secondary complications related to diabetes (Li, 2014). The tissues and pancreatic β-cells, which are the targets of insulin, express significantly well-defined miRNAs sets. Majority of the miRNAs are essentially not cell-specific and these are distributed widely throughout tissues in the human bodies. The miRNA expression profile of β-cells and tissues that are targeted by insulin is changed in patients with T2DM and T1DM. These perhaps have a contribution to imparted functions of the tissues under diseased states (Kumar, Nath, Prasad, Sharma, & Li, 2012).

Certainly, islets of the prediabetic nonobese diabetic (NOD) mice, a model of T1DM, contain an increased level of some miRNAs and this includes miR-34a, miR-21, miR-146a and miR-29. All these have deleterious impacts on the functions of the β-cells (Roggli et al., 2012). In most of the cases these miRNAs along with other miRNAs, there is an alteration of the expression in the islets of the db/db or ob/ob mice, which are the model's related to T2DM and obesity. It is quite interesting that expressions of the miR-34a and miR-29 also are increased in the tissues that are targeted by the insulin within these mouse models. Possibly this has significant contribution in case of the resistance of insulin. Other miRNAs that are dysregulated in tissues targeted by insulin in ob/ob mice, dietary mouse models of obesity and diabetic Goto-Kakizaki rats include miR-143, miR-802 and two closely related miRNAs, miR-103 and miR-107. It is indicated by the strong experimental evidence that there is a significant contribution of all these miRNAs in case of the growth of the insulin resistance within these models of obesity (Trajkovski et al., 2011b). The alterations in case of the profiles of miRNA, which are closely connected with diabetes mellitus, also are reported to exist in the human tissues. There was a detection of over than 60 differently expressed forms of miRNAs within the biopsy samples of human skeletal muscles from the patients with having T2DM. This include miR-143 is up-regulated and other two muscle-specific miRNAs, miR-133a and miR-206, that are downregulated. It is also quite interesting that levels of almost
15% of the miRNAs have already been modified in case of the individuals with an impaired glucose tolerance, which essentially suggests the involvement of the miRNAs in the initial phases regarding the disease process. Some of these miRNAs expression is controlled by insulin, but the mechanism of regulations is reflected to be impaired in the patients suffering from diabetes mellitus. In case of the above-discussed changes in the tissues, which are targeted by insulin, considerable modifications of the expression of miRNAs in case of blood vessels, retina, kidneys and hearts are resulted by diabetes mellitus. The indication from all these findings reflects the involvement of non-coding RNAs in case of the development regarding the long-term complications of diabetes mellitus (Guay & Regazzi, 2013).

2.11 A functional role for circulating miRNAs
Numerous miRNAs are found in blood (plasma or serum) and in other body fluids like urine, breast milk, saliva, amniotic fluid, colostrum, bronchial lavage, cerebrospinal fluids, pleural fluids and seminal fluids, in close association with microvesicles, proteins and lipoprotein complexes (Vickers, Palmisano, Shoucri, Shamburek, & Remaley, 2015).

The miRNAs are found to be very stable in body fluids and these are resistant to the treatments with pH fluctuations, ribonucleases, boiling temperatures and extended storage at the temperature of -20°C. Also, miRNAs have been isolated from the unrefrigerated as well as dried serum spots which are stored for a duration of 5 months or at a temperature of 37°C for 4 weeks (Patnaik, Mallick, & Yendamuri, 2010).

On the basis of the facts regarding abundance of RNases in the circulating blood, a requirement was proposed regarding the distinct mechanisms for protecting miRNAs from being degraded. This was shown for the first time by El-Hefnawy along with his colleagues (El-Hefnawy et al., 2004) that plasma RNA has a protection from the degradation through its inclusion within protein and lipid vesicles. Based on the size and release modes from cells, the classification of these particles can be done as microvesicles (0.1-1 mm), exosomes (50-100) and apoptotic bodies (0.5-2 mm) (Kosaka et al., 2010). It is also possible for the circulating miRNAs to be released in
complexes along with the RNA-binding proteins like lipoprotein complexes or Argonaute 2 with lipoproteins of higher density (Vickers et al., 2015).

From the above observations, this can be concluded that biofluids like plasma and serum are possible to be stored at -80°C or -20 without any substantial degradation of the miRNAs. The availability of the sensitive and simple approaches for the characterization along with quantitation of the miRNAs (as discussed above) turns them into suitable biomarkers for appropriate detection of several diseases (Guay & Regazzi, 2013).

2.12 miRNAs serves as biomarkers in cardiovascular disease

The interest taken by the scientists in studying the circulating miRNAs in case of peripheral blood and to be used potentially as the clinical biomarkers of CVD is recently increased. The usage of miRNAs as the circulating biomarkers in case of prognosis and diagnosis of the cardiovascular diseases have been reported by several studies during the past four years. These uses are in case of cardiovascular diseases like strokes, hypertension, heart failure (HF), Coronary Artery Disease (CAD) and also DM Figure (2.6) (Maegdefessel, 2014).

Figure (2.6): Overview of Circulating miRNAs in case of Cardiovascular Diseases (Maegdefessel, 2014).
The miRNA signatures in each of the cardiovascular condition are unique and separate. Results from several relevant studies were combined to give an overview of which miRNAs are elevated in plasma and which ones are decreased in specific cardiovascular diseases (Maegdefessel, 2014).

T2DM that is a major risk factor for CVD may initiate significant with increased levels of glucose and insulin resistance that promotes endothelial dysfunction and complications in the vascular membranes (Frankel, Meigs, & Massaro, 2009). Many risk factors such as age, sex, HDL, hypertension, cholesterol, triglycerides, parental diabetes, impaired glucose tolerance and ethnicity are identified (Herder, Karakas, & Koenig, 2011). HDL, CRP, insulin, adiponectin and different types of chemokines consider biomarkers in diabetes diagnosis. Nevertheless, diabetes risk screenings have not yet been generally implemented and it is becoming clear that these should also account for the risk of CVD. This approach would allow the identification of patient subgroups and the development of more exclusive therapies. For the reason above, there is a need for the identification of novel biomarkers that will render feasibility and credibility to support of miRNAs that have the possibility to predict T2DM more accurately and it is cardiovascular risks (Empel, Windt, & Martins, 2012).

2.13 MiRNAs serve as circulating DM biomarkers

The miRNAs represents a form of stability being present in the circulating system and act as a medium of monitoring disease progression. Recent reports suggest that expression patterns of circulating miRNAs are ideal materials for reflection of the undergone pathological and physiological processes (Ai et al., 2010). The miRNAs that are present in the plasma or serum may term as biomarkers since they shows promising nature in utilizing and identifying initiation of disease and their nature of progression. miRNAs are potent enough to act as biomarkers for various diseases like CVD, AMI, hepatocellular carcinoma (HCC) and cancer. Wang et al. (2010) found that circulating miR-208a in individuals with AMI have 90.0% sensitivity and 100% specificity (Wang et al., 2010). Li et al. (2010) demonstrated three essential serum miRNAs (miR-25, miR-375 and let-7) as potent biomarkers separated hepatitis B virus-positive HCC from the controls having sensitivity 97.9%
and specificity 99.1%, whereas miR-375 alone restricted HCC of 96% specificity and 100% sensitivity (Li et al., 2010). Though, the diagnostic potential of miRNAs in diabetes is largely unexplored. Recently, miRNAs with the distinct profile are found to get circulated to patients with features of T2DM and DM complications in comparison to patients of non-DM patients. Several studies have shown the circulating of miRNAs in blood can serve as potential biomarkers for diabetes and also have shown that specific miRNA profiles are correlated to DM pathology. While miRNAs are still responsible for monitoring the regulation of insulin production, the sensitivity of insulin, metabolism of lipid and glucose homeostasis that directs towards T2DM pathology (Guay & Regazzi, 2013). Patients with DM complications can be identified with the help of miRNAs since they have potential involvement in regulating homeostasis of tissues, which are the place where DM complication resides (Kantharidis et al., 2011).

2.14 Circulating miRNAs serve as T2DM biomarkers

Due to lack of availability of specific biomarkers, the progression of T2DM is not predictable with accuracy. The traditional biomarkers such as fasting blood sugar, HbA1c, and novel biomarkers such as adipokines, cytokines and C-reactive protein that are available to date cannot efficiently detect the individuals who are going through the phase of developing T2DM, because they identify subjects already displaying metabolic alterations. The ability of potential biomarker is shown within the circulating miRNAs as promising diagnostic biomarkers also in patients with T2DM and in individuals at risk of developing the disease, showing distinct expression profiles (Sebastiani et al., 2017).

Zampetaki et al. (Zampetaki et al., 2010) were the first to identify the signature of specified circulating miRNAs through a prospective study where 800 individuals were selected on a random basis from the cohort of Bruneck. Authors found several miRNAs (miR-21, miR-24, miR-15a, miR-20b, miR-126, miR-191, miR-197, miR-223, miR-320, and miR-486) were found to be down-regulated and miR-28-3p which was found to be up-regulated in T2DM patients compared to non-diabetic subjects. Based on the levels of circulation of five most differentiable miRNAs (miR-15a, miR-320, miR-126, miR-223, and miR-28-3p), it was able to detect 70% of the
patients affected by T2DM irrespective of any other parameter. Furthermore, subjects who were normoglycemic at baseline but who developed diabetes over a 10-year follow-up period could be identified by reduced miR-15a, miR-29b, miR-126, miR-223, and elevated miR-28-3p, indicating that specific expression profiles may represent potential markers for both diagnosis and prediction of T2DM. Along with that, some miRNAs were detected as differentially expressed, e.g. miR-126 (a miRNA highly enriched in endothelial cells, which contributes to the maintenance and repair of vascular integrity and angiogenesis) which caused the most T2DM related (Fish et al., 2008). Patients having the risk of developing of T2DM can be detected by tracing the miRNAs as per Zampetaki and this information was assessed by Zhang et al. in a study including patients with T2DM, as well as subjects with impaired fasting glucose (IFG) and normoglycemic individuals. Only miR-126 was found to be the miRNA that has significantly reduced expression in IFG and T2DM in comparison to normoglycemic subjects regardless of age and sex, confirming that miRNAs are the only entities that behave as the potent biomarker for proper therapeutic diagnosis and prediction of T2DM (Zhang et al., 2013). The same authors analyzed two groups of normoglycemic subjects, to confirm the decrease expression of circulating miR-126 as a predictive factor for T2DM onset in susceptible individuals. During the follow-up of 2 consecutive years, one group was diagnosed as T2DM while the other remained normoglycemic, the result showed that plasma miR-126 have reduced expression before the onset of the patients of T2DM (Zhang et al., 2015).

Ortega et al. (Ortega et al., 2014a) through study revealed that circulating miRNAs have various nature of expression in case of T2DM particularly in the case of patients having normal glucose tolerance level and decreased plasma levels of miR-423-5p, miR-192, miR-195, miR-130b, miR-532-5p and miR-125b and specifically miR-126, together with increased levels of miR-140-5p, miR-142-3p, and miR-222 in T2DM patients. In actual, miR-140-5p, miR-423-50, miR-195 and miR-126 resulted to discriminate T2DM patients from control subjects with an accuracy of 89.2%.

Decreased miR-126, along with the promotion of apoptosis, may inhibit endothelial progenitor cells (EPCs) proliferation and migration, as well as, impairing formation
of new blood vessels or repair of the pre-existing vasculature. Therefore, the fact remains intact that miRNAs have the potential for diagnosis and also the prediction of DM, as well as showed significant ability to work as potent biomarkers (Empel et al., 2012).

In the recent study by Rong et al. (Rong et al., 2013), the cross-sectional data revealed that miR-146a can significantly be increased in the plasma of newly diagnosed T2DM patients in comparison to age and sex-matched the individuals having normal glucose tolerance level, suggesting a role for this miRNA in T2DM pathophysiology, with particular reference to its involvement in the hemeoxygenase-1 expression and indirectly in the oxidative stress caused by iron metabolism that is implicated in T2DM development (in this study, increased levels of circulating miR-146a were considerably related with increased concentration of plasma hemeoxygenase-1).

Baldeón et al. (Baldeón et al., 2014) incorporated that significant reduction in the level of serum miR-146a particularly in Ecuadorian patients with T2DM compared to obese and dyslipidemic non-diabetic subjects. It may be due to differences in study population including ethnicity or indicative parametric change in the attributed potential factors taken into consideration that could affect different expression profiles of microRNAs.

A different approach was taken by Wang et al. (Wang et al., 2016), where evaluation of 14 selected miRNAs in plasma was done taking the sample from 84 Iraqis among which 19 were found with T2DM and 68 Swedes among which 14 were found with T2DM symptoms. In the whole study population, the expression of miR-24 and miR-29b was significantly different between T2DM patients and non-diabetic subjects, while High expressions of miR-144 were found with T2DM in Swedes patients though not in the Iraqis patients, suggesting a phenomenon of association in terms of ethnicity.

Kong et al. (Kong et al., 2011) researched the expression of seven different miRNAs (namely: miR-9, miR-29a, miR-30d, miR-34a, miR-124a, miR-146a and miR-375) that were found to be involved in the pathogenesis of T2DM, in serum of newly diagnosed T2DM patients, of individuals with IFG and/or impaired glucose tolerance (IGT) and in subjects with normal glucose tolerance. The result of seven miRNAs
increased in serum of newly T2DM patient’s vs normoglycemic individuals, while five miRNAs were increased in T2DM patients vs subjects with IFG and/or IGT.

Liu et al. (Liu et al., 2014) examined the expression of miR-126 in 160 T2DM patients among which 75 individuals were IFG, 85 were found to be IGT and 138 were with normal glucose tolerance level. The level of miR-126 was low in IGT, IFG and T2DM individuals in comparison to healthy individuals, more importantly, after 6 months of treatment (diet and exercise in IFG/IGT patients; insulin plus diet and exercise in T2DM patients), it was observed that T2DM patients respect to IFG or IGT patients have a significantly highly increased level of miR-126.

Peripheral blood mononuclear cells (PBMCs) and whole-blood samples are employed to investigate circulating microRNAs in T2DM. Karolina et al. (Karolina et al., 2012) have characterized the miRNAs expression in whole blood and in exosomes from 265 affected individuals with metabolic syndrome and observed a hyper-expression of miR-150, miR-192, miR-320a, miR-375 and miR-27a in patients with T2DM. Apart from this, the report from the same author suggests that eight miRNAs (namely: miR-144, miR-150, miR-182, miR-192, miR-29a, miR-30d and miR-320a) expressed differential in whole blood within patients affected by T2DM and IFG.

2.14.1 MiR-375

These types of microRNAs are plentiful in numbers as they are present in the islet-specific microRNAs that are located in the intergenic region of the structure of chromosome 2 within human cells. Specifically it has been identified with the unique feature of regulating capability of insulin release and β-cell function. The genetic ingredients of Myotrophin (one of the targets of miR-375) are responsible for secretion of insulin and miR-375 acts by reducing the insulin levels within the body by glucose stimulation. Exocytosis of insulin granules are induced by the protein of Myotrophin, also, releasing of catecholamines are controlled by Myotrophin ((Esguerra, Mollet, Salunkhe, Wendt, & Eliasson, 2014). Moreover, protein 3-phosphoinositide-dependent protein kinase-1 (PDK1) is essential for activation of the PI3-Akt pathway. It has been expressed as another major target for miR-375 in the pancreatic islet of Langerhans. The PI3K-Akt cascade show a decreased activation
state when over expression of miR-375 directs towards reduction in the PDK1 levels. This results in the reduced activation of PI3K-Akt cascade leads to reduce in the mass of β-cell and then decreased production of insulin (El Ouamari et al., 2008). Diabetic phenotype is caused by depletion in the miR-375 due to reduction in the production of insulin levels and mass of β-cells. Notably, miR-375 have shown properties of biomarkers in case of samples of serum of T2DM patients collected from the Japan and China (Higuchi et al., 2015).

2.14.2 MiR-222

Important properties are exhibited by miR-222, when they were firstly discovered in human umbilical vein endothelial cells (HUVECs). Epithelial tumors got reduced due to increased expression of miR-222 as they inhibit cell proliferation and induce mitochondrial-mediated apoptosis through directly targeting the p53 up-regulated modulator of apoptosis (PUMA) in breast cancer (Zhang et al., 2010). Its function on proliferation has also been established in glioblastomas, lungs cancer, thyroid papillary cancer, hepatocellular carcinoma, breast cancer, and pancreatic cancer (Fu et al., 2011).

On the other hand, miR-222 can play roles in tumor suppressive through the down regulation of c-kit in erythroleukemia cells. Apart from its role in cancer progress, miR-222 has been found to participate in many physiological and pathological processes in the cardiovascular system. The necessary properties that can be exhibited by miR-222 is its role as a potential biomarker for cardiovascular disease. Recent articles are reviewed thoroughly to check the viability of miR-222 as a new therapeutic option in the context of potential biomarker in case of cardio-vascular disease (Ding, Huang, Xu, Zhu, & Zhong, 2017).

MiR-221 and miR-222 are expressed in many human organs such as the brain, and participate in the development of metabolic pathway, diabetes, and tumors (Dentelli et al., 2014). Abnormal expression of these miRNAs is observed in cases of obesity, cancer, and anoxia. Studies have shown that expression of miR-221/222 is up-regulated in rats and humans with fatty liver disease. They are also positively correlated with the insulin resistance index (Dentelli et al., 2014). Additionally, they are highly expressed in breast cancer tissues, and elevated serum level of miR-221
and miR-222 is correlated with tumor metastasis. Higher expression of miR-221/222 is suggestive of poor breast cancer prognosis, and is used as one of the indexes for prediction of disease progression (Hwang et al., 2013). The survival rate in breast cancer patients with over-expressed miR-221/222 is lower compared with patients with low miR-221/222 lowly expression (Li, Pan, & Qiu, 2016).

2.14.3 MiR-146a
In human chromosome 5, one of the intergenic microRNA is miR-146a that is involved in regulating inflammation and monitor processes regarding the innate immune system. An extra-cellular micro-protein known as Fibronectin, Interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) are being targeted by miR-146a as they confer signaling of NF-κB and IL-1β (Dong et al., 2013). Within retina, heart and kidney the fibronectin gets increased due to induction of glucose. This type of fibronectin accompanied by chronic, low-level inflammation is features of diabetes (Feng et al., 2011).

The miRNA-146a (miR-146a) is one of the most important miRNAs that its deregulation has been concerned in T2DM pathogenesis. Although upregulation/deregulation of miR-146a expression level has been reported in T2DM recurrently, the direction of deregulation events (up or down) remained to be inconsistent in literatures. There is much evidence showing that the expression levels of miR-146a that decreased significantly in PBMCs, serum and plasma samples individuals with T2DM in comparison to controlled individuals (Duan et al., 2014). In contrast to these results, it has been reported that miR-146a serum level was significantly upregulated in newly diagnosed T2DM patients compared to individuals with normal glucose tolerance (Kong et al., 2011).

The miR-146a is one of the most important miRNAs that its deregulation has been implicated in diabetes. Accumulating evidence has reported that miR-146a regulates the genes involved in the pathogenesis of T2DM and its related complications. It has been suggested that chronic inflammation is an important determinant in insulin resistance and also microvascular complications of T2DM including nephropathy, neuropathy and retinopathy (McClelland & Kantharidis, 2014). MiR-146a is involved in the regulation of toll like receptors (TLRs) signaling pathway in innate...
immune system. So, the reduction in miR-146a expression could lead to less efficient inhibition of target genes involved in the TLRs and other cytokine production and signaling pathways (Alipoor et al., 2017).

Studies have shown that miR-146a interacts directly with interleukin-1 receptor-associated kinase-1 (IRAK1)/TNF receptor-associated factor 6 (TRAF6), thus attenuating the inflammatory cytokines production in macrophages (He, Jing, & Cheng, 2014). It has been reported that patients suffering from T2DM have significantly decreased levels of miR-146a (Lenin, Sankaramoorthy, Mohan, & Balasubramanyam, 2015).

Further, the subgroup analysis by sample type showed that the association between miR-146a expression level and T2DM was significant in PBMCs and whole blood samples, whereas it was not significant for serum and plasma samples. As a result, determination of miR-146a expression level as molecular marker for T2DM can be more useful in PBMCs than in serum and plasma. One possible reason for this finding may be due to the fact that miRNAs expressions are cell and tissue specific. It has been shown that miR-146a levels are much more abundant in the PBMCs including lymphocytes and macrophages/monocytes (Saba, Sorensen, & Booth, 2014).

Seyhan did a pilot study taking cross-sectional data where it was found that miR-126, and miR-146a down regulated significantly within the plasma membrane of the individuals among the pre-T2DM stage which is individuals having IFG or IGT or a mildly increased level of HbA1c in comparison to individuals that possess normal glucose tolerance level. However, in another research by Rong et al. (Rong et al., 2013), it was found that within the plasma of diagnosed T2DM individuals there was high increase in circulation of miR-146a. It identified the indirect attachment of this microRNA with hemeooxygenase-1 expression in the detection of T2DM pathophysiology. The oxidative state caused due to iron metabolism in sex-matched normal glucose tolerant individuals is not significant as compared to individuals with T2DM having higher circulation of microRNAs in the highly concentrated plasma of hemeooxygenase-1 (Rong et al., 2013). Contrast to this study another study was put forth by Baldeón et al. (Baldeón et al., 2014) that revealed, Ecuadorian T2DM
patients have significantly diminished level of circulation of microRNAs in comparison individuals with dyslipidemic non-diabetes.

2.14.4 MiR-124-3p
Neurodevelopment and stem cells regulation is hugely dependent on the microRNA-124-3p that is present in the brain (Ryul Lee, Soo Kim, & Kim, 2010). Moreover, Brain Tumor, CNS development, neurodegeneration, CNS stress, stroke, neuroimmunity, such biological process are largely determined by the miR-124-3p (Sun, Luo, Guo, Su, & Liu, 2015). From various biological aspects this miR-124-3p have the ability to inhibit tumor progression by targeting various genes in the segments of glioma and specifically inhibits cell proliferation by targeting IQ motif containing GTPase activating protein 1 (IQGAP1), son of sevenless homolog 1 (SOS1), cell differentiation agent-2 (CDA-2), protein phosphatase 1 regulatory subunit 13 like (PPP1R13L), (Deng et al., 2016). miR-124-3p instigates migration of cells and invade them by targeting IQGAP1 (Lu, Jiang, Xiao, Liu, & Yuan, 2014), rho-associated coiled-coil containing protein kinase 1 (An, Liu, Wu, & Guan, 2013), PPP1R13L (Zhao, Wu, & Zhang, 2013), and calpain small subunit (Cai et al., 2016) in the segments of glioma. miR-124-3p also prompts cell differentiation in glioma by targeting CDA-2 or with reduced Cell division protein kinase 6 (CDK6) expression and inhibits glioma stem-like features through Snail family zinc finger 2 (Deng et al., 2016). Glioma angiogenesis and enhanced chemosensitivity is inhibited by miR-124-3p as they target the R-Ras and N-Ras (Shi et al., 2014). It also inhibits tumor progression by counteracting pro-survival stress responses in glioblastoma multiforme (GBM) (Mucaj et al., 2015). The antitumor effect of miR-124-3p in GBM cells is improved through gap junctions by relatively decreasing CDK6 expression (Suzhi et al., 2015). Additionally, miR-124-3p also associate with poor prognosis and malignant tumor progression in glioma patients (Teng Chen, Wang, Li, & Xu, 2015). Along with these, miR-124-3p is attached with resisting recurrent gliomas of high grade in Mexican children (Eguía-Aguilar et al., 2014). This indicates that this micro RNAs are potential biomarkers in the case of glioma. This incorporates the fact that PIM1 is also an important target gene of miR-124-3p as it invade multiple cellular pathways for cell proliferation to suppress the evil effect of
astrocytoma and enhance bioenergetics including apoptosis by PIM1 suppression. In the way to conclude, miR-124-3p reflects its properties as a tumor suppressor through channelized targets like PIM1 in case of astrocytoma and it also provides therapeutic measures for treatment and prevention of astrocytoma (Deng et al., 2016). Recently, miR-124-3p is detected as a suppressor of tumor in case of some cancerous cells including the specific phenomenon of hepatocellular carcinoma, gastric and cervical cancers (Wang et al., 2016; Xia et al., 2012).

2.14.5 MiR-155-5p

MiR-155 is intragenic and is kept within the only phylogenetically region of β-cell cluster of integration composed of 3 exons located in chromosome 21q21 and reflects properties that are oncogenic in nature. As a wider range of inflammatory mediator it is a common target that can be up-regulated by many inflammatory factors such as polyinosinic-polycytidylic acid, a synthetic analog of double-stranded RNA, interferon-β, lipopolysaccharides (LPSs), tumor necrosis factor-α (TNF-α), and interleukin-1β, by acting on TLR. In response to LPS, it is found in a lot of studies that the miR-155 reveals presence in macrophages and dendritic cells (Dai et al., 2011). It targets Suppressor of Cytokine Signaling 1 (SOCS1) and SH-2 containing inositol 5' polyphosphatase 1 (SHIP1). Targeting SHIP1 negatively regulation of PI3K/Akt by SHIP1 through downregulation of Akt genes (O'Connell, Rao, & Baltimore, 2012). In Mexico, miR-155 down regulated the PBMCs that are being isolated from the T2DM entities (Fernández, Bustamante, Leija, et al., 2013). Moreover, miR-155 is highly expressed in activated B cells, T cells, macrophages, and DCs (Faraoni, Antonetti, Cardone, & Bonmassar, 2009). It is up-regulated in primary murine macrophage and oxidized low density lipoprotein (oxLDL)-stimulated monocytes (Chen et al., 2009).

MiR-155, a key player in the regulation of adaptive immunity and antibody-related T-cell response, is upregulated in multiple immune cell lineages by toll-like receptor ligands and inflammatory cytokines (Thai et al., 2007) and is a mediator of inflammatory response (O'connell et al., 2010). Local inflammation associated with rejection is tightly regulated by T helper (Th) balance, and miR-155 controls differentiation of CD4+T cells into Th cells (Banerjee, Schambach, DeJong,
Hammond, & Reiner, 2010) and also participates in the development of regulatory T cells (Kohlhaas et al., 2009). miR-155 was overexpressed in renal allografts during acute rejection (AR), and a study of renal allograft biopsies and normal PBMCs found that several miRNAs, including miR-155, overexpressed in AR biopsies were also highly expressed in PBMCs. Moreover, intragraft expression of miR-155 and other miRNAs were reliable prognostic markers of AR and allograft function (Anglicheau et al., 2009).

Evidence from recent studies revealed that miR-155 exhibits crucial impact while monitoring tumor genesis, progression of tumor and colorectal cancer (CRC). Svrcek et al. (Svrcek et al., 2013) incorporated that identification of regulation of miR-155 may enhance the treatment and prevention of inflammatory bowel disease within CRCs and the case of instability of microsatellite. Valeri et al (Valeri et al., 2010) reported that miR-155 is inversely correlated to the expression of MLH1 or MSH2 proteins in case of human CRC. Hiroyuki et al (Yamamoto et al., 2012) added that the over-expression of miR-155 have the ability to down regulate MLH1, MSH2 as well as MSH6 that renders tumor genesis as outcome. Zhang et al (Zhang, Zhao, & Deng, 2013) justified that miR-155 targets tumor protein 53 present protein 1 and promotes proliferation of human breast cancer. Yu et al (Yu et al., 2013) added that miR-155 have the ability to promote metastasis of bone marrow. Gasparini et al (Gasparini et al., 2014) proved that miR-155 exhibits protective role by targeting homologous recombination by RAD51 followed by irradiation in case of breast cancer. Li et al (Li et al., 2014) incorporated the fact that in case of gastric cancer miR-155 is down regulated due to heterogeneity in the biological function. Thus, it can be concluded that miR-155 acts differently, even rendering an opposite effect in various types of cancer due to variety in their location within the body and stage of cancer.

As a whole, miR-155-5p reveals superior characteristics while regulating malignant tissues with CRC in impaired patients. miR-155-5p expresses correlated malignant phenotype of CRC based on the distant metastasis, TNM staging and tumor location. Modulation of the CRC cells can be made through cell proliferation, metastasis by the expression of miR-155. miR-155 have the potential to proliferate cells, invade them and execute metastasis of CRC cells through up regulated advancement. Hence,
it have vital role as a diagnostic factor in regulating tumor genesis and progression of tumor on the basis of different biological mechanism (Qu et al., 2015).

2.14.6 MiR-126

In the endothelial cells, miR-126 is one of the most available micro RNAs that helps in blood vessels formation and pro-angiogenesis of Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF) (Sinkam & Croce, 2010). At a matter of astonishment Zhang et al. proved that the miR-126 present in the endothelial cells decreases athero-protective laminar shear stress (Zhang et al., 2014). Within intron 7 and chromosome 9 there is presence of miR-126 that are the major epidermal growth factors (EGF) in the domain of EGF like domain multiple 7 (EGFL7) gene. Endothelium cells also secrete EGFL7 that are responsible for vasculogenesis (Saito et al., 2009). MiR-126-3p and miR-126-5p are highly expressive in the endothelium due to their binding of two transcription factor: EST1 and EST2 (Harris, Yamakuchi, Kondo, Oettgen, & Lowenstein, 2010). SNP within the base of miR-126 prevents the pri-micro RNA to mature it into the micro-RNA by reduced suppression of different targeted cells (Harnprasopwat et al., 2010).

In case of diabetic patients the circulation of miR-126 is very high within the endothelial cells of the plasma. Hence, their roles are pivotal in developing homeostasis and vascular development. Moreover, due to its secretion in diabetic patients the condition of the patients can be tested thoroughly (Zhang et al., 2013). Zampetaki et al. incorporated that negative correlation of miR-126 with the disease of artery reveals it as a sensitive biomarker for vascular complications for diabetes patients (Zampetaki et al., 2010). The level of miR-126 that are improvised in human cells with T2DM suggested the suitableness of circulating micro RNAs as the early forecaster of T2DM in case of vascular complications (Li et al., 2011).

Prior to this, numerous disease have been linked with miR-126 including cancer due to the fact that change in proportion of the circulating miR-126 is found to be increased or decreased depending on the nature of cancer that incorporates miR-126 to be one of the parametric indicators of the disease. It not only controls but also regulates the nature of variation of different proteins with prominent roles in multiple diseases, including the anti-inflammatory TOM1 (target of Myb protein 1), the
growth factor VEGF-A, and the cell cycle regulatory and signaling protein IRS-1 (insulin receptor substrate 1). Hence, movement of miR-126 can be suppressed by the presence of high blood glucose and can be stimulated with the presence of insulin (Liu et al., 2014).

2.14.7 MiR-103a-3p
MiR-103a-3p is under investigation in several cancerous cells. Papillary thyroid cancer cells was found to get decreased due to the presence of miR-103a-3p (Liu et al., 2013). However, it increased in endometrial cancer (Boren et al., 2008), nasopharyngeal carcinoma (Wang et al., 2014), pancreatic cancer (Chakraborty, Priya, & Bandyopadhyay, 2013), bladder cancer (Scheffer et al., 2014), colorectal cancer4 and lung cancer cells (Zhu et al., 2012). Cells that induced apoptosis of cancerous cells (PER3) and genes that are regarded as tumor suppressor like DICER and PTEN (Geng et al., 2014). In case of cellular proliferation and differentiation, silencing miR-103-3p eventually decreased the fat by reduction of the adipocyte size. (Trajkovski et al., 2011c), and inhibited proliferation of mouse intestinal cell by targeting the CCNE1, CDK2, and CREB1 genes (Liao & Lönnerdal, 2010). miR-103/miR-107 regulates the human metabolic pathways hence miR-103/miR-107 exhibits a crucial role by methodizing the movement of lipids and metabolizing the intrinsic energy within cells (Wilfred, Wang, & Nelson, 2007).

2.14.8 MiR-29a-3p
MiR-29a and miR-29b is a part of the miR-29a/b/c family within the group of the micro RNAs. It exhibits properties of high insulin prioritized intake of glucose and activate Akt that leads resistance within insulin in return. Akt is not at all the direct gene targeted by miR-29. However, over-expression of this micro RNA leads to down regulated secretion of insulin as it targets OC-2. It is one of the transcription factors that is responsible in the activation of granuphilin, that it is an inhibitor of the process of insulin exocytosis (Roggli et al., 2012). Insig1 is one of the other targets of miR-29, which is an endoplasmic reticulum membrane protein responsible for the biosynthesis of cholesterol. The Caveolin-2 (Cav2) induced during the phase of differentiation of adipocyte and Syntaxin-1, a receptor in the fusion of vesicle of
GLUT4 to the plasma membrane that is requisite for further exocytosis in pancreatic β-cells in insulin (Kumar et al., 2012). Progressive renal inflammation and fibrosis are controllable by miR-29b (Chen et al., 2014). miR-29a in the serum samples of China are found to up regulate the T2DM individuals (Kong et al., 2011). Also, reduction in the level of miR-29a is reported in certain plasma samples of the T2DM entities (Anna Zampetaki et al., 2010).

Kurtz et al. (Kurtz et al., 2014) incorporated that within livers of the DIO mice and in the Zucker Diabetic Fatty (fa/fa) rats miR-29 was found to be up regulated. The transcription factors are the determinant factors through which miR-29 regulates the FOXA2, PPARGC1A, HMGCS2 and ABHD5. Treatment of pioglitazone normalizes miR-29 through the murine models. Obesity in the sheep that are pregnant rejuvenates increased expression of miR-29 in the tissues of liver in case of offspring lambs. Apart from decreased markers in the signaling of insulin suggests fetal programming of miR-29 expression (Nicholas et al., 2013). However, in T cells, miR-29 represses Tbet and Eomes through transcription factors involved in IFN. The sensitivity of the T cells, and upregulation of the immune cells works as an anti-inflammatory agent due to the presence of miR-29. On the other hand, miR-29b promises as a prominent biomarker for atherosclerotic disease and T2DM. Zampetaki et al. (Zampetaki et al., 2010) scrutinized that the micro RNA expressions in the plasma membranes takes upregulation as the superior active and participatory responsibilities when it comes to the prospective human cohort in case of T2DM and albuminuria patients. The measure of diabetic nephropathy in comparison to the normoalbuminurimic patients are affected in a reduced manner with the presence of miR-223 expression. Hence, it can be concluded that miR-29 is a useful tool while regulating cardiometabolic disease specifically those with atherogenic risk and obesity of human.
2.15 MiRNAs as biomarkers of diabetic complications

Atherosclerotic cardiovascular disease is macro vascular complications and diseases like nephropathy, retinopathy, neuropathy, etc. are micro vascular complications. Both of these comprises of the symptoms of diabetes. Both of these have a high risk of mortality and morbidity. Cardiovascular risk worldwide can be given a score index that represents the estimation of hypertension or dyslipidemic individuals with cardiovascular disease due to smoking or anything else. Apart from these the problems, disease that are cardiovascular in nature may be medically tested thoroughly on the basis of clinical symptoms and provide biomarkers of these disease process by micro RNAs (Raffort, Hinault, Dumortier, & Van Obberghen, 2015).

The complications of human blood cells that are affected by T2DM may get several lines of controls with the help of the circulation micro RNAs in the plasma membranes. It will help to keep a thorough follow-up and track the nature of change and the direction of change within the affected patients (Sebastiani et al., 2017).

Being a novel source of biomarkers the miRNAs are the responsible indicators and determining factors of the variability of the undetected symptoms and the feasibility of the detected ones. About the nature and diversity of the miR-126 (Zampetaki et al., 2010), it is also important to reframe the integrity of vascular structure significantly since that are the major transcriptional factors of the change. T2D patients are inversely correlated with the vasculopathy and the predictive potential of the micro RNAs in the chronic complications are noteworthy. CAD proved to be decreased in terms of expression when it comes to the peripheral measurement of the whole blood compared to non-diabetic controls (Al-Kafaji et al., 2017). Along with that the distinguishing factor of the T2D patients with the CAD and without it is increased due to the presence of miR-126 circulation. To detect T2D with chronic complications there exist significant roles of circulated micro RNAs. Since they have higher predictive potential and detectable nature hence the source of operation of them in the endothelial functions and macro vascular operations are trustworthy (Al-Kafaji et al., 2016). In case of T2D patients 50 samples were taken and investigated among them 29 was T2D with microalbuminuria and 21 with macroalbuminuria.
The results were in accordance with the notions and protocols or diabetic nephropathy based on five micro RNAs namely miR-126, miR-661, miR-770, miR-892b, miR-1303. Notably, the outcome significantly up regulated the T2DM patients with chronic complications (micro vascular and macro vascular both) in comparison to diabetic patients with chronic complications (Wang et al., 2016). Sebastiani et al. incorporated that miR-31 exhibits hyper expression in the serum of T2D patients with the symptoms of microvascular complications rather than in 2D patients with macrovascular complications (Sebastiani et al., 2013). The vascular permeability is predictable with the E-selectin that are involved in the leukostasis and integrin-alpha 5 that are involved in angiogenesis being experimentally validated along with NOS1 which is oxidative stress (Sebastiani et al., 2017).

Diabetes is challenged by the most severe form of complications in the form of diabetic nephropathy (DN). miR-21, miR-29a/b/c and miR-192 all are responsible for DN pathogenesis with clinical significance to prevent and monitor DN advancement (Feng, Xing, & Xie, 2016). miR-21 was found to supersid the PTEN as a key modulator in the diabetic nephropathy where mir-192 revealed to be up regulated and responsible for controlling of the TGF-β-cells matrix proteins accumulations. miR-29c was found to exhibit SPRY1 that involves albuminuria and kidney mesangial phenomenon on the basis of the matrix structure of the diabetic mice models (Feng et al., 2016).

Investigation through several studies proved that micro RNAs are found to be present in different biological fluids (Fehlmann et al., 2016), like urine can get eventually correlated with the diabetic complications or urinary microRNAs have the potential to help in following up the changes in a diabetic patients. Moreover, diabetic nephropathy can be highly predictable with the help of these biological fluids and forecasted activities can be executed under scientific methodology that will reduce renal failure before onset of the microalbuminuria (Sebastiani et al., 2017). As a matter of fact, miR-320c if get isolated from the urinary exosomes then it may impact the TGF-β pathway of signaling through the medium of targeting THBS1 and could be regarded as a credible biomarker for progression of diabetic nephropathy (Delić et al., 2016).
A test was conducted on 8 non diabetic subjects, 8 T2D patients with nephropathy and 8 without nephropathy and the analysis showed that expression of micro RNAs in the context of the urinary exosomes are different. 16 micro RNAs reacted less responsively in case of T2D patients without non-diabetic nephropathy and more responsively in case of patients with diabetic nephropathy. The comparative analysis revealed that micro RNAs on case of T2D patients are deregulated with microalbuminuric patients rather than in case of T2D patients with normoalbuminuria proving strong correlation of progressive renal disease within the T2DM patients (Pomatto, Gai, Bussolati, & Camussi, 2017).
Chapter 3

Materials and Methods
Chapter 3
Materials and Methods

3.1 Materials

3.1.1 Equipments

The practical part of the present work was carried out in the Molecular Biology lab at Al-Aqsa University - Gaza. The major equipments used in the study are listed in Table (3.1)

Table (3.1): The equipments used in this study

<table>
<thead>
<tr>
<th>#</th>
<th>Instrument</th>
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<tr>
<td>1</td>
<td>Rotor-Gene Q Real Time PCR system</td>
<td>Qiagene - Germany</td>
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<tr>
<td>2</td>
<td>C1000 touch thermal cycler</td>
<td>BIO-RAD – Germany</td>
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<tr>
<td>3</td>
<td>Horizontal electrophoresis chambers / tanks</td>
<td>BIO-RAD – Germany</td>
</tr>
<tr>
<td>4</td>
<td>Power supply consort 200V, 200mA, 20W</td>
<td>Anachem – UK</td>
</tr>
<tr>
<td>5</td>
<td>Digital balance</td>
<td>AE Adam – USA</td>
</tr>
<tr>
<td>6</td>
<td>Multi-vortex V-32</td>
<td>Biosan – USA</td>
</tr>
<tr>
<td>7</td>
<td>Thermomixer blate</td>
<td>Eppendorf – Germany</td>
</tr>
<tr>
<td>8</td>
<td>Nanodrop 2000 C spectrophotometer</td>
<td>Thermo scientific – USA</td>
</tr>
<tr>
<td>9</td>
<td>UV transilluminator Gel documentation system</td>
<td>DNR BIO-Imaging–USA</td>
</tr>
<tr>
<td>10</td>
<td>PCR work station</td>
<td>Top Air - Israel</td>
</tr>
<tr>
<td>11</td>
<td>Chemistry auto-analyzer</td>
<td>Diasys response 910</td>
</tr>
<tr>
<td>12</td>
<td>Spectrophotometer(semi-analyzer)</td>
<td>Mindray BA-88 ,china</td>
</tr>
<tr>
<td>13</td>
<td>Water Bath</td>
<td>Memmetr , Germany</td>
</tr>
<tr>
<td>14</td>
<td>Cooling block or ice bucket suitable for PCR plates</td>
<td>Qiagen - Germany</td>
</tr>
<tr>
<td>15</td>
<td>Microcentrifuge</td>
<td>Hethec – Germany</td>
</tr>
<tr>
<td>16</td>
<td>Freezer, refrigerator</td>
<td>LG – USA</td>
</tr>
<tr>
<td>17</td>
<td>Microwave oven</td>
<td>Samsung – South Korean</td>
</tr>
<tr>
<td>18</td>
<td>Micropipettes 0.5–5 μl, 1–10 μl, 5–50 μl, 20–200 μl, 100-1000 μl</td>
<td>Thermo scientific – USA</td>
</tr>
</tbody>
</table>
### 3.1.2 Chemicals and Kits

Chemicals and Kits used in this study are listed in Table (3.2).

All chemicals are of molecular grade.

**Table (3.2):** Chemicals and Kits used in this study

<table>
<thead>
<tr>
<th>#</th>
<th>Chemicals and Kits</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ISOLATE II Biofluids RNA Kit</td>
<td>Bioline – UK</td>
</tr>
<tr>
<td></td>
<td><strong>Components:</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1. ISOLATE II RNA Columns</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. ISOLATE II Genomic DNA Removal Columns</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. Collection Tubes (2ml)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4. Elution Tubes (1.7ml)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5. Lysis Buffer RX</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6. Wash Buffer W1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7. DNase I Solution (RNase-free)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8. DNase I Reaction Buffer (DRB)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9. RNA Elution Buffer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11. Bench Protocol Sheet</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>EPIK™ miRNA Lo/Hi-ROX Kit</td>
<td>Bioline – UK</td>
</tr>
<tr>
<td></td>
<td><strong>Components:</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1. RT primer-Assay#1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. RT primer-Assay#2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. RT primer-Assay#3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4. RT primer-Assay#4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5. PCR primer mix-Assay#1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6. PCR primer mix-Assay#2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7. PCR primer mix-Assay#3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8. PCR primer mix-Assay#4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9. EPIK™ 5x RT Buffer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10. EPIK™ RT Enzyme</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11. 2x SensiSMART™ SYBR Master Mix</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12. DEPC Water</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>β-Mercaptoethanol (for Lysis Buffer RX)</td>
<td>Sigma – Aldrich,</td>
</tr>
<tr>
<td>4</td>
<td>96-100% Ethanol (for Wash Buffer W1)</td>
<td>Sigma – Aldrich,</td>
</tr>
<tr>
<td>5</td>
<td>Liquid nitrogen</td>
<td>Sigma – Aldrich,</td>
</tr>
</tbody>
</table>
6. Molecular biology grade water | Qiagen – Germany  
7. Glucose | Diasys, Germany  
8. HbA1c | Diasys, Germany  
9. Aspartate transaminase | Diasys, Germany  
10. Lactate dehydrogenase | Diasys, Germany  
11. Creatinine kinase (CPK) | Diasys, Germany  
12. Urea | Diasys, Germany  
13. Creatinine | Diasys, Germany  
14. Uric acid | Diasys, Germany  
15. Total Cholesterol | Diasys, Germany  
16. Total Triglycerides | Diasys, Germany  
17. HDL-C | Diasys, Germany  
18. hs. CRP | Diasys, Germany

### 3.1.3 Disposables

Disposables used in this study are listed in Table (3.3).

**Table (3.3): Disposables used in this study**

<table>
<thead>
<tr>
<th>#</th>
<th>Disposables</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nuclease-free disposable plastic ware</td>
<td>Bioline – UK</td>
</tr>
<tr>
<td>2</td>
<td>RNase-free microcentrifuge tubes (1.5ml)</td>
<td>Qiagen - Germany</td>
</tr>
<tr>
<td>3</td>
<td>Needle and syringe (both RNase free)</td>
<td>Homed – china</td>
</tr>
<tr>
<td>4</td>
<td>K3 EDTA tubes</td>
<td>BD Vacutainer –</td>
</tr>
<tr>
<td>5</td>
<td>Microfuge tubes for PCR - thin wall 0.1 mL capacity</td>
<td>Labcon, USA</td>
</tr>
</tbody>
</table>
Sterile RNase-free tips
0.5–5 μl
1–10 μl
5–50 μl
20–200 μl
100–1000 μl

Thermo scientific – USA

3.1.4 miRNA markers
All miRNA targets were selected based on previously published studies and are listed in Table (3.4).

Table (3.4): Information about targets miRNA

<table>
<thead>
<tr>
<th>#</th>
<th>Accession No</th>
<th>ID</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MIMAT0000728</td>
<td>has-miR-375</td>
<td>UUUGUUCGUUCGGCUCGCGUGA</td>
</tr>
<tr>
<td>2</td>
<td>MIMAT0000279</td>
<td>has-miR-222-3p</td>
<td>AGCUACAUCCUGGUACUGGGGU</td>
</tr>
<tr>
<td>3</td>
<td>MIMAT0000449</td>
<td>has-miR-146a-5p</td>
<td>UGAGAACUGAAAUCCAUGGGUU</td>
</tr>
<tr>
<td>4</td>
<td>MIMAT0000669</td>
<td>has-miR-16-5p</td>
<td>UAGCAGCAGUAAAUAUUGGC</td>
</tr>
<tr>
<td>5</td>
<td>MIMAT0000422</td>
<td>has-miR-124-3p</td>
<td>UAAGGACGCGGUGAUGCC</td>
</tr>
<tr>
<td>6</td>
<td>MIMAT0000646</td>
<td>has-miR-155-5p</td>
<td>UUAUGCUAAUCUGAUAGGGGU</td>
</tr>
<tr>
<td>7</td>
<td>MIMAT0000646</td>
<td>has-miR-126-5p</td>
<td>UUAUGCUAAUCUGAUAGGGGU</td>
</tr>
<tr>
<td>8</td>
<td>MIMAT0000101</td>
<td>has-miR-103a-3p</td>
<td>AGCAGCAUUUGUACAGGGCUAUGA</td>
</tr>
<tr>
<td>9</td>
<td>MIMAT0000093</td>
<td>has-miR-93-5p</td>
<td>CAAAGUGCUGUUCGUGCAGGUAG</td>
</tr>
<tr>
<td>10</td>
<td>MIMAT0000086</td>
<td>has-miR-29a-3p</td>
<td>UAGCAACACUGAAACUGGUUA</td>
</tr>
</tbody>
</table>
3.2 Methodology

3.2.1 Study design
The present study is a case control study, in which patients (cases) with type 2 DM were compared to healthy individuals (controls) without any evidence of DM.

3.2.2 Study setting
Study was in the Molecular genetics lab at Al-Aqsa University of Gaza.

3.2.3 Study population
The study population included (cases) who have T2DM and aged between 31-78 years from outpatient’s diabetic clinics of Ministry Health in Gaza Strip. Controls were normal persons with normal fasting blood glucose and negative family history of T2DM was matched in terms of sex and matched in age to a certain degree. The age couldn't be matched completely because the patient’s age ranged from 40 to 70, and we couldn't find individuals who are in the same age and not suffering from diabetes. Moreover we collect a large number of control samples which turned to be actually having elevated fasting blood glucose level.

3.2.4 Sampling and sample size
A total of 85 blood samples (47 males and 38 females) and age between 31-78 years were recruited from outpatient’s of the hospitals and a primary health care centres of the Ministry of Health in Gaza Strip, at which their diagnoses was according to the current WHO diagnostic criteria for diabetes (MOH, 2016; WHO, 2016). Thirty blood samples (14 males and 16 females) were also collected from healthy non diabetic persons who served as controls. The sample covered the five main Governorates of Gaza strip; Northern, Gaza, Mid-Zone, Khan Younis and Rafah.
3.3 Selection criteria

3.3.1 Inclusion criteria
• Cases: T2DM aged between 40-70 years.
• Controls: Normal fasting blood glucose aged between 40-70 years.
• T2DM Patients with or without CVD.

3.3.2 Exclusion criteria
• Cases and controls who are aged less than 40 years and more than 70 years old.
• T1DM patients.
• Controls with fasting blood glucose ≥100 mg/dl.

3.4 Ethical Consideration
The present study was approved by the Local Ethical Committee in Gaza Strip and performed according to the principles of the declaration of Helsinki (Appendix 1). Also, the approval of Ministry of Health (Appendix 2) was obtained to collect patient's blood sample and data from outpatient’s diabetic clinics of the Ministry of Health in Gaza Strip. Informed consent was obtained verbally from all participants to obtain their data and blood samples for analysis, after explaining the aim and objectives of this study.

3.5 Pilot study
Pilot study was performed by using 10 questionnaire and blood samples for cases and control to identify ambiguities and difficulties in data and sample collection from the study population, and validating the efficacy of reagents and the techniques.

3.6 Tools of the study
A questionnaire was completed for each patient by direct interview by the researcher. The data and the results obtained through blood samples were analyzed.
3.6.1 Questionnaire
All interviews were conducted face to face by the researcher himself. The questionnaire (Annex ) included questions about: Personal, demographic and socio-economic data such as: address, marital status, educational level, physical activity, smoking, family history of diabetes and the most important complications of diabetes (retinopathy, CVD, hypertension (HTN), neuropathy and others were covered in the first part. The second part included the Clinical and anthropometric data obtained from the patient and/or his/her medical records. The data included age, gender, residency, height, weight, type and duration of disease. During the study, the researcher explained to the participants any of the confused questions that were not clear to them.

3.6.2 Blood sample collection and processing
Fasting overnight venous blood samples (about 10 ml) were collected from 85 T2DM patients and 30 healthy non diabetic controls into sterile EDTA vacutainer and plain tubes and mixed gently, under quality control and safety procedure. The blood samples were drawn by the researcher himself and the help of the medical technologists of the centers outpatient’s of Ministry of Health. The blood samples were processed to collect serum and plasma by centrifugation at 2500 ×g for 10 min and freezing at -20 ºC until determination of the biochemical and molecular parameters. Two EDTA samples, each of which contains approximately 2.5 ml venous blood were collected. The first was used to perform FBG, HbA1c, complete blood count (CBC) that were analyzed for patients and controls with no delay. Plasma was prepared immediately from the other EDTA sample and stored in liquid nitrogen and later at -80ºC for RNA analysis for patients and controls. Serum samples were used for the determination of hs-CRP, CK, AST, LDH, TCH, TG, HDL-c, LDL-c, Urea, Creatinine and Uric Acid. Extracted RNA was stored at -80ºC until analysis.

3.6.3 Total RNA purification from plasma including miRNA
To harvest cell-free plasma, whole blood samples were centrifuged at 1200×g for 5 min at room temperature. RNA was extracted immediately after sample collection
using ISOLATE II Biofluids RNA Kit (Bioline, UK), according to the manufacturer protocol, which can be summarized as follows:

1. Buffer preparation:
   - Wash Buffer W1 Concentrate was reconstituted by addition of 90 ml of 96-100% ethanol to give a final volume of 128ml.
   - Ten microliter of β-ME were added to each 1ml of the lysis Buffer RX in a fume hood.
   - DNase 1–buffer mix was prepared by adding 15μl of the supplied DNase 1 Solution to 100μl of DNase 1 Reaction Buffer DRB and mixing gently by inverting the tube.

2. Two hundreds microliters of plasma were transferred to a 1.5ml RNase-free microcentrifuge tube.

3. Three hundred microliters of Lysis Buffer RX were added to 200μl plasma and mixing by vortexing for 10s.

4. Eight hundred microliters of 96-100% ethanol were added to lysate and mixing by vortexing for 10s.

5. Assemble an ISOLATE II RNA Column (black ring) with a provided Collection Tube.

6. Six hundred microliters of the ethanolic lysate were applied onto the column and centrifuge for 1 min at ≥8,000 xg, then discard the flow-through. Reassemble the spine column with its Collection Tube. Depending on the lysate volume, repeat steps 6 and 7 as required.

7. Four hundred microliters of Wash Buffer (W1) were applied to the column and centrifuge for 1 min at 14,000 xg, then Discard the flow-through and reassemble the spine column with its Collection Tube.

8. One hundred and fifteen microliters of the DNase1 – buffer mix were added to the column and centrifuge for 1 min at 14,000 xg, then Pipette the flow-through present in the Collection Tube back onto the top of the column. Incubation at room temperature (18-25 °C) for 15 min without any further centrifugation.

9. Four hundred microliters of Wash Buffer W1 were transferred to the column containing DNase1 – buffer mix and then centrifuge for 1 min at 14,000 xg,
then discard the flow-through and reassemble the spine column with its Collection Tube.

10. Column washed a third time by adding 400μl of 96-100% ethanol and centrifuge for 1 min at 14,000 xg, then discard the flow-through and reassemble the spine column with its Collection Tube.

11. Spin column for 2 min at 14,000 xg in order to dry the column thoroughly, then discard the Collection Tube.

12. Place the column into a fresh 1.7 ml Elution Tube.

13. Fifty microliters of RNA Elution Buffer were added to the column, then Centrifuge for 2 min at 200 xg, followed by 1 min at 14,000 xg.

14. The isolated RNA can be stored at -20°C for a few days or at -20°C for long-term storage.

The concentration of miRNA was determined by spectrophotometer measurement at 260 nm using a Nanodrop spectrophotometer (Thermo Fisher, USA).
In addition the purity of the extracted RNA was assessed by comparing the A260/A280 using the same instrument.

### 3.6.4 Quality control
Positive, negative-template and blank controls were applied in each run of PCR along with patients’ samples. The controls were treated the same as the test samples including extraction, amplification and electrophoresis to exclude false positive and false negative test results.

### 3.6.5 miRNA assays
The miRNA assays were performed for 8 miRNA targets selected based on literature reviewing for those most probably associated with diabetes and CVD. The miRNAs are (hsa-miR-29a-3p, hsa-miR-155-5p, hsa-miR-126-5p, hsa-miR-103a-3p, hsa-miR-124-3p, hsa-miR-146a-5p, hsa-miR-222-3p and hsa-miR-375). All assays were performed in duplicates.
The EPIK™ miRNA Select protocol (Bioline, UK) is a two-step protocol (Fig 3.1) consisting of:
**Step 1:** Reverse transcription with miRNA-specific RT-oligonucleotide and EPIK™ cDNA synthesis kit. The principle of the protocol relies on selectively converting only mature miRNAs into cDNA using a specific RT-primer for each target miRNA. The primer is conformationally restricted with a stem-loop structure in its 5’-end to allow efficient hybridization only to the mature form of the target miRNA.

**Step 2:** Real-Time PCR using SensiSMART™ SYBR Master Mix and amplification primers. In this step, a commonly available DNA-binding dye (SYBR Green) is used, rather than a probe-based system. Discrimination between miRNA sequences with high similarity is possible with melting curve analysis. The amplification process of target miRNAs is performed with a miRNA-specific forward primer and a nested reverse primer annealing to the RT-primer.

**Figure (3.1):** The EPIK™ miRNA Select Assay comprises of a set of three specific primers. The conformationally restricted RT primer allows efficient hybridization only to the mature form of the target miRNA (Stage 1). The miRNA-specific forward and nested reverse real-time PCR primers confer further specificity and enable robust amplification.

First-strand cDNA synthesis (Step 1)

1. All components and reactions were kept on ice during the procedure.
2. Gently thaw template RNA on ice. We recommend the use of 100 ng or less of total RNA per 20 μl RT reaction.
3. Gently thaw the EPIK 5x RT Buffer and RT Primer tubes on ice. Mix by vortexing (1 second) and spin down.
4. Assemble the reaction as indicated in Table (3.5): The most consistent results can be obtained by preparing a master mix with template RNA, EPIK 5x RT buffer, water and EPIK RT enzyme in the proportions shown. The EPIK RT Enzyme should be added to the master mix last, right before dispensing of the master mix into the PCR tubes.
5. Thoroughly mix the reagents by gently pipetting up and down. Spin down after mixing.
6. Incubate reaction at 42 °C for 30 min, followed by heat-inactivation of the reverse transcriptase at 90 °C for 5 min.
7. Keep the undiluted cDNA reactions on ice until the assembly of real-time PCR reaction.
8. If desired, undiluted cDNA reactions can be stored at 20 °C for up to three days.

Table (3.5): RT-PCR reaction mixture

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume μl / 1 sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPIK 5x RT Buffer</td>
<td>3</td>
</tr>
<tr>
<td>RT primer-has-miR-375</td>
<td>0.3</td>
</tr>
<tr>
<td>RT primer-has-miR-222-3p</td>
<td>0.3</td>
</tr>
<tr>
<td>RT primer-has-miR-146a-5p</td>
<td>0.3</td>
</tr>
<tr>
<td>RT primer-has-miR-16-5p</td>
<td>0.3</td>
</tr>
<tr>
<td>RT primer-has-miR-124-3p</td>
<td>0.3</td>
</tr>
<tr>
<td>RT primer-has-miR-155-5p</td>
<td>0.3</td>
</tr>
<tr>
<td>RT primer-has-miR-126-5p</td>
<td>0.3</td>
</tr>
<tr>
<td>RT primer-has-miR-103a-3p</td>
<td>0.3</td>
</tr>
<tr>
<td>Reagent</td>
<td>Volume μl / 1 sample</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>2x SensiSMART™ SYBR Master Mix</td>
<td>7.5</td>
</tr>
<tr>
<td>PCR primers (10X)</td>
<td>1.5</td>
</tr>
<tr>
<td>Nuclease-free water (DEPC)</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table (3.6):** SYBR green PCR mixture

**3.6.6 Real-Time PCR amplification and detection (step 2)**

1. In this step, the cDNA is amplified by real-time PCR using SensiSMART SYBR Master Mix.
2. Keep all reagents on ice (or at 4 °C) at all times during set up.
3. Thaw 2x SensiSMART™ SYBR Master Mix and cDNA reactions from Step 1 on ice.
4. Mix by quickly vortexing and spin down.
5. Dilute the cDNA reaction 10 fold with DEPC-water.
6. Prepare each qPCR reaction in the proportion indicated in Table (3.6): Mix by vortexing and spin down.

**For each target miRNA perform each sample in duplicate**
1. Centrifuge briefly (30 s at 200 x g in a suitable plate centrifuge). Spin down after Centrifugation.

2. Perform real-time PCR amplification according to the following cycling parameters that illustrate in Table (3.7).

Table (3.7): cycling parameters for each run

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95 °C</td>
<td>10 min</td>
<td>Polymerase activation</td>
</tr>
<tr>
<td></td>
<td>40 °C</td>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>95 °C</td>
<td>10 s</td>
<td>Denaturation</td>
</tr>
<tr>
<td></td>
<td>60 °C</td>
<td>30 s</td>
<td>Annealing/extension</td>
</tr>
</tbody>
</table>

3. We recommend adding a melt-curve analysis step to your reaction conditions. This is normally added as a set module during qPCR machine programming.

4. To obtain accurate, specific results for the miRNA and control assays, you must ensure that the real-time PCR amplification is performed exactly as set out above. Deviation from the protocol will yield poor results.

3.6.7 Determination of hematological parameters

The hematological parameters including WBCs count, RBCs count, Hb, HCT, mean cell volume (MCV), red blood cells distribution width (RDW) and PLTs count were measured. A complete system of reagents, control and calibrator of ABX Micros E560 (Horiba ABX-USA) was used to determine these parameters according to the manufacturer recommendations.

Principle

The procedure is based on electrical impedance (Coulter principle) in which whole blood is passed between two electrodes through an aperture so narrow that only one cell can pass through at a time. The impedance changes as a cell passes through. The change in impedance is proportional to cell volume, resulting in a cell count and
measure of volume. Impedance analysis returns CBCs and three-part WBC differentials (granulocytes, lymphocytes, and monocytes) but cannot distinguish between the similarly sized granular leukocytes.

**Expected values**
The reference range for WBCs: 4-10.9X10^9/L, RBCs: 4.5-6.1X10^12/L, Hb: Male (13-17g/dL), Female (12-16 g/dL), HCT: 0.40-0.50 L/L, MCV: 80-95 fL, RDW SD: 11.5-14.5% and PLTs: 150-450X10^9/L.

**3.6.8 Determination of FBS level**
Plasma FBS was quantitatively determined by enzymatic colorimetric method using Diasys diagnostics system international kit.

**Principle**
Glucose is oxidized by glucose oxidase (GOD) into gluconic acid and hydrogen peroxide. In presence of peroxidase (POD), hydrogen peroxides reacts with phenol and 4-aminoantipirine to form Quinoneimine, whose intensity at 500 nm is proportional to the glucose concentration in the sample.

\[
\text{GOD} \quad \text{Glucose} + \text{O}_2 \rightarrow \text{Gluconic acid} + \text{H}_2\text{O}_2 \\
\text{POD} \quad 2\text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine Phenol} \rightarrow \text{Quinoneimine} + 4\text{H}_2\text{O}
\]

**Table (3. 8):** list of reagents used for determination of FBG

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer pH 7.5</td>
<td>250 mmol/l</td>
</tr>
<tr>
<td>Phenol</td>
<td>5 mmol/l</td>
</tr>
<tr>
<td>4-Aminoantipyrine</td>
<td>0.5 mmol/l</td>
</tr>
<tr>
<td>GOD</td>
<td>≥ 15 kU/l</td>
</tr>
<tr>
<td>POD</td>
<td>≥ 1 kU/l</td>
</tr>
<tr>
<td>Standard</td>
<td>100 mg/dl</td>
</tr>
</tbody>
</table>
Assay Procedure
1. Ten microliters of standard (sample or calibrator) were added to 1 ml of the reagent and mixed well.
2. The mixture was incubated for 10 min at 37 ºC.
3. The absorbance was measured within 30 min against reagent blank.

Calculation
The glucose concentration in the sample was calculated using the following general formula:
Glucose (mg/dl) = \( \frac{\Delta A_{\text{sample}} \times \text{concentration of standard}}{\Delta A_{\text{standard}}} \)

Expected values
The reference range of plasma FBS was considered as 74-127 mg/dL for child and 70-106 mg/dL for adult as recommended by the kit.

3.6.9 Determination of TC
Serum TC was quantitatively determined by enzymatic colorimetric method using Diasys diagnostics system international kit.

Principle
Determination of TC after enzymatic hydrolysis and oxidation. The colorimetric indicator is quinoneimine which is generated from 4-aminoantipyrine and phenol by hydrogen peroxide under the catalytic action of peroxidase. The measurement is based on the following enzymatic reactions:

\[
\begin{align*}
\text{Cholesterol ester} + \text{H}_2\text{O} & \xrightarrow{\text{CHE}} \text{cholesterol} + \text{fatty acid} \\
\text{Cholesterol} + \text{O}_2 & \xrightarrow{\text{CHO}} \text{cholesterol-3-one} + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + 4\text{- aminoantipyrine} + \text{Phenol} & \xrightarrow{\text{POD}} \text{Quinoneimine} + 4\text{ H}_2\text{O}
\end{align*}
\]
Table (3.9): list of reagents used for determination of TC

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (pH 6.7)</td>
<td>50 mmol/l</td>
</tr>
<tr>
<td>Phenol</td>
<td>5 mmol/l</td>
</tr>
<tr>
<td>4- Aminoantipyrine</td>
<td>0.3 mmol/l</td>
</tr>
<tr>
<td>Cholesterol esterase (CHE)</td>
<td>≥ 200 u/l</td>
</tr>
<tr>
<td>Cholesterol oxidase (CHO)</td>
<td>≥ 100 u/l</td>
</tr>
<tr>
<td>Peroxidase (POD)</td>
<td>≥ 3 ku/l</td>
</tr>
<tr>
<td>Standard</td>
<td>200 mg</td>
</tr>
</tbody>
</table>

Assay Procedure
1. Ten μl of standard (sample or calibrator) was added to 1ml of working reagent and mixed well.
2. The mixture was incubated for 5 min at 37 ºC.
3. The absorbance was measured within 60 min against reagent blank.

Calculation
The TC concentration in the sample was calculated using the following general formula:

\[
TC (\text{mg/dl}) = \frac{\Delta A_{\text{sample}} \times \text{concentration of standard}}{\Delta A_{\text{standard}}} 
\]

Expected values
The reference range of serum TC was considered as: Desirable ≤ 200 mg/dl, Borderline high risk 200 – 240 mg/dL and High risk > 240 mg/dL as recommended by the kit.

3.6.10 Determination of TG
Serum TG was quantitatively determined by enzymatic colorimetric method using Diasys diagnostics system international kit {Cole, 2000 #101}. 
**Principle**

Determination of triglycerides after enzymatic splitting with lipoprotein lipase. Indicator is quinoneimine which is generated from 4-aminoantipyrine and 4-chlorophenol by hydrogen peroxide under the catalytic action of peroxidase.

\[
\text{Triglycerides} \xrightarrow{\text{LPL}} \text{Glycerol + fatty acid}
\]

\[
\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate + ADP}
\]

\[
\text{Glycerol-3-phosphate} + \text{O}_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone phosphate + H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + \text{Aminoantipyrine} + 4\text{-Chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + \text{HCl} + 4\text{H}_2\text{O}
\]

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cood's buffer (pH 7.2)</td>
<td>50 mmol/l</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
<td>4 mmol/l</td>
</tr>
<tr>
<td>ATP</td>
<td>2 mmol/l</td>
</tr>
<tr>
<td>Mg2+</td>
<td>15 mmol/l</td>
</tr>
<tr>
<td>Glycerokinase (GK)</td>
<td>≥ 0.4 KU/I</td>
</tr>
<tr>
<td>Peroxidase (POD)</td>
<td>≥ 2 KU/I</td>
</tr>
<tr>
<td>Lipoprotein lipase (LPL)</td>
<td>≥ 2 KU/I</td>
</tr>
<tr>
<td>4-Aminoantipyrine</td>
<td>0.5 mmol/l</td>
</tr>
<tr>
<td>Glycerol-3-phosphate-oxidase (GPO)</td>
<td>≥ 0.5 KU/I</td>
</tr>
<tr>
<td>Standard</td>
<td>200 mg/d</td>
</tr>
</tbody>
</table>
Assay procedure

1. Ten μl of standard (sample or calibrator) was added to 1ml of working reagent and mixed well.
2. The mixture was incubated for 5 min at 37 ºC.
3. The absorbance was measured within 60 min against reagent blank.

Calculation

The TG concentration in the sample was calculated using the following general formula:

\[
\text{TG (mg/dl)} = \frac{\Delta A_{\text{sample}} \times \text{concentration of standard}}{\Delta A_{\text{standard}}}
\]

Expected values

The reference range of serum TG was considered as: 40-160 mg/dl in mail, 35-135 mg/dL in female and High risk > 400 mg/dL as recommended by the kit.

3.6.11 Determination of HDL-c

Immunotertbidty diagnostic reagent for quantitative in vitro determination of HDL-c in DiaSys respons 910 {Bachorik, 2000 #102}.

HDL-c Immune FS is a homogeneous method for HDL-c measurement without centrifugation steps. Antibodies against human lipoproteins are used to form antigen-antibody complexes with LDL, VLDL and chylomicrons in a way that only HDL-c is selectively determined by an enzymatic cholesterol measurement.

Principle

\[
\text{Anti-human}{}^\beta\text{-lipoprotein antibodies} \rightarrow \text{LDL, VLDL, Chylomicrons} \rightarrow \text{Antigen-antibody complexes + HDL}
\]

\[
\text{HDL-cholesterol + H}_2\text{O} + \text{O}_2 \rightarrow \text{cholesterol-3-one + fatty acid +H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + \text{F-DAOS + 4-Aminoantipyrine} \rightarrow \text{Blue complex + H}_2\text{O}
\]
Table (3.11): list of reagents used for determination of (HDL-c)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1 Good’s buffer PH 7.0</td>
<td>25mmol/L</td>
</tr>
<tr>
<td>4-Aminoantipyrine</td>
<td>0.75mmol/L</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>2000 U/L</td>
</tr>
<tr>
<td>Ascorbate oxidase</td>
<td></td>
</tr>
<tr>
<td>Anti-human β-lipoprotein Antibody (sheep)</td>
<td>2250 U/L</td>
</tr>
<tr>
<td>R2 Good’s buffer vPH 7.0</td>
<td>30 mmol/L</td>
</tr>
<tr>
<td>Cholesterol esterase</td>
<td></td>
</tr>
<tr>
<td>Cholesterol oxidase</td>
<td>4000 U/L</td>
</tr>
<tr>
<td>N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxy-4-fluoroaniline, sodium salt (F-DPASOS)</td>
<td>20000 U/L 0.8mmol/L</td>
</tr>
</tbody>
</table>

**Assay Procedure**

2.4 μl of standard (sample or calibrator) was added to 240μl of R1, after mixing and incubating at 37 °C for 5 min, Read absorbance A1 then add reagent 60 μl of R2. Read absorbance A2. The absorbance was measured within 45 min against reagent blank.

**Calculation**

The HDL concentration in the sample was calculated using the following general formula:

\[
\Delta A = (A2 - A1) \text{ sample or calibrator}
\]

\[
\text{HDL (mg/dl)} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{concentration of standard}
\]

**Expected values**

The reference range of serum HDL was considered High risk of CHD ≤ 35 mg/dL as recommended by the kit.
3.6.12 Determination of LDL-c
LDL-c can be calculated using the empirical relationship of Friedewald Principle [Friedewald, 1972 #103]
LDL-c is most commonly estimated from quantitative measurements of total and HDL-c and triglycerides (TG) using the empirical relationship of Friedewald.

Equation
LDL-c = Total Cholesterol - HDL-c - TG/5

Expected values
The reference range of serum LDL was considered as: Desirable 130 mg/dl, Borderline high risk 130-160 mg/dL and High risk > 160 mg/dL as recommended by the kit.

3.6.13 Determination of aspartate aminotransferase (AST)
Serum AST activity is measured by using optimized ultraviolet-test according to International Federation of Clinical Chemistry and Laboratory Medicine [Huang, 2006 #105], using DiaSys reagent kit.

Principle
AST was transferred an amino acid from aspartate to 2-oxoglutarate and form oxaloacetate which reduced to malate and NADH was oxidized to NAD+ in presence of malate dehydrogenase. Addition of pyridoxal-5-phosphate (P-5-P) stabilizes the transaminases and avoids falsely low values in samples containing insufficient endogenous P-5-P, e.g. from patients with myocardial infarction, liver disease and intensive care patients.

L-Aspartate + 2-Oxoglutarate $\xrightarrow{\text{AST}}$ L-Glutamate + Oxaloacetate
Oxaloacetate + NADH+H+ $\xrightarrow{\text{MAD}}$ L-Malate + NAD+
Table (3.12): list of reagents used for determination of (AST)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R1</strong></td>
<td></td>
</tr>
<tr>
<td>TRIS pH 7.65</td>
<td>110 mmol/L</td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>320 mmol/L</td>
</tr>
<tr>
<td>MDH (malate dehydrogenase)</td>
<td>≥ 800 U/L</td>
</tr>
<tr>
<td>LDH (lactate dehydrogenase)</td>
<td>≥ 1200 U/L</td>
</tr>
<tr>
<td><strong>R2</strong></td>
<td></td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>65 mmol/L</td>
</tr>
<tr>
<td>NADH</td>
<td>1 mmol/L</td>
</tr>
<tr>
<td>Pyridoxal-5-phosphate FS</td>
<td>10 mmol/L</td>
</tr>
<tr>
<td>Good's buffer pH 9.6</td>
<td>100 mmol/L</td>
</tr>
<tr>
<td>Pyridoxal-5-phosphate</td>
<td>13 mmol/L</td>
</tr>
</tbody>
</table>

**Reagent Preparation**
Mix 4 parts of R1 + 1 part of R2 (e.g. 20 mL R1 + 5 mL R2) = mono-reagent
Stability: 4 weeks at 2 – 8 °C, 5 days at 15 – 25 °C, the mono-reagent must be protected from light.

**Assay Procedure**
After mixing and incubating 100 μl (calibrator/sample) with one ml of mono-reagent at test temperature for 1 min. Initial absorbance (A1) was read and reading was repeated at constant intervals of 1 minute, for 3 minutes. at wavelength 340 nm.

**Calculation**
From absorbance readings calculate ΔA/min and multiply by the corresponding factor from below:
A/min x factor = AST activity [U/L] Factor 1745
**Expected values**
The reference range of serum AST was considered < 35 U/L in adult as recommended by the kit.

**3.6.14 Determination of serum lactate dehydrogenase (LDH)**
Determination of LDH in serum on DiaSys reagent {Kricka, 2000 #106}.
Level LDH was determined by kinetic method (Optimized UV test).

**Principle**
The enzyme LDH converted pyruvate to lactate while oxidized NADH to NAD+. The reaction was monitored kinetically at 340 nm by the rate of decrease in absorbance resulting from the oxidation of NADH to NAD+ proportional to the activity of LDH.

L-Lactate + NAD+ \[\rightleftharpoons\] Pyruvate + NADH+

**Table (3. 13): list of reagents used for determination of (LDH)**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>N-Methyl-D-Glucamine pH 9.5</td>
</tr>
<tr>
<td></td>
<td>L-Lactate</td>
</tr>
<tr>
<td>R2</td>
<td>NAD+ pH 4.5</td>
</tr>
</tbody>
</table>

**Reagent Preparation**
Mix 4 parts of R1 + 1 part of R2 (e.g. 20 mL R1 + 5 mL R2) = mono-reagent

**Assay Procedure**
After mixing and incubating ten μl of (calibrator/sample) with one ml of mono-reagent at test temperature for 1 min. Initial absorbance (A1) was read and reading was repeated at constant intervals of 1 minute, for 3 minutes, at wavelength 340 nm against air.
**Calculation**
From absorbance readings calculate $\Delta A/\text{min}$ and multiply by the corresponding factor from below:

$\Delta A/\text{min} \times \text{factor} = \text{LDH activity [U/L]} \quad \text{Factor 16030}$

**Expected values**
The reference range of serum LDH was considered $< 480 \text{ U/L}$ in adult as recommended by the kit.

**3.6.15 Determination of serum CK**
Determination of Creatine kinas (CK) in serum was determined by kinetic method optimized UV-test {Bishop, 2010 #110}

**Principle**
CPK was catalysed the reversible transfer of phosphate from adenosine triphosphate (ATP) to Creatine then using ATP to form glucose-6-phosphate in presence hexokinase (HK). Glucose-6-phosphate was reacted with NADP+ in presence of glucose-6-phosphate dehydrogenase and rate of formation NADPH was measured at 340 nm, the amount of NADPH produced is proportional to the CPK activity present in the serum sample.

\[
\begin{align*}
\text{Creatine phosphate} + \text{ADP} & \quad \xrightleftharpoons{\text{CK}} \quad \text{Creatine} + \text{ATP} \\
\text{Glucose} + \text{ATP} & \quad \xrightleftharpoons{\text{HK}} \quad \text{Glucose-6-phosphate} + \text{ADP} \\
\text{Glucose-6-phosphate} + \text{NADP}^+ & \quad \xrightleftharpoons{\text{G6P-DH}} \quad \text{Glucose-6-phosphate} + \text{NADPH} + \text{H}^+
\end{align*}
\]
### Table (3.14): list of reagents used for determination of (CK)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R1</strong></td>
<td></td>
</tr>
<tr>
<td>Imidazole pH 6.5</td>
<td>60 mmol/L</td>
</tr>
<tr>
<td>Glucose</td>
<td>27 mmol/L</td>
</tr>
<tr>
<td>N-Acetylcysteine (NAC)</td>
<td>27 mmol/L</td>
</tr>
<tr>
<td>Magnesium acetate</td>
<td>14 mmol/L</td>
</tr>
<tr>
<td>EDTA-Na+2</td>
<td>2 mmol/L</td>
</tr>
<tr>
<td>NADP</td>
<td>2.7 mmol/L</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>5 kU/L</td>
</tr>
<tr>
<td><strong>R2</strong></td>
<td></td>
</tr>
<tr>
<td>Imidazole</td>
<td>160 mmol/L</td>
</tr>
<tr>
<td>ADP</td>
<td>11 mmol/L</td>
</tr>
<tr>
<td>AMP</td>
<td>28 mmol/L</td>
</tr>
<tr>
<td>Diadenosine pentaphosphate</td>
<td>55 mmol/L</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase (G6P-DH)</td>
<td>14 kU/L</td>
</tr>
<tr>
<td>EDTA-Na+2</td>
<td>2 mmol/L</td>
</tr>
<tr>
<td>Creatine phosphate</td>
<td>160 mmol/L</td>
</tr>
</tbody>
</table>

### Assay Procedure
Against reagent blank (40 μl of distilled water and 1 ml mono-reagent).

### Reagent Preparation
Mix 4 parts of R1 + 1 part of R2 (e.g. 20 mL R1 + 5 mL R2) = mono-reagent
Stability: 3 weeks at 2 – 8 °C, 2 days at 15 – 25 °C
After mixing and incubating forty μl (calibrator /sample) with one ml of mono-reagent at test temperature for 1 min. Initial absorbance (A1) was read and reading
was repeated at constant intervals of 1 minute, for 3 minutes at 340 nm against reagent blank.

**Calculation**
From absorbance readings calculate ΔA/min and multiply by the corresponding factor from below:

ΔA/min x factor = CPK activity [U/L]

Factor 4127

**Expected values**
The reference range of serum CK was considered < 190 U/L in adult as recommended by the kit.

**3.7 Quality control**
Normal and abnormal control were used in each run through all tests along with patient’s samples. The controls were treated the same as the test samples to verify the performance of measurement procedures. The results of patient’s samples were accepted only when the controls results were found inside the defined range.

**3.8 Data analysis**
The Threshold Cycle (Ct) reflects the cycle number at which the fluorescence curve generated within a reaction crosses the threshold in RT-qPCR. Raw Ct values were collected using the Rotor-Gene Q software supplied with the real-time PCR instrument (Qiagen, Germany). The background fluorescence was automatically detected using the Dynamic tube method and the threshold line was set to a value that is one tenth of the average maximal fluorescence value. The data was exported as an Excel file for further analysis. Relative expression levels of the 8 selected miRNAs was determined using the ΔΔCt method. In this method, Ct values for every miRNA target were normalized to the endogenous hsa-miR-93-5p target from the same case or control samples. The ΔCt was calculated, for all target miRNAs in each case and control sample, by subtracting the Ct values of hsa-miR-93-5p endogenous normalizer miRNA from the Ct values of the miRNA of interest (ΔCt = Ct miR of interest - Ct hsa-miR-93-5p). ΔΔCt was then calculated by subtracting the ΔCt of
samples from cases (patients with known diseased, either diabetic or diabetic with cardiovascular manifestations) from the ΔCt of the healthy controls (ΔΔCt = ΔCt_{control} – C_{patients}). This method determines the change in expression of a nucleic acid sequence in a diseased sample relative to the same sequence in a healthy control sample. The fold-change in miRNAs was calculated for each case relative to the control by the equation 2^{ΔΔCt} (Livak & Schmittgen, 2001). The unpaired t-test with Welch's correction as well as the Area Under the Curve (AUC) values for each miRNA to assess its suitability as a single biomarker were computed using GraphPad software, version 5.0 for Windows (GraphPad Software Inc, CA). Differences were considered significant at P-values below 0.05 and an AUC value close to 0 (or 1) indicates a high diagnostic value, because the expression values of one group are in this case mostly higher (or lower) than in the compared group.

Clinical and expression data were tabulated and analyzed using the SPSS software (Statistical Package for the Social Science Inc. Chicago, Illinois USA, version 20). Simple distribution of the study variables and the cross tabulation were applied. Chi-square (x2) was used to identify the significance of the relations, associations, and interactions among various categorical variables. One-way ANOVA was used to determine association and comparisons between independent groups. Correlation analysis using Spearman’s correlations coefficient for nonparametric variables was used. The statistical significance of differences between groups were considered statistically significant when the P values <0.05 with a false discovery rate (FDR) for all data (clinical data and miRNAs expression levels).
Chapter 4

Results and Discussion
miRNAs are a group of specific highly conserved sequences composed of single strand non-coding RNA (Sebastiani et al., 2017). The main function of miRNAs is to regulate gene expression by binding to complementary sequences on the 3′-UTR regions of their target mRNAs. This leads to translational inhibition and/or mRNA degradation (Bhatia et al., 2015; Parrizas & Novials, 2016; G. Sebastiani et al., 2017). There is a significant role played by the miRNAs in case of secretion of insulin, development and differentiation of the pancreatic β-cell and indirect control of the lipid and glucose metabolism, along with risk for secondary complications related to diabetes (Li, 2014).

The traditional biomarkers such as fasting blood sugar, HbA1c, and novel biomarkers such as adipokines, cytokines and C-reactive protein cannot efficiently detect the individuals who are going through the phase of developing T2DM because they identify subjects already displaying metabolic alterations. Circulating miRNAs represent promising diagnostic biomarkers in patients with T2DM as well as in individuals at risk of developing the disease, showing distinct miRNAs expression profiles (Sebastiani et al., 2017).

4.1 Description and clinicopathological characteristics of the study population

The present study is a case-control study, in which patients (cases) with T2DM were compared to normal persons (controls) without any evidence of DM. A total of 116 individuals were recruited and divided into two groups; the case group included 85 patients and the control group included 30 healthy subjects. The case group was further divided into two groups: first group included 62 T2DM patients without cardiovascular complications and the second group included 24 T2DM with cardiovascular complications (T2DM/CVD). Patients were recruited from outpatients of the hospitals and a primary health care centres of the Ministry of Health in Gaza Strip, at which their diagnoses was according to the current WHO diagnostic criteria for diabetes.
4.1.1 Gender:
Population of the case group, included 48 males (55.8%) and 38 females (44.2%). The control group included 30 healthy subjects who don't have any evidence of DM: 14 (46.7%) were males and 16 (53.3%) were females. Table 4.2 illustrates distribution of the study population according to gender.

Table 4.2: distribution of the study sample by gender (n=116).

<table>
<thead>
<tr>
<th>Gender</th>
<th>Control</th>
<th>Case</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Count</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
<td>48</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>46.7%</td>
<td>55.8%</td>
<td>53.5%</td>
</tr>
<tr>
<td>Female</td>
<td>16</td>
<td>38</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>53.3%</td>
<td>44.2%</td>
<td>46.5%</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>86</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>100.0%</td>
<td>100.0%</td>
<td>100%</td>
</tr>
</tbody>
</table>

4.1.2 Age:
One of the most common risk factors for T2DM is the increasing of age. In our study the age range of the study population was 31 to 78 years as shown in Table (4.3). The mean age for T2DM patients group was 56 ± 10 years, the mean age for T2DM/CVD patients group was 58 ± 7 years and the mean age for control groups was 47 ± 10. In our study the independent sample t-test showed significant difference between mean ages of cases and controls (P=< 0.001) as shown in Table (4.3). The age differences between the case and control groups might be attributed to the nature of experiments. T2DM is very common in the old persons and naturally the majority of T2DM patients this study were relatively old. On the other hand, it was difficult to recruit a large number of T2DM-free healthy persons with ages comparable to the case group. Importantly, our result didn't show any significant correlation between tested miRNA and age (see Table 4.17: Correlation of selected miRNA and clinical data, page 95). Additionally, several studies showed that there is no significant association between the selected miRNAs and age. For example, recent study showed that there is no significant association between miR-29a expression level and other
clinicopathological features (gender, age, smoking history and tumour differentiation) was identified, ($P=0.449$) (Wang, Li, & Jing, 2017).

**Table 4.3: Age of the study population:**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Mean Diabetic</th>
<th>Mean Cardiovascular</th>
<th>Control</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>56 ± 10</td>
<td>58 ± 7</td>
<td>47 ± 10</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

**Note:** Anova analysis test, data presented as mean ± SD; $P<0.05$ was considered to be significant; $P<0.001$ was considered to be highly significant.

### 4.1.3 Body mass index (BMI) of the study population:

Table 4.4 shows the mean BMI for T2DM patients group was $32.45 \pm 10.80$ Kg/m², the mean BMI for T2DM/CVD patients group was $33.11 \pm 11.50$ Kg/m², and the mean BMI for control groups was $29.85 \pm 6.43$ Kg/m². The Anova analysis test shows that there was no significant difference observed in mean BMI of cases (T2DM and CVD) compared to control ($P=0.403$).

BMI is a measurement that compares individual's weight for height (kg/m²), and is usually used to assess obesity. Obesity is a major risk factor for chronic disease including diabetes (Sunyer, 2009). Obesity is commonly defined as a BMI of 30 Kg/m² or higher. This definition distinguishes obesity from being pre-obese or overweight, which is classified as a BMI of 25 kg/m² or more but less than 30 Kg/m² (WHO, 2015, 2016). Accordingly, in our study about 51.2% of type 2 diabetics are obese. But, there was no significant difference between cases (T2DM and CVD) and controls in BMI. In accordance with our results, a Scottish study has previously shown no significant correlation between random blood sugar levels and BMI (Ihanghorbani, Hedley, Iones, & Gilmour, 1992). However, other studies showed that BMI correlates with blood glucose levels. For example, a cross-sectional study have shown that high BMI is associated with a higher level of total cholesterol, glucose and unfavorable lipid pattern showing low concentrations of HDL cholesterol and high triglyceride concentrations (Rainwater et al., 2000; Wild & Byrne, 2006). Lack of significant difference in our case may have resulted from the
number of groups. Ethnic and other biological factors may be responsible for these differences as was suggested by other studies (Dowling & Sunyer, 1993).

**Table 4.4: Body mass index (BMI) of the study population:**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Mean Diabetic</th>
<th>Mean Cardiovascular</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (Kg/m²)</td>
<td>32.45 ± 10.80</td>
<td>33.11 ± 11.50</td>
<td>29.85 ± 6.43</td>
<td>0.403</td>
</tr>
</tbody>
</table>

**Note:** Anova analysis test, data presented as mean ± SD; P<0.05 was considered to be significant; P<0.001 was considered to be highly significant.

**BMI:** Body mass index (Kg/m²): People with BMI=18.5-24.9 were considered to have normal weight, people with BMI=25.0-29.9 were classified overweight, people with BM≥30.0 were considered obese (Organization, 2015, 2016).

4.2 Clinicopathological characteristics of the study population:

4.2.1 FBS and HbA1c level of the study population

According to the WHO, the most important biomarkers for T2DM is FBS and HbA1c (ADA, 2016; Ogurtsova et al., 2017; WHO, 2016) Table 4.4 illustrates the mean levels of FBS and HbA1c between case and control of the study population. In our study the mean FBS for T2DM patients group was 179 ± 70 mg/dl, the mean FBS for T2DM/CVD patients group was 191 ± 77 mg/dl and the mean FBS for control groups was 84 ± 7 mg/dl. On the other hand, the mean HbA1c for T2DM patient group was 8.61 ± 1.86 %, the mean HbA1c for T2DM with cardiovascular patients group was 8.97 ± 1.61 % and the mean HbA1c for control groups was 5.77 ± 0.49 %. In our study the ANOVA test showed highly significant difference between mean FBS and HbA1c of cases (T2DM and CVD) and control (P=< 0.001) and (P=< 0.001) respectively as shown in Table 4.4.

Our results were similar to the most recent reports published by (ADA, 2016; Florkowski, 2013; WHO, 2015) who found that type 2 diabetic patients had higher fasting glucose and HbA1c levels than non-diabetics. Moreover, in a previous population-based retrospective cohort study by Chen et al. (Chen et al., 2015), participants were selected from the 2002 Taiwan's Triple High Survey. Chen et al. association was found between the HbA1c level in patients with or without T2DM
and future risks of CVD and death. In the same study, the levels of HbA1c were associated with increased risks of CVDs and death, particularly in suboptimal glycemic control with HbA1c level over 7.5% (Chen et al., 2015). In addition, in a previous study by Eeg-Olofsson et al. (Eeg-Olofsson et al., 2010) investigated the association between HbA1c and CVD in patients with T2DM in the Swedish National Diabetes Register (NDR). This observational study exhibited progressively increasing risks of CHD, CVD and total mortality with higher HbA1c, and no risk increase at low HbA1c levels even with longer diabetes duration, previous CVD or treatment with either insulin or oral hypoglycemic agents (OHAs). Additionally, patients reaching HbA1c <7% showed benefits for risk reduction (Eeg-Olofsson et al., 2010). In diabetes, Hyperglycaemia promotes the development of microvascular complications by activating six major pathways, including enhanced polyol pathway flux, increased formation of advanced glycation end products (AGEs), increased AGE receptor expression, activation of PKC isoforms, enhanced hexosamine flux and increased intracellular reactive oxygen species (Giacco & Brownlee, 2010). T2DM also affects the macrovasculature and the incidence of myocardial infarction and peripheral vascular disease and stroke is markedly increased (Marja-Riitta Taskinen & Borén, 2015). The level of HbA1c indicates any case of chronic hyperglycaemia existing postdating a period of six to eight weeks rather than at the time the test is taken (Chen et al., 2015). The HbA1c test offers a number of advantages over the FBS and OGTT, including greater convenience (fasting not required), greater pre-analytical stability, stronger correlation with microvascular complications, especially that of retinopathy, a marker for glycaemic control and protein glycation (Kharroubi & Darwish, 2015).

**Table 4.4:** FBS and HbA1C level of the study population:

<table>
<thead>
<tr>
<th>Feature</th>
<th>Mean Diabetic</th>
<th>Mean Cardiovascular</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBS (mg/dl)</td>
<td>179 ± 70</td>
<td>191 ± 77</td>
<td>84 ± 7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.61 ± 1.86</td>
<td>8.97 ± 1.61</td>
<td>5.77 ± 0.49</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

**Note:** Anova analysis test, data presented as mean ± SD; P<0.05 was considered to be significant; P<0.001 was considered to be highly significant.
4.2.2 Serum urea, creatinine and uric acid of the study population

Serum urea, creatinine and uric acid concentrations are important parameters in the management of chronic diseases such as diabetes mellitus. Diabetes mellitus has an effect on the excretion of uric acid and creatinine in both sexes of DM patients (Richard et al., 2017). The concentrations of serum urea, creatinine and uric acid (presented in Table 4.5) was used as indication reflection of kidney function (Nirwan, Vyas, & Jain, 2017). In our study the mean urea, creatinine and uric acid for T2DM patients group was 34 ± 20, 0.91 ± 0.34 and 4.36 ± 1.22 mg/dl respectively, the mean urea, creatinine, uric acid for T2DM/CVD patients group was 33 ± 8, 0.88 ± 0.16 and 4.90 ± 1.36 mg/dl respectively, and the mean urea, creatinine, uric acid for control groups was 28 ± 5, 0.80 ± 0.09 and 3.80 ± 0.98 mg/dl respectively. The Anova analysis test revealed no significant difference was observed between mean urea and creatinine of cases (T2DM and CVD) and control (P=0.293) and (P=0.158) respectively. On the other hand, the serum uric acid concentration was higher in diabetic with CVD than in the healthy and there was significant difference between mean uric acid of cases (T2DM and CVD) and control (P=0.006). A similar result was documented by Joshua Richard et al (Richard et al., 2017) in a prospective study comprised of 76 type 2 diabetes mellitus patients and 74 non diabetic control subjects. They found no significant correlation observed between urea and creatinine of cases compared to controls. The increase in the serum urea, uric acid and creatinine levels may be an implication of general diseases (Toyama et al., 2015) like renal insufficiency which can cause increased creatinine and uric acid level as a result of reduction of blood flow to the kidneys. Renal artery stenosis also decreases blood flow to the kidney (Richard et al., 2017).

However, urea is a waste product from protein cleavage in the liver and is filtered out in the blood via the kidneys. This is considered as one marker of the kidney function (Edelstein, 2017). Furthermore, urea concentrations in microalbuminuric and macroalbuminuric disease and with progressive disease show that the kidney function declines (Pan, Guo, & Jin, 2008).

Creatinine is a waste product that is usually filtered from the blood and excreted in the urine. High creatinine levels in diabetic patients may indicate failure of their kidney function (Manzella, 2008). Uric acid is the product of human purine
metabolism. The increase of uric acid in the body or and the decrease in excretion of uric acid can cause the increase of serum uric acid level (Li, Jiao, & Cheng, 2018). Elevated levels of uric acid may indicate kidney failure or kidney stones which in several prior studies reported that increased uric acid levels are associated with hypertension and the risk of T2DM (Li et al., 2018). Increased serum uric acid may also lead to cardiovascular disease (Yoo et al., 2005), this agreement with the result in our study that show in (table 4.5)

**Table 4.5: Serum urea, creatinine and uric acid of the study population:**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Mean Diabetic</th>
<th>Mean Cardiovascular</th>
<th>Control Cardiovascular</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea (mg/dl)</td>
<td>34 ± 20</td>
<td>33 ± 8</td>
<td>28 ± 5</td>
<td>0.293</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.91 ± 0.34</td>
<td>0.88 ± 0.16</td>
<td>0.80 ± 0.09</td>
<td>0.158</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>4.36 ± 1.22</td>
<td>4.90 ± 1.36</td>
<td>3.80 ± 0.98</td>
<td>0.006</td>
</tr>
</tbody>
</table>

**Note:** Anova analysis test, data presented as mean ± SD; P<0.05 was considered to be significant; P<0.001 was considered to be highly significant.

### 4.2.3 Serum lipid profile of the study population

Table 4.6 illustrates the serum lipid profile including Triglycerides (TG), total Cholesterol (TCH), low-density lipoprotein cholesterol (LDL-c), and high-density lipoprotein cholesterol (HDL-c) of the study population. In our study the mean level of TG, TCH, LDL-c and HDL-c for T2DM patients group was 193 ± 95, 202 ± 39, 121 ± 39 and 56 ± 26 mg/dl respectively. The mean level of TG, TCH, LDL-c and HDL-c for T2DM/CVD patients group was 186 ± 87, 192 ± 31, 108 ± 30 and 58 ± 25 mg/dl respectively, while the mean level of TG, TCH, LDL-c and HDL-c for control groups was 157 ± 88, 190 ± 28, 111 ± 30 and 50 ± 25 mg/dl respectively. The Anova analysis test shows that although there were increase in the serum level of TG, TCH and LDL-c, the HDL-c was decrease. However, data presented in this study revealed no statically significant difference was observed between mean TG, TCH, LDL-c, and HDL-c of cases (T2DM and CVD) compared to control (P=0.224), (P=0.209), (P=0.231) and (P=0.309) respectively.

The result in this study demonstrates the typical diabetic dyslipidemia which is characterized by high triglyceride, high cholesterol, low HDL (Table 4.6). Various
national and international epidemiological studies on lipid profile have also shown this pattern of dyslipidemia (Bhatti et al., 2007; Gupta, Arora, & Sharma, 2015). Furthermore, no significant difference were observed in total cholesterol and LDL-c levels in cases and controls in this study. Even the concentration of LDL-c is not significantly increased, generally there is a minor increase in LDL levels. These changes might be attributed to the increased free fatty acid flux and insulin resistance (Morgantini et al., 2011; Eckardstein & Widmann, 2014).

In patients with diabetes, many studies have recognized that complications are mainly due to chronic hyperglycaemia that exerts its harmful health effects through several mechanisms: dyslipidemia, platelet activation, and altered endothelial metabolism. Both abnormal lipid profile and diabetes have been shown to be important predictors for metabolic disturbances including dyslipidaemia, hypertension and cardiovascular diseases. Lipids play a vital role in the pathogenesis of diabetes mellitus (Ozder, 2014). Dyslipidemia in diabetes commonly manifests as raised LDL-c, decreased HDL-c levels, or elevated TG and TCH levels. Additionally, data from the United Kingdom Prospective Diabetes Study suggest that both decreased HDL-c and elevated LDL-c predict CVD in diabetes (Sultania, Thakur, & Kulshreshtha, 2017).

Table 4.6: Lipid profile of the study population:

<table>
<thead>
<tr>
<th>Feature</th>
<th>Mean Diabetic</th>
<th>Mean Cardiovascular</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>193 ± 95</td>
<td>186 ± 87</td>
<td>157 ± 88</td>
<td>0.224</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>202 ± 39</td>
<td>192 ± 31</td>
<td>190 ± 28</td>
<td>0.209</td>
</tr>
<tr>
<td>LDL-c (mg/dl)</td>
<td>121 ± 39</td>
<td>108 ± 30</td>
<td>111 ± 30</td>
<td>0.231</td>
</tr>
<tr>
<td>HDL-c (mg/dl)</td>
<td>56 ± 26</td>
<td>58 ± 25</td>
<td>50 ± 25</td>
<td>0.309</td>
</tr>
</tbody>
</table>

Note: Anova analysis test, data presented as mean ± SD; P<0.05 was considered to be significant; P<0.001 was considered to be highly significant.
4.2.4 Serum AST of the study population

Table 4.7 shows the mean AST for T2DM patients group was 23 ± 12 U/L, the mean BMI for T2DM/CVD patients group was 21 ± 6 U/L, and the mean BMI for control groups was 29 ± 12 U/L. In our study, the Anova analysis test shows that there was no significant difference observed in mean BMI of cases (T2DM and CVD) compared to control (P=0.073). Our result are consistent with those reported by (Ahn et al., 2014; Nakanishi et al., 2003), who found no association of AST with diabetes risk after adjustment for age, family history of diabetes, BMI, alcohol intake, cigarette smoking, physical activity, systolic blood pressure, lipid profile, fasting glucose, and white blood cell count in a study of Japanese office workers. In contrast, Hanley et al. (Hanley et al., 2004) report that AST could independently predicted type 2 diabetes after adjustment for covariates, including metabolic syndrome variables, directly measured insulin sensitivity, acute insulin response, and CRP. Although AST is a marker of hepatocellular health, it is a less specific marker of liver function. Therefore, AST may be a less specific marker of liver pathology related to development T2DM. (Ahn et al., 2014).

Table 4.7: Serum AST of the study population:

<table>
<thead>
<tr>
<th>Feature</th>
<th>Mean Diabetic</th>
<th>Mean Cardiovascular</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>23 ± 12</td>
<td>21 ± 6</td>
<td>29 ± 12</td>
<td>0.073</td>
</tr>
</tbody>
</table>

Note: Anova analysis test, data presented as mean ± SD; P<0.05 was considered to be significant; P<0.001 was considered to be highly significant.

4.2.5 Cardiac function enzymes of the study populations:

Table 4.8 illustrates the serum cardiac function enzymes including Creatinine phosphate kinase (CPK) and lactate dehydrogenase (LDH) of the study population. In our study the mean level of CPK and LDH for T2DM patients group was 29 ± 12 and 347 ± 64 U/L respectively, the mean level of CPK and LDH for T2DM/CVD patients group was 123 ± 38 and 333 ± 62 U/L respectively, the mean level of CPK and LDH for control groups was 149 ± 47 and 338 ± 83 U/L respectively. The Anova analysis test in presented study shows that there was significant differences observed between mean CPK of cases (T2DM and CVD) compared to control (P=0.027). On
the other hand, there was no significant differences observed between mean LDH of cases (T2DM and CVD) compared to control (P=0.697).

Table 4.8: Cardiac function enzymes of the study populations:

<table>
<thead>
<tr>
<th>Feature</th>
<th>Mean Diabetic</th>
<th>Mean Cardiovascular</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPK (U/L)</td>
<td>123 ± 45</td>
<td>123 ± 38</td>
<td>149 ± 47</td>
<td>0.027</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>347 ± 64</td>
<td>333 ± 62</td>
<td>338 ± 83</td>
<td>0.697</td>
</tr>
</tbody>
</table>

Note: Anova analysis test, data presented as mean ± SD; P<0.05 was considered to be significant; P<0.001 was considered to be highly significant.

4.3 Differentially expressed miRNAs using RT-qPCR:

4.3.1 Expression of miR-29a-3p:

MiR-29a is a part of the miR-29a/b/c family within the group of the miRNAs. It exhibits properties of high insulin based intake of glucose, and activates Akt that leads to insulin resistance in return. Akt is not at all the direct gene targeted by miR-29. Insig1 is another target of miR-29, which is an endoplasmic reticulum membrane protein responsible for the biosynthesis of cholesterol (Kumar et al., 2012). Table 4.9 represents the descriptive statistics for the expression of miR-29a-3p, in our study population, and shows that miR-29a-3p is down-regulated in diabetic patients and T2DM/CVD compared to controls, with fold expression of 0.24 and 0.27 respectively. Furthermore, the Anova analysis test in the present study shows that there are highly significant differences observed in miR-29a-3p expression between diabetic patients and diabetic patients with cardiovascular diseases compared to control (P=0.001) and (P=0.004) respectively. The AUC results obtained indicate that this miRNA could potentially be used as a predictive biomarkers in diabetic (AUC=0.71) than in diabetic patients with cardiovascular manifestations (AUC=0.69).

Our results are consistent with a recent study that profiled miRNA expression of T2DM (Zhu & Leung, 2015). The later study confirmed that miR-29a-3p is significantly downregulated in type 2 diabetes. Furthermore, it proposed miR-29a-3p
as a potential blood biomarker of T2DM. Also, reduction in the level of miR-29a is reported in certain plasma samples of the T2DM entities (Zampetaki et al., 2010). In contrast, miR-29a together and miR-29c were shown to be overexpressed during hyperglycaemia and hyperinsulinaemia. MiR-29a in the serum samples of T2DM individuals from China were found to be up-regulated (Kong et al., 2011). Widlansky et al. investigated the role of miRNAs in endothelial dysfunction in the setting of cardiometabolic disorders represented by T2DM (Widlansky et al., 2018). They showed that miR-29 was dysregulated in resistant arterioles obtained by biopsy in T2DM patients. Intraluminal delivery of miR-29a-3p or miR-29b-3p restored normal endothelium-dependent vasodilation (EDVD) in T2DM arterioles that otherwise exhibited impaired EDVD. On the other hand, overexpression of the miR-29 family impairs insulin-stimulated glucose uptake by inhibiting insulin signaling via the Akt (protein kinase B) signaling pathway (He, Zhu, Gupta, Chang, & Fang, 2007). Similarly, the miR-29 family which directly targets Mmp2 (the matrix metalloproteinase 2 gene), Col1a1 (the collagen, type I, alpha 1 gene) and Col3a1 (the collagen, type III, alpha 1 gene) is involved in renal and cardiovascular disease (Kriegel, Liu, Fang, Ding, & Liang, 2012). On the other hand, Roncarati et al reported that miR-29a is correlated with both fibrosis and hypertrophy (Roncarati et al., 2014).

Table 4.9: Fold expression of miR-29a-3p and area under the curve (AUC) values in patients compared with those from healthy controls as determined by RT-qPCR (P<0.05, FDR adjusted).

<table>
<thead>
<tr>
<th>miR-29a-3p</th>
<th>Mean Diabetic ΔCt</th>
<th>Mean Control ΔCt</th>
<th>Log Mean</th>
<th>Fold change 2(^{-ΔΔCt})</th>
<th>P-value</th>
<th>Corrected P-value</th>
<th>AUC</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-1.14</td>
<td>-3.19</td>
<td>2.05</td>
<td>0.24</td>
<td>0.001</td>
<td>0.002</td>
<td>0.71</td>
<td>Down</td>
</tr>
<tr>
<td>Mean Diabetic With CVD ΔCt</td>
<td>Mean Control ΔCt</td>
<td>Log Mean</td>
<td>Fold change 2(^{-ΔΔCt})</td>
<td>P-value</td>
<td>Corrected P-value</td>
<td>AUC</td>
<td>Regulation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-1.28</td>
<td>-3.19</td>
<td>1.90</td>
<td>0.27</td>
<td>0.004</td>
<td>0.032</td>
<td>0.69</td>
<td>Down</td>
</tr>
</tbody>
</table>

Note: Anova analysis test, data presented as mean ± SD; P<0.05 was considered to be significant; P<0.001 was considered to be highly significant.
4.3.2 Expression of miR-155-5p:

Table 4.10 illustrates the data for the expression of miR-155-5p. The results observed in Table 4.10 show that miR-155-5p is down-regulated in diabetic patients and diabetic patients with cardiovascular manifestation with fold expression 0.54 and 0.66 respectively. Anova test results show that there is a significant difference in miR-155-5p levels between diabetic patients compared to control (P=0.021) group. However, there is no significant difference in miR-155-5p level between T2DM/CVD compared to control (P=0.190). The AUC results in our study observed that this miRNA might have a slight predictive value for diabetic only (AUC=0.69).

Ali et al. (Ali et al., 2016) reported that miR-155-5p is downregulated in various CVDs like hypertension, congestive heart failure (CHF), congenital heart defects (CHDs), coronary artery disease (CAD) and stroke. A research by D. Li et al. (Li et al., 2014) tested 36 miRNAs in patients with ventricular septal defect (VSD), one of the most common CHDs, and found that miR-155-5p is downregulated. Recent studies showed that miR-126, miR-146a, and miR-155 were downregulated in PBMCs in response to hyperglycemic/diabetic conditions, suggesting that miR-mediated impaired proangiogenic effect and deregulated cellular metabolic control could be underlying molecular mechanisms for T2DM pathogenesis (Collares et al., 2013; Fernández, Bustamante, Leija, et al., 2013).

Table 4.10: Fold expression of miR-155-5p and area under the curve (AUC) values in patients compared with those from healthy controls as determined by RT-qPCR (P<0.05, FDR adjusted).

<table>
<thead>
<tr>
<th>miR-155-5p</th>
<th>Mean Diabetic ΔCt</th>
<th>Mean Control ΔCt</th>
<th>Log Mean</th>
<th>Fold change 2^ΔΔCt</th>
<th>P-value</th>
<th>Corrected P-value</th>
<th>AUC</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.74</td>
<td>1.84</td>
<td>0.90</td>
<td>0.54</td>
<td>0.021</td>
<td>0.024</td>
<td>0.69</td>
<td>Down</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Diabetic With CVD ΔCt</td>
<td>Mean Control ΔCt</td>
<td>Log Mean</td>
<td>Fold change 2^ΔΔCt</td>
<td>P-value</td>
<td>Corrected P-value</td>
<td>AUC</td>
<td>Regulation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.43</td>
<td>1.84</td>
<td>0.59</td>
<td>0.66</td>
<td>0.190</td>
<td>0.190</td>
<td>0.61</td>
<td>Down</td>
</tr>
</tbody>
</table>

Note: Anova analysis test, data presented as mean ± SD; P<0.05 was considered to be significant; P<0.001 was considered to be highly significant.
4.3.3 **Expression of miR-126-5p:**

Table 4.11 shows the statistical analysis for the expression of miR-126-5p. These results show that miR-126-5p is down-regulated in diabetic patients and diabetic with cardiovascular diseases with fold expression 0.39 and 0.49 respectively. Likewise, the Anova test shows that there is a highly significant difference in miR-126-5p level between diabetic patients compared to control (P =0.001), and significant difference between T2DM/CVD compared to control (P=0.021). The AUC results obtained indicate that this miRNA could potentially be used as a predictive biomarker in diabetic (AUC=0.77) than in diabetic patients with cardiovascular manifestations (AUC=0.70). Our results are similar to data obtained from recent pilot cross sectional study by Seyhan et al (Seyhan et al., 2016), where it was observed that miR-126 and miR-146a are downregulated significantly within the plasma of the individuals with pre-T2DM compared to individuals that possess normal glucose tolerance level. Several studies considered miR-126 as a potential biomarker of T2DM and its loss was considered an elevated risk of peripheral artery disease, vascular leakage and abnormal angiogenesis (Zampetaki et al., 2010; Zhang et al., 2013; Y. Liu et al., 2014). Furthermore, loss of plasma-miR-126 is considered as type 2 diabetes mellitus-related miRNA signature used for early prediction of T2DM in susceptible individuals (Zhang et al., 2013; Zampetaki et al., 2010). MiR-126 regulates gene expression by targeting mRNAs for cleavage or translational repression and both strands miR126-3p and miR126-5p have distinctive roles in the cardiovascular system (Boon & Dimmeler, 2014). MiR-126-3p and miR-126-5p are highly expressed in the endothelium due to their binding of two transcription factor: EST1 and EST2 (Harris et al., 2010). In this regard other researchers such as Zampetaki et al. suggested that dysregulated miR-126 is a sensitive biomarker for vascular complications in diabetes patients (Zampetaki et al., 2010).
Expression of miR-103a-3p:

Table 4.12 shows that miR-103a-3p is down-regulated in diabetic patients and diabetic patients with cardiovascular diseases with fold expression 0.59 and 0.61 respectively. Furthermore, the Anova analysis shows that there are significant differences in miR-103a-3p levels between diabetic patients and T2DM/CVD compared to control (healthy) (P=0.021) and (P=0.018) respectively. The AUC results obtained indicate that this miRNA could potentially be used as a predictive biomarker in diabetic patients (AUC=0.71) than diabetic patients with cardiovascular manifestations (AUC=0.66). The results of the present study are similar to results of Zhu & Leung (Zhu & Leung, 2015), which is one of the first meta-analysis of T2DM miRNA expression profiling studies. The later study showed that miR-103a-3p is significantly downregulated in T2DM. The same study suggested miR-103a-3p as potential circulating biomarker of T2DM. MiR-103 plays a central role in regulating insulin sensitivity by targeting caveolin-1 (a regulator of the insulin receptor) (Trajkovski et al., 2011a). Also, miR-103 is shown to be associated with glucose metabolism or insulin sensitivity, and abnormal expressions of this miRNA is important in the development of diabetes mellitus (Jordan et al., 2011; Trajkovski et al., 2011a).
Table 4.12: Fold expression of miR-103a-3p and area under the curve (AUC) values in patients compared with those from healthy controls as determined by RT-qPCR (P<0.05, FDR adjusted).

<table>
<thead>
<tr>
<th>miR-103a-3p</th>
<th>Mean Diabetic ΔCt</th>
<th>Mean Control ΔCt</th>
<th>Log Mean</th>
<th>Fold change $2^{\Delta\Delta C_t}$</th>
<th>P-value</th>
<th>Corrected P-value</th>
<th>AUC</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-3.17</td>
<td>-3.93</td>
<td>0.76</td>
<td>0.59</td>
<td>0.021</td>
<td>0.024</td>
<td>0.71</td>
<td>Down</td>
</tr>
<tr>
<td>Mean Diabetic With CVD ΔCt</td>
<td>Mean Control ΔCt</td>
<td>Log Mean</td>
<td>Fold change $2^{\Delta\Delta C_t}$</td>
<td>P-value</td>
<td>Corrected P-value</td>
<td>AUC</td>
<td>Regulation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-3.22</td>
<td>-3.93</td>
<td>0.71</td>
<td>0.61</td>
<td>0.018</td>
<td>0.044</td>
<td>0.66</td>
<td>Down</td>
</tr>
</tbody>
</table>

Note: Anova analysis test, data presented as mean ± SD; P<0.05 was considered to be significant; P<0.001 was considered to be highly significant.

4.3.5 Expression of miR-124-3p:

Table 4.13 shows that miR-124-3p is down-regulated in diabetic patients and diabetic patients with cardiovascular manifestation with fold expression 0.31 and 0.32 respectively. Additionally, the Anova test in our study shows that there were significant differences observed in miR-124-3p levels between diabetic patients and T2DM/CVD compared to control (P=0.003) and (P=0.030) respectively. The AUC results obtained indicate that this miRNA could potentially be used as a predictive biomarkers in diabetic patients (AUC=0.68) and diabetic patients with cardiovascular manifestations (AUC=0.67).

Our results are similar to observations reported by Zhu et al. who reported that the expression levels of miR-124-3p, and other miRNAs were downregulated in T2DM (Zhu et al., 2017). Similar reports were also obtained by Kong et al. (Kong et al., 2011) among Chinese patients.
Table 4.13: Fold expression of miR-124-3p and area under the curve (AUC) values in patients compared with those from healthy controls as determined by RT-qPCR (P<0.05, FDR adjusted).

<table>
<thead>
<tr>
<th>miR-124-3p</th>
<th>Mean Diabetic ΔCt</th>
<th>Mean Control ΔCt</th>
<th>Log Fold change 2^ΔΔCt</th>
<th>P-value</th>
<th>Corrected P-value</th>
<th>AUC</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic</td>
<td>5.48</td>
<td>3.80</td>
<td>1.68</td>
<td>0.31</td>
<td>0.003</td>
<td>0.06</td>
<td>0.68</td>
</tr>
<tr>
<td>With CVD</td>
<td>5.44</td>
<td>3.80</td>
<td>1.64</td>
<td>0.32</td>
<td>0.030</td>
<td>0.044</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Note: Anova analysis test, data presented as mean ± SD; P<0.05 was considered to be significant; P<0.001 was considered to be highly significant.

4.3.6 Expression of miR-146a-5p:

Table 4.14 shows the expression of miR-146a-5p. These results show that miR-146a-5p is down-regulated in diabetic patients and diabetic with cardiovascular diseases with fold expression of 0.38 and 0.46 respectively. Furthermore, the Anova test in our study shows that there were highly significant differences in miR-146-5p levels between diabetic patients compared to control (P=0.001), and significant differences between T2DM/CVD compared to control (P=0.031) respectively. The AUC results obtained in our study indicate that this miRNA could potentially be used as greater predictive biomarkers in diabetic (AUC=0.75) than in diabetic patients with cardiovascular manifestations (AUC=0.69).

Our results are consistent with results of Rong et al. study, which included 90 new-T2DM cases and 90 controls (Rong et al., 2013). This study observed a significant difference between levels of circulating miRNA-146a in risk of new-T2DM patients compared with controls (P=0.001) and (AUC=0.725). On the other hand, in recent pilot cross sectional study by Seyhan et al (Seyhan et al., 2016), it was observed that miR-126 and miR-146a are downregulated significantly within the plasma of the individuals among the pre-T2DM compared to individuals that possess normal glucose tolerance level. However, in another research by Rong et al. levels of circulation miR-146 of T2DM individuals were increased compared to controls. In the same regard, the role of miRNA-146a in the pathogenesis of inflammation and
other degenerative aspects may make it participate in the course of T2DM (Rong et al., 2013).

**Table 4.14:** Fold expression of miR-146a-5p and area under the curve (AUC) values in patients compared with those from healthy controls as determined by RT-qPCR (P<0.05, FDR adjusted).

<table>
<thead>
<tr>
<th>miR-146a-5p</th>
<th>Mean Diabetic ΔCt</th>
<th>Mean Control ΔCt</th>
<th>Log Mean</th>
<th>Fold change $2^{-\Delta\Delta C_t}$</th>
<th>P-value</th>
<th>Corrected P-value</th>
<th>AUC</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-1.62</td>
<td>-3.01</td>
<td>1.39</td>
<td>0.38</td>
<td>0.001</td>
<td>0.001</td>
<td>0.75</td>
<td>Down</td>
</tr>
<tr>
<td></td>
<td>-1.90</td>
<td>-3.01</td>
<td>1.11</td>
<td>0.46</td>
<td>0.031</td>
<td>0.044</td>
<td>0.69</td>
<td>Down</td>
</tr>
</tbody>
</table>

Note: Anova analysis test, data presented as mean ± SD; P<0.05 was considered to be significant; P<0.001 was considered to be highly significant.

**4.3.7 Expression of miR-222-3p:**
Table 4.15 illustrates that miR-222-3p is down-regulated in diabetic patients and diabetic patients with cardiovascular diseases with fold expression of 0.42 and 0.46 respectively. The Anova test shows that there is significant difference observed in miR-222-3p level between diabetic patients compared to control (P=0.002), and significant difference between T2DM/CVD compared to control (P=0.033). The AUC results obtained in the present study indicate that this miRNA could potentially be used as greater predictive biomarker in diabetic (AUC=0.74) than in diabetic patients with cardiovascular manifestations (AUC=0.69). Importantly, miR-222-3p has been reported to play important roles in a variety of physiological and pathological processes in the heart. MiR-222-3p may represent a potential cardiovascular biomarker and a new therapeutic target in cardiovascular diseases (Ding, Huang, Xu, Zhu, & Zhong, 2017). A research by D. Li et al. (Li et al., 2014) reported that miR-222-3p is downregulated in CVD. These data are totally in agreement with ours.

Our results are different from those reported in previous Cross-sectional study by Ortega et al., which showed that the level of circulating miR-222-3p in T2DM patients is higher than in healthy individuals (P=0.001) (Ortega et al., 2014).
Furthermore, other previous studies were registered in the global meta-analysis showed similar results of differential expression of miR-222-3p. For example, ten miRNAs in blood of T2DM patients and 7 miRNAs in blood of obese subjects were tested. Both obese and T2DM patients showed elevated concentrations of miR-222-3p (Villard, Marchand, Thivolet, & Rome, 2015). Differences between these observations might be attributed to the different ethnicities of the studied populations.

Table 4.15: Fold expression of miR-222-3p and area under the curve (AUC) values in patients compared with those from healthy controls as determined by RT-qPCR (P<0.05, FDR adjusted).

<table>
<thead>
<tr>
<th>miR-222-3p</th>
<th>Mean Diabetic ΔCt</th>
<th>Mean Control ΔCt</th>
<th>Log Mean</th>
<th>Fold change 2^ΔΔCt</th>
<th>P-value</th>
<th>Corrected P-value</th>
<th>AUC</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.46</td>
<td>-0.79</td>
<td>1.25</td>
<td>0.42</td>
<td>0.002</td>
<td>0.005</td>
<td>0.74</td>
<td>Down</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>miR-222-3p</th>
<th>Mean Diabetic With CVD ΔCt</th>
<th>Mean Control ΔCt</th>
<th>Log Mean</th>
<th>Fold change 2^ΔΔCt</th>
<th>P-value</th>
<th>Corrected P-value</th>
<th>AUC</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.32</td>
<td>-0.79</td>
<td>1.11</td>
<td>0.46</td>
<td>0.033</td>
<td>0.044</td>
<td>0.69</td>
<td>Down</td>
</tr>
</tbody>
</table>

Note: Anova analysis test, data presented as mean ± SD; P<0.05 was considered to be significant; P<0.001 was considered to be highly significant.

4.3.8 Expression of miR-375:

Table 4.16 shows that miR-375 is down-regulated in diabetic patients and diabetic patients with cardiovascular diseases with fold expression 0.57 and 0.49 respectively. On the other hand, the Anova test in our study shows that there was no significant differences observed in miR-375 between diabetic patients (P=0.083) and T2DM/CVD (P=0.062) compared to control. The AUC results that obtained in presented study indicates that miR-375 could not be used as predictive biomarkers in diabetic (AUC=0.61) and diabetic with cardiovascular manifestations (AUC=0.65). Our results are different from the study reported by Sun et al. (Sun et al., 2014). The later study found that the plasma level of miR-375 was significantly upregulated in T2DM samples compared with normal (healthy) samples. Similar results were shown by Zhu & Leung (Zhu & Leung, 2015). Additionally these studies proposed miR-375 as a potential blood biomarker of T2DM (Zhu & Leung, 2015). Recent reports
suggested that the levels of circulating miR-375 were significantly increased in T2DM patients compared to control individuals and they may become the new potential biomarkers for T2DM patients (Higuchi et al., 2015). Studies showed that inhibition of miR-375 enhances insulin secretion, while miR-375 overexpression impairs the insulin secretory pathway by reducing expression of myotrophin (Mtpn), a protein involved in insulin–granule fusion (El Ouaamari et al., 2008; Poy et al., 2004). Differences between our results and these results might be attributed to the size of our population.

**Table 4.16.** Fold expression of miR-375 and area under the curve (AUC) values in patients compared with those from healthy controls as determined by RT-qPCR (P<0.05, FDR adjusted).

<table>
<thead>
<tr>
<th>miR-375</th>
<th>Mean Diabetic ΔCt</th>
<th>Mean Control ΔCt</th>
<th>Log Mean</th>
<th>Fold change 2^ΔΔCt</th>
<th>P-value</th>
<th>Corrected P-value</th>
<th>AU C</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.89</td>
<td>2.07</td>
<td>0.82</td>
<td>0.57</td>
<td>0.083</td>
<td>0.083</td>
<td>0.61</td>
<td>Down</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Diabetic With CVD ΔCt</td>
<td>Mean Control ΔCt</td>
<td>Log Mean</td>
<td>Fold change 2^ΔΔCt</td>
<td>P-value</td>
<td>Corrected P-value</td>
<td>AU C</td>
<td>Regulation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.09</td>
<td>2.07</td>
<td>1.02</td>
<td>0.49</td>
<td>0.062</td>
<td>0.070</td>
<td>0.65</td>
<td>Down</td>
</tr>
</tbody>
</table>

**Note:** Anova analysis test, data presented as mean ± SD; P<0.05 was considered to be significant; P<0.001 was considered to be highly significant.
4.4 Correlations between selected miRNAs and clinical data

We further analyzed the correlations between the 8 selected miRNAs and various clinical parameters. The results are summarized in Table 4.17.

Pearson’s correlation test found that the expression levels of miR-29a-3p, miR-126-5p, miR-103a-3p, miR-124-3p and miR-146a-5p were significantly positively correlated with glucose and HBA1c levels (r=0.229, p=0.021, r=0.236, p=0.017, r=0.253, p=0.010, r=0.206, p=0.038 and r=0.213, p=0.031, respectively). In contrast, no significant correlation was observed between the expression levels of miR-155-5p, miR-222-3p and miR-375 and glucose and HBA1c levels (r=0.165, p=0.098, r=0.172, p=0.084 and r=0.068, p=0.494, respectively). There were also a significant negative correlations between the expression level of miR-155-5p, miR-126-5p, miR-124-3p, miR-146a-5p and miR-222-3p and uric acid level (r=-0.327, p=0.001, r=-0.241, p=0.015, r=-0.230, p=0.020, r=-0.290, p=0.003 and r=-0.206, p=0.037, respectively). On the other hand, there were no statistically significant correlation between the expression level of miR29a-3p, miR-103a-3p and miR-375 and uric acid level (r=-0.057, p=0.571, r=-0.070, p=0.486 and r=-0.153, p=0.124, respectively). Additionally, we found that the expression level of miR-126-5p, miR-124-3p, miR-146a-5p, miR-222-3p and miR-375 were also a significant negative correlations to CPK level (r=-0.214, p=0.031, r=-0.202, p=0.042, r=-0.228, p=0.021, r=-0.270, p=0.006 and r=-0.206, p=0.038, respectively). In contrast, there were no significant correlation between the expression level of miR-29a-3p, miR-155-5p and miR-103a-3p, respectively).

Recent study observed a relationship between miRNAs and fasting glucose (miR-103 and miR-126) and HbA1c (miR-103 and miR-375) (Jiménez-Lucena et al., 2018). On the other hand, Ortega et al. (Ortega et al., 2014) show that circulating concentrations of miR-222 and miR-126 were significantly associated with fasting glucose and HbA1c. Additionally, our previous study show that miR-375 was significant positive correlation with fasting glucose and HbA1c (Al-Muhtaresh & Al-Kafaji, 2018). Importantly, in presented study we didn't find any significant correlation between tested miRNA and age and this is agreement with different earlier studies. For example, recent study showed that there is no significant
association between miR-29a expression level and age was identified \( (P = 0.449) \) (Li et al., 2017).

**Table 4.17:** Correlation of selected miRNA and clinical data

<table>
<thead>
<tr>
<th>miRNA</th>
<th>miR-29a-3p</th>
<th>miR-155-5p</th>
<th>miR-126-5p</th>
<th>miR-103a-3p</th>
<th>miR-124-3p</th>
<th>miR-146a-5p</th>
<th>miR-222-3p</th>
<th>miR-375</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.229</td>
<td>0.165</td>
<td>0.236</td>
<td>0.253</td>
<td>0.206</td>
<td>0.213</td>
<td>0.172</td>
<td>0.068</td>
</tr>
<tr>
<td>P-value</td>
<td>0.021</td>
<td>0.098</td>
<td>0.017</td>
<td>0.010</td>
<td>0.038</td>
<td>0.031</td>
<td>0.084</td>
<td>0.494</td>
</tr>
<tr>
<td>HbA1c</td>
<td>0.242</td>
<td>0.188</td>
<td>0.297</td>
<td>0.236</td>
<td>0.227</td>
<td>0.260</td>
<td>0.189</td>
<td>0.164</td>
</tr>
<tr>
<td>P-value</td>
<td>0.014</td>
<td>0.058</td>
<td>0.002</td>
<td>0.017</td>
<td>0.022</td>
<td>0.008</td>
<td>0.057</td>
<td>0.100</td>
</tr>
<tr>
<td>Uric acid</td>
<td>-0.057</td>
<td>-0.327</td>
<td>-0.241</td>
<td>-0.070</td>
<td>-0.230</td>
<td>-0.290</td>
<td>-0.206</td>
<td>-0.153</td>
</tr>
<tr>
<td>P-value</td>
<td>0.571</td>
<td>0.001</td>
<td>0.015</td>
<td>0.486</td>
<td>0.020</td>
<td>0.003</td>
<td>0.037</td>
<td>0.124</td>
</tr>
<tr>
<td>CPK</td>
<td>-0.167</td>
<td>-0.188</td>
<td>-0.214</td>
<td>-0.037</td>
<td>-0.202</td>
<td>-0.228</td>
<td>-0.270</td>
<td>-0.206</td>
</tr>
<tr>
<td>P-value</td>
<td>0.094</td>
<td>0.059</td>
<td>0.031</td>
<td>0.708</td>
<td>0.042</td>
<td>0.021</td>
<td>0.006</td>
<td>0.038</td>
</tr>
<tr>
<td>Age</td>
<td>0.127</td>
<td>0.032</td>
<td>0.104</td>
<td>0.010</td>
<td>0.031</td>
<td>0.101</td>
<td>0.036</td>
<td>0.077</td>
</tr>
<tr>
<td>P-value</td>
<td>0.205</td>
<td>0.745</td>
<td>0.300</td>
<td>0.921</td>
<td>0.757</td>
<td>0.312</td>
<td>0.720</td>
<td>0.443</td>
</tr>
</tbody>
</table>

**Note:** Anova analysis test, \( P<0.05 \) was considered to be significant, Spearman’s correlation \( r \).
Chapter 5
Conclusions and Recommendations
Chapter 5
Conclusions and Recommendations

The current study investigated the significance of 8 miRNAs (hsa-miR-29a, hsa-miR-375, hsa-miR-103a-3p, hsa-miR-124-3p, hsa-miR-155-5p and hsa-miR-146a-5p, miR-126 and miR-222) as biomarkers of T2DM patient's. The correlations between miRNAs levels and several biochemical markers were tested. Our findings show that:

(1) The expression levels of all tested miRNAs except of miR-375 were significantly decreased in T2DM patients groups in comparison to healthy persons.

(2) The levels of hsCRP, FBS, HbA1c, TC, TG, LDL, LDH, CPK, were significantly increased among T2DM patients compared with the normal control (P≤0.05).

(3) The expression levels of miR-29a-3p, miR-126-5p, miR-103a-3p, miR-124-3p and miR-146a-5p were significantly correlated to glucose and HBA1c levels.

(4) hsa-miR-126-5p showed the highest diagnostic accuracy as revealed by ROC curve analysis and can relatively discriminate between patients groups and controls.

Together, these findings show the importance of the selected miRNA (miR-126 has a special importance) to be candidates biomarkers for T2DM and its cardiovascular complications.

Based on these findings, we recommend:

1- To confirm these results in larger populations of different ethnicities.

2- To study the relations between these miRNAs and other cardiovascular markers.

3- Similar studies to evaluate the significance of these miRNAs in retinopathy and nephropathy patients is also recommended.
References


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diabetes and prediabetes among Palestinian Arab population. *PLoS One, 9*(2), e88123. doi:10.1371/journal.pone.0088123


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Sultania, S., Thakur, D., & Kulshreshtha, M. Study of Lipid Profile in Type 2 Diabetes Mellitus Patients and its Correlation with HbA1c


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Appendix 1

Helsinki Committee
For Ethical Approval

Date: 04/04/2016
Name: Saeb H. Aliwaini
Number: PHRC/HC/92/16

We would like to inform you that the committee had discussed the proposal of your study about:

New Approaches Towards Molecular Diagnosis and Treatment of Type 2 Diabetes Mellitus

The committee has decided to approve the above mentioned research. Approval number PHRC/HC/92/16 in its meeting on 04/04/2016

Signature

Member

Member

Chairman

General Conditions:
1. Valid for 2 years from the date of approval.
2. It is necessary to notify the committee of any change in the approved study protocol.
3. The committee appreciates receiving a copy of your final research when completed.

Specific Conditions:

E-Mail: pahrc@gmail.com

Gaza, Palestine
Appendix 2
Appendix 3

New Approaches Towards Molecular Diagnosis and Treatment of Type 2 Diabetes Mellitus
بسم الله الرحمن الرحيم

أما الباحث: نتعلم قدرة أرجو المساعدة في إتمام هذه الدراسة من خلال تعبي الاستبيان
وتبركنا بعوانهم. تم إجراء التحصيلات من أجل دراسة "تقييم مستوى مصل الدم كمؤشر جزءي لمرض السكر من النوع الثاني في قطاع غزة".

ولكم جزيل الشكر مع ثمنا لنا لكم بالصحة والبقاء.

رقم الاستبيان:

إسم (اختياري):

رقم الجوال (اختياري):

المدينة:

عنوان:

الجنس:

العمر:

الإنساني:

ضغط الدم:

الطول:

BMI:

الوزن:

1. الشهادة الدراسية:

د. ف. جامعي

د. ف. ثانوي

هل تعمل في منصب؟

2. موظفة / موظف

3. الصناعة؟

هل تعمل في منصب?

4. هل أنت على صحة?

إذا كنت الإجابة لا هل تتعذر للتنبؤ بغير مباشر؟

5. هل تمارس التمارين الرياضية؟

إذا كان الإجابة نعم هل تمارس الرياضة بشكل منتظم؟

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انكر عدد المرات أسبوعياً: نعم  لا  أخرى...

6. كيف كن عمرك عند تشخيص السكر لأول مرة لديك؟
7. هل تقوم بفحص السكر في الدم بشكل دوري؟
   النوع الأول  النوع الثاني
   يرجى تجربتها.
8. إذا كنت تدفي عندما يرتفع السكر في الدم:
   نعم  لا

9. هل يعاني أحد أفراد عائلتك من مرض السكري من النوع الثاني؟
   نعم  لا

10. ما هو نوع العلاج الذي تستخدمه في الوقت الحالي؟
    جرعة ثابتة  فوائد
    بانتظام  جمجم

11. هل تعاني من أمراض القلب؟
    نعم  لا

12. إذا كنت تدفي عند تشخيص مرض القلب لديك:
    قصر القلب السمنة 
    النوبة الصدرية  تصلب الشرايين
13. هل تقوم بإجراء فحوصات الدورة والكوليسترول بشكل دوري؟
    نعم  لا

14. هل تقوم بعمل فحوصات إزالة القلب:
    نعم  لا
15. هل يوجد لديك خطة غذائية؟ نعم لا

16. هل لديك قيد على نوع محدد من الغذاء مثل (mel ملح... دهن... سوائل... لا يوجد)

17. استهلاك مشتقات الألبان يكون:

- يوميا (لا
- مرتين في الأسبوع أو أقل

18. استهلاك الخضروات والفاكهة يكون:

- يوميا (لا
- مرتين في الأسبوع أو أقل

19. استهلاك الكربوهيدرات:

- يوميا (لا
- مرتين في الأسبوع أو أقل

20. استهلاك اللحوم والدهون يكون:

- يوميا (لا
- مرتين في الأسبوع أو أقل

21. كمية شرب الماء المناسبة؟ أقل من لتر واحد إلى 2 لتر أو أكثر من 2 لتر

22. تشعر برضا بخصوص صحتك العامة:

- لا
- نعم

23. الضغط النفسي مرتفع لديك؟ نعم لا

24. هل تشعر بالثقة من الخدمات الصحية المقدمة لديك؟ نعم لا

25. من هو مقدم الخدمة؟

- حكومي
- خاص

إقرار: أنا موافق على شراء هذه الاستبان الذي يتغلب بصحتي:

التاريخ: 

شكراً لك على حسن تعاملكم.

الباحث: نجمة فدورة