Investigation of MicroRNAs that can differentiate between pre-diabetes and diabetes

By

Chanelle Schroeder (212226835)

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Supervisors:

Prof. T.E. Matsha: Cape Peninsula University of Technology

A/Prof. G.M. Davison: Cape Peninsula University of Technology

Dr Cecil Weale Cape Peninsula University of Technology

FACULTY OF HEALTH AND WELLNESS SCIENCES, CAPE PENINSULA UNIVERSITY OF TECHNOLOGY

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Declaration

I, Chanelle Schroeder, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. This thesis represents my own opinions and not those of the Cape Peninsula University of Technology.

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Signed **Date**

Abstract

Background: An ever-increasing incidence of Type 2 Diabetes Mellitus (T2DM) in the global population, has caused a public health concern. MicroRNA are small non-coding RNAs which control gene expression and are involved in several pathophysiological processes, therefore allowing them (miRNA) to play a role in disease detection. The scarcity of studies which investigates the role of miRNA in T2DM, especially in a South African population, has encouraged studies such as the preliminary study conducted by Matsha et al. (2018), profiling miRNA in 1989 randomly selected participants where they found that the expression of hsa-miR-486-5p and hsa-miR-novel-chr1_40444 were 2 of the most dysregulated miRNA in individuals with screen detected Type 2 diabetes mellitus (DM), in comparison to those individuals who were either on treatment or were normoglycaemic. This current study further investigates and validates the expression of hsa-miR-486-5p and hsa-miR-novel-chr1_40444 in 1459 participants of the sample cohort who have participated in the miRNA profiling study.

Methods: The Quantum Studio 7 (Life Technologies, USA) was used to perform quantitative reverse-transcription PCR (RT-qPCR) on 1459 whole blood samples provided by participants who partook in the Vascular and Metabolic Health study. All the participants underwent anthropometric measurements, and biochemical parameters were measured at an accredited pathology laboratory. Participant glycaemic status was determined using the oral glucose tolerance test (OGTT).

Results: Hsa-miR-486-5p and hsa-miR-novel-chr1_40444 were significantly overexpressed in diabetics compared to both controls, and prediabetics (all $p \leq 0.001$). Moreover, multivariate regression analysis revealed independent associations between the expression of both miRNAs and the risk of diabetes, with significant retained in unadjusted and adjusted models (all $p \leq 0.001$).

Conclusion: This study has demonstrated the potential capabilities of hsa-miR-486-5p and hsa-miR-novel-chr1_40444 in distinguishing between Type 2 diabetes (both newly-diagnosed and known diabetes) from normoglycaemia and early-stage hyperglycaemia (pre-diabetes). However, further investigation is required.

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Dedication

To my husband, M. Mwenze who has made many sacrifices for me to do this thesis. To my mom, P.D Schroeder, who is no longer part of this world, but her memories continue to influence my life. Lastly, to my father D. De Bruin who has taught me the value of hard work.

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Key words

Type 2 Diabetes, pre-diabetes, dysglycaemia, microRNAs

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List of Abbreviations

AGO2- Argonaute RISC Catalytic Component 2

BCAA- Branched chain amino acid

BMI- Body mass index

cDNA- Complementary DNA

DKD- Diabetic kidney disease

DM- Diabetes Mellitus

FFA- Free fatty acid

FOXO1- Forkhead box protein O1

GLUT4- Glucose transporter type 4

HbA1c- Glycated haemoglobin

HDL-c- High density lipoprotein cholesterol

IFG- Impaired fasting glucose

IGT- Impaired glucose tolerance

LDL-c- Low density lipoprotein cholesterol

MiRISC- miRNA induced silencing complex

OGTT- Oral glucose tolerance test

P13K- Phosphatidyl inositol-3-kinase

PBS- Phosphate buffered solution

PTEN- Phosphatase and tensin homolog

RNA- Ribonucleic Acid

RT q-PCR- Real-Time Quantitative-Polymerase Chain Reaction

T1DM- Type 1 diabetes mellitus

T2DM-Type 2 diabetes mellitus

TG- Triglycerides

TRBP- Tar RNA binding protein

u-CRP- C-reactive protein

WHO- World Health Organisation

Glossary

- **Apoptosis:** Genetically regulated cell death
- **Cardiovascular disease:** Any disorder affecting the blood vessels and heart
- **Hyperglycaemia:** A condition in which the blood plasma contains an excessive concentration of glucose.
- **Inflammation:** A series of biological reactions which occur in the body in response to injury of healthy tissue or when there is an infection caused by virus, toxins or bacteria.
- **Ketosis:** A metabolic state when the body is deprived of carbohydrates and therefore burns fat for energy. This process produces compounds called ketones.
- **Non-communicable diseases:** Chronic diseases which occur due to environmental, physiologic and behavioural factors. These diseases are usually long in duration.
- **Normoglyceamia:** Normal plasma glucose concentration for that age range
- **Pathogenesis:** The sequence of events which occur and result in the development of a disease

Chapter 1

1.1 Introduction

The global incidence of diabetes mellitus (DM), which is estimated to be 537 million people between the ages of 20-79 years, has made this disease a cause for public health concern (International Diabetes Federation, 2021). This number is expected to increase to 783 million by the year 2045, with most cases presenting in developing countries (International Diabetes Federation, 2021). In addition to the burdening economic impact on healthcare systems and individuals, it is also a major contributor to mortality, morbidity, and a poor quality of life (Ibrahim, 2017). The rapid increase requires urgent prevention strategies (Zimmet et al., 2001), yet a complete understanding of the causes of the disease remains unclear (International Diabetes Federation, 2019). While a cure still does not exist, available therapy delays disease progression and could be improved by identifying individuals most at risk and implementing treatment in the initial stages of the disease (Guay and Regazzi, 2013).

Diabetes is a multifactorial, chronic metabolic condition, which occurs due to the interplay between environmental, genetic, and epigenetic factors (Ibrahim, 2017). In this condition, the pancreas does not have the ability to produce enough insulin in response to an increase in glucose. It also occurs when the pancreas produces insulin in response to elevated glucose, but it is unable to perform its function (Saeedi et al., 2019). The immune system responds to the resultant hyperglycaemia, as well as mediators of inflammation and macrophages from the adipose tissue, by initiating an inflammatory response. Although this response is low and chronic, it causes progressive damage to the pancreatic β-cells, resulting in insufficient production of insulin, thus promoting hyperglycaemia (Berbudi et al., 2020). In addition to hyperglycaemia, an impairment in carbohydrate, protein and lipid metabolism occurs (Wu et al., 2014). The persistent hyperglycaemia and low-grade inflammation, results in various secondary micro- and macro-vascular complications such as neuropathy, nephropathy, stroke, and cardiovascular disease (Rangel et al., 2019). These complications can be avoided through early diagnosis and the initiation of prompt intervention measures (Ibrahim, 2017).

Subclassifications of DM include Type 1 diabetes mellitus (T1DM), Type 2 diabetes mellitus (T2DM), gestational DM, monogenic diabetes, and steroid induced diabetes (Punthakee et al., 2018; Solis-Herrera et al., 2000). These subtypes differ in presentation, pathophysiology, and treatment (World Health Organization, 2019). The most common subtypes affecting individuals are T1DM and T2DM (World Health Organization, 2019). Specific modifications of microRNA (miRNA) profiles in the bloodstream have been uncovered in diabetic individuals, which may be detected many years before disease manifestation (Guay and Regazzi, 2013).

There has been a growing interest in the use of these miRNAs as candidate biomarkers (Chen et al., 2014; He et al., 2017; Jiménez-Lucena et al., 2018). These small non-coding RNAs control gene expression and are involved in several pathophysiological processes (Dangwal and Thum, 2012; Wang et al., 2009). It is these behaviours which make them potentially useful for the early detection of disease, and the identification of individuals who are at risk of developing complications (Guay and Regazzi, 2013). A recent study conducted by Matsha et al 2018, compared the miRNA expression profiles among groups of varying glycaemic statuses. Using next generation sequencing (NGS) this study revealed several miRNAs, including hsa-miR-486-5p and hsa-miR-novel-chr1_40444, which were differentially expressed in the Type 2 diabetes and pre-diabetic groups when compared to the normoglycaemic group.

1.2 The rationale of this study

Due to the dramatic surge in the prevalence of DM and the overall burden DM poses in societies, there is an urgency to identify pre-DM, as this group is at the highest risk of developing T2DM (Sidorkiewicz et al., 2020). Although evidence of the diagnostic and prognostic role miRNA play in dysglycemia is growing, studies of this nature in a South African population are scarce (Matsha et al., 2018). Thorough validation of larger, independent clinical studies is needed to better our understanding of the association between DM and miRNA.

1.3 Research aim

This project aims to investigate and validate the aforementioned miRNAs previously identified by Matsha et al. (2018), in whole blood samples.

1.4 Research objectives

The research objectives were:

1) To validate the miRNAs using quantitative reverse-transcription PCR (RT q-PCR) in a larger, independent sample size and to confirm their association with T2DM and/or pre-diabetes.

2) To determine the expression patterns of hsa-miR-486-5p and hsa-miR-novel-chr1_40444 and their capability of diagnosing dysglycaemia, with the intent of promoting their use as potential, non-invasive diagnostic markers for the early detection of Type 2 diabetes mellitus.

1.5 Hypothesis

There is an association between glucose intolerance and dysregulation of circulating miRNAs (hsa-miR-486-5p and hsa-miR-novel-chr1_40444).

Chapter 2: Literature review

2.1 Prevalence and burden of Diabetes

Non-communicable diseases are amongst the top 10 causes of death in the world, and diabetes ranks 9th (World Health Organization, 2019). The prevalence of diabetes continues to increase worldwide, with the highest increases seen in the middle-income countries (Sun et al., 2022). This rapid increase is attributed to an ever-increasing population growth and a steady rise in the average age of individuals. Furthermore, the adoption of westernised lifestyles and rapid urbanisation has contributed to the increased prevalence in the developing world (Ibrahim, 2017). Diabetes is thus a global public health concern, which imposes a heavy burden on socio-economic and public health development (Lin et al., 2020).

An estimated 537 million adults were living with diabetes in 2021, of which 6.7 million died that year (International Diabetes Federation, 2021). A further 46% increase of diabetes sufferers are expected by the year 2045, thus reaching a total of 6.4 billion people worldwide. The economic effect of this increase is a global health expenditure exceeding 1 trillion USD by 2045 (Sun et al., 2022), especially as 240 million people remain undiagnosed. Most cases of impaired glucose tolerance (IGT) occur in low-income countries, with the global prevalence of IGT cases in adults (20-79 years) increasing from 541 million in 2021 to a predicted 730.3 million in 2045 (International Diabetes Federation, 2021). These adults are at a greater risk of developing T2DM and hypertension and therefore require early intervention (Kabootari et al., 2020). This further emphasizes the need for prompt detection to avoid the negative effects of diabetic complications.

Africa had a prevalence of 24 million people with diabetes in 2021, which is predicted to increase by 129% by 2045. This accounts for 55 million people, with an additional 117 million predicted to have impaired glucose tolerance by 2045. More than half of the individuals in Africa are unaware that they have diabetes (53.6%) (International Diabetes Federation, 2021). This situation is unfavourable as it leads to a rise in complications, premature death, and increased healthcare costs (Asmelash and Asmelash, 2019). Further challenges in this region include a lack of research, limited access to healthcare and inequality which contribute to the rising disease burden (International Diabetes Federation, 2021; Mbanya et al., 2010; Pastakia et al., 2017)

South Africa is among the top 5 countries in Africa with the highest prevalence of DM (International Diabetes Federation, 2021). This disease is the cause of many deaths in South Africa, having ranked fifth in 2013 to being the second leading cause of death in 2017

(Statistics South Africa, 2017). In the Western Cape, DM is the leading cause of death (7.5%), particularly in women of mixed ancestry (8.3%) and Asian (14%) descent (Statistics South Africa, 2017). A study by Erasmus et al on the prevalence of diabetes and metabolic syndrome in a population in Western Cape, Cape Town revealed that 18.1 %of their participants had undiagnosed diabetes. They attributed this to the inadequacy of the primary healthcare system to identify those at risk and those who have diabetes (Erasmus et al., 2012). These alarming statistics place a huge burden on the health of economically productive individuals, and with the manifestation of secondary microvascular and macrovascular complications, South Africa's already strained health care systems and economy is unable to cope (Erzse et al., 2019; Ibrahim, 2017; Pheiffer et al., 2018). Most diabetes cases are classified into two major groups (T1DM and T2DM). However, based on aetiology, there are 4 major subtypes (Punthakee et al., 2018). These subtypes are Type 1 diabetes mellitus, Type 2 diabetes mellitus, Gestational diabetes and Other specific types of diabetes mellitus.

2.2 Type 1 diabetes mellitus

Type 1 diabetes mellitus is characterized by the cellular autoimmune destruction of pancreatic β-cells, which leads to a total insulin deficiency (Jospe, 2007). The mechanism by which this occurs is poorly understood, but a combination of genetic (overexpression of HLA molecules), immunologic and environmental factors are at play (Burrack et al., 2017; Paschou et al., 2018). Hyperglycaemia and ketosis occur as a result of the insulin deficiency (Jospe, 2007). Previously thought to be a disorder mainly occurring in children and adolescence, this is no longer the case however it accounts for 5-10% of cases in adolescents and children (Leslie, 2010). These cases include those which occur because of the autoimmune destruction of pancreatic β-cells (Case et al., 2011), and those where the cause of the destruction is unknown (2017 SEMDSA Guideline for the Management of Type 2 Diabetes Guideline Committee, 2017). Diagnosis is generally made late and when 80-85% of the β-cells have already been destroyed (Atkinson et al., 2014).

The clinical manifestation of the disease is dependent on the rate of β-cell destruction. A more rapid loss of β-cell mass has been observed in infants when compared to adults (Klinke, 2008). The autoimmune process which leads to the β-cell destruction is identified by the presence of insulin, islet cell antibodies or anti-glutamic acid decarboxylase (Kharroubi and Darwish, 2015). The detection of genetic markers and the presence of autoantibodies against the β-cell antigens are used to assess at risk individuals for T1DM (Kahanovitz et al., 2017) and identification of more than 2 biomarkers places an individual in the high-risk category (Atkinson et al., 2014). These autoantibodies can also be used as a risk assessment tool and for diagnostic purposes in both the general population and first-degree relatives (Pihoker et al., 2005). No biomarkers are detected in children with new onset of T1DM due to the presence of

many β-cells, therefore this method cannot be used to determine disease progression (Assmann et al., 2017; Goldenberg and Punthakee, 2013).

Increased levels of glucose in the blood and urine with associated urinary fluid loss occur due to a defect in the transport of glucose from blood to tissue. This results in the classical symptoms of polydipsia, polyuria, and polyphagia (Mencher et al., 2019). Ketone bodies form as a product of fat metabolism, which occurs when low levels of insulin is present and can accumulate in blood resulting in metabolic acidosis. To compensate for the acidosis, hyperventilation occurs which results in respiratory alkalosis (Kahanovitz et al., 2017). If left untreated this condition will result in mental confusion, cerebral oedema, coma, unconsciousness, and death (Atkinson et al., 2014; Baynest, 2015). All patients with T1DM eventually have a lifetime dependence on insulin therapy to maintain normoglycaemia (Baynest, 2015). Poorly managed diabetes will result in long term complications such as damage to blood vessels, and an increased risk of stroke, heart disease, microvascular and peripheral vascular diseases (Atkinson et al., 2014; Baynest, 2015).

2.3 Type 2 diabetes mellitus

This is a multifactorial condition which occurs because of a combination of aetiologies (Ibrahim, 2017). Individuals with T2DM include those with partial insulin deficiency and those with insulin resistance (American diabetes Association, 2015). The gradual development of T2DM encompasses an initial insulin resistant phase where normoglycaemia is maintained. This phase transitions to impaired glucose tolerance and/or impaired fasting glucose until diabetes manifests (Lundqvist et al., 2019).

It is only those who cannot sustain the compensation of β-cells in response to hyperglycaemia, who will develop T2DM (Prentki and Nolan, 2006). More than 90% of all diabetes cases are T2DM, and it often occurs in middle aged adults with chronic hyperglycaemia. It is associated with a sedentary lifestyle, family history and obesity (International Diabetes Federation, 2019). Classic symptoms of the disease do not occur in the early stages and thus patients do not seek early medical attention and remain undiagnosed for many years (Baynest, 2015; Labuschagne et al., 2017). It's asymptomatic nature (Beagley et al., 2014), results in a clinical diagnosis taking 4-6 years to be made (Porta et al., 2014). At the time of eventual diagnosis, 20% of patients would have already suffered some disease complications (World Health Organization, 2019).

The main pathological defect occurring in individuals with this condition is dysfunction of the pancreatic β-cells (American Diabetes Association, 2020). However, fat accumulation in the liver due to defects in the inhibition of adipose tissue lipolysis, is also characteristic of T2DM

(Longo et al., 2019). Obese individuals have a higher body fat percentage mainly distributed in the abdominal area (Elffers et al., 2017). The increased release of free fatty acids, inflammation, and adipokine deregulation in adipose tissue, promotes insulin resistance (Galicia-Garcia et al., 2020). B-cell dysfunction stems from insulin resistance in skeletal muscle and adipose tissue, which leads to an initial increased insulin production (Fazakerley et al., 2019). A failed response to the increased demand for insulin results in β-cell failure, glucose toxicity and hyperglycaemia (Mononen et al., 2019). The compensation by β-cells during T2DM progression, allows normoglycaemia to be maintained for years without inducing symptoms before progression to IGT or impaired fasting glucose (IFG) occurs (Vasu et al., 2019). It is for this reason that many people with diabetes are unaware, undiagnosed, and untreated (American Diabetes Association, 2010).

Genetics, environmental and lifestyle factors contribute to the development of T2DM (Baynest, 2015; Labuschagne et al., 2017). Normally in the presence of hyperglycaemia, the brain communicates via nerve impulse signalling to the pancreas and organs, to reduce the hyperglycaemia (Lundqvist et al., 2019). In T2DM, inflammation, endoplasmic reticulum stress and dysfunctional mitochondria causes damage to various neural circuits and brain segments and results in changes in signalling systems (De Felice and Ferreira, 2014). Endothelial dysfunction, inflammation (Maksymets et al., 2018), iron overload (Miranda and Lawson, 2018), inconsistent circadian systems, adipocyte signalling, and incretin abnormalities are other factors contributing to the pathophysiology of T2DM (Guay and Regazzi, 2013). Intra-abdominal obesity in the presence of insulin resistance, together with dyslipidaemia and hypertension, is commonly seen in these patients (Labuschagne et al., 2017).

Diagnosis often occurs because of the development of diabetic complications and may also be incidental. Symptoms such as polydipsia, polyuria, polyphagia, weight loss, nephropathy and blurred vision are classic symptoms of T2DM (2017 SEMDSA Guideline for the Management of Type 2 Diabetes Guideline Committee, 2017). However, several parameters have been identified which can assist in determining the risk of developing T2DM. These include the waist-hip ratio, body mass index (BMI), gender, blood pressure, physical activity, glycated haemoglobin (HbA1c), glucose levels, triglycerides, C-peptide, and cholesterol (Banerjee et al., 2017). The diagnosis of T2DM is based on the WHO diagnostic criteria and is depicted in table 1.

Measuring plasma glucose using the Oral glucose tolerance test (OGTT) is the gold standard in South Africa for the diagnosis of diabetes (Dias et al., 2019). Although more expensive than glycated haemoglobin (HbA1c) (Pheiffer et al., 2021), it is a more sensitive measurement of the body's tolerance to glucose (Azzi et al., 2018). The procedure requires that an initial sample

is drawn after an overnight fast, followed by the oral ingestion of 75g of glucose. Two hours thereafter, a plasma glucose measurement is taken (Pheiffer et al., 2021). Additional disadvantages of the test are the inconvenience of the 2-hour test duration and the requirement to fast overnight (George, 2011).

Table 1: Diabetes diagnostic criteria according to 2006 WHO recommendations (World Health Organization and International Diabetes Federation, 2006).

	Fasting plasma glucose	**2hr plasma glucose after 75g OGTT**	**Single or both tests required for diagnosis**
Diabetes	≥7 mmol/L	≥ 11mmol/L	Single in symptomatic individuals
Impaired glucose tolerance (IGT)	< 7 mmol/L	≥7.8mmol/L & <11.1mmol/L	Both
Impaired fasting glucose (IFG)	6.1mmol/L – 6.9mmol/L	< 7.8mmol/L	Single or both

Progression of this disorder is often managed by lifestyle changes, but β-cell dysfunction continues regardless of management (Jiménez-Lucena et al., 2018). Insulin resistance and/or insufficiency in organs such as the liver, skeletal muscle and adipose tissue is thus inevitable (2017 SEMDSA Guideline for the Management of Type 2 Diabetes Guideline Committee, 2017; He et al., 2017; Jiménez-Lucena et al., 2018). It is therefore essential to identify the condition early, and this was demonstrated by Herman et.al. (2015). In this study, a reduction in the time between the onset of diabetes and the time that clinical diagnosis was made, resulted in earlier treatment for cardiovascular complications and glycaemia (Herman et al., 2015).

2.4 Gestational diabetes

Gestational diabetes is diagnosed when glucose intolerance is first recognised during pregnancy (Rani and Begum, 2016). The detrimental effects of hyperglycaemia in pregnancy are macrosomia, caesarean delivery, babies being delivered pre-term and pre-eclampsia (Kaaja and Rönnemaa, 2008). Babies born in such conditions have an increased risk of future development of diabetes (Sheiner, 2020). Family history, polycystic ovarian syndrome, maternal age, and sedentary lifestyle are among the risk factors for gestational diabetes (Kharroubi and Darwish, 2015).

2.5 Other specific types of diabetes

Single gene defects in the β cells of the pancreas result in other specific types of diabetes. This interferes with the functioning of the β cells or in a reduced number of β cells. This form of diabetes is called monogenic diabetes and its classification is based on the age of onset (Schwitzgebel, 2014). It could be classified as neonatal diabetes if the age of onset is before 6 months or, if diagnosed before 25 years of age, it is classified as Maturity Onset Diabetes of the Young (Kharroubi and Darwish, 2015). Other specific types also include diabetes associated with the use of drugs or other diseases (Punthakee et al., 2018).

2.6 Pathophysiology of Type 2 Diabetes

2.6.1 Glucose homeostasis

Glucose is an important source of energy for humans, thus making it vital for human health, and survival depends on the maintenance of adequate levels (Hruby, 1997). Glucose homeostasis is defined as the process of balancing the rate of glucose entering and removal from the circulation (Ang and Linn, 2014). Circulatory glucose is mainly derived from glycogenolysis (the breakdown of glycogen to glucose), absorption from the intestine in a fed state and gluconeogenesis (the formation of glucose). Pancreatic α-cells produce a hormone called glucagon, which is partially responsible for the control of glycogenolysis and gluconeogenesis (Aronoff et al., 2004).

Multiple mechanisms ensure that plasma glucose is maintained within a narrow range (4-6mmol/L) (Tilburg et al., 2001). The release of insulin occurs in 2 phases following a meal: In response to an elevated concentration of blood glucose there is a rapid release of endogenous insulin as well as an increased synthesis and secretion of insulin (Aronoff et al., 2004; Uluseker et al., 2018). Of the total glucose absorbed the brain uses 50%, intestines and liver 25% and muscle and adipose tissue 25% (DeFronzo, 2004). The muscles and adipocytes are stimulated to absorb glucose and convert it to glycogen or triglycerides. These processes result in the reduction of plasma glucose and the stimulus to secrete insulin is stopped (Hiriart et al., 2014).

In a fasting state, insulin levels are low due to low levels of glucose. The liver controls glycogenolysis and gluconeogenesis and thus the body relies on it as a source of glucose (Hiriart et al., 2014). Glucagon, a hormone produced by pancreatic alpha cells, stimulates this process. The action by glucagon is supressed in the presence of hyperinsulinemia and hyperglycaemia (Gromada et al., 2007). In muscle cells and adipocytes, glucose is stored as glycogen. Insulin binds to its receptors on these cells and glucose uptake results. The uptake of glucose leads to the release of leptin and the inhibition of hunger (Aronoff et al., 2004). High levels of plasma insulin and glucose also inhibits hunger. Leptin is counteracted by Ghrelin

which the empty stomach secretes resulting in feeding (Sakata and Sakai, 2010).

2.6.2 Glucose homeostasis in Type 2 diabetes mellitus

Type 2 diabetes mellitus occurs because of a malfunction of the mechanisms involved in insulin action and insulin secretion. This results in plasma glucose concentrations which are abnormally high (Galicia-Garcia et al., 2020). In the early stages of diabetes, the action of β-cells become abnormal and fail to respond. This results in a decreased availability of insulin, followed by hyperglycaemia (Aronoff et al., 2004). The progressive failure of β-cells initially presents as impaired glucose tolerance and eventually progresses to overt diabetes (Abdul-Ghani, 2013).

When cells of the body are unable to detect insulin and the sensitivity to insulin is lost, this is called insulin resistance (Himanshu et al., 2020). When insulin resistance is present, the uptake and metabolism of glucose in skeletal muscle, the suppression of glucose production in the liver and lipolysis in the adipocytes are impaired (DeFronzo et al., 1989). Inflammatory cytokines (Interleukin-6, tumour necrosis factor) and free fatty acids are increased in the circulation due to the inhibition of adipocyte activity. This alters insulin sensitivity and interrupts the metabolism of glucose (Besler et al., 2012). The progressive dysfunction of β-cells is more severe than Insulin resistance but, in the presence of both processes, the amplified hyperglycaemia results in progression of Type 2 diabetes mellitus (Galicia-Garcia et al., 2020).

Figure 2.1: Normal physiology vs pathophysiology of diabetes (Adopted from (Baynest, 2015)

2.7 Pre-diabetes

Prior to the development of T2DM, patients can be in a pre-diabetic state (Khan et al., 2019). This state is defined as individuals having an impaired fasting glycaemia (IFG) and impaired glucose tolerance (IGT), which are conditions of transition between normoglycaemia and diabetes (Roglic and World Health Organization, 2016). No uniformity exists between organizations who have attempted to define pre-diabetes (Bansal, 2015). The term is however used to categorize individuals who have abnormalities in their glucose parameters but do not fit the criteria of being diabetic, even though being at risk of developing T2DM (Kharroubi and Darwish, 2015). In addition to the presence of IGT and IFG, various individuals with abnormalities in their glucose metabolism can be identified with a more advanced stage of damage to the glucose homeostasis (Banerjee et al., 2017; Bansal, 2015). A diagnosis based on IGT and IFG alone has been challenged, as the cut-off points cannot predict the future development of diabetes (Genuth and Kahn, 2008). Furthermore, diabetes related pathology cannot be determined and reproducibility in adults and children is poor (Bansal, 2015).

Peripheral insulin resistance is a key feature in the pathogenesis of pre-diabetes (Ahmed et al., 2017). An increase in pancreatic islet mass and an increase in insulin release occurs in pre-diabetes as the β-cells compensate for the insulin resistance from target tissue (Prentki

and Nolan, 2006). Adaptive processes are activated during conditions of chronic stress. This leads to eventual β-cell dysfunction and an increased IGT and/or IFG (Jiménez-Lucena et al., 2018). The simultaneous abnormalities in insulin resistance and β-cell dysfunction, start before detectable changes in glucose concentration occur (Tabák et al., 2012). In IFG and IGT, the secretion of insulin in response to glucose, is impaired (Kabadi, 2017). Hyperinsulinaemia occurs in response to fasting hyperglycaemia (Abdul-Ghani et al., 2006). Although β-cell dysfunction occurs in both IFG and IGT (Yip et al., 2017), those with IGT display more insulin resistance in muscle than in the liver and those with IFG show more resistance in the liver than muscle (Abdul-Ghani et al., 2006).

Not all pre-diabetic individuals progress to diabetes however, they are at high risk of progression (Sidorkiewicz et al., 2020). Therefore, the main concern of pre-diabetes is the increased risk of progression to overt diabetes and the high risk of cardiovascular disease (Huang et al., 2016). Erectile dysfunction, neuropathy, retinopathy, glucotoxicity, defects in insulin secretion, genetics, impaired incretin release (Kabadi, 2017), lipotoxicity, oxidative stress, inflammation and β-cell dysfunction are among the multiple factors involved in its development (Dorcely et al., 2017; Gounden, 2012). Individuals are at risk of developing diabetes within 5 years of having pre-diabetes if left untreated (Lee et al., 2019). Reverting to normoglycaemia is however possible (Huang et al., 2016). A continued high calorie diet, sedentary lifestyle, stress, and lack of exercise are among the contributing factors which will result in progression from pre-diabetes to type 2 diabetes mellitus (Indonesian Diabetes Association, 2014). Identifying pre-diabetes confers the opportunity for therapeutic and lifestyle interventions to effectively prevent the progression to diabetes (Aekplakorn et al., 2015). This type of intervention can reduce progression by up to 70%, which highlights the need for early detection (Okwechime et al., 2015).

A diabetes risk model is a means of identifying those individuals who are at risk of diabetes development. It is based on parameters such as age, weight, body mass index, gender, family history etc. This diabetes prediction model is however not universally accepted as ethnicity is an influencing factor in diabetes risk. This means that prediction algorithms would have to be recalibrated when they are used with different populations (Noble et al., 2011).

2.8 Metabolites as biomarkers for the diagnosis of diabetes

The improvement of the diagnosis and prognosis of disease can be achieved by developing improved clinical tests through biomarker identification (Li and Kowdley, 2012). Biological markers (biomarkers) are molecules which can reflect both diseased and healthy states of the body. These markers can be measured in biological media (human fluids, tissue, and cells) and can also serve as indicators of normal and pathogenic biologic processes or the response

to therapeutic intervention (Mayeux, 2004). Two types of biomarkers exist. One being exposure biomarkers, which are used in risk prediction and the other a disease biomarker which is used to screen, diagnose, and monitor the progression of disease (Mandal, 2019). Specificity, sensitivity, and test reproducibility are the characteristics of a good biomarker (Yousef et al., 2014).

2.8.1 Existing metabolites used for the diagnosis of T2DM

Metabolites are end products or intermediates of a metabolic pathway. These compounds are low in molecular weight, and some have been demonstrated as risk factors for T2DM development (Goek et al., 2012).

Albumin is one of the most abundant proteins in human serum (Anguizola et al., 2013), and as such, measuring fructoseamine reflects the glycated albumin concentration (Welsh et al., 2016). Glycosylated albumin is used to evaluate short term (2-3 weeks) glycaemia (Chume et al., 2019) and to indicate diabetic complications (Pan et al., 2014; Pu et al., 2007; Selvin et al., 2014; Wang et al., 2016). The results however, have low accuracy due to interference from other plasma proteins such as bilirubin (Freitas et al., 2017). Furthermore, falsely low serum fructosamine levels are seen in children due to low protein levels and falsely high levels are seen in adults with conditions such as nephrotic syndrome (Danese et al., 2015; Suneja et al., 2021). An increased BMI, visceral fat and body fat mass also produce a falsely low glycated albumin (Furusyo et al., 2011).

Other biomarkers which have been used include Alpha hydroxybuterate (α-HB) which is a by-product produced in the liver (Wang et al., 2011). An increased production occurs under oxidative stress such as insulin resistance and therefore this metabolite is useful to assess the dysfunction of β-cells (Montane et al., 2014) and dysglycaemia (Syed Ikmal et al., 2013). An increased concentration of lysophosphatidylcholine (LPC), as seen in diabetes is associated with insulin resistance and is a negative predictor of the risk of developing T2DM (Yang et al., 2018). Branched chain amino acids (BCAA's) are abundant in dietary protein (Lynch and Adams, 2014) and its long-term increase is negatively associated with insulin resistance and risk of T2DM (Yoon, 2016). The same result is seen with C-reactive protein, where an increase in its concentration is associated with insulin resistance and T2DM development (Hayfron-Benjamin et al., 2020). An increase in concentration occurs in response to tissue damage and inflammation, as seen in T2DM (You et al., 2016). Free fatty acid (FFA) concentration has also been associated with insulin sensitivity, impaired glucose tolerance and insulin resistance (Mandal, 2019). Changes in the concentration of free fatty acids (FFA) and changes in the regulation of FFA metabolism can result in insulin resistance and T2DM (Sobczak et al., 2019).

Low levels of bilirubin, an antioxidant of heme catabolism is linked to T2DM and the development of complications in individuals with impaired glucose metabolism. The result is however, influenced by ethnicity and age (Mandal, 2019).

The early diagnosis of T2DM ensures a better quality of life for patients while the identification of hyperglycaemia in its infancy, will have a significant impact on the prevention of delayed diagnosis and disease related complications and mortality (Herman et al., 2015; Jiménez-Lucena et al., 2018). Current biological tests used to screen and diagnose diabetes based on blood glucose levels are reliable, but the use of these tests cannot detect at-risk patients or monitor diabetes complications or disease progression (Rezk et al., 2016). They are time-consuming, invasive and inconvenient for patients (Chume et al., 2019). Most of these tests are only useful once the disease has been established and not in pre-diabetic conditions. The eventual result of this is complications such as diabetic nephropathy, chronic heart disease and retinopathy (Vaishya et al., 2018). The alarming increase in the incidence of DM and the limitations of current diagnostic tests, places urgency on the need for more specific, stage-related, non-invasive biomarkers (Dorcely et al., 2017). These biomarkers should be accurate in diagnosing the initiation and progression of T2DM, identify β-cell injury and assess the risk of disease (Banerjee et al., 2017; Jiménez-Lucena et al., 2018; Punthakee et al., 2018; Vaishya et al., 2018). Although there has been an increased interest in epigenetic biomarker research (Elliott et al., 2019; Kato and Natarajan, 2014; Shao et al., 2021), to date no biomarker perfect enough to suite all T2DM patients have been found (Ortiz-Martínez et al., 2022) thus further emphasising the need for continued epigenetic research focussing on population specific biomarkers.

2.9 The discovery of microRNAs

The human genome contains less than 25000 genes (Chi, 2016). Since its first discovery, the research focus regarding the regulation of this genome, has shifted from messenger RNA (mRNA) to miRNA (Ardekani and Naeini, 2010). The first miRNA discovered in 1993, was Lin-4 in *Caenorhabditis elegans,* a nematode (Lee et al., 1993). This miRNA was associated with transformation of the larval stage and adult development of *C. elegans*, and its mutation lead to the inability of the organism to lay eggs (Verma et al., 2016). Seven years later, let-7, the second miRNA was discovered in the same organism (Reinhart et al., 2000). This miRNA played a role in the developmental timing of *C. elegans* (Verma et al., 2016). Since then, more than 2000 miRNAs have been discovered in eukaryotes, of which many have been implicated in human disease (Li and Kowdley, 2012; Shen et al., 2017).

miRNAs are conserved, short, non-coding RNA's. They are important regulators of approximately one third of the human genome (Hammond, 2015), and serve as role players in

mediating human disease (Ardekani and Naeini, 2010; Verma et al., 2016). They function post transcriptionally in major cellular processes such as growth, differentiation, apoptosis, development, and metabolism (Ardekani and Naeini, 2010). Gene expression is controlled by repressing the translation of and/or degradation of target mRNA (Catalanotto et al., 2016). This is achieved by binding to target mRNA sequences which are complementary to their sequence. They then interfere with the translational process, thus preventing or changing the occurrence of protein production (Bhaskaran and Mohan, 2014). Intercellular signalling is another role of miRNAs (Bayraktar et al., 2017). This is made possible by a large proportion of miRNAs migrating from the inside of the cell to the outside via extracellular vesicles or protein binding (exosomes and proteins) and are therefore readily available in body fluids (Bayraktar et al., 2017). miRNAs are found in extracellular fluids such as blood, breast milk, urine, seminal fluid and saliva (Weber et al., 2010). Their existence as protein complexes, provides protection against degradation by extracellular enzymes and therefore they are stable in serum and plasma (Wang et al., 2010). Among the previously mentioned roles, is the mediation of cell-to cell communication. This is what makes miRNAs useful as potential biomarkers in human disease (Bayraktar et al., 2017; Condrat et al., 2020).

2.10 Biogenesis and functioning of microRNAs

miRNA genes are dispersed throughout the genome. Some of them are non-coding, while others are in an intron, an untranslated region of a protein coding gene (Bartel, 2004). miRNAs are 22 nucleotides long (Bartel, 2004) and biogenesis starts in the nucleus (O'Brien et al., 2018). Most of the miRNA biogenesis occurs by the dominant canonical pathway, where deoxyribonucleic acid (DNA) is transcribed into primary miRNAs, followed by processing into precursor miRNAs and then mature miRNAs (O'Brien et al., 2018). The biogenesis can also occur in a non-canonical pathway (Ha and Kim, 2014).

Most of the miRNAs in humans are processed from the introns and only a few from exons of protein coding and non-protein coding genes (O'Brien et al., 2018). RNA polymerase II transcribes a non-coding miRNA gene to form primary miRNA (pri-miRNA). The pri-miRNA has a hairpin like structure for the embedding of miRNA sequences (Ha and Kim, 2014). Pri-miRNA is then cleaved by an enzyme, DROSHA and its associated binding protein DGCR8 (Di George syndrome critical region 8), which is required for the cleaving of the pri-miRNA (Han et al., 2004). Exportin 5 then transports the resultant precursor (pre-miRNA) transcript out of the nucleus, to the cytoplasm, where a mature miRNA duplex will be produced (Bhaskaran and Mohan, 2014). The pre-miRNA is then processed by Dicer (Ribonuclease 3 enzyme) and its associated TRBP protein (TAR RNA binding protein) in mammals to form the RNA duplex. The duplex strands are separated into 2 strands (5P and 3P), where one is loaded onto an argonaute (AGO2) family protein containing miRNA- induced silencing complex

(miRISC) (Gregory et al., 2005). The other strand is degraded (Han et al., 2004). The miRNA molecule can now silence the complementary target mRNA. The RISC is directed by miRNA to either cleave the target mRNA or the repression of translation to achieve the downregulation of gene expression (Bartel, 2004).

The canonical pathway of miRNA biogenesis involves the miRISC containing one of the duplex strands, acts by binding to the 3'-untranslated region of the target mRNA (Condrat et al., 2020). The non-canonical pathway involves miRISC-mRNA interaction which are not always complementary. It is via the non-canonical pathway that 1 miRNA may target multiple mRNA and a single mRNA can bind numerous miRNAs (Chevillet et al., 2014). This characteristic of miRNA allows for their involvement in many biological processes and pathways such as apoptosis, cellular signalling, cell proliferation and cell development (Sohel, 2016).

2.11 MicroRNAs as biomarkers in disease

The presence and role of miRNAs in human disease, make them important molecules to be understood (Ardekani and Naeini, 2010). The tissue specific expression pattern of miRNA (overexpression or under expression) provides information about a cell's physiologic state (Lim et al., 2005). miRNAs do not only exist intracellularly, but also extracellularly as circulating miRNAs (Mori et al., 2019). These circulating miRNAs have been linked to the pathophysiology of many diseases such as cancer (Khan et al., 2019), nervous system disorders (Baloun et al., 2020; Swarbrick et al., 2019), cardiovascular disease (Jones Buie et al., 2016), T2DM and others (Pordzik et al., 2019). In healthy and diseased states, organs such as the ovary, heart, mammary glands, brain, uterus, endothelial cells etc., release miRNAs into the bloodstream, making certain miRNAs specific for diseases and could therefore have value in predicting prognosis (Condrat et al., 2020; Li and Kowdley, 2012).

Disease association occurs when there is a deviation from the tightly controlled biogenesis of miRNA (Sohel, 2016). Extracellular miRNAs have been the focus in disease association due to their stability in extracellular fluids and the ability to detect them easily (Mori et al., 2019). Not only have circulating miRNAs been associated with many diseases (Baloun et al., 2020; Jones Buie et al., 2016; Khan et al., 2019; Pordzik et al., 2019), but changes in their level have also been correlated with exercise and other lifestyle activities such as diet (Flowers et al., 2015). A combination of molecular biology techniques, bioinformatics, genomics, and animal models have suggested that miRNAs possess the potential to transition from laboratories to clinics and contribute to improving public health (Li and Kowdley, 2012). They possess all the traits of a good biomarker, and therefore they have gained increased popularity in diabetic research (Guay et al., 2011; Prattichizzo et al., 2016).

2.12 MicroRNAs and Diabetes

The expression patterns of miRNAs in T2DM have been researched by many (Condrat et al., 2020; Wang et al., 2014). The dramatic surge in the prevalence of T2DM, has evoked an urgency to identify pre-DM, as this group is at the highest risk of developing T2DM (Sidorkiewicz et al., 2020). New opportunities for the early diagnosis of diabetes have been created by the stability and the significant role miRNA plays in metabolic haemostasis (Pordzik et al., 2019). In T2DM, miRNA play vital roles in the control of the metabolic pathways involved in adipocyte differentiation, lipid metabolism, inflammation, insulin secretion, and energy homeostasis (Deng and Guo, 2019; Rome, 2013). Clinical hyperglycaemia can therefore occur due to the aberrant expression of miRNA as the regulation of homeostasis is achieved by the involvement of miRNA in producing and secreting insulin and in the survival of β-cells (Kim and Zhang, 2019).

The changes which circulatory cells undergo in diseased states can be reflected by the profiling of miRNAs (Kim and Zhang, 2019). In a complex process, circulatory miRNA arises from various tissue sources during inflammatory and metabolic disease (Vasu et al., 2019). The complexity of this process prevents pre-diabetic conditions from being accurately diagnosed by singular miRNAs. Diagnosis by groups (signatures) of miRNAs is more accurate (Kim and Zhang, 2019). These signatures are specific for inflammatory or metabolic stress (Vasu et al., 2019). Inflammation is involved in the pathogenesis of T2DM (Tsalamandris et al., 2019). The mechanism of inflammation can affect miRNA expression, just as changes in miRNA expression can affect the occurrence of inflammation in T2DM pathogenesis (Miao et al., 2018). An alteration in the levels of miRNAs contribute to the inflammation seen in obese diabetics and contributes to insulin resistance in tissue and β-cell dysfunction (Landrier et al., 2019). These primary events further lead to the development of diabetic complications affecting the eyes, kidney and heart (McClelland and Kantharidis, 2014).

The regulation of inflammation during the development of T2DM is however, not well understood (Pordzik et al., 2019). Previous research has demonstrated that miRNA is involved in inflammatory and metabolic stress (Barutta et al., 2017; Kamalden et al., 2017; Sebastiani et al., 2017). An example is miR-15a which originated in one cell type and induced cell injury and oxidative stress in another cell type (Miao et al., 2018). This miRNA has been found to play a significant role in β-cell insulin secretion. Furthermore, it was found that during T2DM development, miR-15a which originated in β-cells, contributed to retinal injury upon entering the bloodstream (Kamalden et al., 2017).

The demonstration of changes in serum miRNAs in response to various phases of glycaemia have been conducted. However, less research has been conducted on miRNAs in pre-

diabetes (Mononen et al., 2019). Studies which have focussed on pre-diabetes (Kong et al., 2011; Liu et al., 2014) have identified low levels of miR-15a and miR-126 (Liu et al., 2014; Sun et al., 2011). Kong et al. (2015), further attempted to identify miRNAs which could distinguish between T2DM and pre-DM and demonstrated that miR-29a, miR-124a, miR-9, miR-30d, miR-375, miR-34a, miR-146a, although playing a significant role in the functioning of β-cells, could not distinguish between T2DM and pre-DM susceptible participants with a normal glucose tolerance (Kong et al., 2011). However, Yang et al did identify miR-23a as a marker which could be utilised to detect pre-DM and early T2DM (Yang et al., 2014).

A recent study conducted by Matsha et al. (2018), assessed miRNA expression profiles of 12 individuals with pre-diabetes, 12 with screen-detected diabetes and 12 with normal glucose tolerance, using high throughput sequencing (Matsha et al., 2018). Differentially expressed miRNA profiles were identified between the study groups and a comparison was made. The comparison revealed dysregulation of novel miRNA expression profiles in pre-diabetic profiles, as compared to screen detected diabetic profiles. Amongst the dysregulated miRNA found was hsa-miR-486-5p and hsa-miR-novel-chr1_40444 (novel), which was found to be dysregulated in pre-diabetes and not screen-detected diabetes. These 2 miRNAs were found to be amongst those miRNAs which displayed over regulation (they were either over upregulated or over downregulated) (Matsha et al., 2018).

The tumour suppressor role of hsa-miR-486-5p has been vastly explored in malignancies (Qi et al., 2019). These include cancers of the endometrium (Zheng et al., 2020), lung (J. Wang et al., 2014). and breast (Zhang et al., 2014). The involvement of hsa-miR-486-5p in the pathogenesis of T2DM has however not been thoroughly examined (Tian et al., 2018). Although there are few studies, Tian et al. found that the overexpression of hsa-miR-486-5p promoted cell proliferation, increased insulin sensitivity and inhibited apoptosis of β-cells by targeting Phosphatase and tensin homolog (PTEN) and Forkhead Box O1 (FOXO1) genes. These results highlight the protective role hsa-miR-486-5p plays in the pathogenesis of T2DM by preventing β-cell dysfunction (Tian et al., 2018). Another study found hsa-miR-486-5p to be involved in diabetic nephropathy, playing a regulatory role in inflammation, oxidative stress, and apoptosis (Kato and Natarajan, 2014). These 2 studies along with the study by Matsha et al. is indicative of the role which hsa-miR-486-5p plays in the pathogenesis of T2DM. The limiting factor is the small sample size (Matsha et al., 2018).

The inconsistency of results among studies has led to the absence of standardized protocols for the use of miRNA in clinical practice (Wang et al., 2014). Factors contributing to inconsistent results include differences in biological source of the sample, measurement platform and

glucose tolerance (Dias et al., 2017). Despite this, there is promise for the future use of miRNAs as a reliable tool for the diagnosis of diabetes (Condrat et al., 2020).

Due to the limitations of current diagnostic tests (Sebastiani et al., 2017) and the surge in the prevalence of T2DM (International Diabetes Federation, 2019), there is an urgency to find a non-invasive biomarker to predict the onset of T2DM (Bansal, 2015). The early diagnosis of diabetes can result in early intervention, which can delay or prevent the development of T2DM (Hostalek, 2019). Thorough validation of larger, independent clinical studies is needed, as the knowledge and understanding of the association between miRNA and DM is still limited (Barutta et al., 2018; Chen et al., 2008).

This study aims to investigate if hsa-miR-486-5p and hsa-miR-novel-chr1_40444 can differentiate between pre-diabetes and T2DM. We aim to investigate and validate these miRNAs in whole blood samples employing quantitative reverse transcription-polymerase chain reaction (RT-PCR) to confirm the association between the miRNA and T2DM and/or pre-diabetes. The objectives of this study are to measure the expression of hsa-miR-486-5p and hsa-miR-novel-chr1_40444 in 1989 samples of individuals with normal glucose tolerance status, pre-diabetes and T2DM. This will be followed by comparing the expression of these miRNA with glucose tolerance status of these individuals and correlating the expression with routine biochemical analytes.

Chapter 3: Methodology

3.1 Ethical clearance

This project was a sub-study of the Cape Town Vascular and Metabolic Health (VMH) study, where approval was obtained from the Cape Peninsula University of Technology (CPUT) Research Ethics Committee, and the Stellenbosch University Research Ethics Committee (respectively, NHREC: REC - 230 408 – 014 and N14/01/003). The samples for this study were selected from participants who were recruited between 2014 and 2016. A written, Informed consent was obtained from these participants for genetic testing. Information about the participants and details about the study were kept confidential. Ethical clearance for this sub-study was sought from and granted by the CPUT Research Ethics Committee (REC Approval Reference No: CPUT/HW-REC 2019/H3, CPUT/HW-REC 2019/H3 (renewal). The study was performed according to the Code of Ethics of the World Medical Association Declaration of Helsinki (World Medical Association, 2013).

3.2 Study setting and population

During April 2014 until November 2016, the total target of 1989 participants were enrolled for the VMH study. These participants were of mixed ancestry descent and reside in an urban township area in Cape Town South Africa, called Bellville South. This cross-sectional study used quantitative methods to explore the expression of known miRNAs (hsa-miR-486-5p and hsa-miR-novel-chr1_40444). Although a total 1989 participants from the VMH study was enrolled, data from only 1459 participants were used for this study due to clinically available data. These participants were grouped according to the lab results which tested their glycaemic parameters. They included 974 normoglycaemic, 206 with pre-diabetes and 279 with Type 2 diabetes. The 279 participants in the Type 2 diabetes group included those who were screen-detected diabetics as well as known diabetics on treatment.

3.2.1 Inclusion criteria

- Participants who consented to participating in genetic testing.
- Participants between 18 and 70 years, who were not ill or pregnant.

3.2.2 Exclusion criteria

- Individuals who did not consent to participating in genetic analysis.
- Individuals who were too ill to participate, pregnant as to avoid the inclusion of gestational diabetes since T2DM is our focus, or less than 18 years of age as they cannot give consent.

3.3 Study procedure

3.3.1 Anthropometric measurements

The anthropometric measurements were performed in triplicate and the average value of the measurements were used for the final analysis. Height was measured using a portable stadiometer and recorded in centimetres (cm). The body weight of participants (to the nearest 0.1 kg) was measured using a pre-calibrated Omron body fat meter HBF-511 digital bathroom scale. The body mass (Kg) of each participant was divided by their height (m) squared to determine their body mass index (BMI) (Zierle-Ghosh and Jan, 2022). A non-elastic tape was put around the natural waist (narrowest part of the torso as seen from the anterior view), to measure waist circumference. (Tolonen et al., 2002). Hip circumference was measured using a non-elastic tape, on the widest region at the maximal circumference of the buttocks, (Tolonen et al., 2002).

3.3.2 Blood pressure

The measurement of blood pressure was performed using a semi-automatic digital blood pressure monitor (Omron M6 comfort-preformed cuff BP Monitor). The World Health Organisation (WHO) guidelines were followed to conduct the blood pressure measurements (World Health Organization, 1999). Three readings at one-minute intervals were recorded, and the lowest reading was selected as the participant's blood pressure. (Adams et al., 2002).

3.3.3 Sample collection and biochemical testing

Participants samples were collected using six blood tubes (three fasting and three postprandial). Medical records or medication was used to confirm those self- reported cases of T2DM as these participants had blood fasting samples, without 2-hour blood samples drawn. The tubes used were one sodium fluoride (grey capped) for the measurement of glucose, a tube absent of clotting factors (plain) for the isolation of serum for serological tests and a purple-capped tube for measuring glycosylated haemoglobin (HbA1c).

Whole blood samples were collected in Tempus® Blood RNA Tubes containing 6 mL of stabilizing reagent. These tubes were used for the extraction of circulating miRNAs. Upon interaction with the stabilizing reagent, the whole blood would lyse while the proteins would remain in the solution. This resulted in a higher total ribonucleic acid (RNA) recovery yield.

All participant blood samples were transported in an icebox for the analysis of biochemical parameters at an ISO 15189 accredited pathology laboratory (PathCare, Reference

Laboratory, Cape Town, South Africa). All the biochemical parameters except serum cotinine were analysed using the Beckman AU (Beckman Coulter, South Africa) automated analyser. Serum cotinine was measured on the Immulite 2000 (Siemens, South Africa). The following biochemical analytes were measured using standard operating procedures (SOP's) followed by Pathcare:

- Plasma glucose (mmol/L) using the Enzymatic hexokinase method which is based on phosphorylating and the catalysation of glucose by hexokinase, through a series of reactions. The resultant increased NADPH (Dihydronicotinamide-adenine dinucleotide phosphate) formation measured at 340nm is directly proportional to the glucose concentration (Sonagra and Motiani, 2022).
- HbA1c (%) using high performance liquid chromatography, a method which separates the haemoglobin components based on differences in particle charges (Lorenzo-Medina et al., 2014).
- Insulin (mmol/L) using the Beckman DXI (Beckman Coulter, Miami, USA) analyser which employs the method as set by the manufacturer for the Access Immunoassay system. This method is based on the Paramagnetic Particle Chemiluminescence Assay
- Total cholesterol (mmol/L) using enzymatic immune-inhibition which is based on the enzymatic measurement of cholesterol in serum or plasma. Cholesteryl esters are hydrolysed and the 3-OH group of cholesterol is oxidised. One of the by-products is hydrogen peroxide which is quantitatively measured in a reaction catalysed by peroxidase and subsequently produces a colour where the intensity is directly proportional to the concentration of the cholesterol in the sample.
- High-density lipoprotein cholesterol (HDL-c) (mmol/L) using enzymatic immune-inhibition where anti-human ß-lipoprotein antibody binds to all lipoproteins except high density lipoprotein (HDL). Cholesterol oxidase and esterase react with HDL-c with the subsequent production of hydrogen peroxide which produces a blue colour complex. The absorbance of this complex is measured at 600nm to obtain the sample HDL-c concentration.
- Low density lipoprotein cholesterol (LDL) (mmol/L) using enzymatic selective protection. Low density lipoprotein (LDL) is protected from enzymatic reactions. Cholesterol oxidase (CO) and Cholesterol esterase (CHE) react with all lipoproteins except LDL with the production of hydrogen peroxide as a by-product. Hydrogen peroxide is subsequently decomposed by catalase. In a second reaction upon removal of the protecting agent from LDL, sodium azide (NaN3) inactivates catalase and CO and CHE only react with LDL-C. Hydrogen peroxide is subsequently produced and forms a colour complex with the oxidative condensation of 4-aminoantipyrine (4-AA) and N-(2-hydroxy-3sulfopropyl)-3,5-dimethoxyaniline (HDAOS).

- Triglycerides (TG) (mmol/L) using Glycerol Phosphate Oxidase-peroxidase which is based on hydrolysing the sample triglycerides to release fatty acids and glycerol. The glycerol is converted to glycerol-3-phosphate in the presence of glycerol kinase and ATP. Glycerol-3-phosphate is subsequently oxidised which results in the production of hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide reacts with N,N-bis(4-sulfobutyl)-3,5-dimethylaniline and 4-aminophenazone, disodium salt and produces a chromophore which to produce a chromophore. The colour intensity of the chromophore is directly proportional to the concentration of triglycerides in the sample.
- Ultra-sensitive (U-CRP) using Latex Particle Immunoturbidimetric method which is based on the latex agglutination principle. The serum sample containing C-reactive proteins is mixed with latex particles complexed with human anti-CRP, resulting in agglutination. The complexes formed scatter light relative to their shape, size, and concentration. The reduced incidence light is measured by turbidimeters (www.beckman coulter.com).
- Serum Cotinine (ng/mL) using the Siemens Immulite 1000 which employed competitive chemiluminescence. The Nicotine Metabolite Assay kit produced by the manufacturers, was used and is based on a solid-phase competitive chemiluminescent immunoassay (Janakiraman et al., 2009).

3.3.4 Oral glucose tolerance test (OGTT)

All participants except those with self-reported diabetes were requested to fast overnight, which was followed by an OGTT test. The test was performed in accordance with the guidelines set by the WHO (World Health Organization, 1999) which guides as follows: Fasting blood samples were drawn. This was followed by the participant orally ingesting 75g of anhydrous glucose dissolved in 200-300 mL of water. After 2 hours, another sample was drawn.

3.3.5 Classification of diabetes status

Participants of the VMH sub study were diagnosed in accordance with the World Health Organization criteria (World Health Organization and International Diabetes Federation, 2006) and were categorized into the following groups according to their glycaemic status as revised by WHO criteria of 1999 (World Health Organization, 1999). These categories were: normoglycaemic, pre-diabetic (IGT and/or IFG), Type 2 diabetes (screen detected diabetics) and Known diabetics on treatment. A history of diabetes, fasting and postprandial glucose concentrations were the basis upon which a diagnosis of diabetes was made. The specifications used were fasting plasma glucose ≥ 7mmol/L or 2-hour plasma glucose ≥

11.1mmol/L. For the purposes of this study, the diabetes group consisted of known diabetics on treatment as well as screen detected diabetics.

3.3.6 Isolation of total RNA

Total RNA extraction and the quantification of target miRNAs were performed on 1459 participants. Whole blood samples (3 mL) mixed with Tempus® stabilizing reagents and stored at -20°C, was used for RNA isolation. The extraction of RNA was performed using the MagMAX™ for Stabilized Blood Tubes RNA Isolation Kit (Applied Biosystems, 2010).

3.3.7 Preparation of reagents

The isolation process required 300µL of prepared Wash Solution 1 concentrate, to which 8mL of 100% isopropanol was added, mixed, and stored at room temperature until it was required for use. A Wash Solution 2 concentrate was also prepared, to which 24mL of 100% ethanol was added, mixed, and stored at room temperature until required for use.

3.3.8 Preparation of Tempus® stabilized blood sample

Whole blood samples were collected for RNA analysis, in Tempus Blood RNA tubes, during 2014-2016, for the VMH study. Those samples were then stored at -80°C until needed. Upon the commencement of this study, the Tempus whole blood samples were freeze-thawed, RNA was isolated, after which subsequent cDNA synthesis and qPCR was performed. Once thawed, the contents were mixed, followed by the addition of 3 mL of whole blood and 5 mL of Tempus® 1X phosphate buffered solution (PBS). Quality control was conducted by using a negative control (NC) containing 3 mL nuclease free water, and treating it in the same way as the samples.

The 15 mL tubes were vortexed for 30 seconds to create pellets of crude RNA. This was followed by centrifugation at 7000 revolutions per minute (rpm) for 15 minutes at 4°C. A 4 mL Tempus® Pre-Digestion Wash was then added to each 15 mL tube. To re-pellet the RNA, the tube was vortexed and centrifuged at 7000 rpm for 10 minutes at 4°C. The resulting supernatant was removed and discarded, followed by centrifugation of the tube at 3000 rpm for 10 minutes to re-pellet the samples. Residual supernatant was drained, and the tubes were stored on ice until required for use.

The washed crude RNA pellets were resuspended using Tempus® Resuspension Solution (117.5 µL) and Tempus® Proteinase (2.5 µL), to a total volume of 120 µL. This suspension was then added to an eppendorf tube, mixed well, and centrifuged for 2 to 3 seconds, followed by placing it on ice until required for use. The resuspension mixture (120 µL) was added to each

of the 15 mL sample tubes and vortexed to resuspend the crude RNA pellets. The contents of each tube were transferred to a 96-well Deep-Well Processing Plate. To each sample well containing the resuspended pellets, 10 µL of TURBO™ DNase was added. Adhesive film was then used to seal the plate, followed by 10 minutes of thorough mixing on an orbital plate-shaker.

3.3.9 Purification of RNA

The following table (Table 2) demonstrates the setup of the MagMAX™ Express 96-well Standard Plates which were set up during the TURBO™ DNase incubation step.

Table 3.1: Preparation of the MagMAX™ Express 96-well Standard Plate

Plate		Reagent	Volume per well (µL)
Name	Position on processor		
Wash 1	2	Wash Solution 1	150
Wash 1	3	Wash Solution 1	150
Wash 2	4	Wash Solution 2	150
Wash 2	5	Wash Solution 2	150
Elution	6	Elution Buffer	50

The RNA Binding Beads which were Stored at 4°C, were removed and vortexed until fully resuspended. The addition of 50 µl of Binding Solution Concentrate and 20 µl of RNA Binding Beads to each well of the 96-well Deep-Well processing plate, followed. After which the plate was sealed and placed on an orbital shaker for 1 minute to mix its contents. A P200 multichannel pipette was used to dispense 200 µl of 100% isopropanol to each well, followed by sealing of the plate and storage on ice until required for use.

3.3.10 Kingfisher™ Flex processor for running plates

Upon powering the machine on, the pre-loaded assay protocol (4451893_Tempus 96DW) was selected. An unused MagMAX™ Express 96-well Standard Plate was selected and combined with the MagMAX™ Express-96 Deep Well Tip Comb. The plate tray on the machine opened, and the processor prompted the loading of each plate into the loading stations/ positions, upon selecting the “Start” button on the processor. Once the loading was completed, the “Start” button was selected again to initiate the run. It took approximately 25 minutes for the run to complete. The eluted total RNA was found to be in position 6 of the processor.

The Nanodrop 1000 (Nanodrop Technologies, Wilmington, USA) was used to assess purity and integrity of the subsequent RNA samples. The only samples accepted for further processing, were those with an RNA concentration >15 ng/mL, and OD (optical density) ratios of $^{A260}/_{A280}$ >1.8. Purified RNA could be stored on ice at −20°C before immediate use, or they could be stored for up to 6 months, at −80°C.

3.3.11 Conversion to cDNA

The RNA required conversion to cDNA, before quantitative analysis. The samples were removed from storage at −20°C and thawed on ice. This was followed by reverse transcription, using the TaqMan™ Advanced cDNA Synthesis Kit. The manufacturer's specifications were followed (Applied Biosystems, 2015). The TaqMan™ Advanced cDNA Synthesis Kit reagents were removed from storage at −20°C and thawed on ice. The number of required reactions was determined and adjusted based on the indicated single-reaction volumes. An extra 10% was added as a buffer for pipetting errors.

3.3.12 Poly(A) tailing reaction

The Poly(A) tailing reaction incorporated the addition of a 3'-adenosine tail to the miRNA present in the total RNA samples. The enzyme, Poly(A) polymerase catalysed the reaction. The thawed RNA samples and cDNA synthesis reagents were vortexed and centrifuged. The table 3 below demonstrates the steps followed when preparing the Poly(A) Reaction Mix in a 1.5 mL Eppendorf tube.

Table 3.2: Reaction mix for Poly(A) tailing

Component	1 Sample	96 samples
10X Poly(A) Buffer	0.5 µL	48 µL
ATP	0.5 µL	48 µL
Poly(A) Enzyme	0.3 µL	28.8 µL
RNase-free water	1.7 µL	163.2 µL
Total Poly(A) Reaction Mix volume	3 µL	288 µL

(Indicated volumes include the 10% extra volume)

The Poly(A) tailing reaction mix was vortexed and centrifuged. This was followed by the transfer of 3 µL of the mix into each well of a MicroAmp™ Optical 96-Well Reaction Plate. In addition, 2 µL of each total RNA sample was added to each well and the plate was sealed. The reaction plate was mixed and centrifuged, after which it was placed into a QuantStudio™ 7 Flex Real-Time PCR System. This PCR system was programmed using the settings and standard cycling, for incubation. The incubation conditions included: polyadenylation for 45

minutes at 37°C; a stop reaction step for 10 minutes at 65°C, and an infinite hold at 4°C. Once incubation was completed, the plate was removed from the PCR system to proceed with the Adapter ligation reaction.

3.3.13 Ligation

Upon completion of poly(A) tailing, the miRNA with the poly(A) tails underwent adaptor ligation at their 5' end. The adaptors played the role of the forward-primer binding sites for the miR-Amp reaction which occurred later. Ligation Reaction Mix was prepared in a 1.5 mL Eppendorf tube, according to the following steps:

Table 3.3: Preparation of the Adapter ligation reaction mix

Component	**1 Sample**	**96 samples**
5X DNA Ligase Buffer	3 µL	288 µL
50% PEG 8000	4.5 µL	432 µL
25X Ligation Adaptor	0.6 µL	57.6 µL
RNA Ligase	1.5 µL	144 µL
RNase-free water	0.4 µL	38.4 µL
Total Ligation Reaction Mix volume	10 µL	960 µL

(Indicated volumes include the 10% extra volume)

Once the reaction mix was prepared, it was vortexed and centrifuged. 10 µl of the reaction mix was transferred into each well of the reaction plate containing the poly(A) tailing reaction product. The total volume of each well was 15 µl following the addition of the reaction mix. The reaction plate was sealed and mixed on an orbital plate-shaker set at 1,900 rpm for 1 minute. The plate was then centrifuged and placed in the QuantStudio™ 7 Flex for incubation with the following settings: 60-minute ligation at 16°C, followed by an infinite hold step at 4°C. Upon completion, the plate was removed, to proceed with the Reverse Transcription step.

3.3.14 Reverse transcription (RT)

A universal RT primer which was bound to the 3' poly(A) tails of the miRNA, resulted in the miRNA being reverse transcribed into cDNA. The table 5 below displays the steps followed to prepare RT reaction mix in a 1.5 mL Eppendorf tube. This was prepared according to the required number of reaction**s**

Table 3.4: Reaction mix for reverse transcription (RT)

Component	1 Sample	96 samples
5X RT Buffer	6 µL	576 µL
dNTP Mix (25 mM each)	1.2 µL	115.2 µL
20X Universal RT Primer	1.5 µL	144 µL
10X RT Enzyme Mix	3 µL	288 µL
RNase-free water	3.3 µL	316.8 µL
Total RT Reaction Mix volume	15 µL	1440 µL

(Indicated volumes include the 10% extra volume)

Following the preparation, the RT reaction mix was vortexed and centrifuged. After centrifugation, 15 µL of the reaction mix was added to each well of the 96-well reaction plate containing the adapter ligation reaction product. After the addition of the reaction mix, the total volume in each well was 30 µL. The plate was sealed, centrifuged, and placed in the QuantStudio™ 7 Flex programmed with the following incubation settings: reverse transcription for 15 minutes at 42°C; a stop reaction step for 5 minutes at 85°C, and an infinite hold at 4°C. The plate was removed upon completion of incubation, to prepare for the the miR-Amp reaction step.

3.3.15 Amplification

The universal forward and reverse primers increased the number of cDNA templates present in each sample by amplification. In a 1.5 ml Eppendorf tube, miR-Amp Reaction Mix was prepared according to the required number of reactions. The following steps were followed:

Table 3.5: Preparation of miR-Amp reaction mix

Component	1 Sample	96 Samples
2X miR-Amp Master Mix	25 µL	2400 µL
20X miR-Amp Primer Mix	2.5 µL	240 µL
RNase-free water	17.5 µL	1680 µL
Total miR-Amp Reaction Mix volume	45 µL	4320 µL

(Indicated volumes include the 10% extra volume)

Following preparation, the miR-Amp Reaction Mix was vortexed, centrifuged and 45 µl was transferred into a fresh 96-well reaction plate. The total volume in each well of the fresh

reaction plate was brought to 50 µl with the addition of 5 µl of the RT reaction product from the previous step. The plate was then sealed and mixed using a plate shaker, followed by centrifugation. The plate was then placed in the QuantStudio™ 7 Flex for incubation which was programmed to have the following cyclic conditions: 5 minutes of enzyme activation (1 cycle) at 95°C for; 14 denaturation cycles at 95°C for 3 seconds; 14 cycles of annealing/ extension at 60°C for 30 seconds; a stop reaction (1 cycle) at 99°C for 10 minutes; and an infinite hold (1 cycle) at 4°C. Once this process was complete and cDNA synthesis was achieved, the samples were stored at −20°C until they were required for quantitative PCR (qPCR) analysis.

3.3.16 Real-time qPCR and miRNA quantification

The target miRNAs present in each of the samples were quantified using Real-time qPCR. Pre-designed primers for each of the miRNAs investigated were used according to the TaqMan™ miRNA Assays and protocol (Applied Biosystems, 2015) so that the miRNA could be quantified.

3.3.17 Preparation of reagents

Upon successful completion of cDNA synthesis, the samples were removed from storage at −20°C and thawed on ice. To ensure optimum quantitative analysis, the original cDNA samples were vortexed, and dilutions of 10^{-1} were made as per manufacturer instructions (Applied Biosystems, 2015).

3.3.18 qPCR reaction plate

Following the removal of the TaqMan™ Advanced miRNA Assays from the −20°C storage, it was thawed on ice. Once thawed, the vials were vortexed and centrifuged. The TaqMan™ Fast Advanced Master Mix was removed from the 4°C storage, and gently inverted to mix. A fresh 1.5 ml Eppendorf tube was used to prepare qPCR Reaction Mix was prepared in a fresh 1.5 ml Eppendorf tube using the steps below:

Table 3.6: qPCR reaction mix preparation

Component	1 Sample	96 Samples
TaqMan™ Fast Advanced Master Mix (2X)	10 µL	960 µL
TaqMan™ Advanced miRNA Assay (20X)	1 µL	96 µL
DNase/Rnase-free water	4 µL	384 µL
Total PCR Reaction Mix volume	15 µL	1440 µL

(Indicated volumes include the 10% extra volume)

One position per reaction plate was reserved for an internal control (miR-16-5p). A separate reaction mix was used to prepare the control however, the same procedure and volumes as was used to prepare the TaqMan™ Advanced miRNA Assays, were applied. The use of an internal control or reference genes is advised in gene expression studies, as it serves to normalize or control for quantification errors (Jian et al., 2008). The expression level of reference genes is presumed to remain constant at various tissue levels, irrespective of what the study treatments and conditions are. In this way, the Internal control ensures that the qPCR results are reliable (Stephens et al., 2011).

The qPCR and the internal control reaction mix was vortexed and centrifuged after being prepared. All wells except well H11 on the MicroAmp™ Optical 96-Well Reaction Plate was used to add 15µl of qPCR reaction mix. The well H11, had 15µl of internal control reaction mix was added to it. Each diluted cDNA sample was assigned a well, and 5 µl of each sample was added to their respective wells. The sample assigned to well H10 also served as a template in well H11, the position of the internal control. Well H12 had the negative template control (NTC) assigned to it to where instead of 5 µl of cDNA template added to it, 5µl of dNase/rNase-free water was used as a replacement. The reaction plate was then sealed, vortexed and centrifuged. Upon completion of the qPCR run, the NTC was assessed. An “undetermined” result flagged by the machine, indicated the absence of a DNA and thus no amplification occurred. This meant that there has been no cross contamination of DNA during the preparation of the reaction plate. Should DNA be present, amplification of the well would be visible upon analysis, which would produce a cycle threshold (Ct) value in the NTC well. This would happen when a possible cross-contamination occurred. The results produced from all the wells were not trustworthy and the preparation of the plate would be repeated.

3.3.19 Programming and Operation of the Real-time PCR instrument

The QuantStudio™ 7 Flex Real-Time PCR System was used to run the RT-PCR. The machine was appropriately programmed for the experiment. A fast-cycling mode with a 0.1 cycle threshold, was selected, the reaction plate was loaded, and the required reaction volume was selected at 20 uL. The table below (table 8) provides additional details regarding the programming of the instrument cycles:

Table 3.7: qPCR cycling conditions

Reaction Step	Temperature (°C)	Time (seconds)	Number of cycles
Enzyme activation	95	20	1
Denaturation	95	30	40
Annealing / Extension	60	30	

3.3.20 Data analysis

Microsoft Excel (Microsoft Office Professional 2010) was used to capture the data generated by the QuantStudio™ 7 Flex Real-Time PCR System. The SPSS v.25 (IBM Corp, 2011) program was used for data analysis. Variables were assessed for normality using Normal QQ plots. Using these results, the general characteristics of the participants were presented as the median (25^{th}-75^{th} percentiles) and/or mean and standard deviation were used to identify and summarise the continuous and ordinal variables. The chi square test, Kruskal-Wallis and analysis of the variance (ANOVA) was used to compare the baseline characteristics among the subgroups of varying glycaemic status. The associations between the miRNAs and the investigated variables, were assessed using Partial Spearman's correlations adjusted for body mass index (BMI), age and sex. The multivariate regression analysis was used to assess the relationship between two continuous variables. Multiple regression analysis was used in instances where there was a relationship between a continuous response variable and many other input variables. Thereafter, multiple correlations were used to measure the strength of these relations. Statistical significance in testing of the hypothesis was represented using a p-value <0.05.

CHAPTER 4: RESULTS

4.1 Descriptive characteristics of study participants

The characteristics of the participants are depicted in table 9. It indicates that of the 1459 participants included in this study, 974 were normoglycaemic with a median age of 46 years (32;57); 206 pre-diabetics with a median age of 55 (48;63); and 279 newly – diagnosed/screen-detected as well as known Type 2 diabetics with a median age of 58 (51;66). In addition, females were over-represented in this population group (73.7% of all subjects) and the majority of participants were either overweight or obese (54.6% of all normoglycaemics, 76.0% of prediabetics and 81.1% of diabetics.

4.1.1 Anthropometric measurements

Weight (kg), height (cm), waist (cm), hip circumference (cm) and body mass index (BMI), were all significantly different between the normal and pre-diabetic group, as well as the normal and the diabetic groups (all, $p < 0.001$). In contrast, no significant differences were observed between the pre-diabetic and diabetic groups. As expected, there was a significant difference in blood pressure between the normal versus pre-diabetic and diabetic group (both, $p < 0.001$). However, no significant differences were observed between those who were pre-diabetic and those who were diabetic ($p \leq 0.760$).

4.1.2 Glucose parameters

As anticipated, there was a significant increase in glycaemic measures such as Fasting Blood Glucose, 2hr Post Prandial Glucose, HbA1c, Fasting Insulin as well as 120min Insulin between normal and prediabetic subjects (all, $p < 0.001$). Similar notable differences were demonstrated between the pre-diabetic subgroup and the diabetic group for all glycaemic parameters ($p < 0.001$), with the exception of Fasting Insulin which did not show any noteworthy differences, $p = 0.149$.

4.1.3 Lipid profile

Lipid markers such as: Total Cholesterol, Triglycerides and LDL-C exhibited marked differences between both the prediabetic and diabetic groups, compared to the normoglycaemic group ($p \leq 0.001$), except for HDL-C, which was only significantly different between Normal versus Diabetes ($p = 0.006$). Furthermore, of all markers of lipidaemia, only Triglycerides demonstrated observable differences between prediabetic and diabetic participants, with $p = 0.002$.

4.1.4 Inflammatory markers

In line with expectations, the measurement of the inflammatory marker C-reactive protein (mg/L) increased with worsening diabetic status however, clinical significance was only observed in the normal versus diabetes as well as the normal versus pre-diabetes subgroups (both, $p \leq 0.001$), with no notable differences observed between pre-diabetes and Diabetes. No clinical significance was observed in the pre-diabetes versus diabetes subgroup, where $p = 0.224$. A similar trend was seen with serum gamma-glutamyltransferase (Gamma GT-S), another common marker of inflammation, with $p < 0.001$ for both normal versus pre-diabetes and normal versus diabetes. Also, the renal function marker, serum creatinine (Creatinine-S), only showed significance between prediabetics and diabetics ($p = 0.025$). Similarly, the percentage of Mets individuals characterized with increased risk of cardiometabolic disease, where clinical significance was observed between the normal versus diabetes and pre-diabetes versus diabetes (both, $p \leq 0.001$).

Table 4.1: General characteristics of participants

	Normal (N=974) Median (25th;75th percentiles)	Pre-diabetes (N=206) Median (25th;75th percentiles)	Diabetes* (N=279) Median (25th;75th percentiles)	P-value: Normal vs Pre-diabetes	P-value: Normal vs Diabetes	P-value: Pre-diabetes vs Diabetes
miR-486-5p $2^{-\Delta CT}$	3.454 (1.620; 6.651)	3.010 (1.320; 5.685)	6.928 (2.565; 15.459)	0.038	<0.001	<0.001
miR- novel-chr1_40444 $2^{-\Delta CT}$	0.061 (0.024; 0.125)	0.048 (0.023; 0.106)	0.135 (0.052; 0.285)	0.061	<0.001	<0.001
Sex				0.003	0.003	0.783
Female, %(N)	70.5(687)	80.6(166)	79.6(222)			
Male, %(N)	29.5(287)	19.4(40)	20.4(57)			
Age (Years)	46 (32; 57)	55 (48; 63)	58 (51; 66)	<0.001	<0.001	0.011
Weight (kg)	66.4 (56.3; 80.8)	74.9 (64.1; 90.4)	76.3 (65.3; 88)	<0.001	<0.001	0.617
Height (cm)	160 (155; 166.5)	157 (153.5; 162.3)	158 (153; 164)	<0.001	<0.001	0.430
Waist (cm)	85.5 (74.5; 99)	96.5 (86.1; 109.7)	98.5 (90; 108.9)	<0.001	<0.001	0.135
Hip (cm)	98.5 (88.9; 110.5)	105.8 (95.4; 119.2)	105.5 (97.5; 116.7)	<0.001	<0.001	0.820
Body Mass Index	25.8 (21.3; 31.8)	30.7 (25.4; 36)	30.5 (26.6; 35.4)	<0.001	<0.001	0.979
Body Mass Index				<0.001	<0.001	<0.001
Normal, %(N)	45.4(439)	24(49)	18.9(52)			
Overweight, %(N)	22.2(215)	21.6(44)	29.5(81)			
Obese, %(N)	32.4(314)	54.4(111)	51.6(142)			
Systolic Blood Pressure (mmHg)	121 (107; 137)	130 (115.8; 152)	135 (118; 152)	<0.001	<0.001	0.185
Diastolic Blood Pressure (mmHg)	80 (71; 89)	85 (76; 94)	83 (77; 94)	<0.001	<0.001	0.760
Glucose Fasting Blood (mmol/L)	4.7 (4.4; 5.1)	5.3 (4.9; 5.8)	8.1 (6.1; 11.8)	<0.001	<0.001	<0.001
Glucose 2 HRs Post Prandial (mmol/L)	5.4 (4.5; 6.3)	8.6 (8; 9.6)	12.9 (11.4; 16.8)	<0.001	<0.001	<0.001
HbA1c (%)	5.6 (5.3; 5.9)	6 (5.6; 6.3)	7.7 (6.5; 10.1)	<0.001	<0.001	<0.001
Insulin Fasting (mIU/L)	5.8 (3.7; 8.9)	8.8 (5.3; 13.9)	9.3 (5.5; 15.6)	<0.001	<0.001	0.149
Insulin 120 Minutes (mIU/L)	30.5 (15.9; 53.6)	71.9 (42.8; 113.2)	51.2 (29.5; 82.5)	<0.001	<0.001	<0.001
Total Cholesterol (mmol/L)	4.9 (4.2; 5.7)	5.3 (4.7; 6)	5.3 (4.6; 6.3)	<0.001	<0.001	0.657
LDL-C (mmol/L)	3 (2.4; 3.6)	3.2 (2.7; 3.8)	3.2 (2.6; 4)	<0.001	0.001	0.955
HDL-C(mmol/L)	1.3 (1.1; 1.6)	1.3 (1.1; 1.5)	1.2 (1.1; 1.5)	0.548	0.006	0.125
Triglycerides (mmol/L)	1.1 (0.8; 1.5)	1.4 (1; 1.8)	1.6 (1.1; 2.2)	<0.001	<0.001	0.002
Cotinine Serum (ng/mL)	121 (10; 286.5)	10 (10; 272.3)	10 (10; 160)	0.075	<0.001	<0.001
us-CRP (mg/L)	3.3 (1.3; 7.7)	5.1 (2.2; 11)	5.3 (2.5; 11)	<0.001	<0.001	0.224
Gamma GT-S (IU/L)	27 (19; 42)	31 (22; 53.3)	36 (22; 67)	<0.001	<0.001	0.145
Creatinine-S (umol/L)	59 (52; 68.5)	57 (50; 68.3)	60.5 (52; 75)	0.215	0.056	0.025
MetS JIS criteria				<0.001	<0.001	<0.001
No, %(N)	68.7(663)	39.5(81)	22.1(61)			
Yes, %(N)	31.3(302)	60.5(124)	77.9(215)			
Alcohol use				0.085	<0.001	<0.001
Non-drinker, %(N)	55.2(535)	63.5(129)	70.7(195)			
Past drinker, %(N)	12.2(118)	10.8(22)	16.7(46)			
Current drinker, %(N)	32.6(316)	25.6(52)	12.7(35)			
Tobacco use				0.011	<0.001	<0.001
Non-smoker, %(N)	41.7(389)	51.5(105)	69(185)			
Current smoker, %(N)	58.3(544)	48.5(99)	31(83)			

Characteristics *Diabetes: Screened and known diabetes *Median (25th, 75th percentile); N, normal glucose tolerance, HDL, high-density lipoprotein, LDL, low-density lipoprotein, usCRP, ultrasensitive C-reactive protein, Gamma GT, Gamma-Glutamyltransferase, metS JIS criteria, Metabolic Syndrome criteria

4.2 The relative expression of miRNA

Both miRNAs were normalized to an endogenous control, miR-16-5p. As illustrated in Figure 4.1, no significance was demonstrated in the expression of miR-16-5p between all the subgroups (all, $p > 0.05$), thus justifying the use of the reference gene as a control.

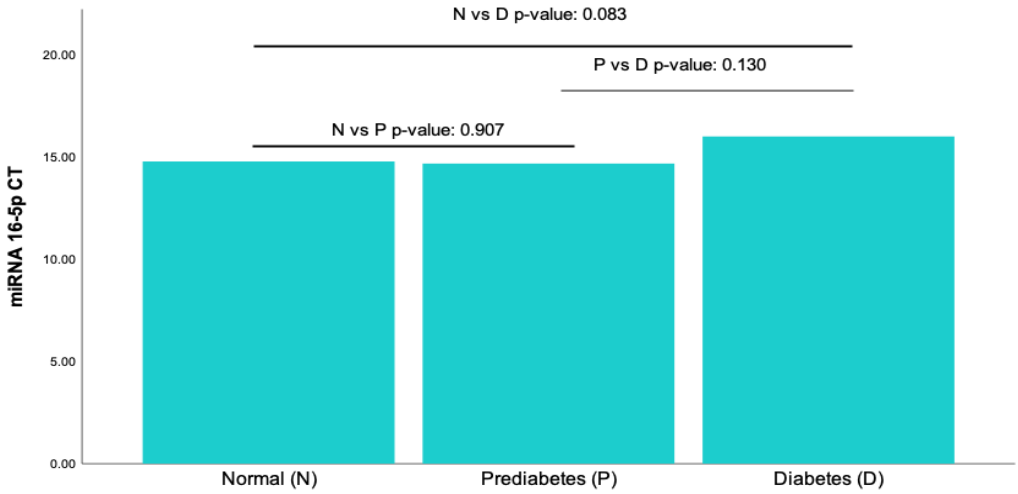

Figure 4.1: Endogenous control- All miRNA were normalized to miR-16-5p. All data is demonstrated as mean ± standard deviation (SD)

4.2.1 Relative expression of hsa-miR-486-5p and hsa-miR-novel-chr1_40444

Due to the largely skewed data with multiple outliers, as illustrated by figure 4.2a and 4.3a, outliers were removed. As such, figures 4.2bb and 4.3b illustrate the corrected relative expression of the investigated miRNA (hsa-miR-486-5p and hsa-miR-novel-chr1_40444) according to glycaemic status. Significantly higher expression levels of hsa-miR486-5p (figure 4.3b) was observed in the diabetes group in comparison to the pre-diabetes and normal ($p < 0.001$). When comparing normotolerants to pre-diabetics, the expression of hsa-miR-486-5p was higher in normotolerants however, this was not significant ($p = 0.038$).

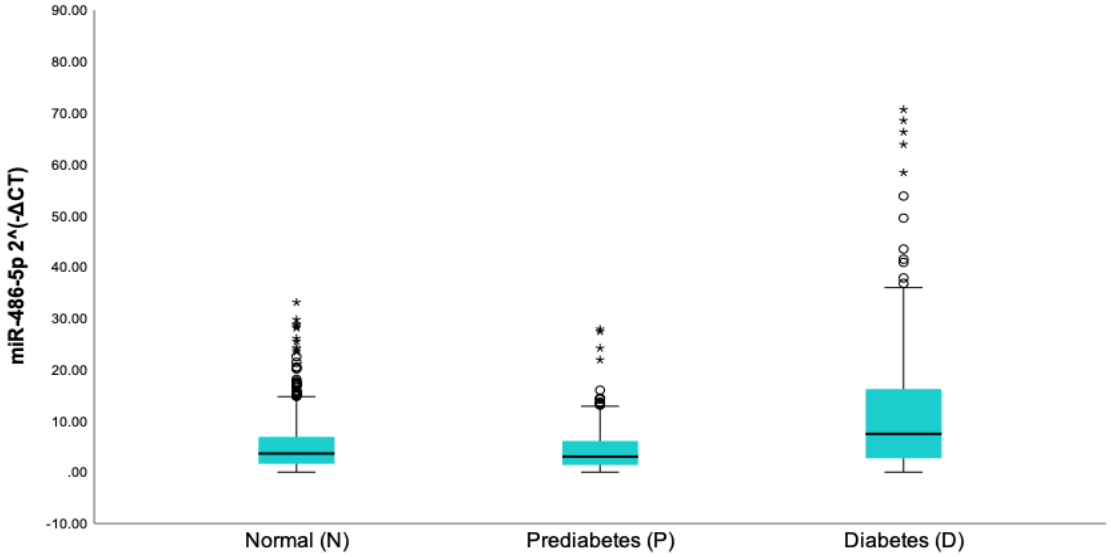

Figure 4.2a: The relative expression of hsa-miR-486-5p vs Diabetes status (all data), containing extreme outliers. [Normal vs PreDM, $p = 0.056$; Normal vs DM, $p < 0.001$; PreDM vs DM, $p < 0.001$].

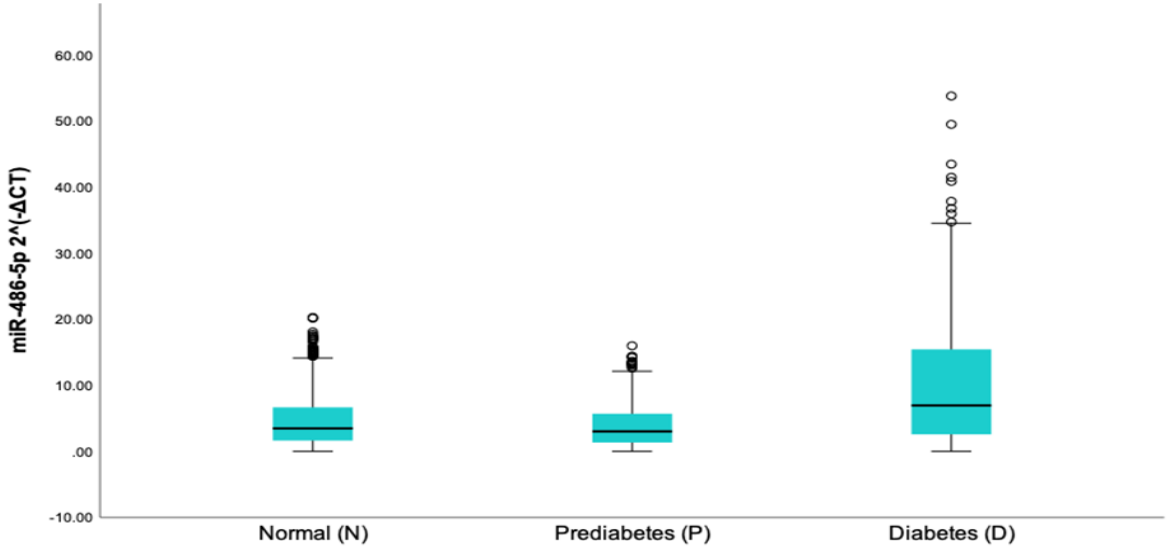

Figure 4.2b: The relative expression of hsa-miR-486-5p vs Diabetes status (Extreme outliers removed*) [Normal vs PreDM, $p = 0.038$; Normal vs DM, $p < 0.001$; PreDM vs DM, $p < 0.001$].

Hsa-miR-novel-chr1_40444 behaved similarly to hsa-miR-486-5p as significantly higher levels of expression was observed in diabetics compared to pre-diabetics ($p < 0.001$). Additionally, although the expression was higher in normoglycaemics than in pre-diabetics, this was not significant, as depicted by Figure 4.2b where $p = 0.061$. Furthermore, minimally increased expression levels of hsa-miR-novel-chr1_40444 were seen in the normal group compared to pre-diabetes, however this was not significant ($p = 0.123$).

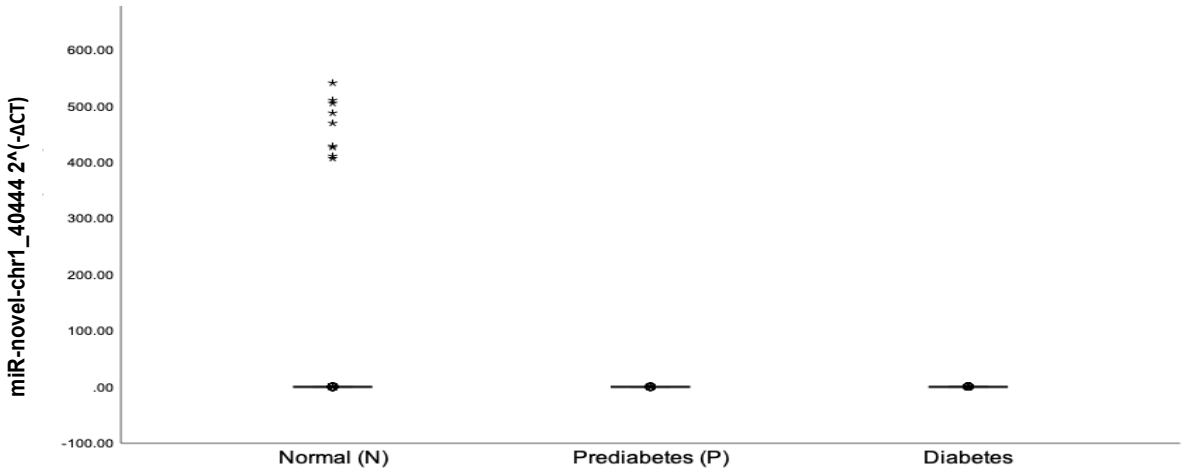

Figure 4.3a: The relative expression of hsa-miR-novel-chr1_40444 vs Diabetes status (all data) [Normal vs PreDM, *p* = 0.082; Normal vs DM, *p* < 0.001; PreDM vs DM, *p* < 0.001]. Extreme outliers are visible.

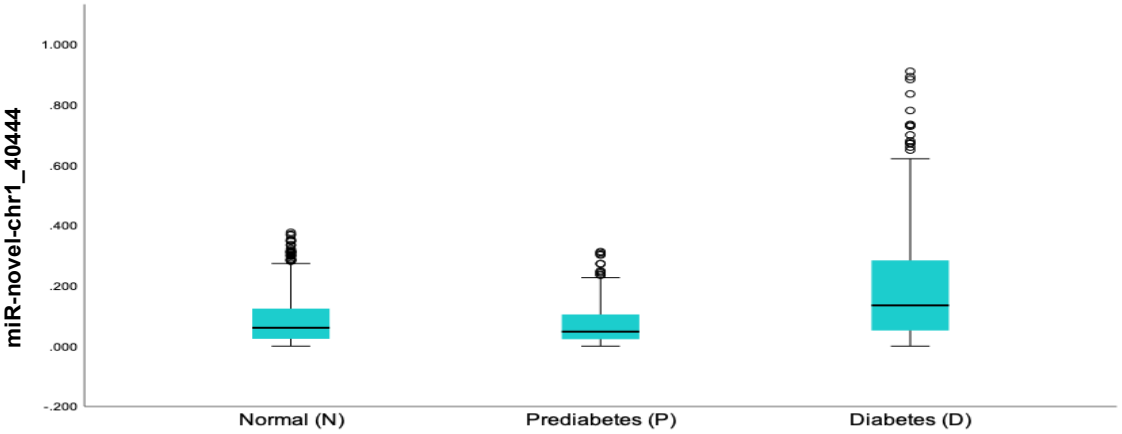

Figure 4.3b: The expression of hsa-miR-novel-chr1_40444 vs Diabetes status (Extreme outliers removed*) [Normal vs PreDM, *p* = 0.061; Normal vs DM, *p* < 0.001; PreDM vs DM, *p* < 0.001]

4.3 Partial Spearman's correlation between hsa-miR-486-5p and hsa-miR-novel-chr1_40444 and participants characteristics in accordance with glycaemic status

Partial Spearman's correlation coefficients adjusted for BMI, were performed to assess the relationship between hsa-miR-486-5p as well as hsa-miR-novel-chr1_40444 and the clinical variables as displayed in tables 10 and 11. Both miRNAs were significantly and positively correlated across all subgroups (both, $r \geq 0.743$, $p < 0.001$).

4.3.1 Correlation between hsa-miR-486-5p and the clinical variables

Table 4.2 shows that when all participants were included, hsa-miR-486-5p had a positive correlation with weight (r = 0.057, p = 0.032), however, no significant correlation was detected when each individual glycaemic group was analysed. A similar pattern was observed with SBP (r = 0.087, p < 0.001). The examination of glycaemic indices revealed that the expression of hsa-miR-486-5p was positively associated with glucose fasting blood (mmol/L) (r = 0.121, p < 0.001), whereas in contrast, a negative correlation was observed with the 2 hours postprandial glucose in the pre-diabetes group (r = -0.277, p < 0.001). Fasting insulin (mIU/L) (r = 0.076, p = 0.005) and HbA1c (r = 0.112, p < 0.001) were both positively correlated when all participants were analysed, whereas no significant correlations were observed when insulin (mIU/L) at 120 minutes was analysed. In addition, a positive correlation with triglycerides was observed when all participants were included (r = 0.071, p = 0.008) (Table 10).

Table 4.2: Partial Spearman's correlation coefficients for hsa-miR-486-5p

	All		Normal		Pre-diabetes		Diabetes	
	r	p-value	r	p-value	r	p-value	r	p-value
miR-486-5p 2^(-ΔCT)	1.000		1.000		1.000		1.000	
miR- novel-chr1_40444 2^(-ΔCT)	0.769	<0.001	0.743	<0.001	0.749	<0.001	0.779	<0.001
Age (Years)	0.024	0.370	-0.051	0.112	0.090	0.204	-0.111	0.069
Weight (kg)	0.057	0.032	0.052	0.109	0.040	0.571	-0.042	0.492
Height (cm)	0.012	0.657	0.036	0.271	0.025	0.730	0.010	0.868
Body Mass Index	0.044	0.096	0.030	0.348	0.025	0.728	-0.066	0.278
Waist (cm)	0.019	0.480	-0.025	0.442	-0.018	0.796	-0.078	0.198
Hip (cm)	-0.006	0.834	-0.038	0.242	-0.007	0.917	-0.065	0.287
Systolic Blood Pressure (mmHg)	0.087	<0.001	0.043	0.185	0.056	0.432	0.058	0.339
Diastolic Blood Pressure (mmHg)	0.014	0.587	0.018	0.568	-0.078	0.268	-0.083	0.172
Glucose Fasting Blood (mmol/L)	0.121	<0.001	-0.026	0.421	0.055	0.435	0.014	0.817
Glucose 2 HRs Post Prandial (mmol/L)	-0.002	0.935	-0.035	0.284	-0.277	<0.001	-0.061	0.562
HbA1c (%)	0.112	<0.001	-0.037	0.251	-0.041	0.559	0.036	0.551
Insulin Fasting (mIU/L)	0.076	0.005	0.069	0.036	-0.063	0.382	0.000	0.995
Insulin 120 Minutes (mIU/L)	-0.012	0.680	0.000	0.997	-0.114	0.112	0.123	0.254
Total Cholesterol (mmol/L)	0.013	0.619	-0.045	0.162	0.106	0.134	0.000	0.997
LDL-C (mmol/L)	0.000	0.993	-0.070	0.033	0.138	0.051	0.053	0.394
HDL-C(mmol/L)	0.028	0.290	0.054	0.095	0.058	0.411	0.011	0.853
Triglycerides (mmol/L)	0.071	0.008	0.028	0.391	0.021	0.763	-0.064	0.295
Cotinine Serum (ng/mL)	-0.046	0.087	-0.019	0.568	0.004	0.955	0.045	0.469
us-CRP (mg/L)	0.009	0.736	0.000	0.989	-0.113	0.111	-0.043	0.481
Gamma GT-S (IU/L)	-0.007	0.786	0.007	0.818	-0.217	0.002	-0.072	0.234
Creatinine-S (umol/L)	-0.035	0.183	-0.036	0.264	-0.122	0.084	-0.025	0.677

4.3.2 Correlation between hsa-miR-novel-chr1_40444 and the clinical variables

Hsa-miR-novel-chr1_40444 was positively associated with weight (r = 0.06, *p* = 0.018) and BMI (r = 0.055, *p* = 0.039) when all participants were included in the analysis. Similarly, highly significant positive correlations with HbA1c (r = 0,086, *p* = 0.001), fasting glucose (r = 0.100, *p* < 0.001), and fasting insulin (r = 0.098, *p* < 0.001) were observed. In contrast, when the Normal group was examined a significantly negative correlation with HbA1c was observed (r = -0.076, *p* = 0.020). The same observations were made when the 2 hours post prandial glucose (mmol/L) was analysed in the pre-diabetes group (r = -0.142, *p* = 0.045).

LDL-Cholesterol demonstrated a significantly positive correlation with the miRNA in diabetes (r = 0.145, *p* = 0.021) but when analysing the Normal participants, a negative correlation was observed (r = -0.142, *p* = 0.045). HDL-Cholesterol was negatively correlated with the miRNA in the Normal group (r = -0.073, *p* = 0.026) while no association could be detected with triglycerides (Table 11).

Table 4.3: Partial Spearman's correlation coefficients for miRNA hsa-miR- novel-chr1_40444

	All		Normal		Pre-diabetes		Diabetes	
	r	p-value	r	p-value	r	p-value	r	p-value
miR-486-5p 2^(-ΔCT)	0.769	<0.001	0.743	<0.001	0.749	<0.001	0.779	<0.001
miR- novel-chr1_40444 2^(-ΔCT)	1.000	.	1.000	.	1.000	.	1.000	.
Age (Years)	0.013	0.617	-0.063	0.053	0.021	0.764	-0.078	0.206
Weight (kg)	0.063	0.018	0.024	0.460	0.124	0.080	0.025	0.682
Height (cm)	0.001	0.983	0.004	0.897	-0.001	0.993	0.064	0.301
Body Mass Index	0.055	0.039	0.019	0.551	0.113	0.111	-0.021	0.733
Waist (cm)	0.030	0.253	-0.042	0.196	0.084	0.234	-0.014	0.822
Hip (cm)	0.022	0.405	-0.032	0.332	0.083	0.243	0.023	0.707
Systolic Blood Pressure (mmHg)	0.033	0.207	-0.031	0.345	-0.015	0.832	0.048	0.433
Diastolic Blood Pressure (mmHg)	-0.018	0.489	-0.056	0.087	-0.040	0.575	-0.048	0.434
Glucose Fasting Blood (mmol/L)	0.100	<0.001	-0.053	0.104	-0.076	0.287	0.001	0.993
Glucose 2 HRs Post Prandial (mmol/L)	0.000	0.995	-0.041	0.207	-0.142	0.045	-0.036	0.739
HbA1c (%)	0.086	0.001	-0.076	0.020	-0.114	0.108	-0.026	0.677
Insulin Fasting (mIU/L)	0.098	<0.001	0.051	0.125	0.085	0.241	0.034	0.591
Insulin 120 Minutes (mIU/L)	-0.012	0.675	-0.028	0.391	0.042	0.563	0.141	0.200
Total Cholesterol (mmol/L)	0.049	0.066	-0.025	0.451	0.077	0.276	0.118	0.054
LDL-C (mmol/L)	0.012	0.647	-0.073	0.026	0.073	0.308	0.145	0.021
HDL-C(mmol/L)	0.033	0.211	0.067	0.040	0.032	0.648	0.042	0.496
Triglycerides (mmol/L)	0.124	<0.001	0.070	0.032	0.117	0.100	0.004	0.953
Cotinine Serum (ng/mL)	-0.054	0.047	-0.020	0.537	-0.017	0.807	0.020	0.746
us-CRP (mg/L)	0.008	0.769	-0.028	0.385	-0.032	0.653	-0.052	0.396
Gamma GT-S (IU/L)	0.018	0.488	0.015	0.644	-0.113	0.110	-0.041	0.500
Creatinine-S (umol/L)	-0.025	0.358	-0.029	0.374	-0.104	0.142	-0.021	0.729

4.4 Multivariate regression analysis

To further assess the associations between the expression patterns of hsa-miR-486-5p with pre-diabetes and Type 2 diabetes, multivariate regression analysis was performed (Table 4.4). Using the reference category as "Normal", the expression of hsa-miR-486-5p was significantly associated with pre-diabetes in the crude or unadjusted model (model 1). Upon further adjustment (models 2 – 4), significance was not retained between the expression of the miRNA and pre-diabetes. Interestingly, when assessing the links between the miRNA and diabetes (screen detected and known diabetics), associations were observed with hsa-miR-486-5p independent of confounding variables (across the crude model and all the adjusted models), all $p < 0.001$. Furthermore, when investigating the associations between hsa-miR-486-5p and diabetes, using "pre-diabetes" as the reference group, similar significant associations were observed with the expression of miRNA in the crude model (model 1) as well as all the adjusted models, further highlighting the independent association between the miRNA and diabetes (all $p < 0.001$).

Table 4.4: Multivariate regression analysis of log hsa-miR-486-5p for the presence of pre-diabetes or diabetes.

log miRNA 486-5p	Odds ratio	95% Confidence interval		p-value
		Lower	Upper	
Pre-diabetes*				
Model 1	0.782	0.628	0.972	0.027
Model 2	0.817	0.651	1.024	0.079
Model 3	0.807	0.647	1.007	0.058
Model 4	0.835	0.65	1.073	0.160
Diabetes*				
Model 1	4.185	2.987	5.863	<0.001
Model 2	4.177	2.957	5.898	<0.001
Model 3	4.462	3.133	6.354	<0.001
Model 4	3.476	2.057	5.875	<0.001
Diabetes#				
Model 1	5.355	3.654	7.847	<0.001
Model 2	5.115	3.486	7.504	<0.001
Model 3	5.526	3.722	8.204	<0.001
Model 4	4.162	2.459	7.043	<0.001

***Reference category: Normal; #Reference category: pre-diabetes; Model 1: Crude, Model 2: Age and sex, Model 3: LDL-C, HDL-C,Gamma GT, Creatinine, Model 4: Weight, BMI, Systolic blood pressure, Fasting blood glucose, HbA1c, Insulin fasting, Triglycerides, Cotinine.**

When assessing the associations depicted in table 4.4 between the expression patterns of hsa-miR-novel-chr1_40444 in pre-diabetes using “Normal” as the reference category, no significant associations were observed between the expression of hsa-miR-novel-chr1_40444 and pre-diabetes (see Table 4.5) across all variables (crude and adjusted models). These significant relations were retained throughout all the adjusted models (2 – 4), all $p < 0.001$). However, when assessing the associations between hsa-miR-novel-chr1_40444 and diabetes, using “pre-diabetes” as the reference category, significant associations were observed across all models (crude and adjusted), all $p < 0.001$.

Table 4.5: Multivariate regression analysis of log miRNA hsa-miR-novel-chr1_40444 for the presence of pre-diabetes and diabetes.

log miR-novel-chr1_40444	Odds ratio	95% Confidence interval		p-value
		Lower	Upper	
Pre-diabetes*				
Model 1	0.849	0.646	1.116	0.240
Model 2	0.890	0.673	1.179	0.418
Model 3	0.900	0.674	1.202	0.476
Model 4	0.937	0.681	1.288	0.687
Diabetes*				
Model 1	4.305	3.107	5.965	<0.001
Model 2	4.223	3.02	5.905	<0.001
Model 3	4.191	2.949	5.956	<0.001
Model 4	2.975	1.77	5.001	<0.001
Diabetes#				
Model 1	5.071	3.416	7.530	<0.001
Model 2	4.742	3.202	7.023	<0.001
Model 3	4.655	3.106	6.979	<0.001
Model 4	3.177	1.876	5.381	<0.001

***Reference category: Normal; #Reference category: pre-diabetes; Model 1: Crude; Model 2: Age and sex; Model 3: Age, Weight, Diastolic blood pressure, LDL-C, HDL-C and Triglycerides; Model 4: Weight, BMI, Fasting blood glucose, HbA1c, Insulin Fasting, Total Cholesterol, Triglycerides, Cotinine**

Chapter 5: Discussion

The purpose of this study was to validate hsa-miR-486-5p and hsa-miR-novel-chr1_40444 as being potential specific biomarkers for pre-diabetes or for T2DM. The key findings are: (i) The expression of both miRNAs were significantly higher in the diabetes group compared to the pre-diabetes and normotolerant groups; (ii) The increased expression was independently associated with the presence of T2DM, even after multiple regression analysis adjustments for age, sex, weight, diastolic blood pressure, LDL-C, HDL-C, triglycerides, BMI, fasting blood glucose, HbA1c, fasting insulin, total cholesterol and Cotinine. (iii) Both miRNAs were however not associated with pre-diabetes, even after adjustments. (iiii) Both miRNAs demonstrated significant positive correlations with each other and with weight, BMI, fasting insulin and triglycerides. Thus, the findings of this study suggests that both hsa-miR-486-5p and hsa-miR-novel-chr1_40444 are involved in the pathogenesis of T2DM and therefore could be used to predict risk of diabetes.

Hsa-miR-486-5p is abundantly found in skeletal muscle, an important site for regulating glucose metabolism (Jensen et al., 2011). Glucose metabolism is modulated by Insulin via the phosphatidyl inositol-3-kinase (PI3K)/Akt signaling pathway (Huang et al., 2018). This pathway contains the negative regulators, phosphatase and tensin homolog (PTEN) as well as Forkhead box O1 (FOXO1) which are downstream targets of hsa-486-5p in pancreatic β-cells (Small et al., 2010). Although PTEN is traditionally known as a tumour suppressor (Lee et al., 2018), the altered expression of PTEN in T2DM, results in defective insulin signalling and a greater chance of insulin resistance (Li et al., 2020). In skeletal muscle, PTEN regulates glucose homeostasis by modulating the expression of the glucose transporter type 4 (GLUT4) (Simpson et al., 2001). The inhibition of PTEN, results in the activation of Akt/P13 signalling pathway, which stimulates GLUT4 activity and promotes glucose uptake (Ramachandran and Saravanan, 2015). On the other hand, FOXO1 play vital roles in the differentiation, growth, and homeostasis of skeletal muscle (Ahmad et al., 2020). The activation of Akt/P13 signalling in muscle tissue results in the inhibition of FOXO1. This promotes protein breakdown and muscle growth (Margolis et al., 2018). The Impairment of glucose metabolism contributes to the pathogenesis of T2DM (Teng and Huang, 2019). Tian et al. (2018), reported on the inhibitory effect the overexpression of hsa-miR-486-5p had on PTEN and FOXO1 in β-cells. The result was an increased insulin secretion, increased β-cell proliferation and the inhibition of cell apoptosis (Tian et al., 2018). miRNA targets allow us to understand how miRNA play a part in T2DM development (Karolina et al., 2011). Thus, based on the important regulatory role hsa-miR-486-5p has on insulin release and glucose homeostasis, its upregulation as seen in our study suggests that its participation in T2DM pathogenesis can serve as a risk factor to assist in diagnosing T2DM.

Although basal miRNA are expressed at low levels, pathological stress results in their upregulation (Thum and Condorelli, 2015). Kong and colleagues demonstrate this in a study which investigates the expression of 7 diabetes related miRNAs. The participants were grouped into newly diagnosed T2DM, pre-diabetics and normotolerants who were susceptible to T2DM. They found the expression of miR30d-5p gradually increasing with worsening glycaemic states (Kong et al., 2011). Although the miRNA targets differed between Kong and co-workers' study and our study, the observed trend was consistent in that in the current study, the increased expression of both hsa-miR-486-5p and hsa-miR-novel-chr1_40444 was directly proportional to worsening diabetes status. In addition, the involvement of both miRNAs in T2DM pathogenesis was later confirmed by the multivariate regression analysis which revealed independent associations with T2DM and not pre-diabetes. These findings are further supported by Kim and colleagues who through real-time PCR, found that the exposure to elevated glucose concentrations, increased the expression of hsa-miR-486-5p (Kim et al., 2012). Lastly, Karolina et al. used microarray and stem-loop real time RT-PCR methods to explore the miRNA expression patterns in T2DM and impaired fasting glucose (IFG) participants. Their results revealed the upregulation of a novel miRNA (miR-144) in T2DM in addition to the level of expression increasing with worsening glycaemic status. They concluded that the circulating miRNA could prove useful in indicating the development of the disease (Karolina et al., 2011). Overall, the abovementioned research endeavours aligned with our own findings, suggesting that both hsa-miR-486-5p and hsa-miR-novel-chr1_40444 are involved in the pathogenesis of T2DM.

Almost all T2DM patients develop from pre-diabetes however, not all pre-diabetic patients progress to diabetes. Preventing T2DM or delaying its progression can be achieved by implementing interventions to one's lifestyle such as adopting healthier diets and physical exercise, as well as pharmacological intervention (Hostalek, 2019; Sidorkiewicz et al., 2020) such as metformin (Baker et al., 2021). Although there was a difference in miRNA expression levels between the normotolerant and pre-diabetic group, the difference was minimal. Only hsa-miR-novel-chr1_40444 displayed a statistically significant difference in expression between the two groups. It is mainly in T2DM as compared to pre-diabetes, that the altered expression of miRNA occurs (Kong et al., 2011). Furthermore, although miRNAs are important regulators of gene expression (Rani and Sengar, 2022), during the pathogenesis of T2DM in "at risk" individuals, there is no significant change in the expression patterns of hsa-miR-novel-chr1_40444 in the pre-diabetic stage, which possibly contributes to the ability to revert from pre-diabetes to normotolerant.

Obesity increases the risk of insulin resistance and T2DM development (Wu and Ballantyne, 2020). In addition, the complications caused by obesity, negatively affects all bodily organs and contribute to a reduced quality of life (Katz et al., 2000). hsa-miR-486-5p has been associated with obesity (Cui et al., 2018; Duggineni, 2013; Prats-Puig et al., 2013) as was shown in a study by Prats-Pruig and colleagues where increased circulation of hsa-miR-486-5p was observed in obese children (Prats-Puig et al., 2013), while another study concluded that the overexpression of circulating hsa-miR-486 can be used as a prognostic marker to screen children who are obese and at risk of developing diabetes in adulthood (Cui et al., 2018). In our study the expression of both hsa-miR-novel-chr1_40444 and hsa-miR-486-5p was upregulated in the T2DM compared to the normotolerant group and significant correlations with weight, BMI, fasting insulin and triglycerides in the "all" group was evident. These correlations suggest that the expression of these miRNAs is associated with obesity (BMI) and insulin resistance (fasting insulin) (Duggineni, 2013). Additional positive correlations between hsa-miR-novel-chr1_40444 and hsa-miR-486-5p and fasting plasma glucose proves that their upregulation contributes to insulin resistance and therefore they can be used as a prognostic tool for T2DM progression. Similar findings were observed in a study by Zaki et al who found the expression of miR-497 to be higher in their Egyptian diabetic subjects than normotolerant control subjects and demonstrated significant positive correlations with fasting plasma glucose (FPG) (Bakr Zaki et al., 2019).

Despite the results of our study correlating with other previous studies (Karolina et al., 2011; Wang et al., 2014) which identified the expression of hsa-miR-486-5p in the pathogenesis of T2DM, we have found contradictions in other studies (Huang et al., 2018, 2015; Liu et al., 2016). For example a study by Duggineni suggested that the dysregulation of miRNAs during obesity may play a fundamental role in regulating insulin resistance and inflammation (Duggineni, 2013). Another study by Regmi et al. showed the downregulation of hsa-miR-486-5p when 4 serum miRNAs were measured in diabetic kidney disease (DKD) patients with T2DM compared to healthy controls (Regmi et al., 2019). This is in contrast to the upregulation observed in our study. In addition, a further study by Tian and colleagues explored the expression of miRNAs in the serum of participants with T2DM. Not only did they demonstrate downregulation of hsa-486-5p in T2DM, they also found negative correlations with blood glucose levels in T2DM participants (Tian et al., 2018). The lack of consistency of results among studies could be attributed to differences in pre-analytic and analytic procedures as well as differences in the inclusion and exclusion criteria (Greco et al., 2020). Differences in sample types can result in significant inconsistencies as was demonstrated by Mononen et al. They showed how the measurement of miRNA differed in different sample types and concluded that the inconsistent measurements was due to the interfering presence of other components of blood in varying amounts (Mononen et al., 2019).

Inflammation is one of the main contributing factors to the development and progression of T2DM (Oguntibeju, 2019; Rehman and Akash, 2016). In our study, the levels of inflammation (u-CRP) were higher in pre-diabetes and diabetes than normotolerants and ¾ of the participants (table 9) in the pre-diabetes and diabetes group were obese, compared to the normal group. Adipocytes in the subcutaneous adipose tissue have a limited storage capacity (Hedbacker et al., 2020). While the cell expands, it is simultaneously infiltrated by macrophages, and the development of fibrosis and obesity (Martinez-Santibañez and Lumeng, 2014). These changes occurring within the adipocyte leads to an increase in circulating, monocytes, neutrophils and cytokines, which contributes to inflammation (Menendez et al., 2022).

Due to the lack of efficient biomarkers and the resultant inaccuracy of the early diagnosis and treatment of patients (Liu et al., 2021), the continual discovery of novel miRNA in disease make valuable contributions to the miRNA pool. For example, Vijayan et al, made valuable contributions to ischaemic stroke when they identified novel miRNAs (PC-3p-57664, PC-5p-12969) which were upregulated in ischaemic stroke patients as compared to healthy controls(Vijayan et al., 2018). Another study by Liu et al used next generation sequencing and bioinformatic approaches and found the downregulation of novel miRNA, hsa-miR-526b-5p, hsa-miR-6516-5p associated with metabolic syndrome (Liu et al., 2021). Furthermore, the detection and overexpression of hsa-miR-nov7 and hsa-miR-nov3 in breast tissue of breast cancer patients was reported by Poduval et al. Although the expression of the miRNA was low, they were overexpressed and therefore concluded that these miRNA are involved in the development and progression of breast cancer (Poduval et al., 2020). Our findings on hsa-miR-novel-chr1_40444 could therefore be important as it implies that its dysregulation is associated with pathophysiological states and that it may be a useful biomarker in patients with hyperglycaemia.

Chapter 6: Conclusion

Advances in miRNA expression studies have contributed to the escalated growth in identifying the diagnostic and prognostic value of epigenetics as a tool in pathophysiology, especially miRNA in diabetes mellitus. The expression patterns of hsa-miR-486-5p have been broadly discussed in human diseases such as cancer, cardiac disease, sepsis and T2DM however, studies within South Africa are few. To the best of our knowledge, there is no evidence of the involvement of hsa-miR-novel-chr1_40444 in disease, making this study the first of its kind to report on the expression of hsa-miR-novel-chr1_40444 in human disease.

6.1 Strengths and limitations of this study

Our study has provided additional evidence that miRNAs possess significant diagnostic and prognostic value in human disease. Compared to other studies, a strength of this study is its large sample size. However, this study is limited by the study population originating from a particular region and ethnic group, therefore (Matsha et al., 2018), making it a poor representation of South Africa as South Africa is diverse and multi-racial. There is also a difference in the number of male and female participants, with more females participating in the study, which is a common observation in studies of this nature. Another limitation is that we have placed those with known diabetes and those who were screen detected, in the diabetic group. This is due to the sample size per subgroup, which caused skewed data. While both groups have been amalgamated into 1 group, some of them could be on medication which could influence the regulation of the miRNA (Weale et al., 2021).

6.2 Future recommendations

The amalgamation of participants who were screen detected and those with known diabetes, creates an opportunity for future studies which places these groups into their own category to assess what influence the medication has on the expression of the miRNA. An additional opportunity for future studies is to detect which pathways hsa-miR-novel-chr1_40444 is involved in and which proteins it targets as this will provide deeper insight into what impact it has on the insulin signalling pathway or glucose homeostasis (Deng and Guo, 2019). This could be achieved using bioinformatic and enrichment analysis techniques (Ramani et al., 2017) or cell type based enriched miRNA expression analysis (Pomper et al., 2020). Furthermore, the crossectional nature of this study creates an opportunity for future longitudinal studies exploring the changes in miRNA expression over time.

6.3 Conclusion

In conclusion, the results of this present study have validated the involvement of miRNA in T2DM in a South African population. The overexpression of both hsa-miR-486-5p and hsa-

miR-novel-chr1_40444 could be useful biomarkers when used concurrently with other risk factors to identify patients who are at risk of developing T2DM

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Appendices

Appendix A: Ethics approval letter

Cape Peninsula University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)

Registration Number NHREC: REC-230408-014

P.O. Box 1906 □ Bellville 7535 South Africa
Symphony Road Bellville 7535
Tel: +27 21 959 6917
Email: simonsy@cput.ac.za

14 November 2019

REC Approval Reference No: CPUT/HW-REC 2019/H26

Dear Ms Chanelle Schroeder

Re: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC to Ms Chanelle Schroeder for ethical clearance on 14 November 2019. This approval is for research activities related to student research in the Department of Informatics of this Institution.

TITLE: **Investigation of MicroRNAs that can differentiate between pre-diabetes and diabetes**

Supervisor: Prof T Matsha and Prof G Davison

Comment:

Approval will not extend beyond 15 November 2020. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Dr. Navindhra Naidoo

Chairperson – Research Ethics Committee
Faculty of Health and Wellness Sciences

Appendix B: Ethics renewal letter

Cape Peninsula University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HWS-REC)

Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South Africa
Symphony Road Bellville 7535
Tel: +27 21 959 6917
Email: simonsy@cput.ac.za

05 April 2022

REC Approval Reference No: CPUT/HW-REC 2019/H26 (renewal)

Dear Ms Chanelle Schroeder,

Re: APPLICATION TO THE HWS-REC FOR ETHICS CLEARANCE - RENEWAL

Approval was granted by the Health and Wellness Sciences-REC to Ms Chanelle Schroeder for ethical clearance on 14 November 2019. This approval is for research activities related to student research in the Department of Biomedical Sciences at this Institution.

Title: Investigation of MicroRNAs that can differentiate between pre-diabetes and diabetes

Supervisors: Prof T Matsha and Prof G Davison

Comment:
Approval will not extend beyond 06 April 2023. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered. Kind Regards

Mrs Carolynn Lackay
Chairperson – Research Ethics
Committee Faculty of Health and Wellness
Sciences