EXPRESSION OF CIRCULATING MICRORNA'S (MIRNAS) IN BLOOD OF MIXED ANCESTRY SUBJECTS WITH GLUCOSE INTOLERANCE

By

Desiree Lem Mbu

(215125975)

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Supervisor: Prof T. E. Matsha

Co-supervisor: Dr S. Hector

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DECLARATION

I, Desiree Lem Mbu, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed **Date**

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DEDICATION

I dedicate this work to God Almighty, my mum, my dad, my husband, my son, my daughter, my sisters, my brother and my entire family.

ABSTRACT

Background: Early detection of individuals who are at risk of developing Glucose Intolerance would decrease the morbidity and mortality associated with this disease. MicroRNA is one of the most widely studied biomolecules involved in epigenetic mechanisms, hence it offers unique opportunities in this regard. Circulating microRNAs are associated with disease pathogenesis during the asymptomatic stage of disease. This has therefore attracted a lot of attention as a potential biomarker for identifying individuals who have an increased risk of developing Glucose Intolerance. The identification of high risk biomarkers for Glucose Intolerance will go a long way to eliminate the possible complications that arise due to late diagnosis and treatment of Glucose Intolerance. This could ultimately lead to better ways to prevent, manage and control the Glucose Intolerance epidemic that is rampant worldwide. The aim of the study is to investigate expression of circulating microRNA's in blood of mixed ancestry subjects with glucose intolerance.

Methods: A quantitative cross-sectional study design involving 36 individuals [who were age, gender and BMI (Body Mass Index) matched] from a total population of 1989 participants of mixed ancestry descent, residing in Bellville South, South Africa was used. Participants were classified as controls (normoglycemic), pre-diabetic (preDM) and diabetic (DM) (screen detected diabetic) according to WHO criteria of 1998. MicroRNAs were extracted from serum using the Qiagen miRNeasy Serum/Plasma Kit (ThermoFisher). The purified micro RNAs were reverse-transcribed to cDNA (complementary deoxyribonucleic acid) using the Qiagen RT^2 First Strand Kit. Then, using Qiagen miScript SYBR Green PCR kit and miScript miRNA PCR arrays (ThermoFisher), the real time polymerase chain reaction was done to determine the expression profile the circulating micro RNAs present in the serum of the participants.

Results: The 36 participants were evenly divided into 3 groups of 12 participants each as mentioned earlier. There were significant differences between groups in the waist (cm) ($p=0.0415$) and waist/hip ratio ($p=0.0011$) with highest values in the DM group and lowest in the normal group. Clinical parameters varied significantly according to glycemic status. As expected, the FBG (mmol/L) ($p<0.0001$), 2 HRs Post Glucose (mmol/L) ($p<0.0001$), HbA1c (%) ($p=0.0009$), Fasting Insulin (mIU/L) ($p=0.0039$), were all highest in the DM and lowest in the control group. In contrast, the 2 HRs Post Insulin (mIU/L) ($p = 0.0027$) was highest in the preDM group and lowest in the normal group, while the Glucose/Insulin ratio ($p=0.0477$) was highest in the normal group and lowest in the preDM group. Triglycerides (mmol/L) ($p=0.0043$) and Total Chol (mmol/L) ($p=0.0429$) were significantly increased through the three groups, with highest values in the DM group and lowest in the normal group. Furthermore, 12 of the 84 miRNAs studied were expressed through all the 3 groups and they exhibited both inverse and positive correlations between the clinical parameters, especially the glucose parameters

(Fasting blood glucose, 2 hours post glucose, Fasting blood insulin, 2 hours post insulin and Glycated Hemoglobin).

Discussion: The expression of circulating micro RNAs varied amongst the controls, preDM and DM groups. The observation of circulating micro RNAs changes in preDM, the asymptomatic stage of T2DM (Type 2 Diabetes Mellitus), and the identification of more pronounced changes in individuals with diabetes suggests that these epigenetic changes could be involved in the progression of T2DM, and holds potential as biomarkers for T2DM.

Conclusion: Our results add to the growing body of evidence that circulating miRNAs are associated with glucose intolerance and can be detected during pre-DM, the asymptomatic stage of T2DM and in T2DM as well. These findings support the use of miRNAs as biomarkers for early detection of T2DM. To our knowledge, this is the first study to assess circulating miRNAs in serum as biomarkers for glucose intolerance in the mixed ancestry population in South Africa and paves the way for future studies in Africa at large.

KEYWORDS

MicroRNAs, biomarker, epigenetics, early detection, control, pre-diabetes, type 2 diabetes.

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LIST OF ABBREVIATIONS

- 2h-PG 2-hour plasma glucose
- 2h-SI 2-hour Serum Insulin
- ADA American Diabetes Association
- ANOVA Analysis of variance
- Beta cell β-cell
- BMI Body mass index
- cDNA Complementary Deoxyribonucleic acid
- CRP C-reactive protein
- CT Threshold cycles
- ΔCT Delta threshold cycles
- ΔΔCT Delta delta threshold cycles
- CTHKG Threshold cycle of housekeeping genes
- CTGOI Threshold cycle of gene of interest
- DKA Diabetic ketoacidosis
- DM Diabetes Mellitus
- DNA Deoxyribonucleic acid
- DROSHA Double-stranded RNA-specific Endoribonucleases
- EDTA Ethylene diamine tetra-acetic acid
- ER Endoplasmic reticulum
- ESSC Endometrial Stromal Stem Cells
- FBI Fasting blood insulin
- GDM Gestational diabetes
- GGT Gamma glutamyl-transferase
- HbA1C Glycated hemoglobin
- HCO_3^- Bicarbonate ions
- HDL High density lipoproteins
- HKG House Keeping Gene
- HHS Hyperglycemic hyperosmolar state
- H^+ Hydrogen ion
- IDDM Insulin Dependent Diabetes Mellitus
- IDF International Diabetes Association
- IFG Impaired Fasting Glucose
- IGT Impaired Glucose Tolerance
- LADA Latent Auto-immune Disease of Adults
- LDL Low density lipoproteins

- Min Minutes
- miRNA MicroRNA
- MODY Maturity Onset Diabetes of the Young
- mRNA Messenger Ribonucleic acid
- NDDG National Diabetes Data Group
- NIDDM Non-Insulin Dependent Diabetes Mellitus
- OGTT Oral Glucose Tolerance Test
- OS Other Studies
- pH Potential of Hydrogen
- PreDM Pre-Diabetes Mellitus
- Pre-miRNA Precursor micro RNA
- Pri-miRNA Primary micro RNA
- PBMC Peripheral blood mononuclear cells
- PS Present study
- RISC RNA Induced Silencing Pathway
- RNA Ribonucleic acid
- ROS Reactive oxygen species
- S Seconds
- SD Standard deviation
- SM Skeletal muscle
- SST Serum-separating tubes
- T1DM Type 1 Diabetes Mellitus
- T2DM Type 2 Diabetes Mellitus
- Trigs Triglycerides
- WB White blood
- WC Waist circumference
- WHO World Health Organisation
- WHR Waist hip ratio

DEFINITIONS

- **Biomarker**: a naturally occurring molecule, gene or characteristic by which a particular pathological or physiological process or disease can be identified.
- **Diabetes**: a condition whereby a person's blood glucose levels are higher than normal and above threshold value
- **Pre-diabetes**: a condition whereby a person's blood glucose levels are higher than normal but not high enough to be classified as diabetic. The condition includes IGT and IFG.
- **Dysglycemia**: abnormality in blood sugar stability
- **Hyperglycemia**: increase in blood glucose level above normal
- **Epigenetics**: heritable changes in gene function which do not involve nucleotide sequence changes.
- **Epigenome**: the overall epigenetic state of an organism.
- **Genome**: the overall gene collection of an organism.
- **microRNA**: miRNAs are short noncoding RNAs
- **Down-regulation** is the process by which a cell decreases the quantity of a cellular component, such as RNA or protein.
- **Up-regulation** is the process by which a cell increases the quantity of a cellular component, such as RNA or protein.

CHAPTER 1: LITERATURE REVIEW

1.1 Glucose intolerance

Glucose intolerance is an umbrella term for metabolic conditions which results in higher than normal blood glucose levels "hyperglycemia" (WHO, 2006). Conditions which can be considered as glucose intolerance include: Impaired fasting glucose (IFG), Impaired glucose tolerance (IGT), pre-diabetes (preDM) and Types 1 and 2 diabetes mellitus (T1DM and T2DM) (WHO, 2006). Other forms include gestational diabetes and maturity onset diabetes of the young (MODY) (Keating and El-Osta, 2013). Diabetes mellitus (DM) is the sixth leading cause of death worldwide with diabetic sufferers having twice the risk of death than someone of the same age without diabetes (WHO, 2014). More than half a million children aged 14 and under are living with T1DM (International Diabetes Federation "IDF", 2015). Furthermore, it is estimated that 415 million adults aged 20-79 now have DM worldwide, including 193 million who are undiagnosed (IDF, 2015). A further 318 million adults are estimated to have impaired glucose tolerance which puts them at risk of developing T2DM (IDF, 2015). Statistically, if this rise is not halted, by 2040 there will be 642 million people living with DM (IDF, 2015). Moreover, DM is the second leading cause of death in South Africa and the first leading cause of death in the Western Cape, with the mixed ancestry population showing the second highest prevalence of diabetes after the Indian population (Erasmus et al., 2012). DM was reported to be significantly increased, hence identified as the number one killer in the Western Cape South Africa (IDF, 2015).

1.2. Diabetes mellitus

DM is a major cause of morbidity and mortality worldwide. The three most common forms are; T1DM, T2DM and gestational diabetes (GDM), accounting for approximately 5 %, 90 % and 3 % of all DM cases, respectively (ADA, 2010; ADA, 2014).

1.2.1. Type 1 Diabetes (T1DM)

Type 1 diabetes, also referred to as insulin dependent diabetes mellitus (IDDM) or juvenile diabetes, is an autoimmune disorder characterized by hyperglycemia due to the inability of the pancreas to secrete insulin (ADA, 2017). It is a diabetes mellitus with early onset, and is due to T-cell mediated autoimmune destruction of beta cells (β-cell) resulting in insulin deficiency and hyperglycemia. Exogenous administration of insulin, usually by injection, and recently via an insulin pump, is required to maintain normoglycemia (Afrand et al., 2012; ADA, 2017).

Furthermore, an insulin patch has been developed for the treatment of T1DM. The insulin patch is an artificial pancreas modeled device, which is fully automated, closed-loop and has the ability to monitor insulin levels without the patient's input (Anhalt and Bohannon, 2010; ADA, 2017). Although the majority of T1DM cases occur due to autoimmunity, in some cases, the disease arises spontaneously with no definite cause (Seino et al., 2010). In certain cases, latent autoimmune diabetes of adults (LADA), also known as type 1.5 diabetes is an adult onset autoimmune diabetes which shares features of both T1DM and T2DM (Afrand et al., 2012). LADA is often misdiagnosed as T2DM because physicians base their diagnosis on patient age rather than on aetiology (Afrand et al., 2012). Although clinical dilemma exists in initial diagnosis, early diagnosis and interventions can influence the speed of progression towards insulin dependency (Fourlanos et al., 2006).

1.2.2. Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is first diagnosed during pregnancy (Seino et al., 2010). The mechanisms underlying GDM are not yet fully elucidated, although progressive insulin resistance during pregnancy has been implicated. About 20 - 50 % of women with GDM develop T2DM later in life (Afrand et al., 2012). Although transient in nature, GDM can adversely affect the health of the fetus and mother. Risks to the baby include macrosomia (high birth weight), congenital cardiac and nervous system abnormalities, skeletal muscle malformations and increased fetal insulin levels, which inhibit fetal surfactant production, possibly resulting in respiratory distress syndrome. In severe cases, perinatal death may occur, most commonly as a result of poor placental perfusion due to vascular system impairment (Afrand et al., 2012).

1.2.3. Other types of diabetes

Other forms of DM include monogenic forms such as neonatal diabetes and MODY, diabetes of the exocrine pancreas such as cystic fibrosis related diabetes, and drug induced or chemical induced diabetes such as steroid diabetes induced by high doses of glucocorticoids (Aguiree et al., 2013; ADA, 2015). These forms of diabetes are rare and collectively account for less than 5 % of all diabetes cases (Bloomgarden, 2004 & ADA, 2015).

1.2.4. Type 2 diabetes

1.2.4.1. Epidemiology in South Africa

T2DM is the major contributor to the DM epidemic accounting for more than 90 % of DM cases globally (ADA, 2014). South Africa is one of the 32 countries with high numbers of diabetic cases, with 2.28 million cases of diabetes recorded (IDF, 2015). Gwebu reported that about

3.5 million South Africans (6 % of the population) have T2DM, while about 5 million South Africans have pre-diabetes, (Gwebu, 2013). Pre-diabetes precedes T2DM with about 5 - 10 % people with pre-diabetes usually progressing to DM annually (Forouhi et al., 2007; Nathan et al., 2007). A prevalence survey conducted between 2008 - 2009 in the Bellville South community, in the Western Cape province of South Africa, reported an age-adjusted T2DM prevalence of 26.3 % in individuals of mixed ethnic ancestry in this setting (Erasmus et al., 2012). A study conducted in the Cape Town suburbs of Langa, Gugulethu, Crossroads, Nyanga and Khayelitsha between 2008 and 2009 reported a prevalence of 13.1 % in black South Africans which is almost half of that reported for individuals of mixed ethnic ancestry in the Bellville South setting during the same period (Erasmus et al., 2012; Peer et al., 2012).

1.2.4.2. Aetiology

The aetiology of T2DM involves both environmental and genetic factors (Tripathy and Chavez, 2010). Studies have reported that only about 5 - 10 % of T2DM cases can be explained by genetics (Voight et al., 2010). Thus environmental factors have received increased attention as major mediators of T2DM progression (Barrès et al., 2009; Hirst and Marra, 2009; Drong et al., 2012; Keating and El-Osta, 2013). In most cases a diabetogenic lifestyle and a susceptible genotype is required for disease development. A diabetogenic lifestyle includes excessive caloric intake, inadequate caloric expenditure such as physical inactivity and obesity (Nolan et al., 2011).

1.2.4.3. Pathophysiology

T2DM is characterized by hyperglycaemia due to defects in insulin action in peripheral tissues, specifically skeletal muscle, liver, adipocytes and brain as well as decreased insulin secretion due to pancreatic β-cell dysfunction (Fernandez-Valverde et al., 2011; Tripathy and Chavez, 2010). Moreover, the pathogenesis of T2DM involves a combination of genetic and environmental/lifestyle factors (Tripathy and Chavez, 2010). Compared with healthy individuals, subjects with impaired glucose tolerance have a 50% to 70% decline in β-cell function, T2DM sets in when 80 to 90% of β-cell function decline (Ward et al., 2003; Gastaldelli et al., 2004; Ferrannini et al., 2008). The hormone insulin is the primary mediator of glucose homeostasis (Leahy, 2005). After the ingestion of food, β-cells in the pancreas secrete insulin to increase glucose uptake in insulin-responsive tissues such as skeletal muscle and adipocytes, and to decrease glucose production from the liver so as to restore normoglycemia. However, insulin resistance, defined as the condition where insulin responsive tissues fail to increase glucose uptake in response to physiological concentrations of insulin leads to persistent hyperglycemia (Reaven, 2005; Yarbrough et al., 1998). Insulin resistance is characterized by high levels of circulating insulin, due to increased insulin secretion by β-cells

as a compensatory mechanism to counteract insulin resistance. A number of factors including insulin resistance, as described above, genetic predisposition, glucotoxicity, lipotoxicity, increased reactive oxygen species (ROS), endoplasmic reticulum (ER) stress and elevated intracellular calcium contribute to β-cell dysfunction, which is a prerequisite for the development of T2DM (Tripathy and Chavez, 2010; Cerf, 2013).
Although the exact mechanisms underlying the development of T2DM is not yet completely understood, insulin resistance in peripheral tissue, and decreased insulin secretion due to pancreatic β-cell dysfunction is accepted to play a major role (Tripathy and Chavez, 2010). Initially β-cells compensate for insulin resistance by increasing insulin secretion, however they subsequently become exhausted and dysfunctional (Poitout and Robertson, 2008). In severe cases of T2DM, β-cells are unable to secrete insulin, requiring the administration of exogenous insulin. T2DM pathophysiology is thought to commence about 10 years prior to clinical diagnosis, at which time insulin resistance is high and β-cell function is already significantly impaired. Moreover, T2DM related microvascular and macrovascular complications are thought to occur about 5 to 10 years before diagnosis (Tripathy and Chavez, 2010).

Lifestyle factors and genetic predispositions leading to insulin resistance account for the glycemic changes from normoglycemia, IGT and finally to DM (Tripathy and Chavez, 2010). This study is aimed to show that epigenetics also has a major role to play in dysglycemia, by displaying the role of circulating miRNAs as biomarkers for this disease process (Kato et al., 2013).

1.2.4.4. Complications of diabetes

Microvascular complications of diabetes include diabetic retinopathy, neuropathy and nephropathy (Afrand et al., 2012). Diabetic retinopathy affects blood vessel formation in the retina of the eye, and can lead to reduced vision and blindness (Afrand et al., 2012). Diabetic neuropathy is the result of the impact of diabetes on the nervous system, most commonly causing numbness, tingling and pain in the feet, and also increases the risk of skin damage due to altered sensation (Afrand et al., 2012). Diabetic nephropathy, due to the impact of diabetes on the kidneys, may lead to scarring in kidney tissue, loss of small and progressively larger amounts of protein in the urine, and eventually chronic kidney disease requiring dialysis (Donaghue et al., 2009). In conjunction with vascular disease in the legs, neuropathy contributes to the risk of diabetes-related foot ailments, such as diabetic foot ulcers, which are difficult to treat and usually require amputation (Afrand et al., 2012; Aguiree et al., 2013). The major long-term macrovascular complications associated with DM relate to blood vessel damage, with DM doubling the risk of cardiovascular disease.

Cardiovascular disease is the most common cause of death and disability in diabetic patients (Aguiree et al., 2013). The main macrovascular diseases (related to atherosclerosis of larger

arteries) in diabetes are ischemic heart disease (angina and myocardial infarction), stroke and peripheral vascular disease. Capillary damage may also occur, causing macro and microangiopathy (Boussageon et al., 2011). Macroangiopathy refers to an angiopathy (blood vessel disease), which affects large and medium size blood vessels (Boussageon et al., 2011). Microangiopathy refers to an angiopathy that affects small blood vessels in the body (Boussageon et al., 2011).

One of the major diabetes complications during T1DM and the late stages of T2DM is diabetic ketoacidosis (DKA). Diabetic ketoacidosis is defined as a state of high anion gap metabolic acidosis due to an excessive concentration of ketone bodies (keto-anions) in the bloodstream (Kitabchi et al., 2009). Due to the body's inability to utilize glucose as an energy source in these individuals, fat is metabolized as an alternative source of energy. Fat or lipid metabolism produces ketone bodies as by-products. Ketone bodies (acetoacetate, beta-hydroxybutyrate and acetone) are released into the bloodstream where they dissociate into ketone anions and hydrogen ions (H+). In an attempt to maintain extracellular pH and ionic balance, H+ ions are coupled with bicarbonate ions (HCO3-), which depletes the body's alkali reserves. The end result is acidosis and failure to regulate the ionic balance, which can result in fatal consequences such as a coma or even death (Fowler, 2009; Kitabchi et al., 2009).

The respiratory system tries to compensate for acidosis by increasing the rate of breathing in order to exhale more carbon dioxide resulting in rapid deep breathing with a smell of acetone known as Kussmaul breathing (Fowler, 2009; Kitabchi et al., 2009). Other symptoms include nausea, vomiting, abdominal pain and an altered state of consciousness (Ali et al., 2012). The fatality of the complication emanates from the fact that it can occur acutely as an initial manifestation of T1DM with no history of the disease manifestation or diagnosis.
An acute attack may also result from an increased insulin requirement in known T1DM patients and sometimes T2DM patients. Hyperglycemic hyperosmolar state (HHS) also known as hyperosmolar non-ketotic state is another less common, but equally fatal complication of T2DM (Afrand et al., 2012; Brenner, 2006). The disorder is characterized by reduced lipolysis (breakdown of triglycerides into glycerol and fatty acids) and ketoacidosis. Hyperglycemic hyperosmolar state has a slower onset than DKA and is characterized by markedly high hyperglycemia, hyperosmolality and severe dehydration (Afrand et al., 2012; Brenner, 2006).

1.2.4.5. Current methods of diagnosis

In 1979, the National Diabetes Data Group (NDDG) issued the first unified classification of diabetes (NDDG, 1979). This was followed by the World Health Organization's (WHO) classification in 1980 (WHO, 1980). These two recommendations ushered a more standardized approach to the diagnosis of diabetes (Alberti and Zimmet, 1998). The WHO criterion was modified in 1985, and again in 1998 (Alberti and Zimmet, 1998; WHO, 1999).

The latest recommendations worldwide are based on the 2006 WHO recommendations (WHO, 2006) modified in 2011 to include glycated haemoglobin type A1c (HbA1c) (WHO, 2011) and the American Diabetes Association (ADA) criteria of 2014 modified in 2016 to include HbA1c (ADA, 2014; ADA, 2016), as seen in the table below.

Table 1.1: WHO and ADA Diagnostic Criteria (Adapted from WHO, 2006; ADA, 2010; WHO, 2011)

	WHO criteria	ADA criteria
T2DM		
FPG	≥ 7.0 mmol/L	≥ 7.0 mmol/L
2h-PG:	≥ 11.1 mmol/L	≥ 11.1 mmol/L
HbA1c:	≥ 6.5%*	≥ 6.5%[†]
RPG	≥ 11.1 mmol/L[¥]	≥ 11.1 mmol/L[¥]
IFG/IGT		
FPG:	6.1-6.9 mmol/L	5.6-6.9 mmol/L
2h-PG:	7.8-10.9 mmol/L	7.8-11.0 mmol/L
HbA1c:	Not specified	5.7-6.4%
Normal		
FPG:	<6.1 mmol/L	<5.6 mmol/L
2h-PG:	<7.8 mmol/L	<7.8 mmol/L
HbA1c:	Not specified	<5.7%

Footnotes

- The FPG test = measures blood glucose concentrations after an overnight fast (about eight hours without food but may have water). Individuals with IFG and/or IGT are defined as pre-diabetic, and they have a higher risk of developing T2DM (ADA, 2014). Pre-diabetes is a condition whereby the blood glucose levels are higher than normal but not high enough to be classified as T2DM according to WHO and ADA, respectively (WHO, 2011; ADA, 2014). The use of a FPG value of 7.0 mmol/L or greater, recommended by both the WHO and ADA as a diagnostic criterion for diabetes has several limitations. The limitations include the need for a confirmatory test on two separate occasions, the need for an eight hour fast and a 12 - 15% day to day variation (Petersen et al., 2005). The criteria recommended by WHO and ADA differ mainly on the lower limit of the FPG's normal levels (WHO: <6.1 mmol/L and ADA: <5.6 mmol/L) (WHO, 2006; ADA, 2010). In South Africa, the WHO criterion of 1998 is adopted and

the recommendations by the Society for Endocrinology, Metabolism and Diabetes of South Africa (SEMDSA) also endorse the WHO criteria (WHO, 1999; Amod et al., 2012). However, the SEMDSA recommendations do not replace professional judgment, experience and appropriate referral where applicable (Amod et al., 2012).

- 2h-PG = 2 hours-plasma glucose: a test which assesses glucose metabolism and clearance from the bloodstream, conducted by ingesting 75 g of glucose after an overnight fast of about eight hours and taking serial blood samples immediately before and two hours after the glucose drink.
- RPG = random plasma glucose: when blood glucose levels are tested without a period of fasting. A value of more than 11.1 mmol/L, together with the classic symptoms of diabetes (polyuria, polyphagia, polydipsia, unexplained weight loss and fatigue) is considered to indicate diabetes, a diagnostic criteria common for both the WHO and ADA (WHO, 2006; ADA, 2010). A RPG value less than 11.1 mmol/L does not exclude diabetes and has to be followed up with another RPG or other diagnostic tests such as fasting plasma glucose (FPG), oral glucose tolerance test (OGTT) and HbA1c. The RPG obviates the requirement for fasting, thus is particularly useful for rapid diagnosis.
- The oral glucose tolerance test (OGTT) is also known as the glucose tolerance test (GTT) or the 2 hour plasma glucose (2h-PG) test. An OGTT is conducted by ingesting 75g of glucose after an overnight fast of about eight hours and taking serial blood samples immediately before and two hours after the glucose drink. The OGTT assesses glucose metabolism and clearance of glucose from the blood stream (Seino et al., 2010). Limitations of the OGTT include poor reproducibility and the requirement for fasting (Ko et al., 1998; Patel & Macerollo, 2010). However, besides the limitations, the OGTT is considered as the gold standard of diabetes diagnosis (Ko et al., 1998).
- The HbA1c test measures the percentage of glycated hemoglobin. This test assesses glycemic control over a period of about three months, the lifespan of red blood cells (Franco, 2012). During the lifespan of a red blood cell, glucose molecules bind to hemoglobin molecules through a process called glycosylation. The process, though gradual, is believed to be directly proportional to the amount of glucose in the bloodstream (Markris & Spanou, 2011). Therefore the test reflects average glucose control over a period of about three months. The HbA1c test has several advantages over FPG and OGTT, such as obviating the need for fasting; no fluctuations due to recent meals (HbA1c is a three month estimate) thus free of the 12 -15% day to day variance and decreased susceptibility to measurement variation due to conditions such as stress and illness) (Saudek et al., 2008; ADA, 2014). However, limitations of HbA1c testing include greater cost, incomplete correlation between HbA1c and glucose concentrations in some individuals, limited availability and standardization in certain regions of the developing world, possible disparities due to race/ethnicity and

interference by anemia, as well as some medications (Saudek et al., 2008; Kumar et al., Ziemer et al., 2010; ADA, 2014). Furthermore conditions which shorten red blood cell survival, such as recovery from acute blood loss, pregnancy, blood transfusion and hemolytic anemia will falsely decrease HbA1c results (Markris & Spanou, 2011; ADA, 2014). Therefore apparent that, like other current methods of diagnosis, HbA1c testing has limitations.

- Taken together, it is apparent that the current methods of diabetes diagnosis are not without limitations, creating the need for the identification of other methods of diagnosis. Epigenetic changes especially in miRNA expression offers an option as a good marker for diabetes, and also as a promising diagnostic and prognostic marker of glucose intolerance (Bartel, 2004; Engels and Hutvagner, 2006 and Pillai et al., 2007).

1.3. Epigenetics

1.3.2. Definition of epigenetics

This word was first defined as "interaction between genes and their environment" emphasizing that epigenetic mechanisms are different in response to a given environment (Bird, 2002). Even though there is no uniform definition for epigenetics, it has been widely described as heritable changes in gene function and/or expression which do not change the nucleotide sequence (Bird, 2007; Christensen and Marsit, 2011). miRNAs are short noncoding RNAs that have elicited immense interest in recent years (Kato et al., 2013). They have been found to be associated in the molecular mechanisms of epigenetics, which also involve DNA methylation, lysine methylation in histones, histone acetylation, histone phosphorylation and RNA interference such as RNA induced silencing complex (RISC) and small interfering RNA (siRNA) (Kato et al., 2013). miRNA is also involved in chromosomal silencing and gene activation via binding protein complexes (Tchurikov, 2005).

Changes in the expression of miRNAs have also been associated with a number of human diseases, indicating that miRNA expression levels are closely associated with the developmental, physiological, as well as disease processes (Betel et al., 2008; Li et al., 2009; Almen et al., 2012; Chen et al., 2012). They function mainly by regulating post-transcriptional gene expression by binding to their target messenger RNAs (mRNAs), leading to mRNA degradation and suppression of translation or even gene activation (Chen et al., 2012). In recent years, a technique using real-time quantitative reverse transcription-PCR (RT-PCR) was developed to investigate miRNA in serum/plasma (Dasi et al., 2001; Tsui et al., 2002 and Izzotti et al., 2018), which can facilitate research in this area, including the possible identification of individuals with an increased risk of developing T2DM. This could prevent the

clinical onset of the multiple malfunctions and complications of T2DM. However, there is scarcity of studies on the involvement of circulating miRNAs in the serum of people with glucose intolerance (Izzotti et al., 2018).

1.3.3. MicroRNAs (miRNA)

miRNAs are a class of small, endogenous, noncoding RNAs approximately 19-22 nucleotides in length that modulate gene expression (Poy et al., 2004). MicroRNA was first identified in 1993, by Ambros and his group. These molecules are found in plants, animals and some viruses and were only identified in humans in the last decade (Rooij, 2011). Generally, miRNAs exert inhibitory effects on gene expression by binding to the 3′- untranslated (UTR) of their mRNA targets hence causing gene silencing by mRNA cleavage or degradation as seen in Figure 1.1, thus modulating physiological and pathological processes (Rooij, 2011). Currently the biological functions of miRNAs are actively being sought. Some studies have notably uncovered roles for miRNAs in cellular processes including apoptosis, proliferation, stress resistance, tumorigenesis, defense against pathogenic infections, neural development and metabolism (Izzotti et al., 2018 and Matsha et al., 2018).

1.3.4. Biogenesis of microRNAs

Figure 1.1, shows the biogenesis of miRNA which begins in the nucleus. The primary miRNAs are transcribed by RNA polymerase II (Bartel, 2004) and cleaved by Drosha, a nuclear RNAse III enzyme (Han et al., 2004). The resulting pre-miRNA is transported into the cytoplasm and processed into double-stranded miRNA by Dicer, an RNAse III enzyme. One strand of this duplex represents the mature miRNA and is assembled into the RNA-induced silencing complex (RISC), while the other strand of this duplex is degraded. MicroRNA incorporated into the RISC regulates expression of genes by translational repression or degradation of mRNA, which depends on complementarity of sequence between mRNA 3′ UTR region and miRNA 5′ region (Bartel, 2004; Engels and Hutvagner, 2006; and Pillai et al., 2007). This target recognition occurs through the miR “seed” sequence, which is comprised of the first 8 nucleotides of the mature miRNA molecule (Bartel, 2009).

Targets for miRNAs may be approximately 60% of mRNAs. miRNAs have a role in controlling the signaling pathways in many types of cells and in phenotype and development of cells of the immune system as well as regulation of the inflammatory response in many tissues (Perry et al., 2015). In addition, miRNAs are emerging as markers of disease diagnosis and prognosis, and as new therapeutic targets. Following the discovery of extracellular miRNA in blood plasma and serum, miRNAs are also found in all other body fluids including; saliva, urine, breast milk, seminal fluid, tears, amniotic fluid, colostrum, bronchial lavage, cerebrospinal fluid, peritoneal fluid, and pleural fluid (Gregory et al., 2006). Both total miRNA concentration and its

relative composition vary significantly among different fluid types (Perry et al., 2015). Furthermore, the changes in miRNA spectra observed in certain fluids correlate with various pathological conditions, suggesting that extracellular miRNAs can serve as informative biomarkers to assess the pathological status of the body (Gregory et al., 2006).

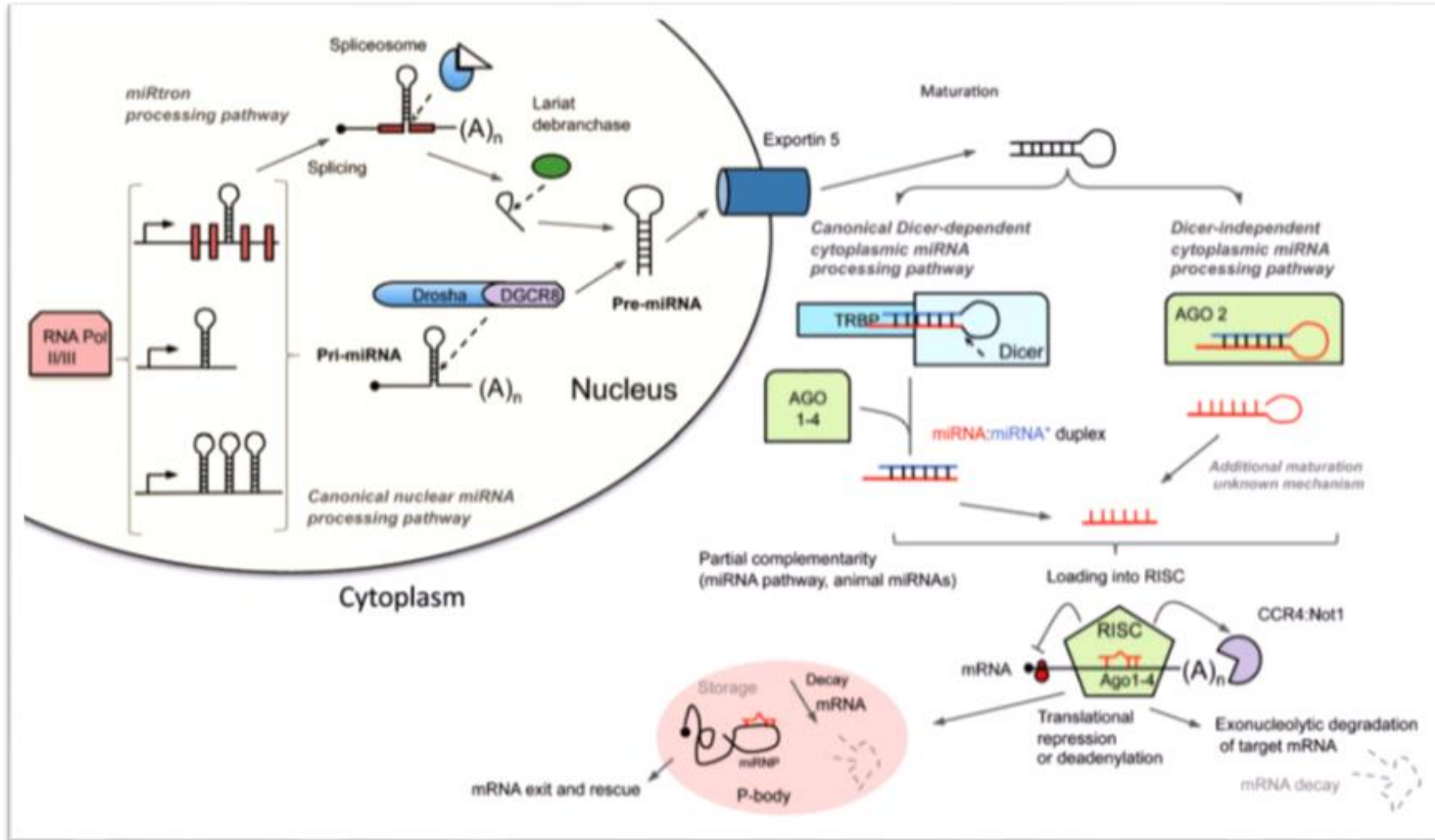


Figure 1.1: Biogenesis of micro RNA (Adapted from: Moore et al., 2011)

Foot notes

Figure 1.1, shows the miRNA biogenesis pathway. MicroRNAs are transcribed in the nucleus by RNA polymerase from independent miRNA genes located in polycistronic transcripts or introns of protein-coding genes into primary transcripts (pri-miRNAs) (Kathryn et al., 2011). Pri-miRNAs are processed in two steps in the nucleus and cytoplasm by the RNase III–type endonucleases Drosha and Dicer, respectively, in complexes with dsRNA-binding domain proteins DGCR8 and TRBP, as indicated (Kathryn et al., 2011). In the canonical pathway, Drosha-DGCR8 processes the transcript to a stem-loop-structured precursor (premiRNA) (Kathryn et al., 2011). Alternatively, miRtrons, a subset of miRNAs derived from introns, may be processed into premiRNAs by the spliceosome and the debranching enzyme (Kathryn et al., 2011). Both canonical miRNAs and miRtrons are exported to the cytoplasm via Exportin 5, where they are further processed by Dicer to yield ≈20-bp miRNA duplexes (Kathryn et al., 2011). One strand is selected to function as the mature miRNA and loaded into the RNA-induced silencing complex (RISC) containing components of the Argonaute family (Ago 1–4), while the partner miRNA∗ strand is preferentially degraded (Kathryn et al., 2011). A subset of miRNAs, exemplified by miR-451, is produced independently of Dicer through recognition by Ago2 (Kathryn et al., 2011). The mature miRNA produced by these two mechanisms leads to translational repression or degradation of the target mRNA (Kathryn et al., 2011). Animal miRNAs usually show only partial complementarity to the target mRNA promoting translational repression or deadenylation coupled to exonucleolytic degradation of target mRNA (Kathryn et al., 2011).

1.4. Role of microRNAs in diabetes

The role of miRNAs in the function of β-cells in T2DM patients has been extensively studied but is not yet fully understood (Poy et al., 2004). A role for miRNAs in T2DM was first established in 2004 showing that miR-375 is directly involved in the regulation of insulin secretion (Poy et al., 2004). This study was one of the first to demonstrate that a miRNA could be tightly linked to a disease phenotype. In recent years, many additional miRNAs have been identified as components of pathways triggered by, or contributing to, the pathology of both T1DM and T2DM (ADA, 2011). Due to the multifactorial and polysystemic nature of this disease and the increased interest in miRNAs, many more miRNAs will be identified as important markers in diabetes (ADA, 2011). Recently, many other miRNAs have been found to be associated with diabetes and the complications that result from hyperglycemia (Matsha et al., 2018). In this study, a good number of miRNAs were investigated. This will undoubtedly lead to a greater understanding of the disease and provide novel diagnostic, prognostic alternative.

1.5. Motivation for this study

1.5.1. Problem Statement

Type 2 diabetes mellitus, a complex, multifactorial metabolic disorder that is caused by genetic and environmental factors, is a major source of morbidity and mortality worldwide. Intensive research is aimed at improved diagnosis and management of the disease. The mixed ancestry population of South Africa, commonly referred to as 'coloureds' has a high prevalence of diabetes (Erasmus et al., 2012). Furthermore, this population has also been reported to have high rates of obesity and metabolic syndrome, and therefore at a high risk of developing CVD (Matsha et al., 2012). Follow-up studies have shown that the accelerated deterioration of glucose tolerance status over time is not only explained by the known determinants of diabetes occurrence, rather by other yet to be identified factors which include those involved in epigenetic mechanisms such as miRNAs (Matsha et al., 2013).

1.5.2. Rationale

- The identification of individuals who are at risk of developing T2DM could facilitate intervention to delay or prevent the progress of the disease, thus minimizing the burden of T2DM. Interventions to protect against insulin resistance and β-cell dysfunction by lifestyle modifications or therapeutic drugs may reverse the disease phenotype and prevent the progression to T2DM. Identifying individuals with an increased risk of developing T2DM, before the clinical onset of these multiple malfunctions and complications is a major priority in glucose intolerance. To date there are no biomarkers that can facilitate identification of individuals who are at risk of developing T2DM. Biomarkers available are used for testing and monitoring T2DM, and these include blood glucose and glycated haemoglobin. Investigating the role of miRNAs in the development of T2DM may contribute to the better understanding of the mechanisms involved in the initial stages of T2DM, and hopefully identification of at risk individuals prior to occurrence of associated complication. To date there are no biomarkers that can facilitate identification of individuals who are at risk of developing T2DM. Biomarkers available are used for testing and monitoring T2DM, and these include blood glucose and glycated hemoglobin. Investigating the role of miRNAs in the development of T2DM may contribute to the better understanding of the mechanisms involved in the initial stages of T2DM, and hopefully identification of at risk individuals prior to occurrence of associated complication.

1.5.3. Hypothesis

Glucose intolerance is associated with altered expression of circulating miRNA which may be an early detection marker

1.5.4. Aim

To investigate the expression profile of circulating miRNAs as a prognostic marker in subjects with glucose intolerance

1.5.5. Objective

- To identify miRNAs that are differentially expressed in individuals with glucose intolerance
- To determine whether miRNAs expression is affected by diabetes associated conditions such as obesity

2: RESEARCH DESIGN AND METHODOLOGY

2.1. Ethics

Ethical clearance for conducting the study in human subjects was sought and obtained from the Cape Peninsula University of Technology Health and Wellness Sciences Research Ethics Committee (HW-REC)+,Registration Number NHREC: REC- 230408-014

2.2. Study design

A quantitative cross sectional study design involving 36 individuals [who were age, gender and BMI (Body Mass Index) matched] from a total population of 1989 participants of mixed ancestry descent, residing in Bellville South, South Africa was used. Quantitative methods were used to investigate the epigenetic changes corresponding with the expression of circulating miRNA in the serum of participants from the Belhar and Bellville South community, Cape Town, South Africa. miRNA present in serum was the dependent variable and was compared to the independent variable, disease status (diabetic, pre-diabetic and controls). The association of other variable factors such as gender and age was also compared to the results obtained above.

2.3. Study population

The enrolment of participants for this study was conducted from 2014 to 2016. A total of 1989 subjects were enrolled. Participants for this study were selected from participants recruited from Bellville South and Belhar, Cape Town, South Africa.The inclusion criteria were: all coloured participants who enrolled in the study on a voluntary basis and gave informed consent. The exclusion criteria were; subjects less than 16 or older than 95 years of age, pregnant women, those who did not volunteer to participate in the study, and acutely ill participants.

All participants signed informed written consent and were informed about their rights, and their involvement in the research. Furthermore, all study procedures were explained to participants in their language of choice. Participants were also informed of their right to freely withdraw from the study whenever they wanted to. Information about the participants and aspects of the study were kept confidential. The study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). The total number of participants used were, 36 females, making a group of three (with 12 participants each), according to the three status' under investigation in this study [Diabetic (DM), pre-diabetic (preDM) and (control) individuals] and they were age, gender and BMI matched.

2.4. Experimental outline

Stored serum samples collected from participants from Belhar or Bellville South were used in this study for investigating the expression of circulating miRNA in mixed ancestry subjects who were diabetic, pre-diabetic and control. This involved the purification of circulating miRNA from serum, followed by identification of the various miRNAs which could possibly serve as biomarkers for glucose intolerance.

2.5. Data collection

Clinical and biochemical data were collected by the research team which comprised of professional nurses and field workers. Field workers were pre-trained individuals involved in the recruitment of participants and for the collection of clinical and biochemical data. The clinical and biochemical data were collected as follows;

2.6. Clinical data

2.6.1. Questionnaire

The questionnaire requested information about the participant's demographics such as gender, age, previous medical history of diabetes, dietary, as well as lifestyle aspects. The four components of the questionnaire were: i) personal demographics, ii) family health history, iii) diet and iv) cigarette smoking. The questionnaire was adopted from other standard and recognized sources such as the CAGE Questionnaire (Ewing, 1984), the South African Demographic and Health Survey Report by the South African Medical Research Council (SADHSR) (Bradshaw et al., 1995; Goedecke et al., 2005). The SADHSR questionnaire focuses on chronic and lifestyle diseases by addressing the diabetic status, nutritional status, hypertension and physical activity of the participants. The CAGE questionnaire is mainly used in alcohol related studies. The questionnaire used for this study was also adopted from a previous study to measure the prevalence of DM amongst learners attending schools in the Belhar, Delft and Mfuleni suburbs in the Cape Town metropole (Somers et al., 2006). The family health history section of the questionnaire covered the presence or previous treatment of family members, up to third degree relatives, for related inheritable diseases. The section of the questionnaire on cigarette smoking focused on: a) the particular habit, b) duration of the habit, c) quantity or amounts consumed, and d) when the habit was stopped. A detailed medication history was obtained by asking participants, as well as examining their clinic cards, and recording the drugs which they brought to the study site.

2.6.2. Clinical measurements

Clinical measurements obtained included: height, weight, waist and hip circumference, blood pressure measurements and blood collection. All anthropometric measurements were done three times and the average of these measurements used for the final analysis.

2.6.2.1. Height

A portable stadiometer was used to measure height with the readings recorded in centimeters (cm). A participant was asked to stand on the flat surface of the stadiometer, without their shoes, at right angles to the vertical sliding lever of the stadiometer. The head was then placed in the Frankfort plane with hands freely at the sides. The scapular and buttocks were placed as close to the vertical sliding metallic bar as possible to ensure accurate readings. The sliding metallic bar was then allowed to gently rest on the subject's head. If the participant was taller than the investigator, the investigator stood on a platform to enable an accurate reading.

2.6.2.2. Weight

A calibrated and standardized Sunbeam EB710 digital bathroom scale was used to measure participants' weight in kilograms (kg). Participants were weighed in light clothing and were also asked to remove shoes and socks. The weight of all participants, except wheelchair bound or those who were posturally impaired were measured. The participant would stand on the middle of the flat surface of the scale after it had been zeroed. Hands were placed on the sides and after ensuring that the subject's weight was evenly distributed, the reading was taken. Readings less than 0.5 kg were rounded off to the nearest lower kilogram while those above 0.5 kg were rounded off to the nearest higher kilogram. The body mass index (BMI) was calculated by dividing the weight and height squared [weight/height^2] (kg/m^2).

2.6.2.3. Waist circumference

A non-elastic tape that had been inspected for calibrations and stretch was used to measure the waist circumference (WC). Subjects were asked to stand in an erect position with hands placed on their sides and with their feet and abdominal muscles relaxed. Measurements were taken with the investigator in front of the participant, and by placing the measuring tape around the natural waist (narrowest part of the torso as seen from the anterior view). For obese participants, the narrowest circumference between the ribs and the iliac crest was measured (Tolonen et al., 2002).

2.6.2.4. Hip circumference

The hip circumference (HC) was measured at the maximal circumference over the buttocks. A non-elastic tape was also used for this measurement. The investigator would squat before the subject with the tape placed around the buttocks on the widest area over the horizontal plane without pressing tightly against the skin, and the measurement taken. When the hip circumference exceeded the tape size, the measurement was not recorded, but noted (Tolonen et al., 2002)

2.6.2.5. Blood pressure measurements

Measurements used to assess the blood pressure were systolic blood pressure and diastolic blood pressure. Systolic blood pressure (SBP) refers to the highest arterial pressure as a result of the exertion of the blood upon the walls of the blood vessels (arteries) immediately after the pumping action of the left ventricle of the heart. Diastolic blood pressure, in contrast, refers to the lowest arterial blood pressure when the heart muscles contract after a systolic event (Pickering et al., 2005). Blood pressure is expressed as SBP over DBP, with the ratio (as a fraction) taken as the blood pressure and the units are millimeters of mercury (mm Hg) (Pickering et al., 2005). Blood pressure measurements were done according to WHO guidelines (WHO, 1999). Blood pressure measurements were taken using a calibrated baumanometer (Rossamax; Berneck, Switzerland) with the participant in a relaxed sitting position. Participants were allowed to sit with their back supported on the chair backrest, while their arms were exposed and rested on the table at the same level as their heart. The correct adult cuff size was placed 2cm above the elbow joint to ensure accurate readings (Pickering et al., 2005). Three readings, at one minute intervals, were taken and the lowest reading was chosen as the participant's blood pressure. During the time of taking the measurement, the participants were not allowed to speak as this has been found to affect the readings (Adams et al., 2002; Panchón et al., 2004).

2.6.2.6. Blood collection

Six blood tubes were collected for each participant: three fasting and three postprandial bloods. Self-reported T2DM participants (confirmed by either medical records or medications) had only the fasting blood samples taken and no 2 hour bloods were drawn. Both the fasting and postprandial blood samples were collected in one grey capped-tube (sodium fluoride), one plain tube (no clotting factors) and one purple capped-tube Ethylene diamine tetra-acetic acid (EDTA). The grey top tubes were used to measure blood glucose concentrations, while the plain top tubes were used to obtain serum for miRNA extraction and serological tests such as serum cotinine, insulin and lipid profile. The purple capped-tubes were used to measure HbA1c.

2.6.2.7. The oral glucose tolerance test (OGTT)

All participants, excluding the self-reported diabetic subjects (confirmed by either participant medical card record or drug use), underwent the oral glucose tolerance test (OGTT). Subjects were asked to fast overnight where after the OGTT was conducted according to WHO guidelines (WHO, 1999) as follows: i) investigators asked participants whether they had fasted, ii) collected fasting blood samples, iii) gave participants 75 grams of anhydrous glucose dissolved in 250 - 300ml of water, which was drunk within 3 - 5 min, and the time recorded, iv) collected a second blood sample after 2 hours (2h-PG). Phlebotomy was conducted by trained nurses.

2.7. Biochemical data

Biochemical measurements including fasting plasma glucose (FPG) and 2h-PG plasma concerntrations, glycosylated haemoglobin (HbA1c), serum insulin, triglycerides, cholesterol, gamma glutamyl-transferase (GGT) enzyme and cotinine were conducted as described previously (Matsha et al., 2012). Plasma glucose concentrations were measured using the hexokinase method (Cobas 6000, Roche Diagnostics; Mannheim, Germany). The HbA1c was measured using the turbidimetric inhibition immunoassay (Cobas 6000, Roche Diagnostics). This method is certified by the National Glycohemoglobin Standardization Programme (NGSP) according to Roche Diagnostics. High density lipoproteins (HDL), gamma glutamyl-transferase (GGT) and triglycerides were estimated using enzymatic colorimetric methods (Cobas 6000, Roche Diagnostics). Low density lipoproteins (LDL) were calculated using the Friedwald's formula (Friedewald et al., 1972). C-reactive protein (CRP) concentration was measured with a Beckman nephelometer auto-analyzer (Beckman, South Africa). Insulin was measured using a microparticle enzyme immunoassay (Axsym, Abbot, Princeton, USA). Serum cotinine was measured using a chemiluminescent assay (Immulite 1000, Siemens).

2.8. Quality control procedures

The study was conducted adhering to strict standard operating procedures (SOPs). Field workers and nurses involved in the study were briefed on how to take blood pressure, blood collection, anthropometric measurements and questionnaire administration. Researchers were required to sign off on questionnaires, blood pressure and collection, as well as anthropometric measurements that they conducted in order to trace and verify measurements and data entries. Spot field checks were also conducted by project supervisors in order to verify the accuracy of measurements and enhance quality control (Adams et al., 2002). Equipments were routinely calibrated according to the manufacturer's instructions. Scales were routinely checked for accuracy using standard weights. The work-load of all staff involved in the study was kept within acceptable limits in order to avoid false measurements due to staff fatigue.

Each team had a supervisor who was responsible for monitoring performance and who was also responsible for equipment calibration. Weekly meetings were held to assess progress, problem-solving, and retraining if necessary.

2.9. Sample for the study

Blood samples for this study were collected from mixed ancestry participants from Ward 009, Cape Town, South Africa, from 2014 to 2016. For the extraction of circulating miRNAs, whole blood samples were collected from participants in BD Vacutainer (SST), subjected to centrifugation at 1900 × g (3000rpm) for 10 minutes at room temperature. Serum aliquots were stored in RNAse free freezing vials at -80ºC, to be used for the extraction of circulating miRNAs in this study. After which a quantitative analysis of the circulating miRNA present in the various samples were ascertained using Real Time Polymerase Chain Reaction as follows.

2.10. MicroRNA extraction/purification

Frozen serum samples which were stored at -80^{0}C were thawed and used for miRNA extraction. The QiagenmiRNeasy Serum/Plasma Kit (Whitehead Scientific, Cape Town) was used for the extraction:

> 1000 µl of QIAzolLysis Reagent Mix was added to 200µl serum samples. Samples were vortexed and incubated at room temperature (15–25°C) for 5min. 3.5µl miRNeasy Serum Spike-In Control (1.6 x 108copies/µl working solution) was added and mixed thoroughly. Then 200µl Chloroform was added to the tube containing the lysate and capped securely. The samples were then vortexed for 15s and incubated at room temperature (15–25°C) for 2–3min. Furthermore, samples were centrifuged for 15min at 12,000 x g at 4°C. After centrifugation, the centrifuge was heated up to room temperature (15–25°C). The upper aqueous phase was then transferred to a new collection tube. 300µl of 100% ethanol was added and mixed thoroughly by pipetting up and down several times. Immediately 700µl of the samples was pipetted, including any precipitate that may have formed, into an RNeasyMinElute spin column in a 2 ml collection tube.
>
> The lid was gently closed and centrifuged at 8000xg (10,000 rpm) for 15s at room temperature (15–25°C) and the flow-through was discarded. This step was repeated with the remaining sample.700µl Buffer RWT was added to the RNeasyMinElute spin column and centrifuged for 15s at 8000xg (10,000 rpm) to wash the column and the flow-through discarded. Then 500µl buffer RPE was pipetted onto the RNeasyMinElute spin column and centrifuged for 15s at 8000xg (10,000 rpm) to wash the column and the flow-through was discarded. 500µl of 80% ethanol was pipette onto the RNeasyMinElute spin column and centrifuged for 2min at 8000xg (10,000rpm) to wash

the spin column membrane. The collection tube with the flow through were discarded. The RNeasyMinElute spin column was then placed into a new 2ml collection tube, the spin column was left open while centrifuging at full speed for 5min to dry the membrane. The collection tube and flow-through were then discarded and RNeasyMinElute spin column was placed in a new 1.5ml collection tube and 14µl RNase-free water was added directly to the center of the spin column membrane and centrifuged for 1min at full speed to elute the miRNA. Elute contained extracted miRNA as seen in Figure 2.1 below. Extracted miRNA was then reverse-transcribed to obtain cDNA for RT-PCR.

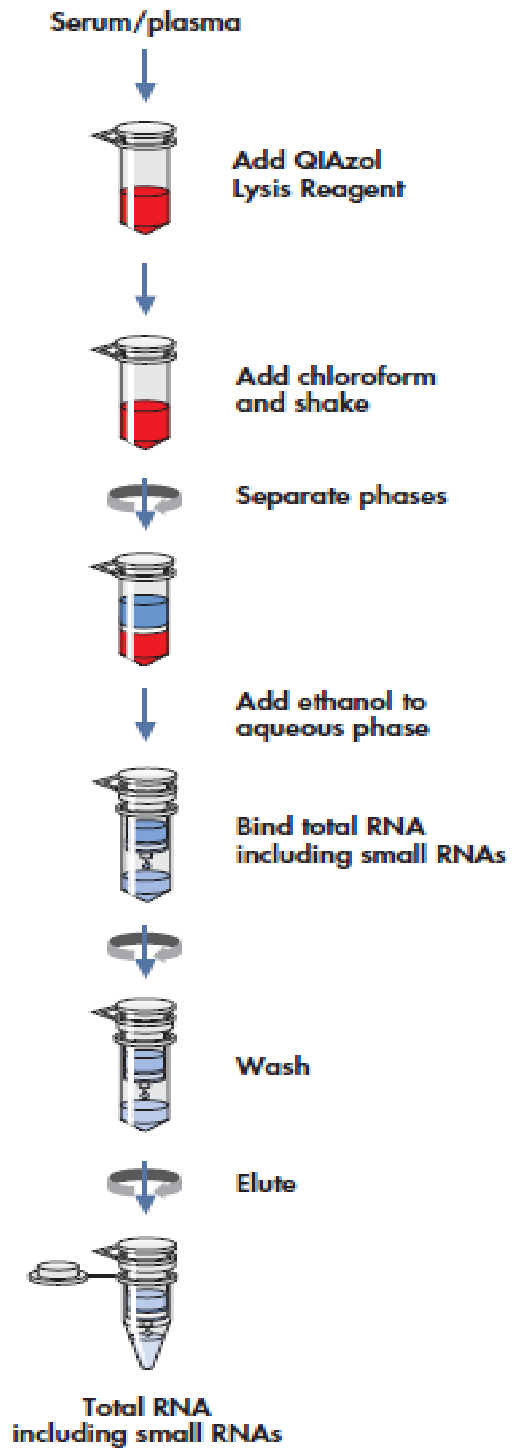


Fig 2.1: Micro RNA Extraction/Purification (Adapted from Qiagen miRNeasy Serum/Plasma 2014 Manual, ThermoFisher Scientific, Massachusetts, USA)

2.11. Reverse transcription (cDNA synthesis)

The cDNA synthesis was done using the Qiagen RT^2 First Strand Kit (ThermoFisher Scientific). The reagents of the RT2 First Strand Kit were thawed and briefly centrifuged (10–15s) to bring the contents to the bottom of the tubes. The genomic DNA elimination mix was prepared for each RNA sample and mixed gently by pipetting up and down and then briefly centrifuged (samples were amplified in singletons). It was incubated for 5min at 42°C, then immediately placed on ice for at least 1min. The reverse-transcription mix was prepared and 10µl of it was added to each tube containing 10µl genomic DNA elimination mix and mixed gently by pipetting up and down. Mixture was incubated at 42°C for 15min and reaction was stopped by immediately incubating at 95°C for 5min using BIO RAD (T100THThermal Cycler). cDNA concentrations and purity were then analyzed using nano-drop machine and the average purity of samples were 1.8 for the 260/280 OD and 2.0 for the 260/230 OD. Dilutions of cDNA yielded were made at (3ng of cDNA in 300µl of nuclease free water). Diluted samples were then stored at the -20°C prior to real-time PCR.

2.12. Real time polymerase chain reaction

The Qiagen miScript SYBR Green PCR kit (ThermoFisher Scientific) was used with miScript miRNA PCR Arrays for the real time polymerase chain reaction. Prior to preparing real-time PCR reaction mixes all reagents (2x QuantiTectRSYBR Green PCR Master Mix, 10x miScript Universal Primer and RNase-free water) (ThermoFisher Scientific) were allowed to thaw. The master mix was prepared as seen on table 2.1.

Table 2.1: Contents of PCR Master Mix

Array Components	**96 wells**
2x QuantiTect SYBR Green PCR Master Mix	1375 µl
10x miScript Universal Primer	275 µl
RNase-free water	1000 µl
Template cDNA	100 µl
Total volume	**2750 µl**

The array was removed from its sealed bag, and 25µl reaction mix added per well and tightly sealed with caps which were then centrifuged for 1min at 1000x to bring content to the bottom and to take out all the bubbles. Array plates were placed into the QUANT STUDIO 7 machine for the RT-PCR. Initial activation and pre-heating step was done at 95^{0}C for 15 min to activate

the HotStarTaq DNA Polymerase, followed by the Real Time PCR with cycle conditions as seen below.

3-step cycling; Denaturation 15s at 94ºC
Annealing 30s at 55 ºC
Extension 30 s at 70ºC, 40 cycles were done for all miRNAs

2.13. Differential expression miRNAs

Differential expression of the miRNAs was done by quantifying amplified cDNA as follows;
At the end of the 40 cycles of the RT-PCR, the cycle threshold values (CT values) of each miRNA were read from 1 and above. All CT values greater than 35 or N/A (not detected /undetermined) were reported as 35. At this point, any CT value equal to 35 was considered a negative call.

The ΔCT for each pathway-focused gene in each plate was calculated using the CT values for the gene of interest (GOI) and the housekeeping genes (HKG) used for normalization using the formula: $\Delta CT = CTGOI - CTAVG\ HKG$.
When biological experiments were performed, the average ΔCT value of each gene (each well) was calculated across the replicate arrays for each treatment group.

The ΔΔCT for each gene across 2 RT^2 Profiler PCR Arrays (or groups of samples) was calculated using the formula: $\Delta\Delta CT = \Delta CT\ (group\ 2) - \Delta CT\ (group\ 1)$, where group 1 was group of control samples and group 2 was group of experimental samples.
The fold-change for each gene from group 1 to group 2 was calculated as: $2^{(-\Delta\Delta CT)}$.

The results were reported as; if the fold-change was greater than 1, the result was reported as a fold up-regulation. If the fold-change was less than 1, the negative inverse of the result was reported as a fold down-regulation. Fold-change ratio calculations were unreliable when raw CT values from both groups were greater than 35.

2.14. Statistical analysis

Statistical analysis of circulating miRNA data was performed using STATISTICA 13 (StatSoft Inc., Tulsa, USA). The selected samples were age, gender and BMI matched. The mean ± SD (used for clinical parameters apart from glucose tests) and where applicable the median ± range (used for glucose tests) were used to establish differences between groups, with a p-value of <0.05 considered to be statistically significant, using ANOVA and the Kruskal-Wallis test respectively. Correlations were done using the Spearman Rank Order Correlations (r).

CHAPTER 3: RESULTS

3.1. The clinical characteristics of participants

The clinical characteristics of the study participants and other biochemical parameters according to glucose tolerance status are summarized in Table 3.1: The participants for this study were chosen from the 2014-2016 study with a total of 1989 participants. Participants were both male and female, with the female population more than the males. Only females were involved in this study and they were age and BMI matched. They were chosen chronologically from the first participants to the number needed for this study and according to their glycemic status' [control, pre-diabetes (preDM) and diabetes (DM)]. Serum from 36 female participants selected was used as the sample for the study. Participants were evenly distributed amongst the three groups, that is, 12 participants per group.

There were significant differences between groups in the waist (cm) ($p=0.0415$) and waist/hip ratio ($p=0.0011$) with highest values in the DM group and lowest in the normal group. Clinical parameters varied significantly according to glycemic status. As expected, the FBG (mmol/L) ($p<0.0001$), 2 HRs Post Glucose (mmol/L) ($p<0.0001$), HbA1c (%) ($p=0.0009$), Fasting Insulin (mIU/L) ($p=0.0039$), were all highest in the DM and lowest in the control group. In contrast, the 2 HRs Post Insulin (mIU/L) ($p = 0.0027$) was highest in the pre-DM group and lowest in the normal group, while the Glucose/Insulin ratio ($p=0.0477$) was highest in the normal group and lowest in the pre-DM group. Trigs (mmol/L) ($p=0.0043$) and Total Chol (mmol/L) ($p=0.0429$) were significantly increased throughout the three groups, with highest values in the DM group and lowest in the normal group.

Table 3.1: Clinical characteristics of participants

	Total, N36	Normal, N12	PreDM, N12	DM, N12	
	Mean±SD [Median (25Q; 75Q)]*				P-value
Age (years)	53.4±7.8	52.1±7.8	53.5±8.5	54.8±7.5	0.7171
BMI	31.5±8.4	27.3±5.8	33.3±9.1	33.5±8.9	0.1410
Waist circumference (cm)	93.9±18.7	83.2±18.5	97.1±13.8	101.3±19.7	**0.0415**
Hip circumference (cm)	107.1±17.7	102.0±18.4	109.9±18.6	109.4±16.6	0.4875
WHR (waist/hip ratio)	0.87±0.08	0.81±0.07	0.89±0.06	0.92±0.07	**0.0011**
SBP (mmHg)	138.4±27.5	135.3±30.8	137.0±18.5	142.9±32.9	0.7843
DBP (mmHg)	87.0±18.0	78.0±16.1	88.9±11.7	94.2±22.1	0.0768
Pulse (bpm)	72.1±13.5	64.8±12.2	71.3±5.8	80.1±16.4	**0.0163**
FBG (mmol/L)*	5.40 (4.80; 6.35)	4.75 (4.25; 4.90)	5.30 (4.95; 5.75)	7.90 (6.25; 11.15)	**<0.0001**
2 HRs Post Glucose (mmol/L)*	9.00 (7.20; 13.30)	5.20 (4.00; 7.20)	9.00 (8.55; 10.00)	15.10 (12.90; 19.50)	**<0.0001**
HbA1c (%)*	6.00 (5.45; 6.65)	5.55 (5.20; 5.75)	6.00 (5.35; 6.50)	7.05 (6.45; 9.15)	**0.0009**
Fasting Insulin (mIU/L)*	8.10 (5.70; 12.50)	5.65 (2.95; 7.85)	9.45 (7.55; 12.50)	14.10 (5.90; 21.20)	**0.0039**
2 HRs Post Insulin (mIU/L)*	53.20 (29.20; 97.90)	28.50 (17.80; 40.40)	111.20 (60.20; 187.20)	48.80 (31.45; 83.30)	**0.0027**
Glucose/Insulin ratio*	0.70 (0.40; 0.90)	0.90 (0.65; 1.60)	0.60 (0.40; 0.70)	0.70 (0.40; 1.50)	**0.0477**
Trigs (mmol/L)*	1.40 (0.94; 1.72)	0.94 (0.73; 1.26)	1.50 (1.11; 1.90)	1.72 (1.40; 2.84)	**0.0043**
LDL-Chol (mmol/L)	3.64±0.98	3.30±0.96	3.46±0.96	4.18±0.84	0.0602
HDL-Chol (mmol/L)	1.42±0.50	1.57±0.50	1.32±0.41	1.38±0.58	0.4514
Total Chol (mmol/L)	5.75±1.05	5.51±1.10	5.39±0.98	6.36±0.85	**0.0429**
Chol/HDL ratio	4.35±1.38	3.73±1.16	4.35±1.35	5.02±1.42	0.0786
CRP (mg/L)	7.84±8.56	3.12±2.73	9.28±5.93	11.11±12.34	0.0520
Cotinine (ng/mL)	129.8±144.1	131.8±150.9	130.2±144.7	127.4±149.5	0.9974
Smokers (even no's selected)	18/36	6/12	6/12	6/12	Not applicable
Gamma GT-S (IU/L)	53.6±56.4	39.8±63.3	56.4±49.9	64.5±57.4	0.5623
Drinkers, Yes %(N)	19.4% (7/36)	33.3% (4/12)	8.3% (1/12)	16.7% (2/12)	0.2890

Parameters with (*) indicate median, while the means have no (*) attached
Bold values indicate statistical significance ($p < 0.05$)

3.2. Micro RNAs expressed in current study population

In this study, a total of 84 miRNAs were used to evaluate the relationship between glucose intolerance and the circulating miRNAs in serum. A total of 54 miRNAs were expressed of which; 12 were expressed in all three groups (control, preDM and DM groups), 3 were expressed in two groups (control and DM groups) and 39 expressed in only one group (DM group) and are summarised in Table 3.2.

Table 3.2: MicroRNAs expressed

MicroRNAs expressed in all groups	MicroRNAs expressed in normal and DM groups (not in PreDM)	MicroRNAs expressed in DM group only
let-7i-5p	miR-324-5p	miR-103a-3p, miR-125a-5p, miR-129-2-3p, miR-130a-3p,
miR-125b-5p	miR-34c-5p	miR-130b-3p, miR-135b-5p, miR-152-3p, miR-184,
miR-126b-5p	miR-490-3p	miR-194-5p, miR-195-5p, miR-196-5p, miR-199a-5p,
miR-129b-5p	-	miR-19a-3p, miR-206, miR-212-3p,
miR-143-3p	-	miR-214-3p, miR-24-3p, miR-27a-3p,
miR-146a-5p	-	miR-27b-3p, miR-296-5p, miR-29b-3p,
miR-200a-3p	-	miR-29c-3p, miR-30a-5p, miR-30c-5p,
miR-330-5p	-	miR-320b, miR-324-3p, miR-335-5p,
miR-382-5p	-	miR-34a-5p, miR-361-5p, miR-370-3p,
miR-451a	-	miR-375, miR-377-3p, miR-380-5p,
miR-542-3p	-	miR-381-3p, miR-424-5p, miR-433-3p,
miR-99b-5p	-	miR-4458, miR-4500, miR-7-5p, miR-96-5

3.3. Correlations

3.3.1. Summary of correlations between expressed miRNAs and the clinical parameters are seen below

These results were presented in 3 separate tables (tables 3.3, 3.4 and 3.5) for convenience because of the large number of variables:

Correlation with anthropometric measurements (Table 3.3): Age (years) (r=-0.07928; p=0.0334) was significantly inversely correlated with miR-324-3p. The BMI was inversely correlated with miR-135-5p (r=-0.9000; p=0.0374) and miR-206 (r=-0.8286; p=0.0416). The waist (cm) was inversely correlated with miR-212-3p (r=-1.000; p<0.0001), miR-335-5p (r=-0.8117; p=0.0499), miR-381-3p (r= -0.7928; p=0.0334) and miR-7-5p (r= -1.000; p<0.0001). The hip (cm) correlated inversely with miR-206 (r=-0.8117; p=0.0499) and miR-7-5p (r=-0.9747; p=0.0048), while the WHR were negatively correlated with miR-324-3p (r=-0.9370; p=0.0019), miR-4458 (r=-0.8697; p=0.0244) and miR-96-5p (r=-0.8697; p=0.0244) but positively with miR-214-3p (r=0.7857; p=0.0362) and miR-308-5p (r=0.7857; p=0.0362).

Correlations with FBG (mmol/L): The parameters used to determine the glycaemic status were correlated with the expressed miRNAs. There were significant inverse correlations between FBG and miR-130b-3p (r=-0.8469; p=0.0162), miR-146a-5p (r=-0.4595; p=0.0361), miR-200a-3p (r=-0.4622; p=0.0402), miR-330-5p (r=-0.4330; p=0.0271), miR-335-5p (r=-0.8117; p=0.0499), miR-381-3p (r=-0.7748;p=0.0408), miR-4458 (r=-0.8117; p=0.0499) and miR-96-5p (r=-0.8117; p=0.0499).

Correlations with 2 HRs Post Glucose (mmol/L): The 2 HRs Post Glucose showed inverse correlations with let-7i-5p (r=-0.4287; p=0.0366), miR-130b-3p (r=-0.9550; p=0.0008), miR-146a-5p (r=-0.4902; p=0.0282), miR-200a-3p (r=-0.5667; p=0.0114), miR-330-5p (r=-0.4500; p=0.0240), miR-4458 (r=-0.8117; p=0.0499), miR-490-3p (r=-0.7909; p=0.0037) and miR-96-5p (r=-0.8117; p=0.0499).

Correlations with HbA1C (%): The HbA1C showed inverse correlations with miR-130b-3p (r=-0.9550; p= 0.0008), miR-29b-3p (r=-0.7748; p=0.0408), miR-330-5p (r=-0.4753; p=0.0141), miR-335-5p (r=-0.8117; p=0.0499), miR-382-5p (r=-0.4003; p=0.0156), miR-4458 (r=-0.8117; p=0.0499), miR-490-3p (r=-0.6105; p=0.0461) and miR-96-5p (r=-0.8117; p=0.0499).

Correlations with Fasting insulin (mIU/L): The Fasting insulin was inversely correlated with miR-135b-5p (r =-1.000; p<0.0001) and miR-424-5p (r=-0.8407; p=0.0361).

Correlations with 2 HRs Post insulin (mIU/L): The 2 Hrs Post insulin was inversely correlated with miR-184 (r=-0.8117; p=0.0499) and positively correlated with miR-125b-5p (r=0.4687; p=0.0209), miR-129-5p (r=0.5842; p=0.0086), miR-195-5p (r=0.8571; p=0.0137), miR-375 (r=0.7857; p= 0.0362), miR-377-3p (r=0.7545; p=0.0305), miR-4458 (r=0.9276; p=0.0077) and miR-96-5p (r=0.9276; p=0.0077).

Summarily, the miRNAs involved in multiple glycaemic parameters were; miR-130b-3p, miR-330-5p, miR-96-5p as well as miR-4458 were inversely correlated with FBG, 2HRs blood glucose Post Glucose and HbA1C, while, miR-330-5pmiR-96-5p correlated positively with 2 HRs Post Insulin. MiR-146a-5pas well as miR-200a-3p was inversely correlated with FBG and 2 HRs Post Glucose, while miR-335-5p was inversely correlated with FBG and HbA1c. MiR-490-3p was inversely correlated with 2 HRs Post Glucose and HbA1c.

Correlation of miRNAs with lipid profile (Table 3.5): Summarized significant correlations between the lipid test results and the expressed miRNAs. Trigs (mmol/L) (Table 3.4) was positively correlated with miR-129-2-3p (r=0.8117; p=0.0499) and inversely correlated with miR-29c-3p (r=-1.000; p<0.0001).

HDL-Chol (mmol/L) (Table 3.5) was inversely correlated with miR-125b-5p (r=-0.4299; p=0.0284). There was no correlation between Total Chol (mmol/L) and any of the expressed miRNAs, while the Chol/HDL ratio was positively correlated with miR-30c-5p (r=0.9747; p=0.0048).

Correlation of miRNAs with other pathophysiological markers (Table 3.5), the test for inflammation was positively correlated with miR-125b-5p (r=0.4632; p=0.0172) and inversely correlated with miR-206(r=-0.8286; p=0.0416), miR-212-3p (r=-0.9000; p=0.0374), miR-27b-3p (r=-0.8286; p=0.0416), miR-335-5p (r=-0.8117; p=0.0499), miR-381-3p (r=-0.8469; p=0.0162) and miR-7-5p (r=-1.000; p<0.0001). GGT showed statistically inverse correlation with miR-99b-5p (r=-0.3908; p<0.0203)

Summarily miRNAs involved with glycemic and inflammatory status were; miR-381-3p and miR-335-5p were also significantly inversely correlated with CRP, FBG and/or HbA1c

3.4. MicroRNAs fold changes in relation to clinical parameters

Fold change is a measure describing how much a quantity changes, hence it compares between two levels of an entity being measured. In this study the fold change was used to describe the relationship between the miRNAs expressed and the glycemic states (control

group/DM group, control/preDM and preDM/DM). A fold change equal to 1 implies no change, fold changes greater than 1 implies up-regulation and fold changes less than 1 implies down-regulation. Table 4.1 shows a summary of fold changes of miRNAs that were expressed across all groups and those expressed just in two of the groups. The Ct values were the primary values obtained at the end of the RT-PCR cycles, and they indicated how much miRNA was able to produce fluorescence at a particular threshold. Lower Ct values implied more miRNAs were available to produce fluorescence at that threshold cycle reading.

The Ct values were further used to calculate the fold changes of the miRNAs amongst the various groups being investigated in relation to the control group. Figure 4.1 demonstrates the fold changes of the 12 miRNAs expressed through all groups. As demonstrated in Figure 4.1 shows let-7i-5p was up regulated in DM and preDM versus control. Let-7i-5p was found to be expressed more in preDM than in DM. Some miRNAs were expressed more in the controls than in preDM, but were expressed more in DM than in the former states. These miRNAs include; miR-125b-5p, miR-129b-5p, miR-146a-5p, miR-200a-3p, miR-451a and miR-542-3p. Interestingly, miR-126b-5p showed a consistent increase through all three groups; from the control to the preDM and then the DM group. MiR-143-3p, miR-330-5p and miR-382-5p were found to be expressed equally in both control and preDM but up-regulated in DM.
Finally, miR-99b-5p was found to be equally expressed through all three groups.

Table 3.3: Correlations of Clinical Characteristics and expressed miRNAs

		Age (years)		BMI		Waist (cm)		Hip (cm)		WHR		SBP (mmHg)		DBP (mmHg)	
MiRNA	**Number**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**
let-7i-5p	25	-0.1090	0.6041	0.0096	0.9646	-0.176	0.3995	-0.0835	0.6915	-0.0615	0.7701	**-0.4252**	**0.0341**	-0.2773	0.1796
miR-103a-3p	7	-0.2883	0.5307	0.0180	0.9694	0.072	0.8780	0.0364	0.9383	0.3604	0.4271	-0.7091	0.0744	-0.1636	0.7259
miR-125a-5p	7	0.6071	0.1482	0.5357	0.2152	0.643	0.1194	0.2523	0.5852	0.7500	0.0522	0.0714	0.8790	0.0000	1.0000
miR-125b-5p	26	-0.0810	0.6939	0.2915	0.1574	0.064	0.7563	0.2282	0.2623	-0.0797	0.6989	-0.0304	0.8826	-0.0359	0.8616
miR-126-5p	20	0.0996	0.6761	0.2579	0.2864	0.141	0.5522	0.1843	0.4367	-0.0060	0.9799	-0.1662	0.4837	-0.0670	0.7791
miR-129-2-3p	6	0.3189	0.5379	0.2029	0.6998	-0.174	0.7417	-0.0588	0.9119	0.0290	0.9565	-0.1176	0.8243	-0.4118	0.4173
miR-129-5p	20	-0.2045	0.3872	0.4346	0.0555	0.158	0.5061	0.3213	0.1672	0.0541	0.8207	0.1828	0.4404	0.2853	0.2228
miR-130a-3p	8	0.0476	0.9108	0.2143	0.6103	0.095	0.8225	-0.2874	0.4900	0.5000	0.2070	-0.0476	0.9108	-0.1190	0.7789
miR-130b-3p	7	-0.1081	0.8175	0.1441	0.7578	-0.234	0.6132	-0.3424	0.4523	-0.2703	0.5577	0.2000	0.6672	0.0545	0.9075
miR-135b-5p	5	-0.2000	0.7471	**-0.9000**	**0.0374**	-0.600	0.2848	-0.8208	0.0886	0.4000	0.5046	-0.4000	0.5046	0.0000	1.0000
miR-143-3p	23	-0.1007	0.6474	0.0582	0.7971	-0.180	0.4116	-0.0722	0.7434	-0.0711	0.7470	-0.3495	0.1021	-0.0668	0.7620
miR-146a-5p	21	-0.0046	0.9843	0.1278	0.5912	-0.095	0.6827	0.0331	0.8866	0.1039	0.6540	-0.2209	0.3359	-0.1985	0.3884
miR-152-3p	9	-0.1255	0.7476	-0.1667	0.6682	0.283	0.4600	0.3096	0.4175	0.0833	0.8312	-0.2167	0.5755	-0.4435	0.2318
miR-184	6	-0.0290	0.9565	-0.1449	0.7841	-0.203	0.6998	-0.1449	0.7841	0.3769	0.4615	0.1176	0.8243	0.4118	0.4173
miR-194-5p	7	-0.0357	0.9394	0.0000	1.0000	0.071	0.8790	-0.1441	0.7578	0.3929	0.3833	-0.3929	0.3833	-0.5357	0.2152
miR-195-5p	7	-0.1071	0.8192	0.4643	0.2939	0.321	0.4821	0.2162	0.6414	0.1429	0.7599	-0.2143	0.6445	-0.6429	0.1194
miR-196-5p	8	0.3333	0.4198	0.5238	0.1827	0.429	0.2894	0.0359	0.9327	0.5476	0.1600	0.0238	0.9554	-0.3571	0.3851
miR-199a-5p	7	0.3929	0.3833	-0.0714	0.8790	0.071	0.8790	-0.2523	0.5852	0.4643	0.2939	-0.3929	0.3833	**-0.9643**	**0.0005**
miR-19a-3p	7	0.2500	0.5887	0.1429	0.7599	0.393	0.3833	0.0721	0.8780	0.5357	0.2152	-0.6429	0.1194	**-0.8214**	**0.0234**
miR-200a-3p	20	0.0460	0.8472	0.1684	0.4907	-0.006	0.9799	0.0918	0.7003	0.0556	0.8158	0.0241	0.9198	0.0203	0.9322
miR-206	6	0.0286	0.9572	**-0.8286**	**0.0416**	-0.771	0.0724	**-0.8117**	**0.0499**	0.2571	0.6228	-0.2571	0.6228	-0.0286	0.9572
miR-212-3p	5	-0.7000	0.1881	-0.6000	0.2848	**-1.000**	**0.0001**	-0.8721	0.0539	-0.4000	0.5046	-0.3000	0.6238	0.0000	1.0000
miR-214-3p	7	0.5357	0.2152	0.2500	0.5887	0.393	0.3833	-0.2500	0.5887	**0.7857**	**0.0362**	0.1071	0.8192	-0.2857	0.5345
miR-24-3p	7	0.3571	0.4316	0.6786	0.0938	0.607	0.1482	0.4865	0.2682	0.3214	0.4821	0.1071	0.8192	-0.6786	0.0938
miR-27a-3p	5	-0.3000	0.6238	0.1000	0.8729	0.300	0.6238	0.1000	0.8729	0.0000	1.0000	-0.3000	0.6238	-0.8000	0.1041
miR-27b-3p	6	0.2571	0.6228	-0.3714	0.4685	-0.4286	0.3965	-0.6088	0.1997	0.3714	0.4685	-0.2000	0.7040	**-0.8286**	**0.0416**
miR-296-5p	8	0.3114	0.4528	-0.2395	0.5678	-0.1078	0.7995	-0.3735	0.3621	0.2275	0.5878	-0.2289	0.5855	**-0.8795**	**0.0040**
miR-29b-3p	7	0.5045	0.2482	0.1802	0.6990	0.0541	0.9084	-0.3063	0.5040	0.0901	0.8477	0.091	0.8463	-0.2364	0.6099
miR-29c-3p	5	0.4000	0.5046	0.4000	0.5046	0.4000	0.5046	-0.1000	0.8729	0.7000	0.1881	-0.400	0.5046	-0.3000	0.6238
miR-30a-5p	6	0.3189	0.5379	0.0290	0.9565	0.3189	0.5379	0.0290	0.9565	0.4928	0.3206	**-0.941**	**0.0051**	**-0.8235**	**0.0440**
miR-30c-5p	6	0.0290	0.9565	-0.0290	0.9565	0.2029	0.6998	-0.0290	0.9565	0.3189	0.5379	**-1.000**	**0.0001**	-0.7647	0.0765
miR-320b	6	0.0870	0.8699	-0.6667	0.1481	-0.4928	0.3206	-0.6667	0.1481	0.0290	0.9565	-0.412	0.4173	-0.7647	0.0765
miR-324-3p	7	**-0.7928**	**0.0334**	-0.2883	0.5307	-0.4324	0.3325	-0.0364	0.9383	**-0.9370**	**0.0019**	-0.091	0.8463	-0.2364	0.6099
miR-324-5p	13	0.1407	0.6466	0.3147	0.3191	0.1484	0.6286	0.0358	0.9077	0.2253	0.4593	-0.115	0.7074	-0.1926	0.5285
miR-330-5p	26	-0.0069	0.9735	-0.0577	0.7841	-0.1713	0.4028	-0.0964	0.6393	-0.1610	0.4320	-0.081	0.6950	-0.2693	0.1833
miR-335-5p	6	-0.2609	0.6175	-0.5508	0.2574	**-0.8117**	**0.0499**	-0.7941	0.0592	-0.0580	0.9131	-0.529	0.2801	**-0.9412**	**0.0051**

miR-34a-5p	8	0.2857	0.4927	0.6667	0.0710	0.3571	0.3851	0.1437	0.7342	0.2619	0.5309	0.024	0.9554	-0.2381	0.5702
miR-34c-5p	13	0.1022	0.7397	0.3636	0.2453	0.0385	0.9007	0.1515	0.6212	-0.0165	0.9574	-0.085	0.7818	-0.220	0.4700
miR-361-5p	5	0.3000	0.6238	-0.1000	0.8729	0.3000	0.6238	-0.1000	0.8729	0.6000	0.2848	-0.800	0.1041	**-1.000**	**<0.0001**
miR-370-3p	6	-0.0290	0.9565	0.2609	0.6175	-0.1160	0.8268	0.0294	0.9559	0.0580	0.9131	-0.294	0.5715	-0.118	0.8243
miR-375	7	0.2857	0.5345	0.5000	0.2532	0.3571	0.4316	0.2703	0.5577	0.2143	0.6445	0.214	0.6445	-0.607	0.1482
miR-377-3p	8	0.2515	0.5479	0.5270	0.1796	0.1796	0.6703	0.2289	0.5855	0.0120	0.9775	0.216	0.6081	-0.331	0.4227
miR-380-5p	7	0.7143	0.0713	0.3214	0.4821	0.5000	0.2532	-0.1982	0.6701	**0.7857**	**0.0362**	0.000	1.0000	-0.393	0.3833
miR-381-3p	7	-0.6847	0.0897	-0.6487	0.1150	**-0.7928**	**0.0334**	-0.5273	0.2239	-0.6847	0.0897	-0.418	0.3505	-0.745	0.0544
miR-382-5p	36	-0.2623	0.1223	-0.1213	0.4876	-0.1740	0.3101	-0.0417	0.8092	-0.2525	0.1373	0.121	0.4818	0.107	0.5348
miR-424-5p	6	-0.0290	0.9565	-0.4928	0.3206	-0.5508	0.2574	-0.4928	0.3206	0.1449	0.7841	0.294	0.5715	0.235	0.6536
miR-433-3p	8	0.4551	0.2572	-0.1916	0.6494	0.0120	0.9775	-0.2892	0.4873	0.3234	0.4346	-0.205	0.6266	**-0.904**	**0.0021**
miR-4458	6	-0.4928	0.3206	-0.1449	0.7841	-0.5218	0.2883	-0.4706	0.3462	**-0.8697**	**0.0244**	0.118	0.8243	-0.353	0.4926
miR-4500	5	0.4000	0.5046	0.2000	0.7471	0.4000	0.5046	0.2000	0.7471	0.3000	0.6238	0.100	0.8729	-0.500	0.3910
miR-451a	27	-0.2171	0.2768	0.0126	0.9511	-0.020	0.9230	0.0076	0.9699	0.1258	0.5319	0.046	0.8215	0.276	0.1635
miR-490-3p	11	-0.3235	0.3319	0.3909	0.2345	-0.055	0.8734	0.1458	0.6689	-0.5364	0.0890	-0.109	0.7490	-0.419	0.1994
miR-542-3p	32	-0.0136	0.9411	0.0319	0.8649	-0.063	0.7302	0.0202	0.9128	-0.0532	0.7726	0.0479	0.7947	-0.030	0.8693
miR-7-5p	5	0.1000	0.8729	-0.7000	0.1881	**-1.000**	**0.0001**	**-0.9747**	**0.0048**	0.3000	0.6238	-0.3000	0.6238	-0.700	0.1881
miR-96-5p	6	-0.4928	0.3206	-0.1449	0.7841	-0.522	0.2883	-0.4706	0.3462	**-0.8697**	**0.0244**	0.1176	0.8243	-0.353	0.4926
miR-99b-5p	35	-0.0058	0.9738	0.3036	0.0809	0.277	0.1066	0.2988	0.0812	-0.0022	0.9898	0.1521	0.3831	0.085	0.6258

Table 3.4: Correlations of Clinical Characteristics and expressed miRNAs

		FBG (mmol/L)		2 HRs Post Glucose (mmol/L)		HbA1c (%)		Fasting Insulin (mIU/L)		2 HRs Post Insulin (mIU/L)		Trigs (mmol/L)	
miRNA	**Number**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**
let-7i-5p	25	-0.2941	0.1535	**-0.4287**	**0.0366**	-0.3079	0.1343	0.011	0.9598	0.1206	0.5837	-0.3555	0.0812
miR-103a-3p	7	0.2703	0.5577	0.1261	0.7876	0.2703	0.5577	-0.342	0.4523	-0.3063	0.5040	-0.6307	0.1289
miR-125a-5p	7	0.5000	0.2532	-0.2857	0.5345	0.0357	0.9394	0.200	0.7040	0.0714	0.8790	-0.7500	0.0522
miR-125b-5p	26	-0.1868	0.3608	-0.2423	0.2432	-0.1760	0.3897	0.369	0.0698	**0.4687**	**0.0209**	0.1611	0.4319
miR-126-5p	20	-0.1995	0.3991	-0.4509	0.0527	-0.2123	0.3687	-0.067	0.7862	0.2012	0.4233	-0.1534	0.5184
miR-129-2-3p	6	-0.1160	0.8268	-0.0580	0.9131	-0.1160	0.8268	0.580	0.2278	0.2319	0.6584	**0.8117**	**0.0499**
miR-129-5p	20	-0.1694	0.4753	-0.2541	0.2796	-0.0836	0.7261	0.428	0.0672	**0.5842**	**0.0086**	-0.0173	0.9423
miR-130a-3p	8	-0.3810	0.3518	-0.6190	0.1017	-0.6905	0.0580	-0.143	0.7599	0.4286	0.2894	-0.5952	0.1195
miR-130b-3p	7	**-0.8469**	**0.0162**	**-0.9550**	**0.0008**	**-0.9550**	**0.0008**	-0.145	0.7841	0.7027	0.0782	-0.5946	0.1591
miR-135b-5p	5	-0.6000	0.2848	-0.1000	0.8729	-0.6000	0.2848	**-1.000**	**0.0001**	-0.5000	0.3910	-0.8000	0.1041
miR-143-3p	23	-0.2731	0.2074	-0.3902	0.0726	-0.2801	0.1956	0.103	0.6470	0.2325	0.3106	-0.1156	0.5993
miR-146a-5p	21	**-0.4595**	**0.0361**	**-0.4902**	**0.0282**	-0.3909	0.0798	0.114	0.6334	0.4053	0.0852	-0.2118	0.3568
miR-152-3p	9	0.1167	0.7650	0.2000	0.6059	0.2167	0.5755	-0.262	0.5309	-0.0333	0.9322	-0.1500	0.7001
miR-184	6	0.4058	0.4247	0.5798	0.2278	0.4058	0.4247	-0.6088	0.1997	**-0.8117**	**0.0499**	-0.2319	0.6584
miR-194-5p	7	-0.0714	0.8790	-0.3214	0.4821	-0.2857	0.5345	0.0357	0.9394	0.5000	0.2532	-0.4286	0.3374
miR-195-5p	7	-0.3214	0.4821	-0.6429	0.1194	-0.4643	0.2939	0.5000	0.2532	**0.8571**	**0.0137**	0.0357	0.9394
miR-196-5p	8	-0.2619	0.5309	-0.6190	0.1017	-0.5952	0.1195	0.3571	0.4316	0.5952	0.1195	-0.2857	0.4927
miR-199a-5p	7	-0.1429	0.7599	-0.0357	0.9394	-0.2857	0.5345	0.0357	0.9394	0.2857	0.5345	0.1429	0.7599
miR-19a-3p	7	0.2143	0.6445	0.0000	1.0000	0.0714	0.8790	0.0714	0.8790	0.1071	0.8192	-0.3929	0.3833
miR-200a-3p	20	**-0.4622**	**0.0402**	**-0.5667**	**0.0114**	-0.4223	0.0636	0.0088	0.9716	0.3540	0.1496	-0.1918	0.4179
miR-206	6	-0.0857	0.8717	0.1429	0.7872	-0.0857	0.8717	-0.7143	0.1108	-0.3714	0.4685	-0.4286	0.3965
miR-212-3p	5	-0.7000	0.1881	-0.5000	0.3910	-0.7000	0.1881	-0.6000	0.2848	0.2000	0.7471	-0.1000	0.8729
miR-214-3p	7	-0.2857	0.5345	-0.3214	0.4821	-0.6786	0.0938	-0.2000	0.7040	0.2500	0.5887	-0.4643	0.2939
miR-24-3p	7	0.0000	1.0000	-0.2857	0.5345	-0.1071	0.8192	0.7500	0.0522	0.7143	0.0713	0.3571	0.4316
miR-27a-3p	5	-0.5000	0.3910	-0.7000	0.1881	-0.7000	0.1881	0.1000	0.8729	0.5000	0.3910	-0.4000	0.5046
miR-27b-3p	6	-0.3714	0.4685	-0.1429	0.7872	-0.6000	0.2080	-0.1429	0.7872	0.4857	0.3287	0.086	0.8717
miR-296-5p	8	-0.3713	0.3652	-0.3234	0.4346	-0.5389	0.1681	0.0120	0.9775	0.5389	0.1681	-0.168	0.6915
miR-29b-3p	7	-0.5946	0.1591	-0.6667	0.1019	**-0.7748**	**0.0408**	0.0290	0.9565	0.5225	0.2289	-0.450	0.3104
miR-29c-3p	5	-0.1000	0.8729	-0.6000	0.2848	-0.5000	0.3910	-0.2000	0.8000	-0.1000	0.8729	**-1.000**	**0.0001**
miR-30a-5p	6	0.2319	0.6584	0.1160	0.8268	0.2319	0.6584	0.0290	0.9565	-0.1160	0.8268	-0.464	0.3542
miR-30c-5p	6	0.1160	0.8268	-0.0580	0.9131	0.1160	0.8268	-0.0870	0.8699	-0.0580	0.9131	-0.638	0.1731
miR-320b	6	-0.6377	0.1731	-0.2899	0.5774	-0.6377	0.1731	-0.6667	0.1481	-0.0580	0.9131	-0.638	0.1731
miR-324-3p	7	-0.4144	0.3553	-0.1261	0.7876	-0.1622	0.7283	0.4638	0.3542	0.5946	0.1591	0.721	0.0676
miR-324-5p	13	-0.2418	0.4262	-0.5245	0.0800	-0.3516	0.2387	0.0140	0.9656	0.2308	0.4705	-0.242	0.4262
miR-330-5p	26	**-0.4330**	**0.0271**	**-0.4500**	**0.0240**	**-0.4753**	**0.0141**	-0.0400	0.8494	0.3226	0.1242	-0.185	0.3655

miR-335-5p	6	**-0.8117**	**0.0499**	-0.5218	0.2883	**-0.8117**	**0.0499**	-0.5218	0.2883	0.3479	0.4993	-0.203	0.6998
miR-34a-5p	8	-0.0952	0.8225	-0.6190	0.1017	-0.4048	0.3199	0.6429	0.1194	0.6429	0.0856	0.000	1.0000
miR-34c-5p	13	-0.3462	0.2466	-0.5245	0.0800	-0.3714	0.2115	0.0629	0.8459	-0.1329	0.6806	-0.302	0.3156
miR-361-5p	5	-0.1000	0.8729	0.0000	1.0000	-0.1000	0.8729	-0.1000	0.8729	-0.1000	0.8729	-0.300	0.6238
miR-370-3p	6	0.1160	0.8268	-0.0580	0.9131	0.1160	0.8268	0.4058	0.4247	0.1160	0.8268	0.3769	0.4615
miR-375	7	-0.2143	0.6445	-0.3571	0.4316	-0.3214	0.4821	0.6429	0.1194	**0.7857**	**0.0362**	0.5000	0.2532
miR-377-3p	8	-0.0240	0.9551	-0.3473	0.3993	-0.1916	0.6494	0.7066	0.0501	**0.7545**	**0.0305**	0.4431	0.2715
miR-380-5p	7	0.3214	0.4821	-0.0714	0.8790	-0.1429	0.7599	0.0286	0.9572	0.0000	1.0000	-0.4643	0.2939
miR-381-3p	7	**-0.7748**	**0.0408**	-0.1261	0.7876	-0.4505	0.3104	-0.2319	0.6584	0.4144	0.3553	0.4685	0.2890
miR-382-5p	36	-0.2504	0.1408	-0.3045	0.0753	**-0.4003**	**0.0156**	-0.1133	0.5169	0.0000	1.0000	-0.1124	0.5140
miR-424-5p	6	-0.1160	0.8268	0.2899	0.5774	-0.1160	0.8268	**-0.8407**	**0.0361**	-0.6377	0.1731	-0.2899	0.5774
miR-433-3p	8	-0.2515	0.5479	-0.2036	0.6287	-0.4192	0.3013	0.0599	0.8880	0.4431	0.2715	-0.2156	0.6081
miR-4458	6	**-0.8117**	**0.0499**	**-0.8117**	**0.0499**	**-0.8117**	**0.0499**	0.2319	0.6584	**0.9276**	**0.0077**	0.6088	0.1997
miR-4500	5	-0.3000	0.6238	0.0000	1.0000	-0.3000	0.6238	0.2000	0.7471	0.2000	0.7471	0.6000	0.2848
miR-451a	27	-0.2233	0.2629	-0.2695	0.1831	-0.2899	0.1424	0.0133	0.9484	0.1423	0.4974	0.0046	0.9819
miR-490-3p	11	-0.5182	0.1025	**-0.7909**	**0.0037**	**-0.6105**	**0.0461**	0.2000	0.5554	0.3576	0.3104	-0.2455	0.4669
miR-542-3p	32	-0.1482	0.4181	-0.2472	0.1800	-0.1857	0.3090	0.0990	0.5962	0.1483	0.4427	-0.0356	0.8467
miR-7-5p	5	-0.7000	0.1881	-0.2000	0.7471	-0.7000	0.1881	-0.4000	0.5046	0.1000	0.8729	0.0000	1.0000
miR-96-5p	6	**-0.8117**	**0.0499**	**-0.8117**	**0.0499**	**-0.8117**	**0.0499**	0.2319	0.6584	**0.9276**	**0.0077**	0.6088	0.1997
miR-99b-5p	35	-0.1033	0.5548	-0.2352	0.1806	-0.0017	0.9923	-0.0918	0.6054	-0.1342	0.4641	-0.0430	0.8062

Table 3.5: Correlations of clinical characteristics and expressed miRNAs

		LDL-Chol (mmol/L)		HDL-Chol (mmol/L)		Total Chol (mmol/L)		Chol/HDL ratio		CRP (mg/L)		Cotinine (ng/mL)		Gamma GT-S (IU/L)	
miRNA	**Number**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**
let-7i-5p	25	-0.3620	0.0753	0.1650	0.4307	-0.3849	0.0574	-0.3722	0.0733	-0.0185	0.9302	-0.1270	0.5451	-0.1302	0.5350
miR-103a-3p	7	0.2883	0.5307	-0.1636	0.7259	0.2883	0.5307	0.5218	0.2883	0.0000	1.0000	-0.3371	0.4597	0.4144	0.3553
miR-125a-5p	7	0.0357	0.9394	-0.7207	0.0676	0.2883	0.5307	0.3714	0.4685	0.4643	0.2939	-0.0891	0.8494	-0.3214	0.4821
miR-125b-5p	26	0.0445	0.8289	**-0.4299**	**0.0284**	-0.0315	0.8787	0.3010	0.1437	**0.4632**	**0.0172**	0.2615	0.1968	-0.0733	0.7221
miR-126-5p	20	-0.1167	0.6242	-0.2555	0.2770	-0.2611	0.2662	0.0897	0.7151	0.1459	0.5395	-0.0900	0.7061	-0.4000	0.0806
miR-129-2-3p	6	0.6957	0.1248	0.1739	0.7417	0.6957	0.1248	0.1160	0.8268	-0.1739	0.7417	0.0000	1.0000	0.2029	0.6998
miR-129-5p	20	-0.0301	0.8997	-0.3132	0.1787	-0.2152	0.3622	0.1185	0.6289	0.4060	0.0757	0.3021	0.1955	-0.0806	0.7355
miR-130a-3p	8	-0.2381	0.5702	-0.4791	0.2297	0.0000	1.0000	0.6071	0.1482	-0.0714	0.8665	-0.2182	0.6036	-0.5000	0.2070
miR-130b-3p	7	-0.3063	0.5040	-0.5455	0.2054	-0.1982	0.6701	0.5798	0.2278	0.1441	0.7578	-0.3371	0.4597	-0.5766	0.1754
miR-135b-5p	5	-0.3000	0.6238	0.5643	0.3217	-0.3000	0.6238	-0.2000	0.8000	-0.7000	0.1881	-0.7071	0.1817	-0.3000	0.6238
miR-143-3p	23	-0.1177	0.5926	-0.2833	0.1902	-0.1933	0.3768	0.0781	0.7299	0.3113	0.1483	0.2501	0.2498	-0.0015	0.9946
miR-146a-5p	21	-0.0825	0.7220	-0.3101	0.1713	-0.1677	0.4675	0.1220	0.6083	0.2519	0.2706	0.2581	0.2586	-0.1028	0.6574
miR-152-3p	9	0.2500	0.5165	0.2762	0.4720	0.2678	0.4860	-0.0714	0.8665	-0.6167	0.0769	-0.2079	0.5914	0.1667	0.6682
miR-184	6	-0.1449	0.7841	0.3529	0.4926	-0.1449	0.7841	-0.5643	0.3217	-0.1449	0.7841	-0.0171	0.9743	-0.1449	0.7841
miR-194-5p	7	0.2857	0.5345	-0.3604	0.4271	0.3964	0.3786	0.7143	0.1108	-0.4286	0.3374	-0.1336	0.7752	0.0357	0.9394
miR-195-5p	7	0.0357	0.9394	-0.6847	0.0897	0.1081	0.8175	0.7143	0.1108	-0.0357	0.9394	0.2227	0.6312	0.2500	0.5887
miR-196-5p	8	-0.1905	0.6514	-0.6467	0.0831	0.0120	0.9775	0.5714	0.1802	0.2143	0.6103	-0.0779	0.8545	-0.2143	0.6103
miR-199a-5p	7	0.2143	0.6445	0.0360	0.9389	0.2883	0.5307	0.1429	0.7872	-0.2857	0.5345	-0.4009	0.3728	0.3214	0.4821
miR-19a-3p	7	0.2857	0.5345	-0.3424	0.4523	0.3604	0.4271	0.6000	0.2080	0.1429	0.7599	-0.4009	0.3728	0.6429	0.1194
miR-200a-3p	20	-0.0632	0.7911	-0.1379	0.5620	-0.1783	0.4519	-0.0229	0.9260	0.0902	0.7052	0.0772	0.7462	-0.2829	0.2268
miR-206	6	0.5429	0.2657	0.7247	0.1032	0.5429	0.2657	-0.3000	0.6238	**-0.8286**	**0.0416**	-0.6547	0.1583	-0.4286	0.3965
miR-212-3p	5	0.3000	0.6238	0.6000	0.2848	0.3000	0.6238	0.2000	0.8000	**-0.9000**	**0.0374**	-0.3536	0.5594	-0.4000	0.5046
miR-214-3p	7	-0.6071	0.1482	-0.4324	0.3325	-0.3063	0.5040	0.2000	0.7040	0.0714	0.8790	-0.2673	0.5623	-0.6429	0.1194
miR-24-3p	7	-0.0357	0.9394	-0.6667	0.1019	0.0180	0.9694	0.4286	0.3965	0.2500	0.5887	0.4009	0.3728	0.2857	0.5345
miR-27a-3p	5	-0.3000	0.6238	-0.4617	0.4338	-0.2052	0.7406	0.8000	0.2000	0.2000	0.7471	-0.1118	0.8579	0.2000	0.7471
miR-27b-3p	6	0.1429	0.7872	0.1160	0.8268	0.2609	0.6175	-0.1000	0.8729	**-0.8286**	**0.0416**	-0.3381	0.5122	-0.2571	0.6228
miR-296-5p	8	0.1317	0.7558	-0.0482	0.9098	0.2169	0.6059	0.4144	0.3553	-0.5389	0.1681	-0.4116	0.3110	-0.2036	0.6287
miR-29b-3p	7	-0.1622	0.7283	-0.2727	0.5540	-0.0180	0.9694	0.3479	0.4993	0.1802	0.6990	-0.5169	0.2349	-0.4324	0.3325
miR-29c-3p	5	0.1000	0.8729	-0.5643	0.3217	0.5000	0.3910	0.8000	0.2000	0.4000	0.5046	-0.7071	0.1817	-0.3000	0.6238
miR-30a-5p	6	0.5508	0.2574	-0.0294	0.9559	0.5508	0.2574	0.8721	0.0539	0.0290	0.9565	-0.6002	0.2078	0.5508	0.2574
miR-30c-5p	6	0.4928	0.3206	-0.1765	0.7380	0.4928	0.3206	**0.9747**	**0.0048**	-0.0290	0.9565	-0.6002	0.2078	0.4928	0.3206
miR-320b	6	-0.2609	0.6175	0.4412	0.3812	-0.2609	0.6175	-0.0513	0.9347	-0.6667	0.1481	-0.7717	0.0722	-0.2609	0.6175
miR-324-3p	7	0.3604	0.4271	0.0727	0.8769	0.1441	0.7578	0.1160	0.8268	-0.4865	0.2682	0.3371	0.4597	0.3604	0.4271
miR-324-5p	13	0.1843	0.5466	-0.1926	0.5285	0.1926	0.5285	0.3328	0.2906	-0.1209	0.6940	-0.3258	0.2774	-0.3439	0.2500
miR-330-5p	26	-0.2387	0.2402	-0.1413	0.4912	-0.2552	0.2083	-0.0146	0.9446	0.1501	0.4643	0.1484	0.4692	-0.0442	0.8302
miR-335-5p	6	0.2319	0.6584	0.4706	0.3462	0.2319	0.6584	0.1539	0.8048	**-0.8117**	**0.0499**	-0.7717	0.0722	-0.0870	0.8699
miR-34a-5p	8	0.2381	0.5702	-0.6108	0.1077	0.3473	0.3993	0.6071	0.1482	0.3810	0.3518	-0.0156	0.9708	0.0476	0.9108

miR-34c-5p	13	0.0824	0.7890	-0.2146	0.4814	0.1018	0.7407	0.0455	0.8883	-0.0330	0.9149	0.0564	0.8548	-0.3246	0.2792
miR-361-5p	5	0.2000	0.7471	0.0513	0.9347	0.2000	0.7471	0.6000	0.4000	-0.1000	0.8729	-0.7071	0.1817	0.6000	0.2848
miR-370-3p	6	0.6957	0.1248	0.0000	1.0000	0.6957	0.1248	0.3591	0.5528	-0.1160	0.8268	0.0514	0.9229	0.3769	0.4615
miR-375	7	-0.0714	0.8790	-0.4685	0.2890	-0.0180	0.9694	0.2571	0.6228	-0.0357	0.9394	0.4009	0.3728	0.0357	0.9394
miR-377-3p	8	0.1078	0.7995	-0.5602	0.1487	0.1386	0.7435	0.4286	0.3374	0.1078	0.7995	0.5351	0.1718	0.1198	0.7776
miR-380-5p	7	0.0000	1.0000	-0.3424	0.4523	0.2883	0.5307	-0.0857	0.8717	0.2143	0.6445	-0.4009	0.3728	-0.2143	0.6445
miR-381-3p	7	0.2523	0.5852	0.4909	0.2633	0.1441	0.7578	-0.1739	0.7417	**-0.8469**	**0.0162**	-0.2023	0.6636	0.2523	0.5852
miR-382-5p	36	-0.2193	0.1988	0.0490	0.7765	-0.1500	0.3825	-0.1525	0.3817	0.0891	0.6055	0.1903	0.2664	0.0057	0.9738
miR-424-5p	6	-0.4928	0.3206	0.5882	0.2194	-0.4928	0.3206	-0.8721	0.0539	-0.4928	0.3206	-0.1886	0.7204	-0.4928	0.3206
miR-433-3p	8	0.0838	0.8435	-0.0723	0.8649	0.1687	0.6897	0.3424	0.4523	-0.4192	0.3013	-0.4116	0.3110	-0.1557	0.7128
miR-4458	6	0.2319	0.6584	-0.0882	0.8680	0.2319	0.6584	0.1539	0.8048	-0.5218	0.2883	0.0171	0.9743	-0.2029	0.6998
miR-4500	5	-0.4000	0.5046	0.1539	0.8048	-0.4000	0.5046	0.0000	1.0000	0.2000	0.7471	0.0000	1.0000	0.3000	0.6238
miR-451a	27	-0.1346	0.5034	-0.0086	0.9661	-0.1277	0.5257	-0.0613	0.7662	0.1667	0.4060	0.3283	0.0945	0.1588	0.4290
miR-490-3p	11	-0.1468	0.6667	-0.0501	0.8837	-0.2688	0.4242	-0.0182	0.9601	0.1545	0.6500	-0.1053	0.7580	-0.3235	0.3319
miR-542-3p	32	-0.1485	0.4173	-0.0800	0.6636	-0.1522	0.4056	0.0242	0.8971	0.2009	0.2703	0.0990	0.5899	-0.0198	0.9142
miR-7-5p	5	0.4000	0.5046	0.6669	0.2189	0.4000	0.5046	-0.4000	0.6000	**-1.000**	**0.0001**	-0.7071	0.1817	-0.2000	0.7471
miR-96-5p	6	0.2319	0.6584	-0.0882	0.8680	0.2319	0.6584	0.1539	0.8048	-0.5218	0.2883	0.0171	0.9743	-0.2029	0.6998
miR-99b-5p	35	0.0488	0.7806	-0.2389	0.1670	-0.0046	0.9790	0.2621	0.1343	0.0188	0.9148	-0.1637	0.3474	**-0.3908**	**0.0203**

CHAPTER 4: DISCUSSION

MicroRNAs are receiving increased attention as potential biomarkers of many disease conditions including glucose intolerance and studies have reported the detection of miRNAs in blood and other body fluids in relation to pathophysiological status (Chen et al., 2008; Mitchell et al., 2008; Gilad et al., 2008; Weber et al., 2010 and Izzotti et al., 2018). Despite growing support for miRNAs as potential biomarkers for glucose intolerance globally, such evidence is lacking in South Africa. Previous studies from our laboratory have shown that the prevalence of glucose intolerance in the mixed ancestry cohort of Bellville South, South Africa is increasing rapidly (Erasmus et al., 2012 and Matsha et al., 2013) and majority of those with diabetes were undiagnosed.

Successful and accurate identification of people with preDM, who are likely to progress to the full stage of the disease, is the actual means to eradicate DM and its complications (Matsha et al., 2018). Until recently, the diagnosis of preDM and DM for instance, requires individuals to be subjected to the cumbersome oral glucose overload and measurement of blood glucose post 2 hours (Ko et al., 1998; Alberti and Zimmet 1988). As a result, individuals with this category of preDM largely remain undiagnosed. However, the emerging evidence for the role of miRNAs in the development of human diseases including diabetes is encouraging. In the development of diabetes, miRNAs have been reported to regulate metabolism, adipocyte differentiation, pancreatic development, β-cell mass, insulin biosynthesis, secretion and signaling (Poy et al., 2004; Uchida et al 2006; Hausser et al., 2011; Zhu et al., 2011 and Gu et al.,2016) underscoring their important role in the early diagnosis, pathogenesis and treatment of diabetes.

Some of the dysregulated miRNAs found in our study corroborate findings of many other studies that have aimed to characterize miRNAs in different tissue types of individuals with DM and/or preDM (He et al., 2017). A recent systematic study of dysregulated miRNAs in T2DM identified a total of 158 dysregulated miRNAs in adipose, islet, skeletal muscle, whole blood, PBMC, plasma and serum (He et al., 2017). In our study, 84 circulating miRNAs were investigated in the serum of 36 randomly picked mixed ancestry participants (women) in three groups according to their glycemic status' (controls, preDM and DM) to ascertain their biomarker properties in glucose intolerance. Differential expression was observed in 12 miRNAs across all groups, 3 miRNAs in the control and DM groups and 39 miRNAs only in one group (DM group). Table 4.1 shows the summary of micro RNAs expressed in present study compared to other studies.

Table 4.1: A summary of micro RNAs expressed in present study compared to other studies

miRNAs	Study	Sample type	Exp Change	Disease state	Reference
let-7i-5p	PS	Serum	up-regulated	DM	PS
	PS	Serum	up-regulated	preDM	PS
	OS	Serum	down-regulated	DM	Yang et al., 2014
miR-125b-5p	PS	Serum	up-regulated	DM	PS
	PS	Serum	down-regulated	preDM	PS
	OS	Serum	up-regulated	DM	Wang et al., 2016
	OS	Adipose	up-regulated	DM	Klöting et al., 2009
	OS	Plasma	down-regulated	DM	Ortega et al., 2014
miR-126b-5p	PS	Serum	up-regulated	DM	PS
	PS	Serum	up-regulated	preDM	PS
	OS	WB	down-regulated	DM	Karolina et al.,2011
	OS	PBMC	down-regulated	DM	Meng et al.,2012
	OS	Plasma	up-regulated	DM	Lu et al.,2014
miR-129b-5p	PS	Serum	up-regulated	DM	PS
	PS	Serum	down-regulated	preDM	PS
	OS	Islets	up-regulated	DM	Locke et al., 2014
miR-143-3p	PS	Serum	up-regulated	DM	PS
	OS	Adipose	up-regulated	DM	Xie et al.,2009
miR-146a-5p	PS	Serum	up-regulated	DM	PS
	PS	Serum	down-regulated	preDM	PS
	OS	Plasma	up-regulated	DM	Heilmeier et al.,2016
	OS	Serum	down-regulated	DM	Yang et al., 2014
miR-200a-3p	PS	Serum	up-regulated	DM	PS
	PS	Serum	down-regulated	preDM	PS
	OS	Adipose	up-regulated	DM	Zhao et al.,2009
miR-330-5p	PS	Serum	up-regulated	DM	PS
	OS	Liver cells	down-regulated	DM	Zhao et al.,2009
miR-382-5p	PS	Serum	up-regulated	DM	PS
	OS	Bone cells	up-regulated	DM	Heilmeier et al.,2016
miR-451a	PS	Serum	up-regulated	DM	PS
	PS	Serum	down-regulated	preDM	PS
	OS	SM	up-regulated	DM	Yuqing et al.,2017
miR-542-3p	PS	Serum	up-regulated	DM	PS

	PS	Serum	down-regulated	preDM	PS
	OS	ESSC	up-regulated	DM	Yuqing et al.,2017
miR-99b-5p	PS	Serum	no change	DM	PS
	PS	Serum	no change	preDM	PS
	OS		up-regulated	DM	Paramasivam 2015

Key: PS = Present study

OS = Other Studies

WB = White blood

PBMC = Peripheral blood mononuclear cells

SM = Skeletal muscles

Islet = Islets of langerhans

ESSC = Endometrial Stromal Stem Cells

4.1. MicroRNAs expressed in control, preDM and DM

MicroRNA expression is affected by a number of factors. In this study some microRNAs showed significant relationships with one or more glycaemic parameters (FBG, 2HRs post glucose, HbA1C, FBI and 2HRs post insulin). MiR-125b-5p and miR-129b-5p were expressed across all 3 groups and were positively correlated with 2HRs post insulin (mIU/L). Thus, their expression through all groups, indicates they are normally found circulating in serum, but only get altered with changes in the insulin levels, hence making them good markers in the insulin production/proliferation pathway. Furthermore, miR-125b-5p was also positively correlated with CRP (mg/L), a marker for inflammation. Inflammation has been shown to precede the development of diabesity (Herrera et al., 2009). Elevated levels of inflammatory cytokines predict future weight gain, and infusion of inflammatory cytokines into healthy, normal weight participants caused insulin resistance (Herrera et al., 2009). In this study, miR-125b-5p was up-regulated in DM as seen in Table 4.1. Up-regulation of miR-125b-5p in the liver of hyperglycemic participants results in a proinflammatory diabetic phenotype in vascular smooth muscle cells (Herrera et al., 2009).

MiR-146a-5p, miR-200a-3p and miR-330-5p were expressed through all 3 groups and all inversely correlated with both FBG (mmol/L) and 2HRs Post Glucose. Only miR-330-5p showed a negative correlation with HbA1C as well. Their expression through all groups indicates that they are normally found circulating in serum, but only get altered with changes in the glucose levels. Therefore, they can serve as good markers to identify early development of hyper-glycaemia. MiR-146a-5p has been reported to contribute to increased apoptosis of β-cells expression induced by cytokines and sodium palmitate (Roggli et al., 2010). MiR-200a-3p has equally been reported to be up-regulated in the adipose tissue of diabetic participants (Zhao et al., 2009). Also, miR-330-5p was reported to be down-regulated in the liver cells of diabetic participants (Zhao et al., 2009). miRNAs regulate insulin resistance in liver and hepatocytes and this is well documented by many studies. Up-regulation of miRNA in the livers of diabetic participants (Jordan et al., 2011) and obese participants (Takanabe et al., 2008) has been observed. Further study has shown that miRNA down-regulates Oxysterol-binding protein-related protein 8 (Orp8), and inturn impairs the ability of insulin to induce the activation of Protein kinase B (PKB) signaling, a central signaling node of insulin action to induce glucose metabolism (Jordan et al., 2011).
This implies each of these miRNAs affect different pathways leading to glucose intolerance and is differentially expressed in different samples.

MiR-382-5p was expressed through all groups and only inversely correlated with HbA1C. Its expression through all groups indicates that it is normally found circulating in serum, but only gets altered with changes in the HbA1C levels. Even though miR-451a was expressed through all groups, it was expressed more in DM than the control as compared to the preDM (Figure 4.1). It also did not correlate with any clinical characteristics. Furthermore, it is down-regulated in preDM while up-regulated in DM (Figure 4.1). MiR-451a was found to be up-regulated in serum and skeletal muscles in T2DM (Yuqing et al., 2017). MiR-382-5p has not been reported to be directly related to DM, but it has been found increased in diabetic women with osteoporosis (Heilmeier et al., 2016), but interestingly, in this study miR-382-5p shows direct relationships with dysglycemia.

MiR-126-5p, miR-143-3p, miR-542-3p and miR-99b-5p were expressed in all groups, but showed no correlation with any of the clinical characteristics. MiR-99b-5p was identified to be equally expressed through all groups (Table 4.1). There was evidently no change in the expression levels of miR-99b-5p both in the controls and the disglycemic states (Figure 4.1). Therefore miR-99b-5p may serve as a housekeeping gene in further studies concerning glucose intolerance. Contrary to this study, an Asian Indian study reported miR-99b-5p to be slightly up-regulated in DM participants (Paramasivam et al., 2015). This discrepancy in the two studies might be as a result of the different methods or apparatus' used in the different experiments or may be ethnic-specific.

MiR-542-3p has received little attention in relation to metabolic disorders, but it has been reported to have potential as a biomarker or therapeutic target in endometriosis (Yuqing et al., 2017). The endometrium has been reported to provide an easily accessible, renewable, and immunologically identical source of stem cells with potential therapeutic applications in diabetes (Yuqing et al., 2017). Thus implying an up-regulation of miR-542-3p could encourage the differentiation of normal human endometrial stromal stem cells (ESSC) into insulin secreting cells which could actually serve as a remedy for DM, especially T1DM (Shamima et al., 2017).

Though in this study miR-143-3p was up-regulated in DM but not affected in preDM (Figure 4.1), it has been reported to participate in adipocyte differentiation, it is also induced in adipogenesis and down-regulated in obesity (Xie et al., 2009). MiR-126-5p was up-regulated in both DM and preDM and it is expressed a little bit more in DM than in preDM (Figure 4.1). It has been reported to be expressed during pancreatic development and up-regulated in skeletal muscles (Li et al., 2009). Most recently, findings of up-regulation of miR-126 in preDM versus screen-detected DM most probably pointed towards a cascading reduction with respect

to DM related complications suggesting a potential role for miR-126 in distinguishing preDM from DM (Matsha et al., 2018).

Let-7i-5p was expressed through all groups and was inversely correlated with 2HRs Post Glucose (mmol/L). It was expressed more in preDM than in DM (Table 4.1). The let-7 family is ubiquitously expressed and a global knockdown of let-7 protects against glucose intolerance (Frost and Olson, 2011), whereas global and pancreas-specific over-expression impair glucose tolerance by decreasing glucose-stimulated insulin-secretion (Frost and Olson, 2011). In addition, transgenic overexpression of let-7 causes glucose intolerance associated with decreased muscle specific expression of several proteins of the insulin signalling pathway, including IRS2 (insulin receptor substrate 2) and the insulin receptor (Zhu et al., 2011). Hence, the let-7 family orchestrates diverse aspects of glucose metabolism in different tissues (Zhu et al., 2011). This therefore ties with the way let-7i-5p is being expressed in this study, as more of it is expressed in preDM than DM, reflecting the ubiquitous nature of the let-7 family. This may also reflect a compensatory mechanism that only occurs in prediabetic status, but shuts down as the disease progresses to DM stage.

4.2. MicroRNAs expressed in control and DM only

MiR-324-5p, miR-34c-5p and miR-490-3p were expressed in only two groups (control and DM) and were as well all up-regulated in DM. Their Ct values indicate that, they can be found in little quantities in controls, but only get a little elevated in DM, but not preDM. MiR-490-3p was inversely correlated with HbA1c (%) and 2HRS Post Glucose (mmol/L), though up-regulated in DM (Table 4.1). Though little has been reported on miR-324-5p, miR-34c-5p and miR-490-3p and their correlations with metabolic disorders, miR-324-5p has been implicated to be up-regulated in T1DM and as an early biomarker in T1DM (Erener et al., 2017). MiR-34c-5p was validated as significantly highly expressed in circulating monocytes in T2D patients (Baldeón et al., 2015). Hence implying that they are found affecting different metabolic pathways and could serve as possible biomarkers for dysglycemia.

4.3. MicroRNAs expressed in DM only

Amongst the miRNA's expressed only in DM, there are some that show strong correlation with some clinical parameters. MiR-130b-3p and miR-96-5p were both inversely correlated with FBG (mmol/L), 2HRS Post Glucose (mmol/L) and HbA1C (%) but miR-96-5p was equally positively correlated with 2HRS Post Insulin (mIU/L). Hence, they could serve as markers which can only be found in circulation in DM state.

MiR-4458 was inversely correlated with FBG (mmol/L), 2HRS Post Glucose (mmol/L), 2HRS Post Insulin (mIU/L) as well as WHR. Globally, miR-4458 has received very little attention,

especially in studies involving metabolic disorders and particularly glucose intolerance. The results in this study show evidence that miR-4458 has a major role to play in glucose intolerance as it is well expressed in the DM group and its correlations with glycemic parameters. This implies its presence in circulation is indicative of a glucose disorder, especially DM.

Furthermore, miR-135b-5p, miR-184, and miR-424-5p were inversely correlated with 2HRS Post Insulin (mIU/L), while miR-375 and miR-377 were both positively correlated with 2HRS Post Insulin (mIU/L). Previous studies have shown that miR-375 was expressed in pancreatic development and regulated insulin secretion in β-cells and increased their death by lipo-apoptosis, as it regulates cell viability and proliferation (Kloosterman et al., 2007; El et al., 2008 & Li et al, 2010). Moreover, it was found up-regulated in β-cells of T2D participants and its deletion caused severe insulin-deficient diabetes in the participants (Kloosterman et al., 2007 & Li et al, 2010). MiR-129-2-3p and miR-29c-3p were positively and inversely correlated with Trigs (mmol/L) respectively. These miRNAs may have important roles to play in adipogenesis, differentiation and/or proliferation. They could either hinder or promote certain biochemical pathways.

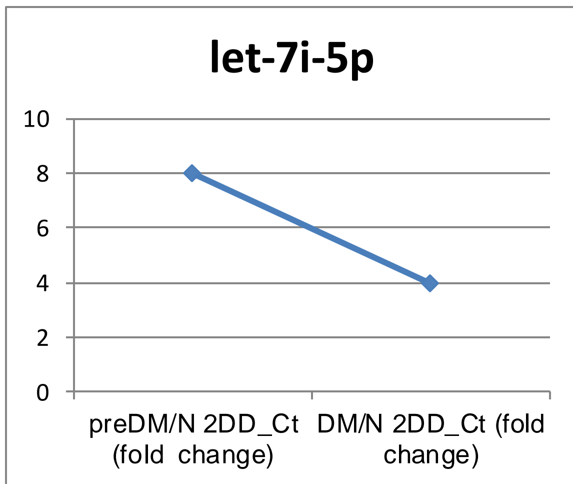
MiR-7-5p, miR-27b-3p, miR-206, miR-212-3p, miR-335-5p, and miR-381-3p were all inversely correlated to CRP (mg/L). These miRNAs may as well have important roles to play in certain inflammatory pathways that might be linked to glucose intolerance and its complications. MiR-335-5p was equally inversely correlated with FBG (mmol/L) but previous findings show that, miR-335 influenced insulin secretion and miR-335 overexpression negatively affects insulin secretion via the reduction in multiple exocytosis protein targets and impaired priming of insulin granule (Wang et al., 2016).

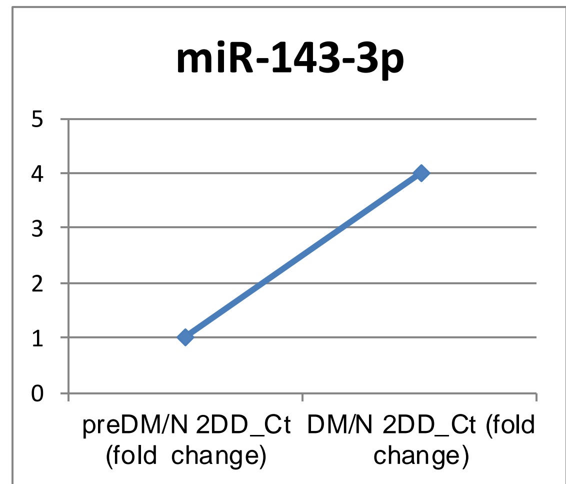
There was no association between age and miRNA as there was no correlation between the age and the miRNAs in this study. Other parameters like WHR, waist (cm) and hip (cm) were scarcely correlated with the miRNAs expressed. This might have been as result of the sample used, since miRNA expression is also affected by the sample type.
Smoking, alcohol and gamma GT have shown no association with the miRNA expression as well, since there were evidently no significant associations/correlations recorded in our study.

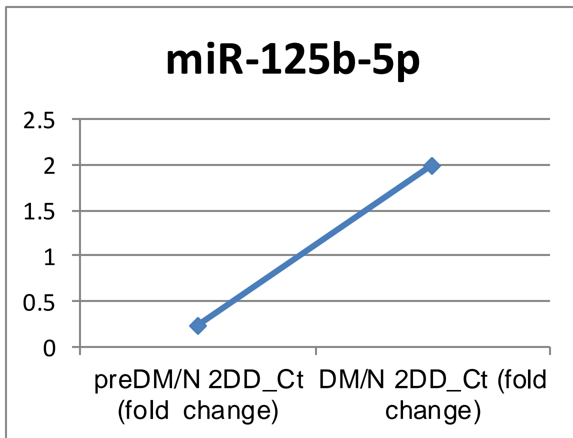
Table 4.2: Fold changes and average Ct values of microRNAs expressed through all groups and between two groups

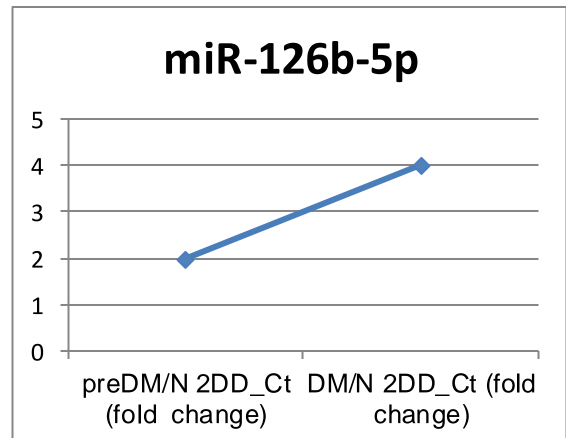
MicroRNA	Normal group Average Ct value	preDM group Average Ct value	DM group Average Ct value	preDM/N 2DD_Ct (fold change)	DM/N 2DD_Ct (fold change)	DM/preDM2 DD_Ct (fold change)
let-7i-5p	31	28	29	8	4	0.5
miR-125b-5p	29	31	28	0.25	2	8
miR-126b-5p	33	32	31	2	4	2
miR-129b-5p	30	31	29	0.5	2	4
miR-143-3p	28	28	26	1	4	4
miR-146a-5p	31	32	29	0.5	4	8
miR-200a-3p	32	33	30	0.5	4	8
miR-330-5p	32	32	30	1	4	4
miR-382-5p	27	27	26	1	2	2
miR-451a	30	32	29	0.25	2	8
miR-542-3p	31	32	30	0.5	2	4
miR-99b-5p	31	31	31	1	1	1
miR-324-5p	31	NA	31	NA	2	NA
miR-34c-5p	32	NA	32	NA	2	NA
miR-490-3p	34	NA	32	NA	4	NA

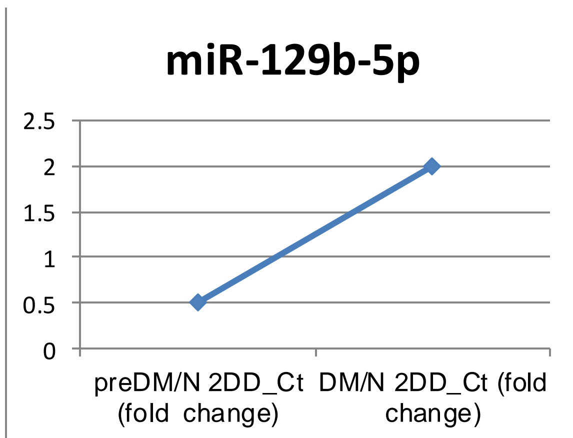
Ct value is inversely proportional to the amount of micro RNA expressed
Fold changes equal to 1 = No change
Fold changes greater than 1 = Up-regulation
Fold changes less than 1 = Down-regulation
NA = Not applicable

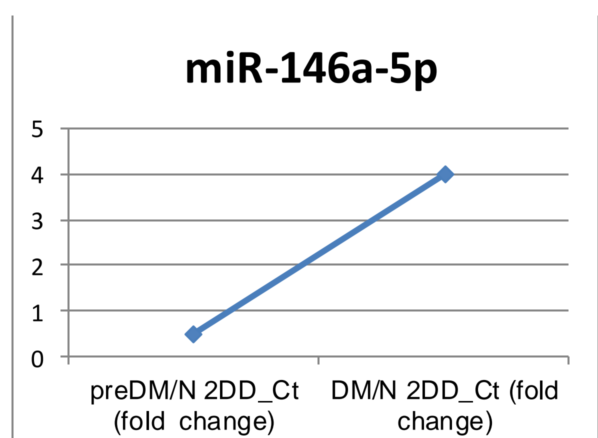

let-7i-5p
10
8
6
4
2
0
preDM/N 2DD_Ct (fold change)
DM/N 2DD_Ct (fold change)

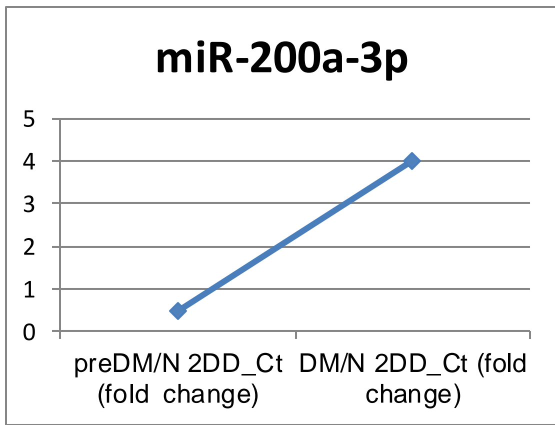

miR-143-3p
5
4
3
2
1
0
preDM/N 2DD_Ct (fold change)
DM/N 2DD_Ct (fold change)

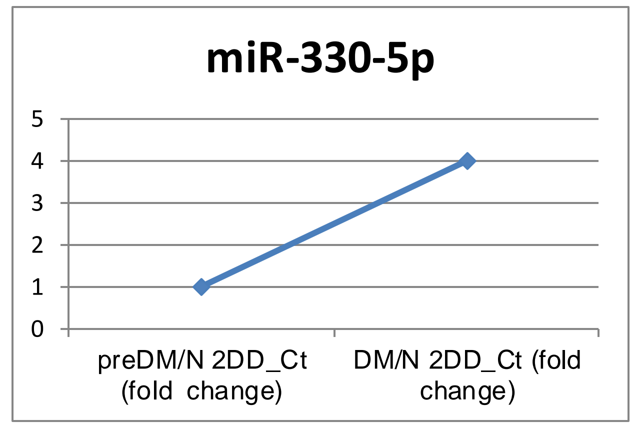

miR-125b-5p
2.5
2
1.5
1
0.5
0
preDM/N 2DD_Ct (fold change)
DM/N 2DD_Ct (fold change)

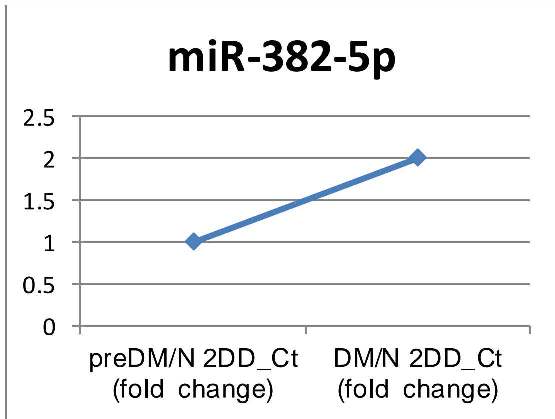

miR-126b-5p
5
4
3
2
1
0
preDM/N 2DD_Ct (fold change)
DM/N 2DD_Ct (fold change)

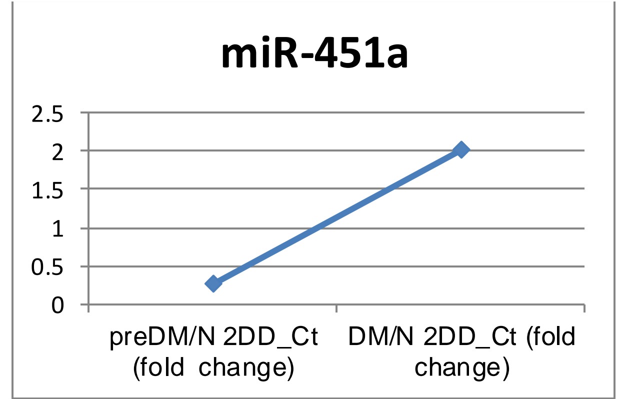

miR-129b-5p
2.5
2
1.5
1
0.5
0
preDM/N 2DD_Ct (fold change)
DM/N 2DD_Ct (fold change)

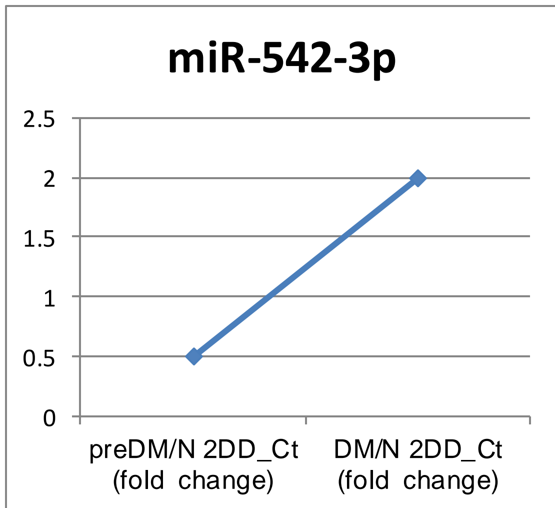

miR-146a-5p
5
4
3
2
1
0
preDM/N 2DD_Ct (fold change)
DM/N 2DD_Ct (fold change)











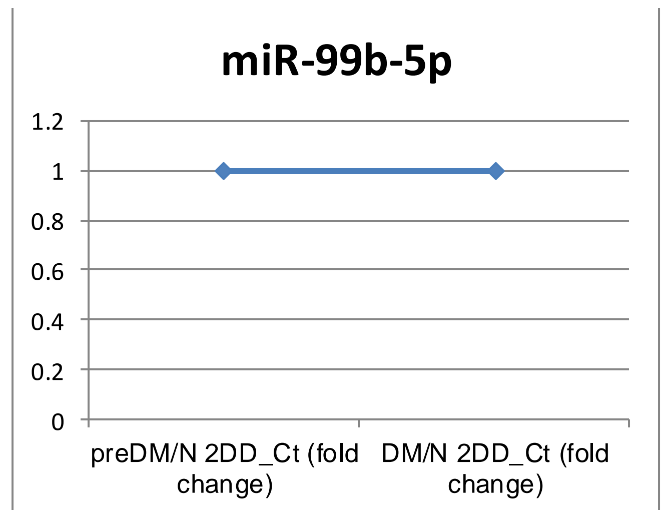


Fig 4.1: Fold changes of the individual 12 micro RNAs expressed through all three groups

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4.4. The strengths of the study

RT-PCR was used to evaluate the expression of circulation miRNAs in the serum of participants which was a lot less time consuming and less cumbersome as the present day's routine tests for glucose tolerance.
Furthermore, circulating miRNAs are expressed in the pre-diabetic state, hence paving a way to manage diabetes, preventing its progress to diabetes and the complications that come with diabetes.

4.5. Weaknesses of the study

RT-PCR is an expensive procedure to be used in a diagnostic laboratory as compared to the routine diagnostics tests in the present day's laboratory; which means it would not be cost-effective in low-economic countries. Also, this study was limited to a small population of selected females only.

4.6. Future perspectives

Although this study showed that miRNAs were associated with glucose intolerance, prospective, longitudinal studies in different populations are required to confirm their potential as special biomarkers for T2DM. More so, a study population that comprises of both males and females at different ages will shed more light on the biomarker capacities of the circulating miRNAs.

CHAPTER 5: CONCLUSION

In conclusion, our results show that circulating miRNAs are associated with glucose intolerance in individuals of mixed ancestry descent, despite the limitations eluded earlier on. Our results add to the growing body of evidence that circulating miRNAs are associated with glucose intolerance and can be detected during pre-diabetes, the asymptomatic stage of T2DM and in T2DM as well. These findings support the use of miRNAs as biomarkers for early detection of T2DM. The quick, precise and less cumbersome procedure used in this study as compared to the procedures of the current diagnostic tests for glucose intolerance previously mentioned, gives it the best standing for the diagnosis, prognosis and possibly treatment of T2DM. To our knowledge, this is the first study to assess circulating miRNAs in serum as biomarkers for glucose intolerance in the mixed ancestry population in South Africa.

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APPENDIX A

CONSENT FORM

PARTICIPANT INFORMATION AND INFORMED CONSENT FORM FOR RESEARCH INVOLVING GENETIC STUDIES

TITLE OF RESEARCH PROJECT: PROGRESSIVE RESEARCH ON RISK FACTORS OF TYPE 2 DIABETES AND CARDIOVASCULAR DISEASES IN SOUTH AFRICA

REFERENCE NUMBER:

PRINCIPAL INVESTIGATORS:	Professor Tandi Matsha (Cape Peninsula University of Technology) Professor Rajiv Erasmus (Stellenbosch University) Professor Andre Kengne (SA Medical Research Council)
Project manager:	Dr Gloudina Maria Hon (Cape Peninsula University of Technology)
Address:	Obesity and chronic diseases of lifestyle Department of Biomedical Sciences Faculty of Health & Wellness Sciences Cape Peninsula University of Technology, Bellville
Contact number:	Prof T Matsha 021 959 6366 or email: matshat@cput.ac.za
Ethics approval:	Cape Peninsula University of Technology Ethics Reference number: CPUT/SW-REC 2015/H01 University of Stellenbosch Ethics Reference number: N14/01/003

We would like to invite you to participate in a research study that involves genetic analysis and possible long-term storage of blood or tissue specimens. Please take some time to read the information presented here which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails

and how you could be involved. Also, your participation is entirely voluntary and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part initially.

This research study has been approved by the ethics Faculty of Health & Wellness Sciences of the Cape Peninsula University of Technology and it will be conducted according to international and locally accepted ethical guidelines for research, namely the Declaration of Helsinki, and the SA Department of Health's 2004 Guidelines: Ethics in Health Research: Principles, Structures and Processes.

1. What is Genetic research?

Genetic material, also called DNA or RNA, is usually obtained from a small blood sample. Occasionally genetic material is obtained from other sources such as saliva or biopsy specimens. (A biopsy is a tiny piece of tissue that is cut out e.g. from the skin or from a lump, to help your doctor make a diagnosis). Genes are found in every cell in the human body. Our genes determine what we look like and sometimes what kind of diseases we may be susceptible to. Worldwide, researchers in the field of genetics are continuously discovering new information that may be of great benefit to future generations and also that may benefit people today, who suffer from particular diseases or conditions.

2. What does this particular research study involve?

This research study seeks to address the increasing problem of diabetes and cardiovascular diseases such as heart attack and stroke amongst the mixed ancestry or coloured population of South Africa. In this study we shall identify people with diabetes and those at high risk of diabetes as well as investigate the environmental and genetic risk factors that predispose some individuals to the development of diabetes and cardiovascular diseases. Examples of environmental factors include body weight, diet, and physical activity. Additionally, this project aims to investigate whether oral health is a risk factor for diabetes and cardiovascular diseases. In this study we shall investigate whether some individuals have early cardiovascular diseases by using an ultrasound machine. This project also aims to collect genetic material (blood) to analyze for certain variants and to store excess material for future research. When a large group of patients with similar diseases has been collected, meaningful research into the disease processes may become possible.

3. Why have you been invited to participate?

Our research team has previously conducted a similar research study involving the coloured community and found out that more that 18 out of 100 individuals had diabetes but did not know. We also found that some of the risk factors associated with diabetes in other populations were not necessary the same as those affecting the coloured population of South Africa. You

have therefore been invited to take part in this research study to assist in establishing the risk factors for diabetes and cardiovascular diseases affecting the coloured people of South Africa.

4. What procedures will be involved in this research?

A. You will be requested to provide information about your medical history, family history and information on eating, drinking and smoking habits. Completion of the questionnaire will take no longer than 30 minutes.

B. You shall be requested to provide a record of the medication you are currently taking, therefore if you are taking chronic medication, you shall be requested to provide this to the research team to record the medication.

C. Measurement such as weight, height, waist and hip will be done.

D. Fasting Venous Blood (20ml) will be collected thereafter you will be asked to drink a glucose solution (glucose content 75g). After two hours another venous blood (10ml) will be collected. The blood will be used to determine whether you have diabetes or you are at high risk for developing diabetes.

E. The other tests that will be determined from your blood sample are: Cholesterol, triglycerides, creatine levels to assess your kidney function, liver enzymes to assess your liver, and biochemical markers for inflammation.

F. A finger prick blood sample (a drop of blood), to be taken at the same time of the first venous blood sample, may also be required from you. The finger prick blood sample will be used to test for diabetes or the risk of developing diabetes on a point-of-care test instrument. Researchers will compare the finger prick point-of-care diabetes test with that of the send away venous blood laboratory test and would be able to establish whether the point-of-care test provides the same accurate results as that of the laboratory. Point-of-care testing may in the future be used to provide fast and accurate results without the need to send blood away to a laboratory for processing. This may be of benefit to people undergoing testing for diabetes as results would be available within a few minutes.

G. The remainder of the blood sample will be used for genetic and future research studies. The serum and DNA may be stored for several years until the technology for meaningful analysis becomes available. No pharmaceutical agent (medication) will be tested in the study.

H. For oral health, research study personnel will extract wooden toothpick, flocked brush, and mouthwash saliva samples from you to test for the presence of Porphyromonas gingivalis as an indicator for periodontal disease. Flocked brush and wood toothpick sampling will involve inserting devices in the subgingival crevice between the last upper premolar and the first upper

molar. The device will sweep down the anterior surface of the first upper molar with the direction of motion away from the gum to minimize any potential discomfort. Mouthwash sampling will involve rinsing with 10 ml sterile saline solution for 20 seconds.

I. Early cardiovascular diseases will be performed by means of an ultrasound machine.

J. The research team will follow up on you on a yearly basis and some of these test may be repeated. The investigators wish to follow you up for your entire life. In the unfortunate event that you are deceased during the study period. The study team will review stats SA data and/or medical records to ascertain whether the cause of death was due to diabetes or cardiovascular diseases.. ***If you do not wish to be followed up on a yearly basis and your Statistics SA and/or medical records not to be accessed in the unfortunate event that you are deceased whilst being a participant in the study, you will have an opportunity to request that it be not accessed when you sign the consent form.***

K. Radio imaging techniques will be done on consenting subjects. These include **(i)** ultra sound to assess whether you have signs of early cardiovascular diseases, **(ii)** computed tomography scan (CT-scan) to accurately assess the fat content that is dangerous for cardiovascular diseases **(iii)** Dual-energy X-ray absorptiometry (DXA) devices will be used to study the morphology of the liver. These radio imaging techniques involve radiation which can be harmful if one is exposed excessively. For this study a low dose radiation will be used for acquisition of the images thereby minimizing radiation exposure to the participant. ***If you do not wish to undergo any of these radio imaging techniques, you will have an opportunity to decline when you sign the consent form.***

L. An eye examination will be done to test your eye vision and any other abnormalities in the eye. For this examination, drops placed in your eyes widen (dilate) your pupils to allow the doctor to better view inside your eyes. The drops may cause your close vision to blur for a short while.

5. Are there any risks involved in genetic research?

A slight bruising might occur after blood has been drawn from the arm but this will heal quickly. After the administration of the glucose solution, you may feel nauseous and dizzy in which case you must notify the medical personnel. A medical nurse or doctor will be present on all occasions. You may also learn that you have diabetes, in which case you will be referred to your health care giver with the results for further treatment and management. If during the study it is discovered that you have changes in your genes that may lead to a serious disease, a genetic counsellor at the expense of the principal investigators will counsel you. Radio imaging techniques such

as the CT-scan involves radiation which can be harmful if one is exposed excessively. For this study a low dose radiation will be used for acquisition of the images thereby minimizing radiation exposure to the participant.

6. Are there any benefits to your taking part in this study and will you get told your results?

Your personal results will be made known to you ***only if they indicate*** that you may:

- Have diabetes, thereafter, you will be referred to your local health centre or general practitioner for further investigations and treatment.
- Have a condition or predisposition to developing diabetes that is treatable or avoidable e.g. by a lifestyle modification.
- Need genetic counselling.

> **However, participants with normal results who wish to know their results are free to contact the research team and their results will be given upon written request.**

7. How long will your blood be stored and where will it be stored?

The blood samples may be stored ***indefinitely*** to accommodate new technologies that may develop. In the event that a technology is not available in South Africa to analyse your blood sample, your blood specimen may be sent to another country with the technology either now or at a later date. However, if your specimen is to be sent to another country, permission to do so will be sought from relevant bodies. Your blood specimen will be stored at the Cape Peninsula University of Technology.

8. If your blood is to be stored is there a chance that it will be used for other research?

Your blood will only be used for genetic research that is directly related to Diabetes and cardiovascular diseases. Also if the researchers wish to use your stored blood for ***additional research in this field*** they will be required to apply for permission to do so from the ethics ***Faculty of Health & Wellness Sciences of the Cape Peninsula University of Technology. If you do not wish your blood specimen to be stored after this research study is completed you will have an opportunity to request that it be discarded when you sign the consent form.***

9. How will your confidentiality be protected?

Your identity will be recorded once and kept confidential throughout. This is to allow the principal investigators to convey information that may be beneficial to you. Access will be limited to the principal investigators by assigning a special study code to all your data and blood samples. This means that your sample will be identified with a special study code that will remain linked to your name and contact details. However, during the entire research study, your blood specimens will be anonymised and the research staff won't be able to associate it with your name and contact details. You shall also be supplied this code so that if at any time the investigators need to contact you, you may only identify yourself using your special code. Any scientific publications, lectures or reports resulting from the study will not identify you.

Some insurance companies may mistakenly assume that taking part in research indicates a higher risk for disease. Thus no information about you or your family will be shared with such companies.

10. Will you or the researchers benefit financially from this research?

You will not be paid to take part in this study ***although your out-of-pocket expenses may be reimbursed.*** The expenses that will be covered by the research team are those that include transportation to a hospital radiography department should you consent to radio imaging.

Important information: In the unlikely event that this research leads to the development of a commercial application or patent, you or your family will not receive any profits or royalties, but profits will be reinvested into supporting the cause of further research which may bring benefits to you or your family and to the community, such as health screening, medical treatment, educational promotions, etc.

11. Is there anything else you should know or do?

You should inform your family practitioner or usual doctor that you are taking part in a research study. You can contact

Prof T Matsha at 021 959 6366 or matshat@cput.ac.za,

If you have any further queries or encounter any problems, you can also contact the Cape Peninsula University of Technology Health and Wellness Sciences Research Ethics Committee,

Chairperson: Prof Engel-hills at 0219596570 or EngelhillsP@cput.ac.za or

You will receive a copy of this information and consent form for your own records if it is requested.

12. Declaration by participant

By signing below, I …………………………………...…………. agree to take part in a research project that includes genetic research study entitled (**PROGRESSIVE RESEARCH ON RISK FACTORS OF TYPE 2 DIABETES AND CARDIOVASCULAR DISEASES IN SOUTH AFRICA**).

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is voluntary and I have not been pressurised to take part.
- I have received a signed duplicate copy of this consent form for my records.

13. Tick the option you choose:

☐ I agree that my blood or tissue sample can be stored ***indefinitely*** after the project is completed but that it is anonymised with all possible links to my identity removed, and that the researchers may then use it for additional research in this or a related field. Once my sample is anonymised, my rights to that sample are waivered. My sample may be shipped to another laboratory in SA or abroad to be used in other research projects in this or a related field

OR

☐ I agree that my blood or tissue sample can be stored ***indefinitely,*** but I can choose to request at any time that my stored sample be destroyed. My sample will be identified with a special study code that will remain linked to my name and contact details. I have the right to receive confirmation that my request has been carried out.

OR ☐

Please destroy my blood sample as soon as the current research project has been completed.

14. Tick the option you choose:

☐ I ***consent*** that the research team may follow me up for yearly check-up ***AND*** in the unfortunate event that I am deceased whilst still part of the study, I ***consent*** that the team may access Statistics SA and/or my medical records to ascertain whether the cause of my death was due to diabetes or cardiovascular diseases.

OR

☐ I ***do not consent*** to follow me up for yearly check-up ***BUT*** in the unfortunate event that I am deceased whilst still part of the study, I ***consent*** that the team may access Statistics SA and/or my medical records to ascertain whether the cause of my death was due to diabetes or cardiovascular diseases.

OR

☐ I **do not consent** to follow me up for yearly check-up ***AND*** in the unfortunate event that I am deceased whilst still part of the study, I ***do not consent*** that the team accessing Statistics SA and/or my medical records to ascertain whether the cause of my death was due to diabetes or cardiovascular diseases.

15. Tick the option you choose: Radio Imaging

☐ I ***consent*** to ultra sound techniques to assess if I have early cardiovascular diseases

☐ I ***do not consent*** to ultra sound techniques that assess if I have early cardiovascular diseases

AND

☐ I ***consent*** computed tomography scan (CT-scan) to accurately assess the fat content that is dangerous for cardiovascular diseases

☐ I ***do not consent*** to computed tomography scan (CT-scan) that accurately assess the fat content that is dangerous for cardiovascular diseases

AND

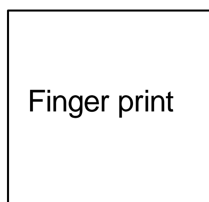
☐ I ***consent*** to Dual-energy X-ray absorptiometry (DXA) used to study body composition.

☐ I ***do not consent*** Dual-energy X-ray absorptiometry (DXA) used to study body composition

Signed at (*place*) .... on (*date*) ....

………………………………………………………… …………………………………………………………

Signature of participant **Signature of witness**



16. Declaration by investigator

I *(name)* ……………………….……….……….……….…… declare that:

- I explained the information in this document to …………………..……………...
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research as discussed above.
- I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) ....…........……………. on (*date*) …………....………. 2016.

……………………………………………………………… ………………………………………………………………

Signature of investigator **Signature of witness**

17. Declaration by Interpreter

I *(name)* ……………………….……….……….……….…… declare that:

- I assisted the investigator (*name*) ………………………….…. to explain the information in this document to (*name of participant*) ……………………….……..…. Using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily

answered.

Signed at (*place*) ....…........…………….… on (*date*) …………....……… 2016.

……………………………………………………………… ....

Signature of interpreter **Signature of witness**

APPENDIX B

Ethics clearance certificate

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: sethn@cput.ac.za

18 April 2018

REC Approval Reference No:

CPUT/HW-REC 2016/H30(extension)

Faculty of Health and Wellness Sciences – Biomedical Sciences Dear Ms Mbu

Re: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC on 15 September 2016 to Ms Desiree Lem Mbu for ethical clearance. This approval is for research activities related to student research in the Department of Biomedical Science at this Institution.

TITLE: Expression of circulating microRNA's (miRNAs) in blood of mixed ancestry subjects with glucose intolerance

Supervisor: Professor T Matsha Comment:

Data collection permission is required and has been obtained.

Approval will not extend beyond 19 April 2019. 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

Mr. Navindhra Naidoo
Chairperson – Research Ethics Committee
Faculty of Health and Wellness Sciences