# MICRORNA PROFILING AND VALIDATION IN MIXED ANCESTRY INDIVIDUALS IN SOUTH AFRICA

By

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BELLVILLE
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**DECLARATION**

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

**14 March 2021**

**Signed** **Date**

## ABSTRACT

**Background:** There has been a gradual increase in the prevalence of type 2 diabetes mellitus (T2DM) in South Africa, and in 2019, the prevalence was reported to be the highest in comparison to all other countries in the African region. Concerns have arisen regarding the efficiency of current methods of diagnosis, such as the invasiveness and cumbersomeness of the oral glucose tolerance test (OGTT), diurnal variations in blood glucose levels limiting the capacity of blood glucose tests and increasing the chances of missing risk groups, as well as the influence of age, ethnicity and haemoglobinopathies on HbA1c levels. In light of this, studies have shifted to small, non-coding RNA transcripts called microRNAs (miRNAs), which are present at consistent and reproducible levels in peripheral blood. They have been touted as potential diagnostic and prognostic biomarkers for various diseases, such as type 2 diabetes mellitus (T2DM), and expression patterns in response to treatment may further promote their use as therapeutic targets in T2DM. Therefore, the objectives of this study were to investigate the expression of miR-30a-5p, -1299, -182-5p, -30e-3p and -126-3p, and their association with- and diagnostic capability for dysglycaemia in a South African population previously reported to have a high prevalence of undiagnosed diabetes, as well as to explore their expression patterns in newly diagnosed and known diabetic individuals on treatment.

**Methods:** This was a cross-sectional study involving a total of 1273 individuals (men, n=345), including 207 prediabetes, 94 screen-detected diabetes and 972 normotolerant individuals, all aged >20 years, from the ongoing Vascular and Metabolic Health (VMH) study. Glycaemic status was assessed using the OGTT and blood pressure measurements were taken, all in accordance with World Health Organization (WHO) guidelines. In addition, HbA1c, insulin levels, obesity indices, lipid profile, C-reactive protein and serum cotinine levels were performed. Five miRNA (miR-30a-5p, -1299, -182-5p, -30e-3p and -126-3p) expression profiles were assessed by TaqMan-based RT-qPCR. These were based on data obtained from whole genome miRNA profiling from the whole blood of 12 individuals with known diabetes, 12 with screen-detected diabetes, 12 with prediabetes and 12 with normal glucose tolerance, matched for age, blood pressure, smoking and body mass index (BMI). Receiver operating characteristic (ROC) curves were used to assess the ability of each miRNA to discriminate dysglycaemia, and univariate as well as multivariable logistic regression analyses were performed to link expression with dysglycaemia.

**Results**: Relative expression between subgroups revealed all miRNAs were significantly highly expressed in prediabetes compared to normotolerant (≥3.1-fold and $p$<0.001), with miR-30a-5p showing the highest significant expression between the two groups (3.5-fold and $p$<0.001). miR-30a-5p, -1299, 182-5p, and -126-3p were significantly overexpressed when compared to screen-detected diabetes and normotolerant (≥1.6-fold and $p$≤0.013), with the exception of -30e-3p (1.3-fold and

$p$=0.145). All miRNAs demonstrated overexpression in known diabetics when compared to normotolerant individuals (≥5.6-fold and $p$<0.001). Comparisons between prediabetes and screen-detected diabetes revealed significantly reduced expression of miR-1299, -182-5p and 126-3p (≤0.56-fold and $p$≤0.020), whilst miR-30e-3p and -30a-5p did not demonstrate significantly reduced levels (≤0.58-fold and $p$≥0.097), additionally a significant elevation was observed in known diabetics versus screen-detected (≥3.1-fold and $p$<0.001). Multivariable logistic regressions, adjusted for age, sex, BMI, systolic blood pressure (SBP), 2-hour blood glucose, HbA1c, triglycerides, high-density lipoprotein cholesterol (HDL-cholesterol) and low-density lipoprotein cholesterol (LDL-cholesterol), revealed all miRNAs were consistently and continuously associated with dysglycaemia when compared to normotolerant, while only miR-126-3p and -182-5p showed associations with screen-detected diabetes versus prediabetes, (odds ratio (OR)≥0.89, 95% confidence interval (CI): 0.83-0.96, $p$≤0.003) and (OR≥0.70, CI: 0.60-0.81, $p$≤0.001) respectively. The diagnostic capabilities of the miRNAs in distinguishing dysglycaemia was assessed using receiver operating characteristic curve (ROC) analyses, whereby miRNAs miR-126-5p and -182-5p in particular, outperformed HbA1c in distinguishing prediabetes, area under the curve (AUC) = 0.76 for miR-126-3p versus 0.70 for HbA1c, ($p$=0.042), and 0.74 for miR-182-5p versus 0.69 for HbA1c ($p$=0.217). Moreover, univariate regression analysis was used to illustrate the relationships between the miRNAs and the duration of T2DM. miR-1299, -182-5p and -126-3p were associated with the duration of diabetes upon models adjusted for age and sex, (OR≥0.076, CI: 0.001 – 0.151, $p$<0.046) however after adjustment for type of treatment, only miR-182-5p remained significantly associated with the duration of the disease (OR: 0.127, CI: 0.018-0.236, $p$=0.023).

**Conclusions**: These results demonstrated altered expression levels and significant associations of the miRNAs in question with dysglycaemia, as well as demonstrating greater diagnostic capabilities of miR-126-3p and -182-5p compared to HbA1c in differentiating prediabetics from normotolerant individuals, implicating their use in potentially contributing to diabetes risk screening strategies. Furthermore, we illustrated important associations and altered expression-patterns of the miRNAs in known diabetics on anti-diabetic treatment compared to newly diagnosed individuals, with miR-182-5p expression decreased with increasing duration of T2DM, indicating the potential value of the miRNA in diabetes management. Further studies are however recommended to shed light on the involvement of the miRNAs in glucose homeostasis, to endorse their use as a therapeutic targets in DM and its associated complications.

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## DEDICATION

To my late parents, Lynette Weale and Rodney Weale. I hope you're looking down from heaven and are proud of how far I've come, and what I've accomplished thus far. I do it in honour of you both. Rest in eternal peace.

**THESIS OUTLINE**

This dissertation is organized into seven chapters.

**Chapter 1** is a general introduction, study rationale, and the study objectives and aims.

**Chapter 2** is an overview of literature review which underlines type 2 diabetes mellitus (T2DM) and its associated risk factors, with focus on genetic risk factors, and the identification of microRNAs (miRNAs) whose regulatory functions contribute to the pathophysiology of various diseases such as diabetes mellitus (DM), as well as their proposed use in diagnostic and/or therapeutic strategies.

**Chapter 3** summarises the foundation of this study, based on whole genome miRNA profiling whereby differential miRNAs were detected in a small sample of prediabetic, newly diagnosed diabetics and known diabetics on anti-diabetic therapy, in comparison to individuals with normal glucose tolerance, and this was done using genome-wide sequencing.

**Chapter 4** is a submitted manuscript for publication. In this manuscript, the association and diagnostic capability of miR-1299, - 126-3p and - 30e-3p and prediabetes is investigated**.**

**Chapter 5** is a published manuscript in which miR-30a-5p and miR-182-5p and dysglycaemia is investigated.

**Chapter 6** is a manuscript investigating miR-30a-5p, -1299, -182-5p, -30e-3p and -126-3p in individuals with diabetes and on anti-diabetes therapy.

**Chapter 7** provides summary of the key novel findings, clinical implications and recommendations arising from this research.

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## LIST OF ABBREVIATIONS, SYMBOLS AND UNITS

$2^{-\Delta Ct}$ – relative quantification of gene expression detailing the difference between expression of the gene of interest versus the endogenous control

$2^{-\Delta\Delta Ct}$ – relative quantification of gene expression detailing the difference between $2^{-\Delta Ct}$ of treated sample and control sample

% – percentage

cm – centimetres

$kg/m^2$ – kilograms per square metre

IU/L – international units per litre

mg/L – milligrams per litre

mIU/L – milli-international units per litre

ml/min – millilitres per minute

mmHg – millimetres

mmol/L – millimoles per litre

µmol/L – micromoles per litre

µg/µl – micrograms per microlitre

ABCA1 – adenosine triphosphate–binding cassette transporter A1

ADA – American Diabetes Association

Ago – argonaute

AMI – acute myocardial infarction

AMPK – adenosine monophosphate activated protein kinase

ANOVA – analysis of the variance

AT1R blockers – angiotensin II type-1 receptor blockers

ATM – ataxia telangiectasia mutated

AUC – area under the curve

BCL2 – B-cell lymphoma 2

BMI – body mass index

CAD – coronary artery disease

CAV2 – caveolin-2

CDK6 – cyclin-dependent kinase 6

CKD-EPI eGFR - Chronic Kidney Disease Epidemiology Collaboration estimated glomerular filtration rate

CI – confidence interval

CLL – chronic lymphocytic leukaemia

CPUT – Cape Peninsula University of Technology

CRP – C-reactive protein
CVD – cardiovascular disease
DBP – diastolic blood pressure
DGCR8 – DiGeorge critical region 8
DKD – diabetic kidney disease
DLBCL – diffuse large B-cell lymphoma
Dlk1 – delta-like 1 homolog
DM – diabetes mellitus
dsRBD – double stranded RNA binding domain
EDTA – ethylenediaminetetraacetic acid
FDA – Food and Drug Administration
GGT – γ-Glutamyltransferase
GLUT4 – glucose transporter type 4
HCC – hepatocellular carcinoma
HDL – high-density lipoprotein
HIF-1 – hypoxia-inducible factor 1
HPLC – high performance liquid chromatography
IFCC – International Federation of Clinical Chemistry and Laboratory Medicine
IFG – impaired fasting glucose
IFN-β – interferon-β
IGF-1 – insulin-like growth factor-1
IGF-1R – insulin-like growth factor-1 receptor
IGT – impaired glucose tolerance
IL-1β – interleukin-1β
IL-6 – interleukin-6
INSIG1 – insulin-induced gene 1
IR – insulin resistance
IRS-1 – insulin receptor substrate 1
KIRCSJ – potassium inwardly rectifying channel subfamily J member 11
LDL – low-density lipoprotein
MAPK – mitogen-activated protein kinase
MDRD eGFR - Modification of Diet in Renal Disease estimated glomerular filtration rate
MEF2A – myocyte enhancer factor 2A
miRISC – miRNA-induced silencing complex
miRNAs – microRNAs
mRNAs – messenger RNAs
Mtpn – myotrophin

NGT – normal glucose tolerance
NHLS – National Health Laboratory Service
NPV – negative predictive value
OD – optical density
OGTT – oral glucose tolerance test
OR – odds ratio
PBMCs – peripheral blood mononuclear cells
PI3K/Akt – phosphoinositide-3-kinase/protein kinase B
PIK3R1 – phosphoinositide-3-kinase regulatory subunit 1
PPV – positive predictive value
pre-miRNA – precursor miRNA
pri-miRNAs – primary miRNAs
RHD – rheumatic heart disease
RNAi – RNA interference
ROC – receiver operating characteristic curve
RT-qPCR – reverse transcriptase quantitative PCR
SAMRC – South African Medical Research Council
SANRF – South African National Research Foundation
SBP – systolic blood pressure
S-creatinine – serum creatinine
SD – standard deviation
SNPs – single nucleotide polymorphisms
T1DM – type 1 diabetes mellitus
T2DM – type 2 diabetes mellitus
TG – triglycerides
TNF-α – tumour necrosis factor-α
TCF7L2 – transcription factor 7-like 2
uLMCAD – unprotected left main coronary artery disease
usCRP – ultra-sensitive C-reactive protein
VMH – Cape Town Vascular and Metabolic Health
VSMC – vascular smooth muscle cells
WHO – World Health Organization

# CHAPTER 1 – INTRODUCTION

### 1.1 Diabetes mellitus (DM)

Diabetes mellitus (DM), is a heterogeneous metabolic disorder characterized by the presence of hyperglycaemia due to insulin secretion impairment, defective insulin action or both. This chronic hyperglycaemia is associated with long-term microvascular complications that affect the eyes, kidneys and nerves, as well as a resultant increased risk for developing cardiovascular disease (CVD) (Punthakee *et al*., 2018). It is a major burden globally, with 463 million people reported to be living with diabetes worldwide in 2019 (International Diabetes Federation, 2019). The International Diabetes Federation reported that Africa in particular may be subject to a 143% increase in the number of people diagnosed with DM by the year 2045. Furthermore, South Africa was described as having the highest age-adjusted prevalence of DM in the entire African region, the highest number of deaths, as well as the highest diabetes-related expenditure (approximately 23% of the total health budget) (International Diabetes Federation, 2019). Diabetes is often preceded by a condition known as prediabetes which describes a state whereby an individual's blood glucose levels are not low enough for them to be considered normoglycaemic, and neither high enough for them to classified as diabetic (Hamar, 2012). According to the World Health Organization (WHO) criteria, these individuals may either be categorized as having impaired fasting glucose (IFG), with fasting blood glucose levels 6.1-6.9 mmol/L, or impaired glucose tolerance (IGT), with 2-hour blood glucose levels (7.8-11.0 mmol/L), both of which are risk factors for the development of type 2 diabetes mellitus (T2DM) (World Health Organization, 2006). It has been shown that in some populations as many as 70% of people categorized as IFG or IGT may progress to overt T2DM, hence the need to accurately identify these individuals, as at this stage intervention measures may contribute to reverting glucose tolerance to normalcy (Li *et al*., 2008).

On the other hand, the American Diabetes Association (ADA), though suggesting the same reference range as the WHO for IGT, suggest a lower cut-off limit for IFG (5.6-6.9 mmol/L), as well as accepting the use of the HbA1c for the diagnosis of prediabetes (5.7% to 6.4%) (American Diabetes Association, 2014). Contrasts such as these, between globally accepted classification bodies, have raised concerns, and the reliability of these cut-off-based tests in anticipating future development of T2DM has been questioned (Genuth & Kahn, 2008). This criticism has extended to the use of the HbA1c in diabetes diagnosis, with reports of various factors that may affect its efficiency (Bloomgarden *et al*., 2008; Florkowski, 2013). Factors such as age have been seen to influence HbA1c readings, with levels generally increasing with age (Davidson & Schriger, 2010). Genetic factors are also known to affect Hb1Ac, such as ethnic differences, with reports that African people usually have higher HbA1c levels as opposed to Caucasian people (Ziemer *et al*., 2010). Moreover, hereditary genetic conditions such as haemoglobinopathies impact the efficiency of HbA1c, resulting in inaccurate results

(Florkowski, 2013). In light of this, it is therefore essential to optimize diabetes screening strategies by identifying innovative techniques, that may eliminate the adverse effects of such factors.

There are various factors contributing to the progression and onset of T2DM, such as age, obesity, hypertension, environmental factors, as well as genetic factors (Kaku, 2010; Habtamu *et al*., 2015), and there has been a considerable growth in research focusing on the role of epigenetics, namely the interactions between the environment and gene expression (Ling & Groop, 2009; Kaku, 2010; Habtamu *et al*., 2015). Particular interest has been drawn to microRNAs (miRNAs), which are non-coding RNA transcripts that regulate gene expression through targeting messenger RNAs (mRNAs) in mammals and plants (Lee *et al*., 1993; Bartel, 2004). These transcripts, which are present in peripheral blood at consistent levels, have been shown to mediate various cellular processes and mechanisms, such as differentiation, proliferation as well as senescence and apoptosis (Kong *et al*., 2011; Ardekani & Naeini, 2010). Furthermore, miRNAs have been revealed to play a role in regulating various processes involved in the pathogenesis of diseases such as cancers, cardiovascular diseases (CVDs), as well as DM (Hanahan & Weinberg, 2011; Zhang *et al*., 2012; Xu *et al*., 2012; Marques *et al*., 2011; Karolina *et al*., 2011; Rezk *et al*., 2016). miRNA regulation has been reported in insulin production and secretion, through maintenance of pancreatic beta-cell growth and development (Poy *et al*., 2004; Xia *et al*., 2011). Moreover, miRNA involvement has been shown in pathways related to insulin action and sensitivity (He *et al*., 2007; Dooley *et al*., 2016), and studies have demonstrated dysregulated miRNAs in prediabetic as well as diabetic individuals in comparison to normotolerant individuals, proposing their use as biomarkers for early detection and as indicators of diabetes risk schemes (Zampetaki *et al*., 2010; Al-Kafaji *et al*., 2016).

### 1.2 Rationale of the study

Numerous investigations have illustrated links between the development of DM and the complex interplay between the effects of the surrounding environment, namely lifestyle habits, and gene expression. Recent propositions suggest that the connection between genetic predisposition and environmental factors contributing to the development of DM, may lie in complex epigenetic pathways and modifications. There is substantial evidence detailing the genetic diversity of the African population, and considering the evolutionary contribution of the continent to the genetic blueprint of the human race as a whole, exploring genomics in African individuals may provide further insight into the pathophysiology of DM, for optimization of screening and therapeutic approaches not only in Africans, but in populations around the world. Examples of successful diabetes-related genomic investigations include the identification of associations between potassium inwardly rectifying channel subfamily J member 11 (KCNJ11) and transcription factor 7-like 2 (TCF7L2) variants and poor patient response to the sulphonylurea class of anti-diabetic treatment (Rastegari *et al*., 2015; Holstein *et al*.,

2011), as well as the role of variants proximal to the ataxia telangiectasia mutated (ATM) gene that may affect glycaemic response to metformin (Zhou *et al.*, 2011). It is therefore essential to expand on epigenetic interactions, especially in Sub-Saharan African populations, which may eventually pave the way for the discovery of novel biomarkers, to facilitate diagnosis/ prognosis and management of DM.

Matsha *et al* recently conducted an investigation involving differentially expressed miRNAs compared across subgroups with differing glucose tolerance status, the first study of its kind in Africa. Differentially expressed miRNAs were identified in the prediabetic and screen-detected (newly-diagnosed) diabetic groups, as well as screen-detected and known diabetic groups, when compared to the normotolerant group (Matsha *et al.*, 2018). This study formed the basis of the current study, ***whose overall objective was to validate the most dysregulated miRNAs found, in a larger sample size, with the purpose of identifying miRNAs that may in future serve as possible markers for the early identification of individuals at risk of progressing to overt T2DM, and also be incorporated in therapy programmes.*** This objective was achieved by the following aims:

(i) Investigating the expression of miR-30a-5p, miR-1299, miR-182-5p, miR-30e-3p and miR-126-3p in individuals with prediabetes and newly diagnosed T2DM
(ii) Investigating the diagnostic ability of these miRNAs to accurately identify individuals with prediabetes and newly diagnosed T2DM
(iii) Investigating the expression of the miRNAs in individuals with known diabetes and on anti-diabetic therapy

### 1.3 References


Al-Kafaji, G., Al-Mahroos, G., Al-Muhtaresh, H.A., Skrypnyk, C., Sabry, M.A. & Ramadan, A.R. 2016. Decreased expression of circulating microRNA-126 in patients with type 2 diabetic nephropathy: A potential blood-based biomarker. *Experimental and Therapeutic Medicine*, 12(2): 815–822.

American Diabetes Association. 2014. Diagnosis and classification of diabetes mellitus. *Diabetes care*, 37(Supplement 1): S81–S90.

Ardekani, A.M. & Naeini, M.M. 2010. The role of microRNAs in human diseases. *Avicenna Journal of Medical Biotechnology*, 2(4): 161–179.

Bartel, D.P. 2004. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell*, 116(2): 281–297.

Bloomgarden, Z.T., Inzucchi, S.E., Karnieli, E. & Le Roith, D. 2008. The proposed terminology 'A 1c-derived average glucose' is inherently imprecise and should not be adopted. *Diabetologia*, 51(7): 1111.

Davidson, M.B. & Schriger, D.L. 2010. Effect of age and race/ethnicity on HbA1c levels in people without known diabetes mellitus: implications for the diagnosis of diabetes. *Diabetes research and clinical practice*, 87(3): 415–421.

Dooley, J., Garcia-Perez, J.E., Sreenivasan, J., Schlenner, S.M., Vangoitsenhoven, R., Papadopoulou, A.S., Tian, L., Schonefeldt, S., Serneels, L., Deroose, C., Staats, K.A., Van Der Schueren, B., De Strooper, B., McGuinness, O.P., Mathieu, C. & Liston, A. 2016. The microRNA-29 family dictates the balance between homeostatic and pathological glucose handling in diabetes and obesity. *Diabetes*, 65(1): 53–61.

Florkowski, C. 2013. HbA1c as a diagnostic test for diabetes mellitus - Reviewing the evidence. *Clinical Biochemist Reviews*, 34(2): 75–83.

Genuth, S. & Kahn, R. 2008. A step backward—or is it forward? *Diabetes Care*, 31(6): 1093–1096.

Habtamu, W.B., Wondifraw, G. & Baynest, J. 2015. Classification, Pathophysiology, Diagnosis and Management of Diabetes Mellitus. *Journal of Diabetes & Metabolism*, 6: 1–9.

Hamar, P. 2012. Role of regulatory microRNAs in type 2 diabetes mellitus-related inflammation. *Nucleic Acid Therapeutics*, 22(5): 289–294.

Hanahan, D. & Weinberg, R.A. 2011. Hallmarks of cancer: The next generation. *Cell*, 144(5): 646–674. doi: 10.1016/j.cell.2011.02.013.

He, A., Zhu, L., Gupta, N., Chang, Y. & Fang, F. 2007. Overexpression of micro ribonucleic acid 29, highly up-regulated in diabetic rats, leads to insulin resistance in 3T3-L1 adipocytes. *Molecular Endocrinology*, 21(11): 2785–2794.

Holstein, A., Hahn, M., Körner, A., Stumvoll, M. & Kovacs, P. 2011. TCF7L2 and therapeutic response to sulfonylureas in patients with type 2 diabetes. *BMC medical genetics*, 12(1): 1–5. https://pubmed.ncbi.nlm.nih.gov/21349175 [21 October 2020].

International Diabetes Federation. 2019. *IDF Diabetes Atlas*. 9th ed. Brussels, Belgium. https://www.diabetesatlas.org [5 January 2020].

Kaku, K. 2010. Pathophysiology of Type 2 Diabetes and Its Treatment Policy. *Journal of the Japan Medical Association*, 53(1): 41–46.

Karolina, D.S., Armugam, A., Tavintharan, S., Wong, M.T.K., Lim, S.C., Sum, C.F. & Jeyaseelan, K. 2011. MicroRNA 144 Impairs Insulin Signaling by Inhibiting the Expression of Insulin Receptor Substrate 1 in Type 2 Diabetes Mellitus. *PloS one*, 6(8): e22839.

Kong, L., Zhu, J., Han, W., Jiang, X., Xu, M., Zhao, Y., Dong, Q., Pang, Z., Guan, Q. & Gao, L. 2011. Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study. *Acta diabetologica*, 48(1): 61–69.

Lee, R.C., Feinbaum, R.L. & Ambros, V. 1993. The C. elegans Heterochronic Gene lin-4 Encodes Small RNAs with Antisense Complementarity to lin-14. *Cell*, 75(5): 843–854.

Li, G., Zhang, P., Wang, J., Gregg, E.W., Yang, W., Gong, Q., Li, Hui, Li, Hongliang, Jiang, Y., An, Y., Shuai, Y., Zhang, B., Zhang, J., Thompson, T.J., Gerzoff, R.B., Roglic, G., Hu, Y. & Bennett, P.H. 2008. The long-term effect of lifestyle interventions to prevent diabetes in the China Da Qing Diabetes Prevention Study: a 20-year follow-up study. *The Lancet*, 371(9626): 1783–1789.

Ling, C. & Groop, L. 2009. Epigenetics: A molecular link between environmental factors and type 2 diabetes. *Diabetes*, 58(12): 2718–2725.

Marques, F.Z., Campain, A.E., Tomaszewski, M., Zukowska-Szczechowska, E., Yang, Y.H.J., Charchar, F.J. & Morris, B.J. 2011. Gene expression profiling reveals renin mRNA overexpression in human hypertensive kidneys and a role for microRNAs. *Hypertension*, 58(6): 1093–1098.

Matsha, T.E., Kengne, A.P., Hector, S., Mbu, D.L., Yako, Y. & Erasmus, R.T. 2018. MicroRNA profiling and their pathways in South African individuals with prediabetes and newly diagnosed type 2 diabetes mellitus. *Oncotarget*, 9(55): 30485–30498.

Poy, M.N., Eliasson, L., Krutzfeldt, J., Kuwajima, S., Ma, X., MacDonald, P.E., Pfeffer, S., Tuschl, T., Rajewsky, N., Rorsman, P. & Stoffel, M. 2004. A pancreatic islet-specific microRNA regulates insulin secretion. *Nature*, 432(7014): 226–230. doi: 10.1038/nature03076.

Punthakee, Z., Goldenberg, R. & Katz, P. 2018. Definition, Classification and Diagnosis of Diabetes, Prediabetes and Metabolic Syndrome. *Canadian Journal of Diabetes*, 42: S10–S15. doi: 10.1016/j.jcjd.2017.10.003.

Rastegari, A., Rabbani, M., Sadeghi, H.M., Imani, E.F., Hasanzadeh, A. & Moazen, F. 2015. Pharmacogenetic association of KCNJ11 (E23K) variant with therapeutic response to sulphonylurea (glibenclamide) in Iranian patients. *International Journal of Diabetes in Developing Countries*, 35: 630–631.

Rezk, N.A., Sabbah, N.A. & Saad, M.S.S. 2016. Role of microRNA 126 in screening, diagnosis, and prognosis of diabetic patients in Egypt. *IUBMB Life*, 68(6): 452–458. doi: 10.1080/15592294.2016.1278097.

World Health Organization. 2006. *Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia: report of a WHO/IDF consultation*. Geneva: World Health Organization.

Xia, H.Q., Pan, Y., Peng, J. & Lu, G.X. 2011. Over-expression of miR375 reduces glucose-induced insulin secretion in Nit-1 cells. *Molecular Biology Reports*, 38(5): 3061–3065.

Xu, M., Wu, H.-D., Li, R.-C., Zhang, H.-B., Wang, M., Tao, J., Feng, X.-H., Guo, Y.-B., Li, S.-F., Lai, S.-T., Zhou, P., Li, L.-L., Yang, H.-Q., Luo, G.-Z., Bai, Y., Xi, J.J., Gao, W., Han, Q.-D., Zhang, Y.-Y., Wang, X.-J., Meng, X. & Wang, S.-Q. 2012. Mir-24 regulates junctophilin-2 expression in cardiomyocytes. *Circulation research*, 111(7): 837–841.

Zampetaki, A., Kiechl, S., Drozdov, I., Willeit, P., Mayr, U., Prokopi, M., Mayr, A., Weger, S., Oberhollenzer, F., Bonora, E., Shah, A., Willeit, J. & Mayr, M. 2010. Plasma MicroRNA profiling reveals loss of endothelial MiR-126 and other MicroRNAs in type 2 diabetes. *Circulation Research*, 107(6): 810–817.

Zhang, Y., Dutta, A. & Abounader, R. 2012. The role of microRNAs in glioma initiation and progression. *Frontiers in bioscience (Landmark edition)*, 17: 700–712.

Zhou, K., Bellenguez, C., Spencer, C.C.A., Bennett, A.J., Coleman, R.L., Tavendale, R., Hawley, S.A., Donnelly, L.A., Schofield, C. & Groves, C.J. 2011. Common variants near ATM are associated with glycemic response to metformin in type 2 diabetes. *Nature genetics*, 43(2): 117–120.

Ziemer, D.C., Kolm, P., Weintraub, W.S., Vaccarino, V., Rhee, M.K., Twombly, J.G., Narayan, K.M.V., Koch, D.D. & Phillips, L.S. 2010. Glucose-independent, black–white differences in hemoglobin A1c levels: a cross-sectional analysis of 2 studies. *Annals of internal medicine*, 152(12): 770–777.

# CHAPTER 2 – LITERATURE REVIEW

## 2.1 Type 2 diabetes mellitus (T2DM)

### *2.1.1 Epidemiology and Aetiology of type 2 diabetes mellitus (T2DM)*

Type 2 diabetes mellitus (T2DM) is the most common form of diabetes, accounting for approximately 90 to 95% of all cases globally, and its development may be attributed to insulin resistance, which is the decreased sensitivity of target tissues to insulin action (Ozougwu *et al.*, 2013; International Diabetes Federation, 2019). Recognized as a major epidemic in most developed countries, T2DM has also begun to manifest in developing countries (Hu, 2011). In 2019, the International Diabetes Federation (IDF) Diabetes Atlas reported that in Africa, countries such as: South Africa, Nigeria, Democratic Republic of Congo and Ethiopia recorded the highest numbers of people aged 20-79 years living with the disease (55.8% of all individuals in this age group, in the entire region) (International Diabetes Federation, 2019).

In South Africa, rapid urbanization and economic change have stimulated a gradual increase in the prevalence of T2DM, with a more westernized lifestyle contributing towards the increase in metabolic syndrome (Erasmus *et al.*, 2012). In 2019, the prevalence of the disease in South Africa was 12.7%, the highest in comparison to all other countries in the African region, and similarly, the number of diabetes-associated deaths were considered the highest on the continent, totalling 89 900 (International Diabetes Federation, 2019). In the past decade, the emergence of non-communicable diseases such as T2DM in both rural and urban settings in South Africa, have resulted in excessive pressure on health-care services (Mayosi *et al.*, 2009). To date, the country has one of the highest percentages of health-care expenditure due to diabetes, with 23.0% of the total health budget spent on the national management of the disease in 2019 (International Diabetes Federation, 2019). In light of this, there is therefore a grave need for new therapeutic approaches to better control glucose homeostasis.

### *2.1.2 Risk factors affecting type 2 diabetes mellitus (T2DM)*

Progression to T2DM occurs as a result of a gradual loss of insulin-dependent glucose uptake by cells. This results in a precursor state, known as prediabetes, which is characterised by blood glucose levels higher than normal, but not elevated enough to be classified as diabetes (Hamar, 2012). People exhibiting this early dysglycaemic state may either be classified as having impaired fasting glucose (IFG), or impaired glucose tolerance (IGT), both of which are risk factors for the development of T2DM. Reports have suggested that in some populations approximately 70% of people diagnosed as IFG or IGT progress to T2DM (Li *et al.*, 2008). The onset of T2DM can also occur as a result of environmental and genetic factors, affecting mainly elderly individuals displaying visceral obesity, as well as presenting with hypertension and dyslipidaemia (high triglyceride and low high-density lipoprotein cholesterol (HDL-cholesterol) levels; postprandial hyperlipidaemia) (Kaku, 2010; Habtamu

*et al.*, 2015). Approximately 80% of diabetic individuals are obese, which occurs subsequent to impaired sensitivity of peripheral tissues, such as muscle and adipose tissues, to insulin, otherwise known as insulin resistance (Parmar, 2018). Ultimately, the pancreatic beta-cells, responsible for secretion of insulin, are unable to compensate for the reduced insulin sensitivity and beta-cell dysfunction ensues (Al-Goblan *et al.*, 2014).

Furthermore, elevated circulating insulin, or hyperinsulinaemia, has also been shown to promote arterial remodelling and stiffness in states of insulin resistance, thereby pointing to a connection with hypertension (Ferrannini & Cushman, 2012; Park *et al.*, 2013). The most widely described mechanisms justifying the associations between hypertension and T2DM are increased oxidative stress and low-grade inflammation, both of which play key roles in endothelial dysfunction, reduced vascular reactivity, excessive peripheral vascular resistance, as well as altered glucose and lipid metabolism. These are all mechanisms which lead to altered haemodynamic and glycolipid metabolism, resulting in elevated blood pressure levels and plasma glucose (Petrie *et al.*, 2018).

#### ***2.1.3 Genetic factors affecting type 2 diabetes mellitus (T2DM)***

T2DM is a metabolic disease whose development is attributed to the complex interplay between environmental and genetic factors, with genome-wide association studies demonstrating several genes accountable for, or linked with the occurrence of T2DM (Saxena *et al.*, 2007; McCarthy & Zeggini, 2009; Scott *et al.*, 2017). Although there has been enthusiasm in view of the identification of genetic risk loci for T2DM (Qi & Hu, 2012; Yu *et al.*, 2012), the incorporation of these genetic markers in clinical settings for T2DM prediction and prevention has remained limited. Increasing evidence has suggested that epigenetic mechanisms may provide a key contribution to the interface between interactions between genes and the surrounding environment in T2DM, as well as other diseases, and studies have demonstrated a congruence of epigenetic loci and gene polymorphisms in diseases (Kathiresan *et al.*, 2008; El-Menyar *et al.*, 2015; Staiger *et al.*, 2007; Dayeh *et al.*, 2013). For instance, 19 single nucleotide polymorphisms (SNPs) associated with T2DM were found to either introduce or remove potential sites for DNA methylation (Dayeh *et al.*, 2013).

##### *2.1.3.1 Epigenetics*

Epigenetics may be defined as “the study of changes in gene function that are mitotically and/or meiotically heritable, and that do not entail a change in DNA sequence’’ (Wu & Morris, 2001). These genomic changes occur as a response to external stimuli, inducing gene silencing or activation, providing plausible links between genetic and environmental determinants of health statuses (Tchurikov, 2005; Sommese *et al.*, 2017). Examples of epigenetic mechanisms include lysine methylation, histone methylation, histone phosphorylation, genomic imprinting, DNA methylation and RNA interference (RNAi) (Tchurikov, 2005; Szyf, 2009; Sharma *et al.*, 2010). Lysine methylation is a

process involving the addition of one or more methyl groups from S-adenosyl-L-methionine to the **ε**-amino group of a lysine residue, and this is catalysed by specific enzymes called lysine methyltransferases (Qian & Zhou, 2006). Histone methylation on the other hand, involves the addition of a methyl group to the amino acids of histone proteins on nucleosomes (Szyf, 2009). Another type of histone modification is histone phosphorylation, whereby a phosphate group is added to histone proteins, and is important in modulating chromatin structure (Rossetto *et al.*, 2012). Genomic imprinting is a mechanism in which the expression of a particular gene is restricted to one of the parental alleles (Barlow & Bartolomei, 2014).

DNA methylation is an epigenetic mechanism involving the covalent addition of a methyl group to the 5'-position of cytosine nucleotides, catalysed by DNA methyltransferase enzymes. This results in a 5-methylcytosine in CpG dinucleotides concentrated in gene promoters (Moore *et al.*, 2013). These CpG dinucleotides in gene promoters are not usually methylated, therefore methylation at these regions correspond with alterations in gene expression. Hypermethylation induces suppressed transcription ultimately decreasing gene expression, whilst hypomethylation promotes transcriptional activation of the genes involved (Phillips, 2008). RNA interference (RNAi) is also a gene regulatory mechanism controlling transcription by either inhibiting transcription, or by initiating sequence-specific RNA-degradation (Agrawal *et al.*, 2003). MicroRNAs (miRNAs), are one of the many RNA transcripts involved in this post-transcriptional gene expression regulatory mechanism, and since they circulate in the bloodstream, they are being heralded as minimally invasive biomarkers for age-related diseases including T2DM and its complications (Mattie *et al.*, 2006; Yang *et al.*, 2013; Rezk *et al.*, 2016).

## 2.2 MicroRNA (miRNA) biogenesis

First discovered in 1993 by Lee *et al*, miRNAs are endogenous RNA transcripts, approximately 22 nucleotides in length, that play important regulatory roles by targeting messenger RNAs (mRNAs) for cleavage or translational repression in animals and plants (Lee *et al.*, 1993; Bartel, 2004). According to miRBase, the human genome encodes for approximately 2000 different miRNA transcripts, divided into two classes, canonical and non-canonical miRNAs, depending on the processing mechanisms of the primary miRNAs (pri-miRNAs) (Kozomara & Griffiths-Jones, 2011; Graves & Zeng, 2012).

Canonical miRNA biogenesis involves several steps which ultimately lead to the final mature miRNA transcript. This process begins with the initial transcription of miRNA genes into longer primary-miRNA (pri-miRNA) transcripts in the nucleus (Abdelfattah *et al.*, 2014). Still in the nucleus, the RNA binding protein DiGeorge Critical Region 8 (DGCR8) recognizes the pri-miRNA, directing the RNase III enzyme Drosha, which subsequently cleaves the pri-miRNA, yielding a precursor miRNA (pre-miRNA) hairpin molecule which is roughly 70 nucleotides in length (Abdelfattah *et al.*, 2014).

Recognising the two nucleotide 3`-overhang from Drosha cleavage, exportin-5 transports the pre-miRNA into the cytoplasm, where a second RNase III named Dicer, paired with the double stranded RNA binding domain (dsRBD) proteins TRBP/PACT/Loquacious (Loqs), cleaves the pre-miRNA, releasing a 22 nucleotide miRNA duplex (Miyoshi *et al.*, 2010). Upon maturation, one of the two duplex strands, destined to become the mature miRNA transcript, associates with an Argonaute (Ago) protein and loads into a miRNA-induced silencing complex (miRISC) (Castellano & Stebbing, 2013). The mature miRNA then guides the miRISC to complementary sites within the 3`-untranslated regions (UTRs) of the target mRNA molecule, leading to repression of that mRNA (Bartel, 2009). Alternatively, miRNA repression can be reversed and the miRISC may even activate expression of the target mRNA (Miyoshi *et al.*, 2010).

Alternative mechanisms of miRNA biogenesis have arisen, otherwise called "non-canonical pathways." Deep sequencing have revealed classes of miRNA molecules which structurally and functionally resemble canonically derived miRNAs (Abdelfattah *et al.*, 2014). Non-canonical miRNA biogenesis differs from the more common canonical pathway in that one or more steps in the conventional canonical biogenesis pathway are bypassed, chiefly the absence of Drosha and DGCR8 processes. Drosha and DGCR8 are therefore only necessary for canonical miRNA processing, while non-canonical miRNAs can be generated in their absence (Abdelfattah *et al.*, 2014).

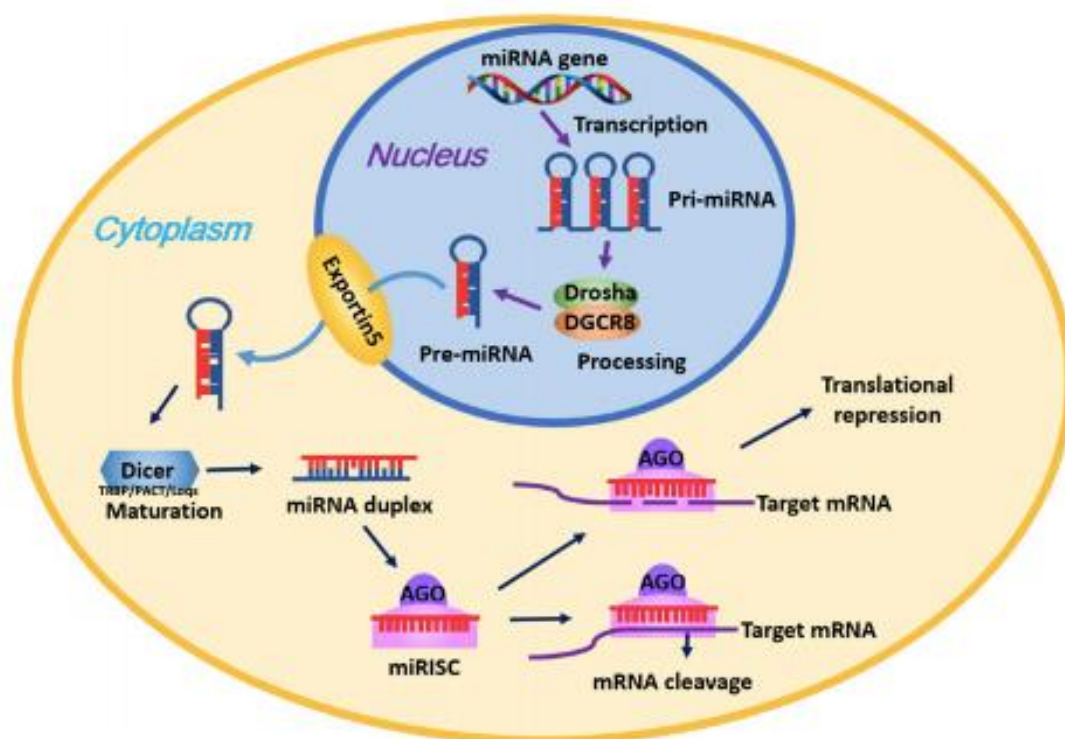



**Figure 1:** Canonical pathway of miRNA biogenesis (Zhang *et al.*, 2017).

## 2.3 MicroRNAs (miRNAs) and disease

Many important cellular process have been shown to be regulated by miRNA transcripts, including differentiation, proliferation and apoptosis (Ardekani & Naeini, 2010). In light of these reports, miRNAs have subsequently been demonstrated to act as vital mediators in the pathogenesis of diseases such as cancers, cardiovascular diseases (CVDs), as well as DM (Hanahan & Weinberg, 2011; Zhang *et al.*, 2012; Xu *et al.*, 2012; Marques *et al.*, 2011; Karolina *et al.*, 2011; Rezk *et al.*, 2016).

### *2.3.1 MicroRNAs (miRNAs) and cancer*

miRNA dysregulation plays a role in the initiation and progression of various human cancers, with overexpressed miRNAs reported to play oncogenic roles by downregulating tumour suppressor genes and/or genes regulating cell differentiation or apoptosis. Alternatively, downregulated miRNAs have been illustrated to act as tumour suppressor genes, negatively regulating oncogenes and/or genes that control cell differentiation or apoptosis (Zhang *et al.*, 2007; L. He *et al.*, 2007). Distinctive miRNA signatures have been identified to be involved in various cancers such as breast, ovarian, endometrial, oesophageal, colon, lung, bladder, as well as haematological cancers (Ardekani & Naeini, 2010).

The first documented association between miRNA dysregulation and cancer was reported by Calin et el in 2002, who reported that a frequently deleted chromosome region (13q14.3) in chronic lymphocytic leukaemia (CLL) encoded for miRNAs miR-15 and miR-16. This chromosomal abnormality consequently led to decreased expression of the miR-15/16 cluster which targets and controls the anti-apoptotic B-cell lymphoma 2 (BCL2) protein. Decreased expression of miR-15/16 consequently resulted in uncontrolled expression of BCL2, decreased apoptosis and increased proliferation of the malignant cells (Calin *et al.*, 2002).

miR-21 has been reported to regulate in vitro proliferation, apoptosis, and migration of tumour cells. Overexpression of this miRNA has also been linked to cancers, namely breast, colorectal, lung, glioblastoma, neuroblastoma, leukaemias and lymphomas. In vivo, the same miRNA has proved useful in prognostic predictions of cancer patients, having been reported to act as a tumour suppressor target (Selcuklu *et al.*, 2009). In contrast, a downregulation of miR-1299 was demonstrated in hepatocellular carcinoma (HCC) tissues by Zhu *et al*, who identified the participation of the miRNA in inhibiting cell proliferation by targeting the cyclin-dependent kinase 6 (CDK6) gene involved in cellular differentiation, proliferation and maintenance (Zhu *et al.*, 2016). In view of reports detailing miRNA dysregulation in cancer development and progression, the use of these transcripts as candidates for diagnostic and prognostic markers, as well as therapeutic targets has been promoted (Peng & Croce, 2016).

### *2.3.2 MicroRNAs (miRNAs) and cardiovascular disease (CVD)*

Cardiovascular diseases (CVDs) are the leading cause of mortality worldwide, and despite substantial advances made in diagnosis, treatment and prognosis of these disorders, there is still a grave need for novel diagnostic biomarkers and therapeutic interventions to reduce their incidences (Zhou *et al.*, 2018). The first publication implicating miRNAs in CVD was in a mouse model, whereby >12 miRNAs were found to be dysregulated in cardiac tissue in response to stimuli that induced pathological cardiac remodelling (van Rooij *et al.*, 2006). Since then, various follow-up studies have detailed changes in miRNA expression in cardiovascular disorders such as myocardial infarction, hypertension, as well as atherosclerosis (Long *et al.*, 2012; Eskildsen *et al.*, 2013; Kumar *et al.*, 2014).

Long *et al* explored plasma miRNA expression in patients with acute myocardial infarction (AMI) at intervals after onset of the condition, and illustrated upregulations of miRNAs miR-30a and -195, whilst a downregulation of miR-let-7b. Moreover, receiver operating characteristic (ROC) curve analyses indicated significant diagnostic capabilities of the miRNAs in differentiating AMI patients from healthy individuals (Long *et al.*, 2012). Links between miRNA expression and hypertension were described by Eskildsen *et al*, demonstrating increased miR-132 and miR-212 levels in the hearts, aortic walls and kidneys of rats with hypertension and cardiac hypertrophy following chronic angiotensin II infusion. Further postulating that angiotensin II type-1 receptor blockers (AT1R blockers) may reduce miR-132 and -212 expression, they analyzed tissue samples of surplus mammary arterial tissue from coronary bypass surgeries and found reduced miR-132 and miR-212 expression levels in patients treated with AT1R blockers (Eskildsen *et al.*, 2013).

Atherosclerosis is a disease occurring at susceptible sites in major conduit arterial walls as a result of lipid retention, oxidation and modification, which give rise to chronic inflammation, eventually leading to thrombosis or stenosis (Insull, 2009). In a study conducted in mice, miR-126-5p was identified as a vital regulator of endothelial integrity, through suppressing the Notch1 inhibitor delta-like 1 homolog (Dlk1), preventing atherosclerotic plaque formation, with elevated expression in endothelial cells pointing to a proliferative reserve, compensating for the antiproliferative effects of hyperlipidaemia (Schober *et al.*, 2014).

miRNAs have been implicated in various developmental mechanisms such as cellular metabolism, proliferation and apoptosis, with emerging evidence suggesting altered levels vital in the progression of disease (Faruq & Vecchione, 2015). In addition to cancers and CVDs, numerous additional miRNAs have been recognized as constituents of pathways triggered by, or contributing to, the pathology of both T1DM and T2DM (Grieco *et al.*, 2018; Patoulias, 2018). Whether the diseases occur as a direct cause of differentially expressed miRNAs, or this dysregulation is triggered by the pathological state, is still unknown (Guay *et al.*, 2011).

## 2.4 MicroRNAs (miRNAs) and type 2 diabetes mellitus (T2DM)

Candidate miRNAs have been implicated in the pathophysiology of T2DM, involved in pathways mediating insulin production, insulin sensitivity, glucose homeostasis, or lipid metabolism (Ferland-McCollough *et al.*, 2010; Erener *et al.*, 2013). miRNAs are present in peripheral blood at consistent and reproducible levels, and unlike mRNA they are stable and resistant to enzymatic digestion by RNAse (Kong *et al.*, 2011).

### *2.4.1 MicroRNAs (miRNAs) and glucose homeostasis*

miRNA regulation is essential in insulin production and secretion, through maintenance of pancreatic beta-cell growth, and the roles of miRNAs such as miR-375 have been identified in beta-cell development (Poy *et al.*, 2004; Xia *et al.*, 2011). Myotrophin (Mtpn) is a protein responsible for facilitating the late stage exocytosis of insulin, with downregulated expression resulting in reduced insulin secretion (Xia *et al.*, 2011). Being one of the most abundantly expressed miRNAs in beta-cells, miR-375 has been shown to target Mtpn, negatively regulating glucose-stimulated insulin secretion, with overexpression inhibiting insulin secretion (Poy *et al.*, 2004; Xia *et al.*, 2011).

Other miRNAs have similarly been shown to interact with Mtpn, such as miR-124a and -let-7b, promoting the notion that multiple miRNAs may regulate the same target (Krek *et al.*, 2005). In addition to regulating insulin production and secretion, miRNAs have also been shown to interact in pathways involved in insulin action and sensitivity. For instance, the expression of miR-29a and miR-29b was shown to be significantly increased in liver, fat and muscle tissues of diabetic rats, and associations with insulin resistance have been demonstrated in cell-based experiments (He *et al.*, 2007; Dooley *et al.*, 2016). Similarly, the regulatory functions of miRNAs have been illustrated, such as the inhibition of certain proteins such as caveolin-2 (CAV2), insulin-induced gene 1 (INSIG1) and phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1) (Kim & Pak, 2005; He *et al.*, 2007; Pandey *et al.*, 2011). Ryu *et al* used reverse transcriptase quantitative PCR (RT-qPCR) to investigate the expression levels of miRNAs proposed to target insulin receptor substrate 1 (IRS-1), and to assess whether genetic or metabolic inhibition led to mitochondrial dysfunction in hepatocytes. The findings illustrated that overexpression of miRNAs such as miR-126, led to a considerable reduction in IRS-1 protein levels, and subsequent insulin signalling impairment (Ryu *et al.*, 2011). Henceforth, evidence supporting the role of miRNAs in modulating insulin production and secretion pathways, as well as glucose homeostasis provides new perspectives for clinical research in the underlying mechanisms of T2DM (Deng & Guo, 2019).

### *2.4.2 MicroRNAs (miRNAs) and diabetes-associated inflammation*

Low-grade chronic inflammation has been known to contribute to the pathogenesis of T2DM, with hyperglycaemia and oxidative stress being the main inducers of the inflammatory response. Resident cells and infiltrating macrophages release inflammatory cytokines contributing to cell damage, promoting inflammation-driven fibrosis (Barutta *et al*., 2018). There are numerous miRNAs implicated in the regulation of inflammatory processes, directing both the innate and adaptive immune response (Barutta *et al*., 2018).

Studies have detailed monocyte migration to the pancreas, liver, as well as adipose tissue, where they become resident macrophages resulting in local inflammation and subsequent insulin resistance (Kraakman *et al*., 2014). In states of insulin resistance, macrophage polarization occurs, namely the shift from anti-inflammatory M2 populations to the pro-inflammatory M1 population, which initiates the low-grade inflammation by secreting cytokines and chemotactic factors (Stoehr & Federici, 2013). miRNAs involved in regulating macrophage activation include miR-144-3p, reported to increase secretion of inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), through targeting the adenosine triphosphate–binding cassette transporter A1 (ABCA1), stimulating differentiation of THP-1 macrophages into pro-inflammatory foam cells (Hu *et al*., 2014). Furthermore, Talari *et al* revealed the significant role of miR-16 in inhibiting inflammation-induced insulin resistance, demonstrating that forced expression of this miRNA in macrophages inhibited production of TNF-α, IL-6 and interferon-β (IFN-β). Ultimately, hindered production of these inflammatory cytokines was observed to enhance glucose uptake in cultured skeletal myoblasts by upregulation of glucose transporter type 4 (GLUT4) and myocyte enhancer factor 2A (MEF2A) (Talari *et al*., 2015). Moreover, in 2012, Reddy *et al* demonstrated overexpression of miR-200 in aortic vascular smooth muscle cells (VSMC) of diabetic mice. It was further determined that subsequent downregulation of Zeb-1, a target of miR-200, resulted in upregulated pro-inflammatory genes such as cyclo-oxygenase-2 and monocyte chemoattractant protein-1, promoting monocyte adhesion to VSMCs (Reddy *et al*., 2012).

Altered miRNA expression has been associated with various mechanisms and pathways associated with T2DM pathogenesis, as well as associated complications (Xia *et al*., 2011; Dooley *et al*., 2016; Reddy *et al*., 2012; Ferland-McCollough *et al*., 2010; Erener *et al*., 2013). In a recent study, miRNAs were found to be associated with prediabetes and diabetes, and the complications resulting from hyperglycaemia (Matsha *et al*., 2018). In this study, high throughput sequencing was used to assess miRNA expression profiles in the whole blood of 12 individuals with known diabetes, 12 screen-detected diabetes, 12 with prediabetes and 12 with normal glucose tolerance. Comparison of the miRNA expression profiles between prediabetes and screen-detected diabetes, as well as screen-

detected versus known diabetes revealed miRNAs (miR-30a-5p, miR-1299, miR-182-5p, miR-30e-3p and miR-126-3p) that were dysregulated between the groups (Matsha *et al.*, 2018).

#### *2.4.3 MicroRNAs (miRNAs) as biomarkers for disease*

Over the years, epigenetic mechanisms have been shown to provide relevant information regarding gene functions, and their involvement in the modulation of important physiological processes therefore implies that modifications in these mechanisms may contribute to predicting and managing disease development, prompting their use as potential biomarkers (García-Giménez *et al.*, 2017). Epigenetic biomarkers have been heralded in their abilities to convey clues about gene functions in specific cell types, illustrating the extent of the genetic control of various processes, thus filling clinical gaps (García-Giménez *et al.*, 2017). These markers also possess the ability to connect environmental factors, elucidating the effects of factors such as diet and lifestyle on physiological and pathological processes (Alegría-Torres *et al.*, 2011). A further advantage of epigenetic biomarkers is their consistent expression and stability across a wide range of tissues and fluids, such as plasma, serum, urine and semen (Li *et al.*, 2014). For instance, a study headed by Bulla and co-workers demonstrated that the integrity of DNA in plasma ethylenediaminetetraacetic acid (EDTA) samples at 4, −20, and −80°C remained unaltered, and that regardless of storage conditions, it was still possible to conduct DNA methylation analysis without consequential impact on measurements (Bulla *et al.*, 2016). A prime example of an epigenetic-based biomarker, which has been approved by the Food and Drug Administration (FDA), is Epi proColon® 2.0 CE (Epigenomics AG, Berlin, Germany), which detects methylated Septin9 in DNA obtained from serum samples, and is the only methylated marker for colorectal cancer screening, with previous comparisons with known tests describing significantly higher sensitivities and specificities for the marker (Song *et al.*, 2017).

Evidence is emerging revolving around the potential benefits of small, non-coding RNAs as markers of disease prediction and progression (Reid *et al.*, 2011). The possible use of miRNAs as non-invasive diagnostic markers has been proposed as early as 2008, by Lawrie *et al*, who demonstrated elevated serum miR-21 levels in patients with diffuse large B-cell lymphoma (DLBCL), when compared to the control group (Lawrie *et al.*, 2008). Numerous subsequent studies have established potential diagnostic capabilities of these small RNA transcripts in other cancers (Wong *et al.*, 2019; Gao *et al.*, 2018; Ouyang *et al.*, 2014), as well as cardiovascular diseases (Wang *et al.*, 2010; Ovchinnikova *et al.*, 2016; C. Li *et al.*, 2014). Research involving the role of epigenetics and metabolic diseases is still at a preliminary stage, however there have been reports detailing the potential diagnostic and prognostic benefits of epigenetic markers in T2DM (Hidalgo *et al.*, 2014; Yuan *et al.*, 2014; Zampetaki *et al.*, 2010). There has been a growth in studies entailing the associations between miRNA expression and DM development, giving rise to prospects of their use as markers of disease prediction and progression in DM (Esguerra *et al.*, 2014; Al-Kafaji *et al.*, 2016; Zampetaki *et al.*, 2010). In 2010,

Zampetaki and colleagues demonstrated a distinctive miRNA signature in people with T2DM, observing significantly decreased miR-126 expression in these individuals (Zampetaki *et al*., 2010). A similar expression pattern was observed in T2D patients in an Egyptian study conducted by Rezk *et al*, concluding that miR-126 expression may be suitable for diagnosing prediabetes and overt diabetes, as well as in monitoring diabetes-associated complications (Rezk *et al*., 2016). A separate study by Karolina *et al*, identified dysregulated miR-144, -146a, -150, as well as miR-182 in the blood of T2D patients, also promoting the use of miRNA signatures in predetermining the risk of developing T2D (Karolina *et al*., 2011).

In routine clinical settings, highly sensitive and highly specific biomarkers are sought after, and these features are considered before incorporating the use of a potential biomarker (Ray *et al*., 2010). Reverse transcription quantitative PCR (RT-qPCR) remains the standard technique in assessing miRNA expression, with very high sensitivity and specificity in comparison to other assays that have been cleared by the FDA (García-Giménez *et al*., 2017). Moreover, miRNAs are presented as promising candidates in their reproducible expressions in a wide spectrum of samples including peripheral blood and other tissues (Kong *et al*., 2011). Early identification of persons at risk of developing T2DM is of the utmost importance, and embracing the use of sensitive and stable epigenetic markers that may give indications to future disease development is desirable (Gillberg & Ling, 2015). Hence the need to further explore the molecular involvement of miRNA transcripts in DM, and subsequent potential clinical benefits, particularly in settings such as Africa with an increasing occurrence of the disease (International Diabetes Federation, 2019).

### 2.5 References


Abdelfattah, A.M., Park, C. & Choi, M.Y. 2014. Update on non-canonical microRNAs. *Biomolecular Concepts*, 5(4): 275–287.

Agrawal, N., Dasaradhi, P.V.N., Mohmmed, A., Malhotra, P., Bhatnagar, R.K. & Mukherjee, S.K. 2003. RNA Interference: Biology, Mechanism, and Applications. *Microbiology and Molecular Biology Reviews*, 67(4): 657–685.

Al-Goblan, A.S., Al-Alfi, M.A. & Khan, M.Z. 2014. Mechanism linking diabetes mellitus and obesity. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, 7: 587–591.

Al-Kafaji, G., Al-Mahroos, G., Al-Muhtaresh, H.A., Skrypnyk, C., Sabry, M.A. & Ramadan, A.R. 2016. Decreased expression of circulating microRNA-126 in patients with type 2 diabetic nephropathy: A potential blood-based biomarker. *Experimental and Therapeutic Medicine*, 12(2): 815–822.

Alegría-Torres, J.A., Baccarelli, A. & Bollati, V. 2011. *Epigenetics and lifestyle. Epigenomics*, 3(3): 267–277.

Ardekani, A.M. & Naeini, M.M. 2010. The role of microRNAs in human diseases. *Avicenna Journal of Medical Biotechnology*, 2(4): 161–179.

Barlow, D.P. & Bartolomei, M.S. 2014. Genomic imprinting in mammals. *Cold Spring Harbor perspectives in biology*, 6(2): a018382.

Bartel, D.P. 2004. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell*, 116(2): 281–297.

Bartel, D.P. 2009. MicroRNAs: Target Recognition and Regulatory Functions. *Cell*, 136(2): 215–233.

Barutta, F., Bellini, S., Mastrocola, R., Bruno, G. & Gruden, G. 2018. MicroRNA and Microvascular Complications of Diabetes. *International Journal of Endocrinology*, 2018: 1–20.

Bulla, A., De Witt, B., Ammerlaan, W., Betsou, F. & Lescuyer, P. 2016. Blood DNA yield but not integrity or methylation is impacted after long-term storage. *Biopreservation and biobanking*, 14(1): 29–38.

Calin, G.A., Dumitru, C.D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., Rassenti, L., Kipps, T., Negrini, M., Bullrich, F. & Croce, C.M. 2002. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proceedings of the National Academy of Sciences of the United States of America*, 99(24): 15524–15529.

Castellano, L. & Stebbing, J. 2013. Deep sequencing of small RNAs identifies canonical and non-canonical miRNA and endogenous siRNAs in mammalian somatic tissues. *Nucleic Acids Research*, 41(5): 3339–3351.

Dayeh, T.A., Olsson, A.H., Volkov, P., Almgren, P., Rönn, T. & Ling, C. 2013. Identification of CpG-SNPs associated with type 2 diabetes and differential DNA methylation in human pancreatic islets. *Diabetologia*, 56(5): 1036–1046.

Deng, J. & Guo, F. 2019. MicroRNAs and type 2 diabetes. *ExRNA*, 1(1): 36. doi: 10.1186/s41544-019-0038-5.

Dooley, J., Garcia-Perez, J.E., Sreenivasan, J., Schlenner, S.M., Vangoitsenhoven, R., Papadopoulou, A.S., Tian, L., Schonefeldt, S., Serneels, L., Deroose, C., Staats, K.A., Van Der Schueren, B., De Strooper, B., McGuinness, O.P., Mathieu, C. & Liston, A. 2016. The microRNA-29 family dictates the balance between homeostatic and pathological glucose handling in diabetes and obesity. *Diabetes*, 65(1): 53–61.

El-Menyar, A.A., Rizk, N.M., Al-Qahtani, A., AlKindi, F., Elyas, A., Farag, F., Bakhsh, F.D., Ebrahim, S., Ahmed, E., Al-Khinji, M., Al-Thani, H. & Suwaidi, J. Al. 2015. The cardiovascular implication of single nucleotide polymorphisms of chromosome 9p21 locus among Arab population. *Journal of research in medical sciences: the official journal of Isfahan University of Medical Sciences*, 20(4): 346–352.

Erasmus, R.T., Soita, D.J., Hassan, M.S., Blanco-Blanco, E., Vergotine, Z., Kengne, A.P. & Matsha, T.E. 2012. High prevalence of diabetes mellitus and metabolic syndrome in a South African coloured population: Baseline data of a study in Bellville, Cape Town. *South African Medical Journal*, 102(11): 841–844.

Erener, S., Mojibian, M., Fox, J.K., Denroche, H.C. & Kieffer, T.J. 2013. Circulating miR-375 as a biomarker of beta-cell death and diabetes in mice. *Endocrinology*, 154(2): 603–608.

Eskildsen, T. V, Jeppesen, P.L., Schneider, M., Nossent, A.Y., Sandberg, M.B., Hansen, P.B.L., Jensen, C.H., Hansen, M.L., Marcussen, N., Rasmussen, L.M., Bie, P., Andersen, D.C. & Sheikh, S.P. 2013. Angiotensin II regulates microRNA-132/-212 in hypertensive rats and humans. *International journal of molecular sciences*, 14(6): 11190–11207. https://pubmed.ncbi.nlm.nih.gov/23712358 [12 November 2019].

Faruq, O. & Vecchione, A. 2015. microRNA: Diagnostic perspective. *Frontiers in Medicine*, 2: 1–10.

Ferland-McCollough, D., Ozanne, S.E., Siddle, K., Willis, A.E. & Bushell, M. 2010. The involvement of microRNAs in Type 2 diabetes. *Biochemical Society Transactions*, 38(6): 1565 LP – 1570. http://www.biochemsoctrans.org/content/38/6/1565.abstract [20 June 2019].

Ferrannini, E. & Cushman, W.C. 2012. Diabetes and hypertension: The bad companions. *The Lancet*, 380(9841): 601–610. doi: 10.1016/S0140-6736(12)60987-8.

Gao, C., Zhou, C., Zhuang, J., Liu, L., Liu, C., Li, H., Liu, G., Wei, J. & Sun, C. 2018. MicroRNA expression in cervical cancer: novel diagnostic and prognostic biomarkers. *Journal of cellular biochemistry*, 119(8): 7080–7090.

García-Giménez, J.L., Seco-Cervera, M., Tollefsbol, T.O., Romá-Mateo, C., Peiró-Chova, L., Lapunzina, P. & Pallardó, F. V. 2017. Epigenetic biomarkers: current strategies and future challenges for their use in the clinical laboratory. *Critical reviews in clinical laboratory sciences*, 54(7–8): 529–550.

Gillberg, L. & Ling, C. 2015. The Potential Use of DNA Methylation Biomarkers to Identify Risk and Progression of Type 2 Diabetes. *Frontiers in Endocrinology*, 6: 43.

Graves, P. & Zeng, Y. 2012. Biogenesis of Mammalian MicroRNAs: A Global View. *Genomics, Proteomics and Bioinformatics*, 10(5): 239–245. doi: 10.1016/j.gpb.2012.06.004.

Grieco, G.E., Cataldo, D., Ceccarelli, E., Nigi, L., Catalano, G., Brusco, N., Mancarella, F., Ventriglia, G., Fondelli, C. & Guarino, E. 2018. Serum levels of miR-148a and miR-21-5p are increased in type 1 diabetic patients and correlated with markers of bone strength and metabolism. *Non-coding RNA*, 4(4): 37.

Guay, C., Roggli, E., Nesca, V., Jacovetti, C. & Regazzi, R. 2011. Diabetes mellitus, a microRNA-related disease? *Translational Research*, 157(4): 253–264.

Habtamu, W.B., Wondifraw, G. & Baynest, J. 2015. Classification, Pathophysiology, Diagnosis and Management of Diabetes Mellitus. *Journal of Diabetes & Metabolism*, 6: 1–9.

Hamar, P. 2012. Role of regulatory microRNAs in type 2 diabetes mellitus-related inflammation. *Nucleic Acid Therapeutics*, 22(5): 289–294.

Hanahan, D. & Weinberg, R.A. 2011. Hallmarks of cancer: The next generation. *Cell*, 144(5): 646–674. doi: 10.1016/j.cell.2011.02.013.

He, A., Zhu, L., Gupta, N., Chang, Y. & Fang, F. 2007. Overexpression of micro ribonucleic acid 29, highly up-regulated in diabetic rats, leads to insulin resistance in 3T3-L1 adipocytes. *Molecular Endocrinology*, 21(11): 2785–2794.

He, L., He, X., Lim, L.P., de Stanchina, E., Xuan, Z., Liang, Y., Xue, W., Zender, L., Magnus, J., Ridzon, D., Jackson, A.L., Linsley, P.S., Chen, C., Lowe, S.W., Cleary, M.A. & Hannon, G.J. 2007. A microRNA component of the p53 tumour suppressor network. *Nature*, 447(7148): 1130–1134. https://pubmed.ncbi.nlm.nih.gov/17554337 [12 June 2019].

Hidalgo, B., Irvin, M.R., Sha, J., Zhi, D., Aslibekyan, S., Absher, D., Tiwari, H.K., Kabagambe, E.K., Ordovas, J.M. & Arnett, D.K. 2014. Epigenome-wide association study of fasting measures of glucose, insulin, and HOMA-IR in the Genetics of Lipid Lowering Drugs and Diet Network study. *Diabetes*, 63(2): 801–807.

Hu, F.B. 2011. Globalization of diabetes: The role of diet, lifestyle, and genes. *Diabetes Care*, 34(6): 1249–1257.

Hu, Y.-W., Hu, Y.-R., Zhao, J.-Y., Li, S.-F., Ma, X., Wu, S.-G., Lu, J.-B., Qiu, Y.-R., Sha, Y.-H., Wang, Y.-C., Gao, J.-J., Zheng, L. & Wang, Q. 2014. An agomir of miR-144-3p accelerates plaque formation through impairing reverse cholesterol transport and promoting pro-inflammatory cytokine production. *PloS one*, 9(4): e94997–e94997. https://pubmed.ncbi.nlm.nih.gov/24733347 [11 April 2020].

Insull, W. 2009. The Pathology of Atherosclerosis: Plaque Development and Plaque Responses to Medical Treatment. *American Journal of Medicine*, 122(Supplement 1): S3–S14. doi: 10.1016/j.amjmed.2008.10.013.

Kaku, K. 2010. Pathophysiology of Type 2 Diabetes and Its Treatment Policy. *Journal of the Japan Medical Association*, 53(1): 41–46.

Karolina, D.S., Armugam, A., Tavintharan, S., Wong, M.T.K., Lim, S.C., Sum, C.F. & Jeyaseelan, K. 2011. MicroRNA 144 Impairs Insulin Signaling by Inhibiting the Expression of Insulin Receptor Substrate 1 in Type 2 Diabetes Mellitus. *PloS one*, 6(8): e22839.

Kathiresan, S., Melander, O., Anevski, D., Guiducci, C., Burtt, N.P., Roos, C., Hirschhorn, J.N., Berglund, G., Hedblad, B. & Groop, L. 2008. Polymorphisms associated with cholesterol and risk of cardiovascular events. *New England Journal of Medicine*, 358(12): 1240–1249.

Kim, S. & Pak, Y. 2005. Caveolin-2 regulation of the cell cycle in response to insulin in Hirc-B fibroblast cells. *Biochemical and Biophysical Research Communications*, 330(1): 88–96. http://www.sciencedirect.com/science/article/pii/S0006291X05003967 [4 April 2020].

Kong, L., Zhu, J., Han, W., Jiang, X., Xu, M., Zhao, Y., Dong, Q., Pang, Z., Guan, Q. & Gao, L. 2011. Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study. *Acta diabetologica*, 48(1): 61–69.

Kozomara, A. & Griffiths-Jones, S. 2011. MiRBase: Integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Research*, 39(Supplement 1): 152–157.

Kraakman, M.J., Murphy, A.J., Jandeleit-Dahm, K. & Kammoun, H.L. 2014. Macrophage polarization in obesity and type 2 diabetes: weighing down our understanding of macrophage function? *Frontiers in immunology*, 5: 470. https://pubmed.ncbi.nlm.nih.gov/25309549 [3 February 2020].

Krek, A., Grün, D., Poy, M.N., Wolf, R., Rosenberg, L., Epstein, E.J., MacMenamin, P., Da Piedade, I., Gunsalus, K.C., Stoffel, M. & Rajewsky, N. 2005. Combinatorial microRNA target predictions. *Nature Genetics*, 37(5): 495–500.

Kumar, S., Kim, C.W., Simmons, R.D. & Jo, H. 2014. Role of flow-sensitive microRNAs in endothelial dysfunction and atherosclerosis mechanosensitive athero-miRs. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 34(10): 2206–2216.

Lawrie, C.H., Gal, S., Dunlop, H.M., Pushkaran, B., Liggins, A.P., Pulford, K., Banham, A.H., Pezzella, F., Boultwood, J. & Wainscoat, J.S. 2008. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. *British journal of haematology*, 141(5): 672–675.

Lee, R.C., Feinbaum, R.L. & Ambros, V. 1993. The C. elegans Heterochronic Gene lin-4 Encodes Small RNAs with Antisense Complementarity to lin-14. *Cell*, 75(5): 843–854.

Li, G., Zhang, P., Wang, J., Gregg, E.W., Yang, W., Gong, Q., Li, Hui, Li, Hongliang, Jiang, Y., An, Y., Shuai, Y., Zhang, B., Zhang, J., Thompson, T.J., Gerzoff, R.B., Roglic, G., Hu, Y. & Bennett, P.H. 2008. The long-term effect of lifestyle interventions to prevent diabetes in the China Da Qing Diabetes Prevention Study: a 20-year follow-up study. *The Lancet*, 371(9626): 1783–1789.

Li, J., Jin, H. & Wang, X. 2014. Epigenetic Biomarkers: Potential Applications in Gastrointestinal Cancers J. Yu & T. Tsukamoto, eds. *ISRN Gastroenterology*, 2014(464015): 1–10. doi: 10.1155/2014/464015.

Long, G., Wang, F., Duan, Q., Yang, S., Chen, F., Gong, W., Yang, X., Wang, Y., Chen, C., Wang, D.W. & Navarro, A. 2012. Circulating miR-30a, miR-195 and let-7b Associated with Acute Myocardial Infarction. *PLoS one*, 7(12): e50926.

Marques, F.Z., Campain, A.E., Tomaszewski, M., Zukowska-Szczechowska, E., Yang, Y.H.J., Charchar, F.J. & Morris, B.J. 2011. Gene expression profiling reveals renin mRNA overexpression in human hypertensive kidneys and a role for microRNAs. *Hypertension*, 58(6): 1093–1098.

Matsha, T.E., Kengne, A.P., Hector, S., Mbu, D.L., Yako, Y. & Erasmus, R.T. 2018. MicroRNA profiling and their pathways in South African individuals with prediabetes and newly diagnosed type 2 diabetes mellitus. *Oncotarget*, 9(55): 30485–30498.

Mattie, M.D., Benz, C.C., Bowers, J., Sensinger, K., Wong, L., Scott, G.K., Fedele, V., Ginzinger, D., Getts, R. & Haqq, C. 2006. Optimized high-throughput microRNA expression profiling provides novel biomarker assessment of clinical prostate and breast cancer biopsies. *Molecular cancer*, 5(1): 24.

Mayosi, B.M., Flisher, A.J., Lalloo, U.G., Sitas, F., Tollman, S.M. & Bradshaw, D. 2009. The burden of non-communicable diseases in South Africa. *The Lancet*, 374(9693): 934–947.

McCarthy, M.I. & Zeggini, E. 2009. Genome-wide association studies in type 2 diabetes. *Current diabetes reports*, 9(2): 164–171. https://pubmed.ncbi.nlm.nih.gov/19323962 [29 October 2019].

Moore, L.D., Le, T. & Fan, G. 2013. DNA methylation and its basic function. *Neuropsychopharmacology*, 38(1): 23–38.

Ouyang, M., Li, Y., Ye, S., Ma, J., Lu, L., Lv, W., Chang, G., Li, X., Li, Q. & Wang, S. 2014. MicroRNA profiling implies new markers of chemoresistance of triple-negative breast cancer. *PloS one*, 9(5): e96228.

Ovchinnikova, E.S., Schmitter, D., Vegter, E.L., ter Maaten, J.M., Valente, M.A.E., Liu, L.C.Y., van der Harst, P., Pinto, Y.M., de Boer, R.A. & Meyer, S. 2016. Signature of circulating microRNAs in patients with acute heart failure. *European journal of heart failure*, 18(4): 414–423.

Ozougwu, J., Obimba, K., Belonwu, C. & Unakalamba, C. 2013. The pathogenesis and pathophysiology of type 1 and type 2 diabetes mellitus. *Journal of Physiology and Pathophysiology*, 4(4): 46–57.

Pandey, A.K., Verma, G., Vig, S., Srivastava, S., Srivastava, A.K. & Datta, M. 2011. miR-29a levels are elevated in the db/db mice liver and its overexpression leads to attenuation of insulin action on PEPCK gene expression in HepG2 cells. *Molecular and Cellular Endocrinology*, 332(1–2): 125–133. http://www.sciencedirect.com/science/article/pii/S0303720710004922 [21 February 2020].

Park, S.E., Rhee, E.J., Park, C.Y., Oh, K.W., Park, S.W., Kim, S.W. & Lee, W.Y. 2013. Impact of hyperinsulinemia on the development of hypertension in normotensive, nondiabetic adults: A 4-year follow-up study. *Metabolism: Clinical and Experimental*, 62(4): 532–538.doi: 10.1016/j.metabol.2012.09.013.

Parmar, M.Y. 2018. Obesity and Type 2 diabetes mellitus. *Integrative Obesity and Diabetes*, 4(4): 1–2.

Patoulias, D.I. 2018. Is miRNA-375 a promising biomarker for early detection and monitoring of patients with type 2 diabetes? Archives of medical sciences. *Atherosclerotic diseases*, 3: e119.

Peng, Y. & Croce, C.M. 2016. The role of MicroRNAs in human cancer. *Signal Transduction and Targeted Therapy*, 1(15004): 1–9. doi: 10.1038/sigtrans.2015.4.

Petrie, J.R., Guzik, T.J. & Touyz, R.M. 2018. Diabetes, Hypertension, and Cardiovascular Disease: Clinical Insights and Vascular Mechanisms. *Canadian Journal of Cardiology*, 34(5): 575–584. doi: 10.1016/j.cjca.2017.12.005.

Phillips, T. 2008. The role of methylation in gene expression. *Nature Education*, 1(1): 116.

Poy, M.N., Eliasson, L., Krutzfeldt, J., Kuwajima, S., Ma, X., MacDonald, P.E., Pfeffer, S., Tuschl, T., Rajewsky, N., Rorsman, P. & Stoffel, M. 2004. A pancreatic islet-specific microRNA regulates insulin secretion. *Nature*, 432(7014): 226–230. doi: 10.1038/nature03076.

Qi, Q. & Hu, F.B. 2012. Genetics of type 2 diabetes in European populations. *Journal of Diabetes*, 4(3): 203–212.

Qian, C. & Zhou, M.-M. 2006. SET domain protein lysine methyltransferases: Structure, specificity and catalysis. *Cellular and molecular life sciences (CMLS)*, 63(23): 2755–2763.

Ray, P., Manach, Y. Le, Riou, B., Houle, T.T. & Warner, D.S. 2010. Statistical evaluation of a biomarker. *The Journal of the American Society of Anesthesiologists*, 112(4): 1023–1040.

Reddy, M.A., Jin, W., Villeneuve, L., Wang, M., Lanting, L., Todorov, I., Kato, M. & Natarajan, R. 2012. Pro-inflammatory role of microRNA-200 in vascular smooth muscle cells from diabetic mice. *Arteriosclerosis, thrombosis, and vascular biology*, 32(3): 721–729.

Reid, G., Kirschner, M.B. & van Zandwijk, N. 2011. Circulating microRNAs: Association with disease and potential use as biomarkers. *Critical reviews in oncology/hematology*, 80(2): 193–208.

Rezk, N.A., Sabbah, N.A. & Saad, M.S.S. 2016. Role of microRNA 126 in screening, diagnosis, and prognosis of diabetic patients in Egypt. *IUBMB Life*, 68(6): 452–458.

van Rooij, E., Sutherland, L.B., Liu, N., Williams, A.H., McAnally, J., Gerard, R.D., Richardson, J.A. & Olson, E.N. 2006. A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proceedings of the National Academy of Sciences of the United States of America*, 103(48): 18255–18260. https://pubmed.ncbi.nlm.nih.gov/17108080 [12 July 2020].

Rossetto, D., Avvakumov, N. & Côté, J. 2012. Histone phosphorylation: a chromatin modification involved in diverse nuclear events. *Epigenetics*, 7(10): 1098–1108.

Ryu, H.S., Park, S.Y., Ma, D., Zhang, J. & Lee, W. 2011. The induction of microRNA targeting IRS-1 is involved in the development of insulin resistance under conditions of mitochondrial dysfunction in hepatocytes. *PLoS one*, 6(3): e17343.

Saxena, R., Voight, B.F., Lyssenko, V., Burtt, N.P., de Bakker, P.I.W., Chen, H., Roix, J.J., Kathiresan, S., Hirschhorn, J.N., Daly, M.J., Hughes, T.E., Groop, L., Altshuler, D., Almgren, P., Florez, J.C., Meyer, J., Ardlie, K., Bengtsson Boström, K., Isomaa, B., Lettre, G., Lindblad, U., Lyon, H.N., Melander, O., Newton-Cheh, C., Nilsson, P., Orho-Melander, M., Råstam, L., Speliotes, E.K., Taskinen, M.-R., Tuomi, T., Guiducci, C., Berglund, A., Carlson, J., Gianniny, L., Hackett, R., Hall, L., Holmkvist, J., Laurila, E., Sjögren, M., Sterner, M., Surti, A., Svensson, Margareta, Svensson, Malin, Tewhey, R., Blumenstiel, B., Parkin, M., DeFelice, M., Barry, R., Brodeur, W., Camarata, J., Chia, N., Fava, M., Gibbons, J., Handsaker, B., Healy, C., Nguyen, K., Gates, C., Sougnez, C.,

Gage, D., Nizzari, M., Gabriel, S.B., Chirn, G.-W., Ma, Q., Parikh, H., Richardson, D., Ricke, D. & Purcell, S. 2007. Genome-Wide Association Analysis Identifies Loci for Type 2 Diabetes and Triglyceride Levels. *Science*, 316(5829): 1331 LP – 1336.

Schober, A., Nazari-Jahantigh, M., Wei, Y., Bidzhekov, K., Gremse, F., Grommes, J., Megens, R.T.A., Heyll, K., Noels, H., Hristov, M., Wang, S., Kiessling, F., Olson, E.N. & Weber, C. 2014. MicroRNA-126-5p promotes endothelial proliferation and limits atherosclerosis by suppressing Dlk1. *Nature medicine*, 20(4): 368–376.

Scott, R.A., Scott, L.J., Mägi, R., Marullo, L., Gaulton, K.J., Kaakinen, M., Pervjakova, N., Pers, T.H., Johnson, A.D., Eicher, J.D., Jackson, A.U., Ferreira, T., Lee, Y., Ma, C., Steinthorsdottir, V., Thorleifsson, G., Qi, L., Van Zuydam, N.R., Mahajan, A., Chen, H., Almgren, P., Voight, B.F., Grallert, H., Müller-Nurasyid, M., Ried, J.S., Rayner, N.W., Robertson, N., Karssen, L.C., van Leeuwen, E.M., Willems, S.M., Fuchsberger, C., Kwan, P., Teslovich, T.M., Chanda, P., Li, M., Lu, Y., Dina, C., Thuillier, D., Yengo, L., Jiang, L., Sparso, T., Kestler, H.A., Chheda, H., Eisele, L., Gustafsson, S., Frånberg, M., Strawbridge, R.J., Benediktsson, R., Hreidarsson, A.B., Kong, A., Sigurðsson, G., Kerrison, N.D., Luan, J., Liang, L., Meitinger, T., Roden, M., Thorand, B., Esko, T., Mihailov, E., Fox, C., Liu, C.T., Rybin, D., Isomaa, B., Lyssenko, V., Tuomi, T., Couper, D.J., Pankow, J.S., Grarup, N., Have, C.T., Jørgensen, M.E., Jørgensen, T., Linneberg, A., Cornelis, M.C., van Dam, R.M., Hunter, D.J., Kraft, P., Sun, Q., Edkins, S., Owen, K.R., Perry, J.R.B., Wood, A.R., Zeggini, E., Tajes-Fernandes, J., Abecasis, G.R., Bonnycastle, L.L., Chines, P.S., Stringham, H.M., Koistinen, H.A., Kinnunen, L., Sennblad, B., Mühleisen, T.W., Nöthen, M.M., Pechlivanis, S., Baldassarre, D., Gertow, K., Humphries, S.E., Tremoli, E., Klopp, N., Meyer, J., Steinbach, G., Wennauer, R., Eriksson, J.G., Männistö, S., Peltonen, L., Tikkanen, E., Charpentier, G., Eury, E., Lobbens, S., Gigante, B., Leander, K., McLeod, O., Bottinger, E.P., Gottesman, O., Ruderfer, D., Blüher, M., Kovacs, P., Tonjes, A., Maruthur, N.M., Scapoli, C., Erbel, R., Jöckel, K.H., Moebus, S., de Faire, U., Hamsten, A., Stumvoll, M., Deloukas, P., Donnelly, P.J., Frayling, T.M., Hattersley, A.T., Ripatti, S., Salomaa, V., Pedersen, N.L., Boehm, B.O., Bergman, R.N., Collins, F.S., Mohlke, K.L., Tuomilehto, J., Hansen, T., Pedersen, O., Barroso, I., Lannfelt, L., Ingelsson, E., Lind, L., Lindgren, C.M., Cauchi, S., Froguel, P., Loos, R.J.F., Balkau, B., Boeing, H., Franks, P.W., Barricarte Gurrea, A., Palli, D., van der Schouw, Y.T., Altshuler, D., Groop, L.C., Langenberg, C., Wareham, N.J., Sijbrands, E., van Duijn, C.M., Florez, J.C., Meigs, J.B., Boerwinkle, E., Gieger, C., Strauch, K., Metspalu, A., Morris, A.D., Palmer, C.N.A., Hu, F.B., Thorsteinsdottir, U., Stefansson, K., Dupuis, J., Morris, A.P., Boehnke, M., McCarthy, M.I. & Prokopenko, I. 2017. An Expanded Genome-Wide Association Study of Type 2 Diabetes in Europeans. *Diabetes*, 66(11): 2888–2902.

Selcuklu, S.D., Donoghue, M.T.A. & Spillane, C. 2009. miR-21 as a key regulator of oncogenic processes. *Biochemical Society Transactions*, 37(4): 918–925.

Sommese, L., Zullo, A., Mancini, F.P., Fabbricini, R., Soricelli, A. & Napoli, C. 2017. Clinical relevance of epigenetics in the onset and management of type 2 diabetes mellitus. *Epigenetics*, 12(6): 401–415. doi: 10.1080/15592294.2016.1278097.

Song, L., Jia, J., Peng, X., Xiao, W. & Li, Y. 2017. The performance of the SEPT9 gene methylation assay and a comparison with other CRC screening tests: a meta-analysis. *Scientific reports*, 7(1): 1–12.

Staiger, H., Machicao, F., Stefan, N., Tschritter, O., Thamer, C., Kantartzis, K., Schäfer, S.A., Kirchhoff, K., Fritsche, A. & Häring, H.-U. 2007. Polymorphisms within Novel Risk Loci for Type 2 Diabetes Determine β-Cell Function. *PloS one*, 2(9): e832. doi: 10.1371/journal.pone.0000832.

Stoehr, R. & Federici, M. 2013. Insulin resistance and atherosclerosis: Convergence between metabolic pathways and inflammatory nodes. *Biochemical Journal*, 454(1): 1–11.

Szyf, M. 2009. Epigenetics, DNA methylation, and chromatin modifying drugs. *Annual review of pharmacology and toxicology*, 49: 243–263.

Talari, M., Kapadia, B., Kain, V., Seshadri, S., Prajapati, B., Rajput, P., Misra, P. & Parsa, K.V.L. 2015. MicroRNA-16 modulates macrophage polarization leading to improved insulin sensitivity in myoblasts. *Biochimie*, 119: 16–26. doi: 10.1016/j.biochi.2015.10.004.

Tchurikov, N.A. 2005. Molecular mechanisms of epigenetics. *Biochemistry (Moscow)*, 70(4): 406–423.

Wang, G.-K., Zhu, J.-Q., Zhang, J.-T., Li, Q., Li, Y., He, J., Qin, Y.-W. & Jing, Q. 2010. Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans. *European heart journal*, 31(6): 659–666.

Wong, C.K., Gromisch, C., Ozturk, S., Papageorgis, P., Abdolmaleky, H.M., Reinhard, B.M., Thiagalingam, A. & Thiagalingam, S. 2019. MicroRNA-4417 is a tumor suppressor and prognostic biomarker for triple-negative breast cancer. *Cancer biology & therapy*, 20(8): 1113–1120.

Wu, C. -t. & Morris, J.R. 2001. Genes, Genetics, and Epigenetics: A Correspondence. *Science*, 293(5532): 1103 LP – 1105.

Xia, H.Q., Pan, Y., Peng, J. & Lu, G.X. 2011. Over-expression of miR375 reduces glucose-induced insulin secretion in Nit-1 cells. *Molecular Biology Reports*, 38(5): 3061–3065.

Xu, M., Wu, H.-D., Li, R.-C., Zhang, H.-B., Wang, M., Tao, J., Feng, X.-H., Guo, Y.-B., Li, S.-F., Lai, S.-T., Zhou, P., Li, L.-L., Yang, H.-Q., Luo, G.-Z., Bai, Y., Xi, J.J., Gao, W., Han, Q.-D., Zhang, Y.-Y., Wang, X.-J., Meng, X. & Wang, S.-Q. 2012. Mir-24 regulates junctophilin-2 expression in cardiomyocytes. *Circulation research*, 111(7): 837–841.

Yang, Y., Xiao, L., Li, J., Kanwar, Y.S., Liu, F. & Sun, L. 2013. Urine miRNAs: potential biomarkers for monitoring progression of early stages of diabetic nephropathy. *Medical hypotheses*, 81(2): 274–278.

Yu, W., Hu, C. & Jia, W. 2012. Genetic advances of type 2 diabetes in Chinese populations. *Journal of diabetes*, 4(3): 213–220.

Yuan, W., Xia, Y., Bell, C.G., Yet, I., Ferreira, T., Ward, K.J., Gao, F., Loomis, A.K., Hyde, C.L. & Wu, H. 2014. An integrated epigenomic analysis for type 2 diabetes susceptibility loci in monozygotic twins. *Nature communications*, 5(1): 1–7.

Zampetaki, A., Kiechl, S., Drozdov, I., Willeit, P., Mayr, U., Prokopi, M., Mayr, A., Weger, S., Oberhollenzer, F., Bonora, E., Shah, A., Willeit, J. & Mayr, M. 2010. Plasma MicroRNA profiling reveals loss of endothelial MiR-126 and other MicroRNAs in type 2 diabetes. *Circulation Research*, 107(6): 810–817.

Zhang, B., Pan, X., Cobb, G.P. & Anderson, T.A. 2007. microRNAs as oncogenes and tumor suppressors. *Developmental Biology*, 302(1): 1–12. http://www.sciencedirect.com/science/article/pii/S0012160606010967 [8 February 2020].

Zhang, Y., Dutta, A. & Abounader, R. 2012. The role of microRNAs in glioma initiation and progression. *Frontiers in bioscience (Landmark edition)*, 17: 700–712.

Zhang, Y., Sun, X., Icli, B. & Feinberg, M.W. 2017. Emerging roles for microRNAs in diabetic microvascular disease: Novel targets for therapy. *Endocrine Reviews*, 38(2): 145–168.

Zhou, S.S., Jin, J.P., Wang, J.Q., Zhang, Z.G., Freedman, J.H., Zheng, Y. & Cai, L. 2018. MiRNAS in cardiovascular diseases: Potential biomarkers, therapeutic targets and challenges review-article. *Acta Pharmacologica Sinica*, 39(7): 1073–1084.

Zhu, H., Wang, G., Zhou, X., Song, X., Gao, H., Ma, C., Chang, H., Li, H., Liu, F. feng, Lu, J. & Ma, J. 2016. miR-1299 suppresses cell proliferation of hepatocellular carcinoma (HCC) by targeting CDK6. *Biomedicine and Pharmacotherapy*, 83: 792–797. doi: 10.1016/j.biopha.2016.07.037.

# CHAPTER 3 – FOUNDATION OF STUDY

## 3.1 Genome-wide miRNA profiling in prediabetes, newly diagnosed DM and known DM

In light of growing indications of the significance and potential diagnostic worth of miRNAs in dysglycaemia, a study headed by Matsha and co-workers aimed at demonstrating this in a South African population reported to be at high risk of DM (Matsha *et al*., 2018; Erasmus *et al*., 2012). The subsequent findings from this investigation prompted the motive for the current research project, and these findings are presented in this chapter.

### *3.1.1 Ethical approval*

This research initiative was based on the Cape Town Vascular and Metabolic Health (VMH) study, which is an extension of the previously described Cape Town Bellville South study (Matsha *et al*., 2012; Kengne *et al*., 2017), of which ethical clearance was approved by the Research Ethics Committee of the Cape Peninsula University of Technology (CPUT) as well as the Research Ethics Committee of Stellenbosch University (NHREC: REC - 230 408 – 014 and N14/01/003, respectively). Ethical clearance for this sub-study, was also sought from and granted by the Health and Wellness Sciences Research Ethics Committee of CPUT (CPUT/ HW-REC 2014/H08).

### *3.1.2 Study population and setting*

The participants involved in this study were enrolled between 2014 and 2016 from Bellville South, Cape Town, with enrolment of 1989 individuals. Bellville South is a township with a population of roughly 29 301 people, and comprises of lower to middle-class residents (Lehohla, 2011). The target population for this particular project comprised largely of individuals of mixed ancestry descent (De Wit *et al*., 2010).

### *3.1.3 Methods*

Matsha *et al* conducted a cross-sectional sub-study from the ongoing VMH study, aimed at identifying dysregulated miRNAs from the whole blood of 12 individuals with impaired glucose tolerance (IGT) (prediabetes), 12 with screen-detected/newly diagnosed diabetes, 12 known diabetics on treatment and 12 with normal glucose tolerance (NGT) matched for age, blood pressure, smoking and body mass index (BMI) (Matsha *et al*., 2018). Blood pressure and anthropometric measurements were performed in triplicate, with the average recorded. Furthermore, all participants underwent an oral glucose tolerance test (OGTT) in accordance with WHO guidelines (Alberti & Zimmet, 1998), and biochemical analysis was conducted at PathCare, Reference Laboratory, Cape Town, South Africa. Separate blood samples were collected in Tempus® RNA tubes, stored at -80°C before RNA isolation and analysis (Applied Biosystems, 2010).

Total RNA was extracted from whole blood using the MagMax Total RNA Isolation Kit (ThermoFisher Scientific, South Africa) in accordance with manufacture guidelines, and the integrity of the

subsequent RNA samples assessed using a NanoDrop ND-1000 spectrophotometer, with samples meeting a $^{A260}/_{A280}$ value of >1.8 and concentration value >20µg/µl deemed eligible for miRNA sequencing. In addition, RNA integrity was further evaluated by denaturing agarose gel electrophoresis (Matsha *et al.*, 2018). Thereafter, miRNA sequencing was performed at Arraystar Inc., Rockville, USA, on an Illumina HiSeq, generating raw sequences which were then trimmed by removing the adaptor sequences, and the resultant reads (≥15 nucleotides in length) were aligned to reference pre-miRNAs in a pre-existing miRBase 21 database, using novoalign software.

### *3.1.4 Key findings*

Heat Maps and Unsupervised Hierarchical Clustering were produced on all miRNAs that expressed per sample to generate miRNA or condition trees, enabling selection of groups of similar miRNAs. Recognizable miRNA expression profiles were seen amongst the samples (Figure 1), Differentially expressed miRNAs were described as having fold changes ≥1.3, *p*-value ≤0.1. A total of 261 differentially expressed miRNA profiles were identified between the study groups, and comparison of miRNA expression profiles revealed miRNAs that were dysregulated in IGT compared to both NGT and screen-detected diabetes, as well as known diabetes versus both NGT and screen-detected diabetes. Amongst these altered miRNAs were: hsa-miR-30a-5p, hsa-miR-1299, hsa-miR-182-5p, hsa-miR-30e-3p and hsa-miR-126-3p. The most differentially expressed miRNAs in the IGT group compared to NGT were miR-1299, -30e-3p and -126-3p, whilst in IGT versus screen-detected diabetes miR-126-3p was the most dysregulated, and in known diabetes versus screen-detected diabetes miR-30e-3p was seen to be the most differentially expressed (Table 1) (Matsha *et al.*, 2018).

Bioinformatics further revealed potential biological pathways linked with the miRNA signatures, such as p53 signalling, phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) and mitogen-activated protein kinase (MAPK), of which the PI3K/Akt/ and MAPK pathways play key roles in regulating glucose homeostasis, cell division as well as and survival (Schultze *et al.*, 2012; Matsha *et al.*, 2018)

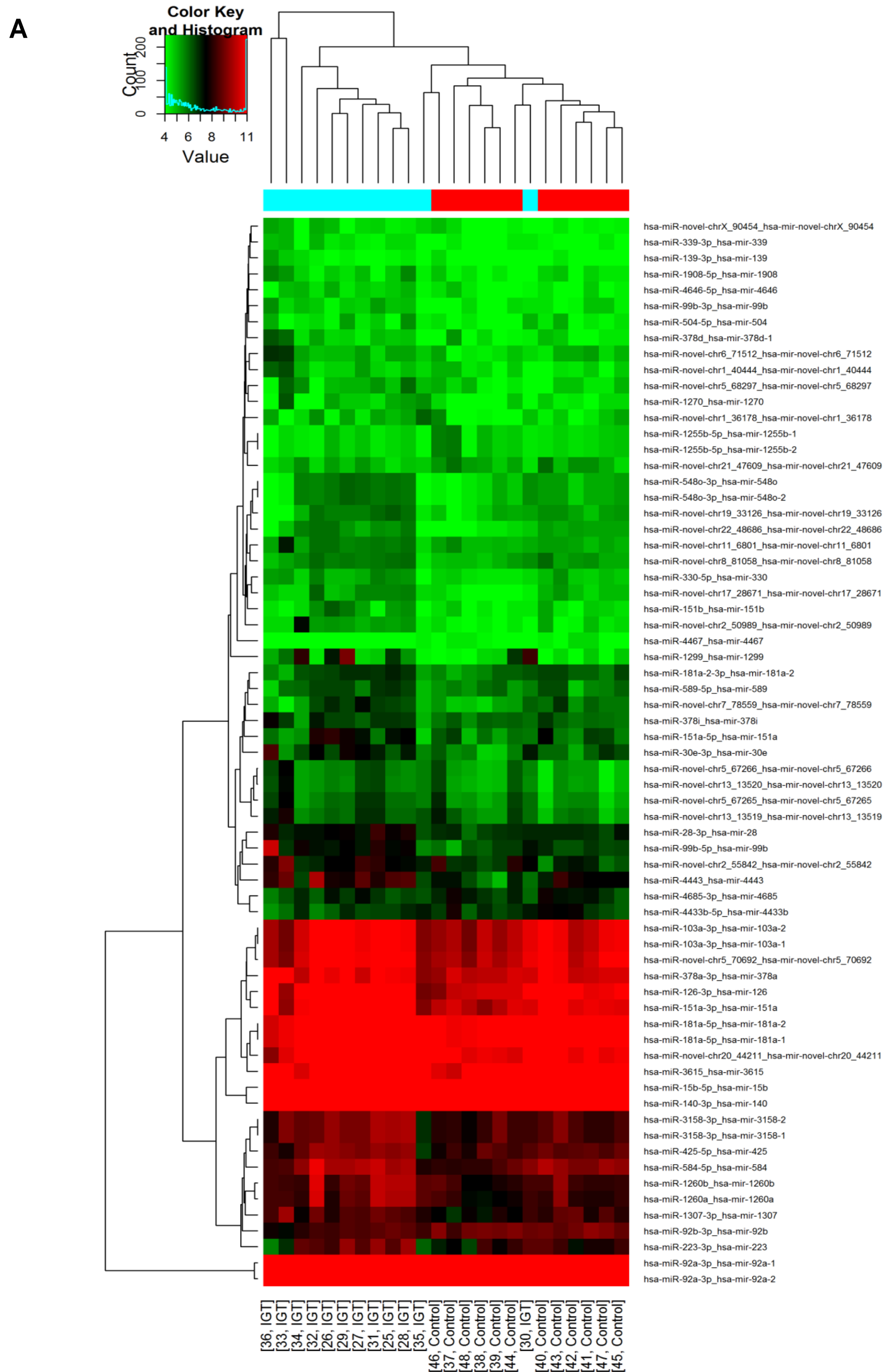
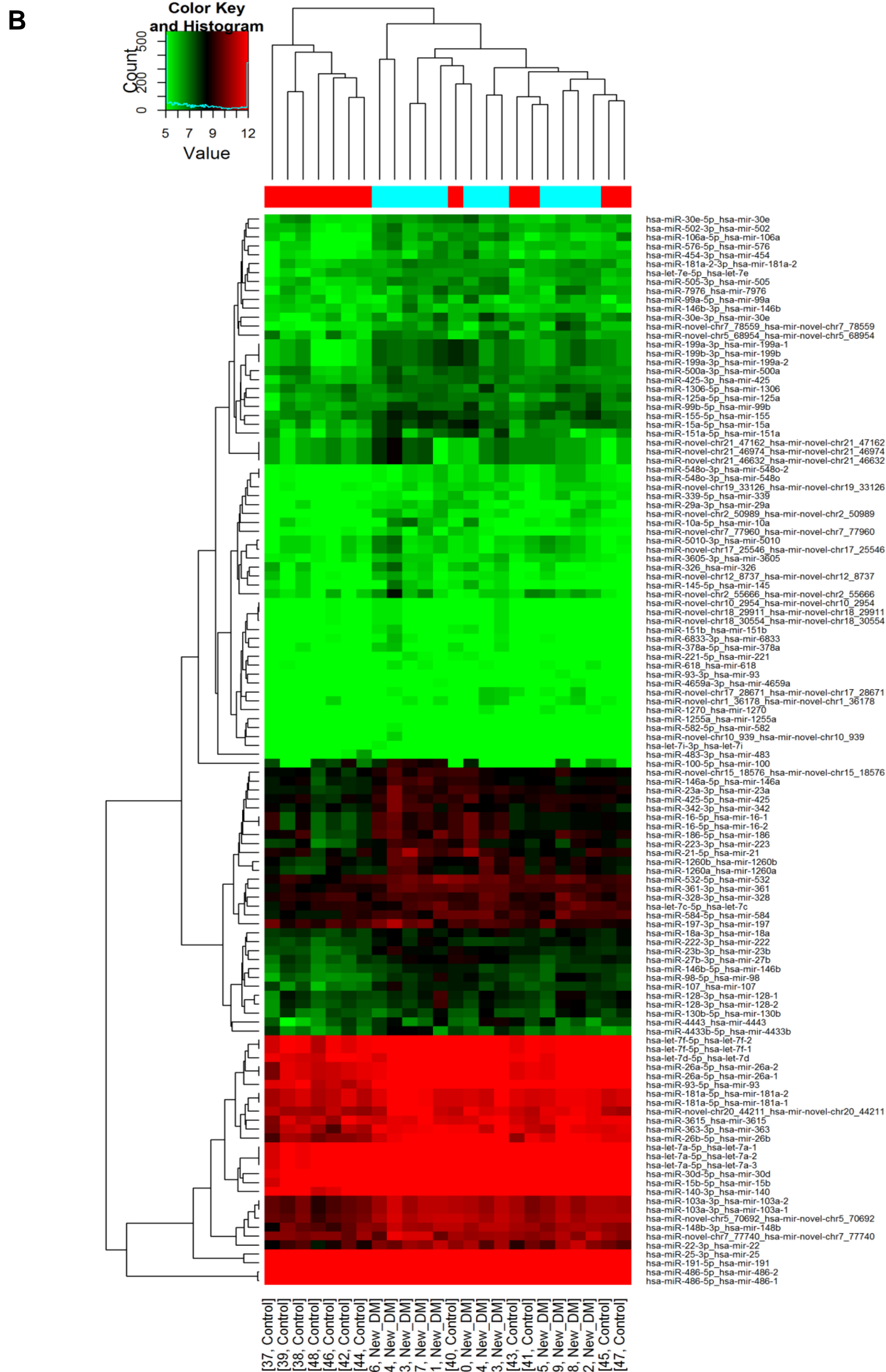



**Figure 1:** Differential miRNA expression in glycaemic groups. The Heat Map illustrated all differentially expressed miRNAs at adjusted *p*-value <0.05. **A.** IGT versus NGT; **B**. screen-detected DM versus NGT; **C.** IGT versus screen-detected DM; **D.** Known DM versus NGT; **E.** Known DM versus screen-detected DM. Signal intensity was expressed as. Hierarchical clustering was used to group samples, based on similar expression profiles. **Red:** underexpression; **White:** no change; **Blue:** overexpression (Matsha *et al*., 2018).

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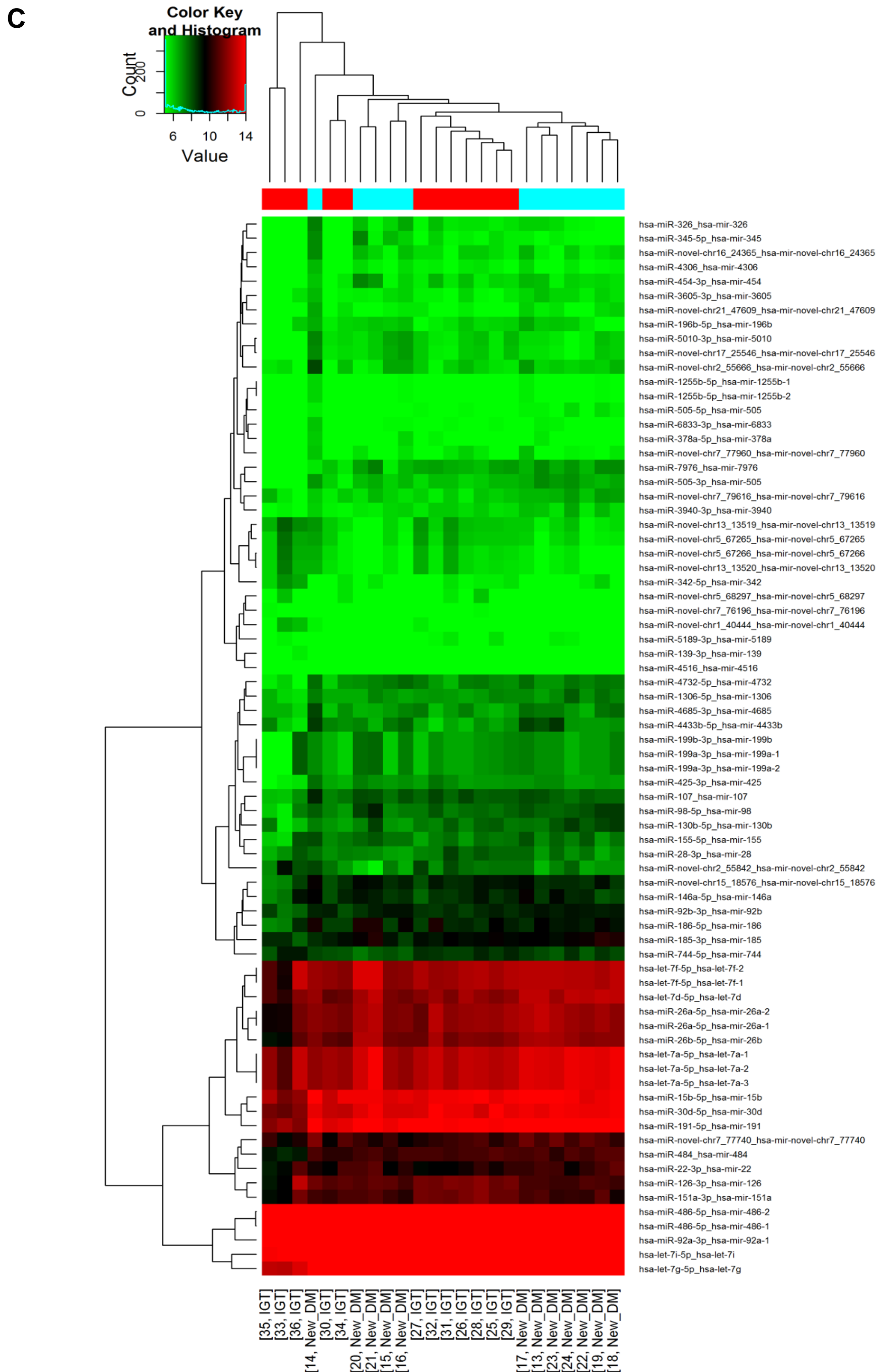


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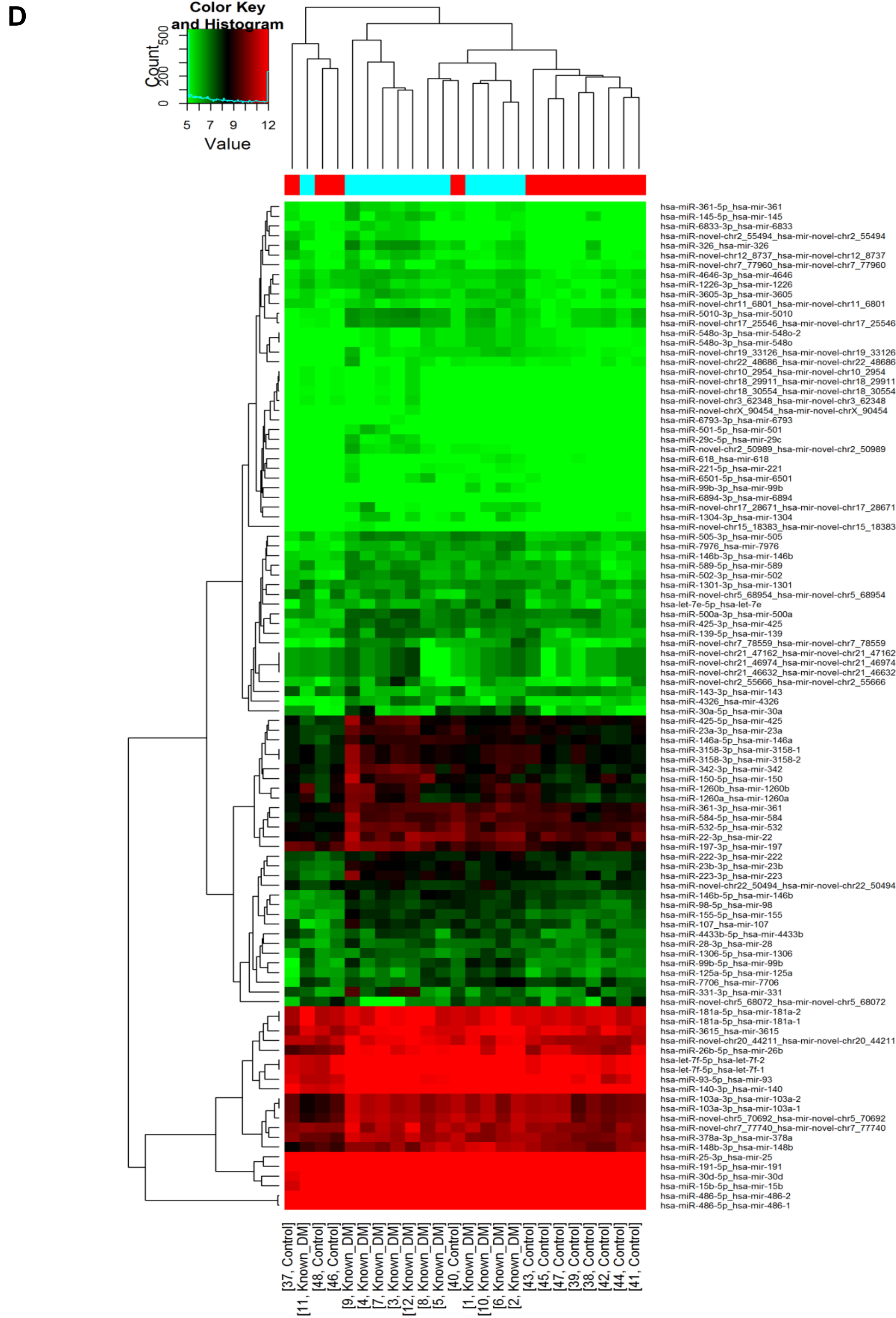


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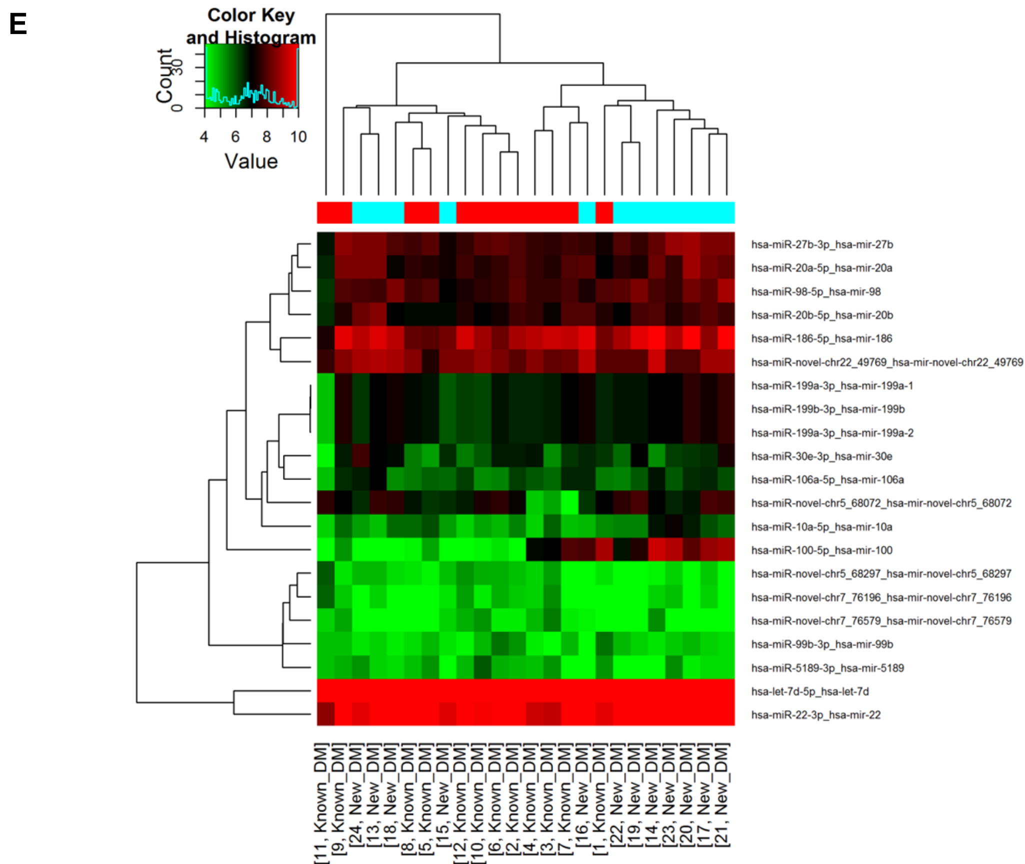


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**Table 1:** Fold change analysis, $2^{-\Delta\Delta Ct}$ between the glycaemic subgroups

| | IGT vs NGT | Screen-detected DM vs NGT | IGT vs Screen-detected DM | Known DM vs NGT | Known DM vs Screen-detected DM |
|---|---|---|---|---|---|
| miR-30a-5p | 2.39 *p*=0.19 | 1.68 *p*=0.13 | 3.24 *p*=0.26 | **2.44 *p*=0.009** | 1.45 *p*=0.23 |
| miR-1299 | **5.38 *p*=0.04** | 2.20 *p*=0.20 | 2.45 *p*=0.15 | 1.18 *p*=0.68 | 0.53 *p*=0.27 |
| miR-182-5p | 0.87 *p*=0.70 | 0.81 *p*=0.56 | 1.07 *p*=0.77 | 0.99 *p*=0.98 | 1.23 *p*=0.24 |
| miR-30e-3p | **2.40 *p*=0.007** | **1.78 *p*=0.007** | 1.34 *p*=0.24 | 1.14 *p*=0.49 | **0.64 *p*=0.03** |
| miR-126-3p | **1.74 *p*=0.03** | 1.14 *p*=0.28 | **1.53 *p*=0.06** | 1.08 *p*=0.63 | 0.95 *p*=0.71 |

### 3.2 Selected microRNAs (miRNAs) and other studies

The differentially expressed miRNAs observed following whole genome-wide sequencing in the Matsha *et al* study (miR-30a-5p, -1299, -miR-182-5p, -miR-30e-3p and -miR-126-3p), were selected for further validation in this current project, to confirm whether similar associations may be demonstrated in a larger, independent sample. Relationships between these miRNAs and DM as well as its associated complications have been reported in other independent studies as well (Maciejak *et al*., 2018; Zhu *et al*., 2016; Karolina *et al*., 2011; Wang *et al*., 2019; Rezk *et al*., 2016).

#### *3.2.1 miR-30a-5p*

Microvascular rarefaction, which is the loss of terminal blood vessels, is consistent with hypertension, and the miR-30a family have been revealed to play a role in the regulation of arteriolar branching, and subsequent arteriole blood pressure (Goligorsky, 2010; Jiang *et al*., 2013). Jiang *et al* used a zebrafish model to illustrate that overexpression of miR-30a promoted angiogenesis and subsequent arteriolar branching, aimed at lowering arteriole blood pressure (Jiang *et al*., 2013). Other studies have demonstrated similar links between miR-30a-5p and CVD (Maciejak *et al*., 2018; Zhang *et al*., 2019), with propositions that miR-30 may act as predictors for acute myocardial infarction (AMI) (Maciejak *et al*., 2018).

Human studies have suggested that altered miRNA patterns probably precede or appear at the early stages of diabetes (Tang *et al*., 2008; Erener *et al*., 2017). Increased levels of miR-30a-5p were observed, and have previously been associated with a higher risk of T2DM development (Jiménez-Lucena *et al*., 2018). In corroboration with the sequencing findings, the CORPIOPREV longitudinal study also described elevated levels of miR-30a-5p in dysglycaemic subjects in comparison to normotolerant, with levels seen to rise several years before the development of diabetes (Jiménez-Lucena *et al*., 2018). In a study conducted in rats, Kim *et al* revealed that miR-30a-5p mediated beta-cell dysfunction induced glucotoxicity by suppressing Beta2/NeuroD gene expression, and that overexpression led to pancreatic beta-cell dysfunction (Kim *et al*., 2013). It is known that persistent exposure of pancreatic beta-cells to elevated glucose levels may result in the subsequent inhibition of glucose-induced insulin secretion, insulin gene expression impairment, as well as induced beta-cell death (Weir *et al*., 2009). Consequently, the inhibition of miR-30a-5p in this animal model was shown to exhibit a protective effect on pancreatic islets and improved glucose tolerance (Kim *et al*., 2013), supporting sequencing data which showed elevated expression of the miRNA, indicating a disruption in glucose tolerance, therefore prompting its selection for further validation.

#### *3.2.2 miR-1299*

miR-1299 has been shown to be linked with cancers such as prostate cancer, as well as hepatocellular carcinoma, and is thought to be involved in tumour suppression, with significantly

reduced expressions described in cancer cells from these patients compared to controls (Felekkis & Papaneophytou, 2020; Wang *et al.*, 2012). miR-1299 has also been seen to play a regulatory role in rheumatic heart disease (RHD), with significant upregulation in the RHD patients compared to healthy controls (Zhang *et al.*, 2019). miRNA sequencing revealed associations between the miRNA and prediabetes, however limited information exists linking the miRNA to DM. One study which compared the mononuclear cell miRNA profiles of type 1 and type 2 diabetes patients to that of healthy controls demonstrated associations between expression levels of miR-1299 and pathways associated with diabetic complications (RET-HIF-1 signalling pathway), according to target prediction and functional analysis (Zhu *et al.*, 2016). Hypoxia-inducible factor 1 (HIF-1) signalling is the main regulatory mechanism in response to hypoxia, targeting various genes involved in angiogenesis, erythropoiesis as well as glucose metabolism (Cerychova & Pavlinkova, 2018). Moreover, studies of cultured cells in hypoxic and hyperglycaemic induced environments revealed increased degradation of the HIF-1α protein (Catrina *et al.*, 2004; Ramalho *et al.*, 2017), and with postulated participation of miR-1299 in HIF-1, this could explain the differential expression observed in the prediabetic group, motivating further assessment of the miRNA in a larger sample size.

#### *3.2.3 miR-182-5p*

Previous investigations have described the involvement of miR-182 in mediating glucose homeostasis, through the targeting of forkhead box protein O1 (FOXO1) (Karolina *et al.*, 2011; Zhang *et al.*, 2016; Zhou *et al.*, 2014). Mammalian cells express four FOXO variants, namely FOXO1, FOXO3, FOXO4 and FOXO6, of which FOXO1 is the most abundantly expressed in liver, adipose tissue as well as in pancreatic beta-cells (Kitamura *et al.*, 2002). FOXO1 is important in controlling pancreatic beta-cell replication and differentiation, as well as maintenance in states of metabolic stress (Kitamura, 2013). Moreover, FOXO1 promotes hepatic gluconeogenesis in states of hypoglycaemia via the PI3K/Akt signalling pathway, whilst in hyperglycaemia, insulin signalling via insulin-like growth factor-1 (IGF-1) and its receptor (IGF-1R) stimulate PI3K/Akt-dependent phosphorylation of FOXO1, leading to subsequent suppression of gluconeogenesis (Tsuchiya & Ogawa, 2017). Karolina *et al* reported miR-182 to be a potential modulator of FOXO1, with upregulation displayed in impaired fasting glycaemia. Downregulation of the FOXO1 mRNA in impaired fasting glycaemia was observed, with an upregulation in T2DM, suggesting potential hepatic gluconeogenesis to have a compensatory reduction in impaired fasting glycaemia, whilst increased in T2DM (Karolina *et al.*, 2011). Hence, in light of the miRNA sequencing findings of dysregulated miR-182-5p, further assessment was necessary in order to elucidate these relationships.

#### *3.2.4 miR-30e-3p*

Reports have shown that the miR-30e family play a key role in cell proliferation inhibition, with studies linking downregulation of the miRNA to various cancers (Li *et al.*, 2015; Massaro *et al.*, 2019). In

addition, miR-30e has also been demonstrated to mediate renal function (Wang *et al.*, 2019). In their study, Wang and co-workers assessed the expression and clinical significance of plasma miRNAs in the development and progression of diabetic nephropathy. They observed significantly reduced miR-30e plasma levels in diabetic patients at early stages of diabetic nephropathy versus healthy controls (Wang *et al.*, 2019). In a similar study comparing plasma miRNA expression between type 1 diabetics with varying stages of diabetic kidney disease (DKD) and those without, miR-30e expression was seen to be reduced in patients with moderate and severe DKD in comparison to T1DM controls (Dieter *et al.*, 2019). Overexpressed miR-30e was observed in dysglycaemic individuals after miRNA sequencing, therefore warranting further exploration to understand the functional role of the miR-30e family in dysglycaemia.

#### *3.2.5 miR-126-3p*

miR-126 has been postulated to regulate angiogenesis, acting as an endothelial cell-restricted miRNA promoting vascular (Zhang *et al.*, 2019; Roy & Sen, 2012). In addition to its role in modulating vascular maintenance and wound repair (Roy & Sen, 2012; Zhang *et al.*, 2013), miR-126 has also been reported to be associated with DM, in particular the micro/macrovascular complications associated with the disease (Zampetaki *et al.*, 2010; Al-Kafaji *et al.*, 2016; Barutta *et al.*, 2018; Qin *et al.*, 2017; Rawal *et al.*, 2017; Rezk *et al.*, 2016). Genome-wide miRNA-profiling demonstrated significantly increased miR-126 levels in prediabetes versus normotolerant, as well as in screen-detected diabetes versus prediabetes (Matsha *et al.*, 2018). Contrarily, studies have shown reduced circulating levels of miR-126 in prediabetes or diabetes versus normotolerant subjects (Zampetaki *et al.*, 2010; Liu *et al.*, 2014; Amr *et al.*, 2018). Liu and co-workers aimed to explore the associations between miR-126 and prediabetes, by performing qPCR on the serum of participants with IGT/ IFG, newly diagnosed T2D individuals and a control group. Their study portrayed reduced serum miR-126 in the prediabetic (IGT/IFG) and diabetic subjects in comparison to the control group. After six months of intervention, diet control and exercise in prediabetics as well as insulin and diet control in diabetics, miR-126 levels were seen to rise, suggesting its use as a potential biomarker for dysglycaemia as well as a therapeutic target (Liu *et al.*, 2014). Hence, the need to validate previous miRNA sequencing findings and assess its associations across different glucose tolerance groups on a larger scale.

Comprehensive analysis of specific miRNA signatures may shed light on the complex mechanisms involved in the progression of DM, and these altered expressions may function as early markers of diabetic homeostasis changes or may offer new therapeutic targets. Therefore, in this study we aimed to validate the abovementioned miRNAs in a South African population previously reported to have a high prevalence of undiagnosed diabetes (Erasmus *et al.*, 2012). This was a cross-sectional study, approved by the Research Ethics Committee of CPUT (CPUT/HW-REC 2019/H3), whereby quantitative methods were used to investigate the expression of known miRNAs (miR-30a-5p, miR-

1299, miR-182-5p, miR-30e-3p and miR-126-3p) in patients with pre-diabetes, newly diagnosed/ screen detected diabetes, and known diabetics on treatment. Results were then compared to normoglycaemic individuals.

### 3.3 References

Al-Kafaji, G., Al-Mahroos, G., Al-Muhtaresh, H.A., Skrypnyk, C., Sabry, M.A. & Ramadan, A.R. 2016. Decreased expression of circulating microRNA-126 in patients with type 2 diabetic nephropathy: A potential blood-based biomarker. *Experimental and Therapeutic Medicine*, 12(2): 815–822.

Alberti, K.G.M.M. & Zimmet, P.Z. 1998. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus. Provisional report of a WHO consultation. *Diabetic Medicine*, 15(7): 539–553.

Amr, K.S., Abdelmawgoud, H., Ali, Z.Y., Shehata, S. & Raslan, H.M. 2018. Potential value of circulating microRNA-126 and microRNA-210 as biomarkers for type 2 diabetes with coronary artery disease. *British Journal of Biomedical Science*, 75(2): 82–87. doi: 10.1080/09674845.2017.1402404.

Applied Biosystems. 2010. MagMAX TM for Stabilized Blood Tubes RNA Isolation Kit (Compatible with Tempus® Blood RNA Tubes) Protocol. https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_084749.pdf [15 February 2019].

Barutta, F., Bellini, S., Mastrocola, R., Bruno, G. & Gruden, G. 2018. MicroRNA and Microvascular Complications of Diabetes. *International Journal of Endocrinology*, 2018: 1–20.

Catrina, S., Okamoto, K., Pereira, T., Brismar, K. & Poellinger, L. 2004. Hyperglycemia Regulates Hypoxia-Inducible Factor-1α Protein Stability and Function. *Diabetes*, 53(12): 3226–3232.

Cerychova, R. & Pavlinkova, G. 2018. HIF-1, metabolism, and diabetes in the embryonic and adult heart. *Frontiers in Endocrinology*, 9: 460.

Dieter, C., Assmann, T.S., Costa, A.R., Canani, L.H., De Souza, B.M., Bauer, A.C. & Crispim, D. 2019. MiR-30e-5p and MiR-15a-5p expressions in plasma and urine of type 1 diabetic patients with diabetic kidney disease. *Frontiers in Genetics*, 10: 563.

Erasmus, R.T., Soita, D.J., Hassan, M.S., Blanco-Blanco, E., Vergotine, Z., Kengne, A.P. & Matsha, T.E. 2012. High prevalence of diabetes mellitus and metabolic syndrome in a South African coloured population: Baseline data of a study in Bellville, Cape Town. *South African Medical Journal*, 102(11): 841–844.

Erener, S., Marwaha, A., Tan, R., Panagiotopoulos, C. & Kieffer, T.J. 2017. Profiling of circulating microRNAs in children with recent onset of type 1 diabetes. *JCI Insight*, 2(4): e89656.

Felekkis, K. & Papaneophytou, C. 2020. Challenges in Using Circulating Micro-RNAs as Biomarkers for Cardiovascular Diseases. *International Journal of Molecular Sciences*, 21(2): 561.

Goligorsky, M.S. 2010. Microvascular rarefaction: The decline and fall of blood vessels. *Organogenesis*, 6(1): 1–10.

Jiang, Q., Lagos-Quintana, M., Liu, D., Shi, Y., Helker, C., Herzog, W. & Le Noble, F. 2013. MiR-30a regulates endothelial tip cell formation and arteriolar branching. *Hypertension*, 62(3): 592–598.

Jiménez-Lucena, R., Camargo, A., Alcalá-Diaz, J.F., Romero-Baldonado, C., Luque, R.M., van Ommen, B., Delgado-Lista, J., Ordovás, J.M., Pérez-Martínez, P., Rangel-Zúñiga, O.A. & López-Miranda, J. 2018. A plasma circulating miRNAs profile predicts type 2 diabetes mellitus and prediabetes: from the CORDIOPREV study. *Experimental and Molecular Medicine*, 50(12): 1–12. doi: 10.1038/s12276-018-0194-y.

Karolina, D.S., Armugam, A., Tavintharan, S., Wong, M.T.K., Lim, S.C., Sum, C.F. & Jeyaseelan, K. 2011. MicroRNA 144 Impairs Insulin Signaling by Inhibiting the Expression of Insulin Receptor Substrate 1 in Type 2 Diabetes Mellitus. *PloS one*, 6(8): e22839.

Kengne, A.P., Erasmus, R.T., Levitt, N.S. & Matsha, T.E. 2017. Alternative indices of glucose homeostasis as biochemical diagnostic tests for abnormal glucose tolerance in an African setting. *Primary Care Diabetes*, 11(2): 119–131. doi: 10.1016/j.pcd.2017.01.004.

Kim, J.-W., You, Y.-H., Jung, S., Suh-Kim, H., Lee, I.-K., Cho, J.-H. & Yoon, K.-H. 2013. miRNA-30a-5p-mediated silencing of Beta2/NeuroD expression is an important initial event of glucotoxicity-induced beta cell dysfunction in rodent models. *Diabetologia*, 56(4): 847–855.

Kitamura, T. 2013. The role of FOXO1 in β-cell failure and type 2 diabetes mellitus. *Nature Reviews Endocrinology*, 9(10): 615. doi: 10.1038/nrendo.2013.157.

Kitamura, T., Nakae, J., Kitamura, Y., Kido, Y., Biggs 3rd, W.H., Wright, C.V.E., White, M.F., Arden, K.C. & Accili, D. 2002. The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. *The Journal of clinical investigation*, 110(12): 1839–1847. doi: 10.1038/nrendo.2013.157.

Lehohla, P. 2011. *South African Statistics*, 2011.

Li, N., Lian, J., Zhao, S., Zheng, D., Yang, X., Huang, X., Shi, X., Sun, L., Zhou, Q., Shi, H., Xu, G., Incoom, E.K., Zhou, J. & Shao, G. 2015. Detection of Differentially Expressed MicroRNAs in Rheumatic Heart Disease: MiR-1183 and miR-1299 as Potential Diagnostic Biomarkers. *BioMed Research International*, 2015(524519): 1-15.

Liu, Y., Gao, G., Yang, C., Zhou, K., Shen, B., Liang, H. & Jiang, X. 2014. The role of circulating microRNA-126 (miR-126): A novel biomarker for screening prediabetes and newly diagnosed type 2 diabetes mellitus. *International Journal of Molecular Sciences*, 15(6): 10567–10577.

Maciejak, A., Kostarska-Srokosz, E., Gierlak, W., Dluzniewski, M., Kuch, M., Marchel, M., Opolski, G., Kiliszek, M., Matlak, K. & Dobrzycki, S. 2018. Circulating miR-30a-5p as a prognostic biomarker of left ventricular dysfunction after acute myocardial infarction. *Scientific reports*, 8(1): 1–11.

Massaro, J.D., Polli, C.D., Costa e Silva, M., Alves, C.C., Passos, G.A., Sakamoto-Hojo, E.T., Rodrigues de Holanda Miranda, W., Bispo Cezar, N.J., Rassi, D.M., Crispim, F., Dib, S.A., Foss-Freitas, M.C., Pinheiro, D.G. & Donadi, E.A. 2019. Post-transcriptional markers associated with clinical complications in Type 1 and Type 2 diabetes mellitus. *Molecular and Cellular Endocrinology*, 490: 1–14. doi: 10.1016/j.mce.2019.03.008.

Matsha, T.E., Hassan, M.S., Kidd, M. & Erasmus, R.T. 2012. The 30-year cardiovascular risk profile of South Africans with diagnosed diabetes, undiagnosed diabetes, pre-diabetes or normoglycaemia: the Bellville, South Africa pilot study. *Cardiovascular journal of Africa*, 23(1): 5–11.

Matsha, T.E., Kengne, A.P., Hector, S., Mbu, D.L., Yako, Y. & Erasmus, R.T. 2018. MicroRNA profiling and their pathways in South African individuals with prediabetes and newly diagnosed type 2 diabetes mellitus. *Oncotarget*, 9(55): 30485–30498.

Qin, L.-L., An, M.-X., Liu, Y.-L., Xu, H.-C. & Lu, Z.-Q. 2017. MicroRNA-126: a promising novel biomarker in peripheral blood for diabetic retinopathy. *International journal of ophthalmology*, 10(4): 530. doi: 10.18240/ijo.2017.04.05.

Ramalho, A.R., Toscano, A., Pereira, P., Girão, H., Gonçalves, L. & Marques, C. 2017. Hyperglycemia-induced degradation of HIF-1α contributes to impaired response of cardiomyocytes to hypoxia. *Revista Portuguesa de Cardiologia*, 36(5): 367–373.

Rawal, S., Munasinghe, P.E., Shindikar, A., Paulin, J., Cameron, V., Manning, P., Williams, M.J.A., Jones, G.T., Bunton, R., Galvin, I. & Katare, R. 2017. Down-regulation of proangiogenic microRNA-126 and microRNA-132 are early modulators of diabetic cardiac microangiopathy. *Cardiovascular Research*, 113(1): 90–101.

Rezk, N.A., Sabbah, N.A. & Saad, M.S.S. 2016. Role of microRNA 126 in screening, diagnosis, and prognosis of diabetic patients in Egypt. *IUBMB Life*, 68(6): 452–458.

Roy, S. & Sen, C.K. 2012. miRNA in Wound Inflammation and Angiogenesis. *Microcirculation*, 19(3): 224–232.

Schultze, S.M., Hemmings, B.A., Niessen, M. & Tschopp, O. 2012. PI3K/AKT, MAPK and AMPK signalling: protein kinases in glucose homeostasis. *Expert reviews in molecular medicine*, 14: e1.

Tang, X., Tang, G. & Özcan, S. 2008. Role of MicroRNAs in Diabetes. *Biochimica Et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*, 1779(11): 697–701.

Tsuchiya, K. & Ogawa, Y. 2017. Forkhead box class O family member proteins: The biology and pathophysiological roles in diabetes. *Journal of Diabetes Investigation*, 8(6): 726–734. doi: 10.1111/jdi.12651.

Wang, J., Wang, G., Liang, Y. & Zhou, X. 2019. Expression profiling and clinical significance of plasma microRNAs in diabetic nephropathy. *Journal of Diabetes Research*, 2019.

Wang, K., Yuan, Y., Cho, J.-H., McClarty, S., Baxter, D. & Galas, D.J. 2012. Comparing the MicroRNA spectrum between serum and plasma. *PloS one*, 7(7): e41561.

Weir, G.C., Marselli, L., Marchetti, P., Katsuta, H., Jung, M.H. & Bonner-Weir, S. 2009. Towards better understanding of the contributions of overwork and glucotoxicity to the β-cell inadequacy of type 2 diabetes. *Diabetes, Obesity and Metabolism*, 11: 82–90.

De Wit, E., Delport, W., Rugamika, C.E., Meintjes, A., Möller, M., Van Helden, P.D., Seoighe, C. & Hoal, E.G. 2010. Genome-wide analysis of the structure of the South African Coloured Population in the Western Cape. *Human Genetics*, 128(2): 145–153.

Zampetaki, A., Kiechl, S., Drozdov, I., Willeit, P., Mayr, U., Prokopi, M., Mayr, A., Weger, S., Oberhollenzer, F., Bonora, E., Shah, A., Willeit, J. & Mayr, M. 2010. Plasma MicroRNA profiling reveals loss of endothelial MiR-126 and other MicroRNAs in type 2 diabetes. *Circulation Research*, 107(6): 810–817.

Zhang, D., Li, Y., Yao, Xuan, Wang, H., Zhao, L., Jiang, H., Yao, Xiaohan, Zhang, S., Ye, C., Liu, W., Cao, H., Yu, S., Wang, Y., Li, Q., Jiang, J., Liu, Yi, Zhang, L., Liu, Yun, Iwai, N., Wang, H., Li, Jingya, Li, Jia, Li, X., Jin, Z.-B. & Ying, H. 2016. miR-182 Regulates Metabolic Homeostasis by Modulating Glucose Utilization in Muscle. *Cell Reports*, 16(3): 757–768.

Zhang, D., Li, Z., Wang, Z., Zeng, F., Xiao, W. & Yu, A. 2019. MicroRNA-126: a promising biomarker for angiogenesis of diabetic wounds treated with negative pressure wound therapy. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, 12: 1685–1696.

Zhang, F.B., Du, Y., Tian, Y., Ji, Z.G. & Yang, P.Q. 2019. MiR-1299 functions as a tumor suppressor to inhibit the proliferation and metastasis of prostate cancer by targeting NEK2. *European Review for Medical and Pharmacological Sciences*, 23(2): 530–538.

Zhang, T., Lv, C., Li, L., Chen, S., Liu, S., Wang, C. & Su, B. 2013. Plasma miR-126 is a potential biomarker for early prediction of type 2 diabetes mellitus in susceptible individuals. *BioMed research international*, 2013(761617): 1-6. doi: 10.1155.2013/761617.

Zhang, X., Dong, S., Jia, Q., Zhang, A., Li, Y., Zhu, Y., Lv, S. & Zhang, J. 2019. The microRNA in ventricular remodeling: the miR-30 family. *Bioscience reports*, 39(8): BSR20190788.

Zhou, J., Meng, Y., Tian, S., Chen, J., Liu, M., Zhuo, M., Zhang, Y., Du, H. & Wang, X. 2014. Comparative MicroRNA Expression Profiles of Cynomolgus Monkeys, Rat, and Human Reveal that miR-182 Is Involved in T2D Pathogenic Processes. *Journal of Diabetes Research*, 2014(760397). doi: 10.1155/2014/760397.

Zhu, H., Wang, G., Zhou, X., Song, X., Gao, H., Ma, C., Chang, H., Li, H., Liu, F. feng, Lu, J. & Ma, J. 2016. miR-1299 suppresses cell proliferation of hepatocellular carcinoma (HCC) by targeting CDK6. *Biomedicine and Pharmacotherapy*, 83: 792–797. doi: 10.1016/j.biopha.2016.07.037.

# CHAPTER 4 – RESULTS (MANUSCRIPT ONE)

**Micro RNAs -1299, - 126-3p and - 30e-3p as potential diagnostic biomarkers for prediabetes**

**Short title:** miRNAs and prediabetes

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## 4.1 Novelty statement

**What is already known?** MicroRNAs are potentially novel sources of biomarkers for diagnosis, prognosis and therapeutic options for a constellation of diseases
**What this study has found?** miR-1299, -126-3p and -30e-3p are associated with prediabetes
**What are the clinical implications of the study?** miR-126-3p has a potential to play a role in diabetes risk screening strategies, particularly in the detection of people with non-diabetic range dysglycaemia

## 4.2 Acknowledgments

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### 4.3 Abstract

**Objective:** To investigate the association of miR-1299, -126-3p and -30e-3p with- and their diagnostic capability for dysglycaemia in South Africans.

**Methods:** Cross-sectional study involving a total of 1273 (men, n=345) individuals, aged >20 years. Glycaemic status of all participants was assessed by oral glucose tolerance test (OGTT). Whole blood miR-1299, -126-3p and -30e-3p expressions were assessed by TaqMan-based RT-qPCR. Receiver operating characteristic (ROC) curves were used to assess the ability of each miRNA to discriminate dysglycaemia. Multivariable logistic regression analyses were used to link expression with dysglycaemia.

**Results:** In all, 207 (16.2%) and 94 (7.4%) participants had prediabetes and type 2 diabetes mellitus (T2DM), respectively. All three micro RNAs were significantly highly expressed in individuals with prediabetes compared to normotolerant, all $p<0.001$. miR-30e-3p and miR-126-3p were also significantly more expressed in T2D compared to normotolerant, $p<0.001$. In multivariable logistic regressions, the three miRNA were consistently and continuously associated with prediabetes, while only miR-126-3p showed an association with T2D. The ROC analysis indicated that all three miRNAs had a significant overall predictive ability to diagnose prediabetes, diabetes and the combination of both (dysglycaemia), with AUC being always significantly higher for miR-126-3p in prediabetes. For prediabetes diagnosis, miR-126-3p (AUC=0.760) outperformed HbA1c (AUC=0.695), $p=0.042$.

**Conclusions:** These results suggest that miR-1299, -126-3p and -30e-3p are associated with prediabetes, and measuring miR-126-3p could potentially contribute to diabetes risk screening strategies.

**Keywords:** miRNA, diabetes, prediabetes, Africa, biomarker

## 4.4 Introduction

MicroRNAs (miRNAs) are endogenous, small (21–25 nucleotides in length), non-protein-coding but functional RNA [1] and their role in various metabolic disorders, including diabetes mellitus (DM) has drawn widespread interest. These miRNAs are present in tissues and several human body fluids, including peripheral blood at consistent and reproducible levels, and they are stable as well as resistant to enzymatic digestion by RNAse [2]. It is for this reason that miRNAs have become potentially novel sources of biomarkers for diagnosis, prognosis and therapeutic options for a constellation of diseases [2, 3].

Diabetes mellitus (DM), a condition affecting 463 million people worldwide [4], continues to pose a diagnostic dilemma in which for the last four decades plasma glucose and recently HbA1c levels remain the diagnostic and prognosis markers. Globally, nearly half of the subjects with diabetes remain undetected contributing to the morbidity and mortality associated with DM. The hyperglycaemic state is a continuum of a prediabetes state which is a term denoting to impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) [5]. Currently, an oral glucose tolerance test (OGTT) is required to identify individuals with IGT, a test that is considered to be invasive, cumbersome and lengthy. The identification of individuals with prediabetes is of importance since at this stage intervention such as diet or physical activity can prevent the progression to overt DM. Emerging evidence suggests that specific miRNAs can serve as potential biomarkers for prediabetes and DM. Among these is miR-126 in which a number of studies have shown its circulating levels to be decreased in subject with type 2 diabetes mellitus (T2DM) [6-8]. We had earlier reported dysregulated miRNAs including miR-126 in subjects with prediabetes or DM using high throughput sequencing [9]. Herein, we selected the three most dysregulated miRNAs (hsa-miR-1299, hsa-miR-126-3p and hsa-miR-30e-3p) and investigated their association with- and their diagnostic capability for dysglycaemia in a large independent sample from an urban community residing in Cape Town, South Africa.

## 4.5 Materials and Methods

### *4.5.1 Ethical statement*

The study was approved by the Research Ethics Committees of the Cape Peninsula University of Technology and Stellenbosch University, respectively (NHREC: REC—230 408–014 and N14/01/003). The proposal for the current study was approved by the Ethics Committee of Cape Peninsula University of Technology (CPUT/HW-REC 2019/H3). The study was conducted in accordance with the Declaration of Helsinki. Permission to conduct the study was obtained from

relevant city and community authorities. All participants voluntarily signed written informed consent after all the procedures were fully explained in their language of choice.

#### *4.5.2 Study design and procedures*

The cross-sectional data presented in this study was obtained from the ongoing Cape Town Vascular and Metabolic Health (VMH) study, described elsewhere [31]. The VMH is a population based study that enrolled mixed-ancestry participants who reside in Bellville South, located in the Northern Suburbs of Cape Town, Western Cape, South Africa. According to South African census data of 2011, the population is comprised of 76.0% mixed ancestry, 18.5% black, 1.0% Asian, 0.5% Caucasian, and 4.0% individuals from other ethnicities [10, 11]. The data collection for the current analysis took place between April 2014 and November 2016 involving only South Africans from Cape Town. A total of 1273 subjects including 207 prediabetes, 94 T2D and 972 normotolerant individuals were enrolled in this study. All participants underwent 75g oral glucose tolerance test (OGTT) using the World Health Organization (WHO) criteria [12], blood pressure and anthropometric measurements. Prediabetes was defined as the presence of impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) [12].

At the time of screening, biochemical parameters were immediately analyzed at an International Organization for Standardization (ISO) 15189 accredited Pathology practice (PathCare, Reference Laboratory, Cape Town, South Africa) as described elsewhere [9]. Blood glucose levels (mmol/L) were determined with an enzymatic hexokinase method (Beckman AU, Beckman Coulter, South Africa) and HbA1c levels were determined with High Performance Liquid Chromatography (HPLC) (Biorad Variant Turbo, BioRad, South Africa). Serum insulin was by a paramagnetic particle chemiluminescence assay (Beckman DXI, Beckman Coulter, South Africa). High-density lipoprotein cholesterol (HDL-cholesterol) (mmol/L) was measured by enzymatic immuno-inhibition – End Point (Beckman AU, Beckman Coulter, South Africa), low-density lipoprotein cholesterol (LDL-cholesterol) (mmol/L) by enzymatic selective protection – End Point (Beckman AU, Beckman Coulter, South Africa) and triglycerides (TG) (mmol/L) were estimated by glycerol phosphate oxidase-peroxidase, End Point (Beckman AU, Beckman Coulter, South Africa). Ultra-sensitive C-reactive protein (usCRP) was measured by Latex Particle immunoturbidimetry. γ-Glutamyltransferase (GGT) were measured using International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) standardized reagents on a Beckman AU (Beckman Coulter, South Africa). serum cotinine was by Competitive Chemiluminescent (Immulite 2000, Siemens, South Africa).

#### *4.5.3 RNA Isolation*

The total RNA including miRNAs was isolated from whole blood that had been collected in a Tempus RNA tube (Applied Biosystems) that had been stored at -80 °C using the MagMAX for Stabilized

Blood Tubes RNA Isolation Kit, was used to perform the extraction as per manufacturer's specifications (Life Technologies, South Africa). The purity and integrity of the subsequent RNA samples was then assessed using a nanodrop (Nanodrop Technologies, Wilmington, USA), and only samples with an RNA concentration >15 ng/mL, and an OD (optical density) ratio $^{A260}/_{A280}$ >1.8 were accepted for further processing.

### *4.5.4 Reverse Transcription Quantitative Real-Time PCR*

Total miRNA extracts were converted to cDNA using the TaqMan MicroRNA Reverse Transcription Kit according to the manufacturer's protocol (Life Technologies, USA). In order to quantify the target miRNAs, pre-designed primers for each miRNA under investigation were used. These primers were miR-1299 (assay number: PM13770); miR-126-3p (assay number: 002228); miR-30e-3p (assay number: 000422) and executed in accordance with the *TaqMan Advanced* miRNA Assays and protocol on QuantStudio 7 Flex (Applied Biosystems, Thermo Fisher Scientific, South Africa). Briefly, dilutions of $10^{-1}$ were made of each cDNA sample and 5µl of each diluted cDNA sample was used for RT-qPCR. These were performed for optimum quantitative analysis. Sufficient reaction mix of the miRNA assay was prepared, according to the required number of reactions, and as per manufacturer specifications. Samples and endogenous controls were all performed in duplicate. Data were obtained as Ct values, and the $2^{-\Delta Ct}$ method was used to assess the miRNA expression levels in each sample analysed whilst the $2^{-\Delta\Delta Ct}$ value was used as the measure of the miRNA expression in each sample analysed compared with control sample [13]. For analysis of miRNA expression levels, an endogenous control (miR-16 5p) (Thermo Fisher Scientific, South Africa), was used. The distribution of miR-16-5p was not significantly different between the glucose tolerance status (Figure 1).

### *4.5.5 Statistical analysis*

Data analysis was performed with SPSS v.25 (IBM Corp, 2011). Results are reported as count (and percentages), mean (and standard deviation) or median ($25^{th}$-$75^{th}$ percentiles). The chi-square test, analysis of the variance and Kruskal-Wallis test were used to compare baseline characteristics across glucose tolerance subgroups. The relationship between miRNAs and other variables was by Spearman's partial correlations adjusted for body mass index (BMI), age and gender. Multivariate logistic regression models were used to assess the association of miRNAs with prediabetes or DM with crude or adjusted odds ratio (OR). The area under the receiver operating characteristic curve (AUC) was used to assess and compare the ability of each miRNA to predict the presence of prediabetes or DM. Then the diagnostic ability of the miRNA with the highest AUC was assessed alongside HbA1c at their optimal sample-specific threshold derived with use of the Youden index methods. AUCs comparison was based on the DeLong method while the 95% confidence interval around the diagnostic performance measures was from bootstrap resampling, based on 2000 replications. A p-value <0.05 was used to characterize statistically significant results.

## 4.6 Results

### *4.6.1 Basic Characteristics of the Study Subjects*

In a total of 1468 participants who consented, 184 with known type 2 diabetes and on treatment as well as 11 with missing data were excluded. The basic clinical characteristics of the remaining 1273 participants are summarised in Table 1. Over 70% of participants across all glucose tolerance statuses were women. Compared to the participants with normal glucose tolerance (NGT), those with prediabetes or diabetes were on average older, they had a higher waist circumference, BMI, blood pressure, triglycerides, usCRP, LDL-cholesterol and GGT, all $p<0.001$. Alcohol and tobacco consumption were significantly more prevalent in the NGT group, $p\leq0.002$.

### *4.6.2 Relative expression of miRNAs*

All three microRNAs were significantly highly expressed in individuals with prediabetes compared to NGT, all $p<0.001$. miR-30e-3p and miR-126-3p were significantly more expressed in T2D compared to NGT, $p<0.001$ whilst miR-1299 and miR-126-3p were significantly increased in prediabetes compared to T2D, $p\leq0.020$ (Fig. 2). These findings were further confirmed by the fold change analysis, $2^{-\Delta\Delta Ct}$. For instance, all three miRNAs were upregulated in prediabetes compared to normal glucose tolerant by ≥3.12-fold change and downregulated in T2D compared to prediabetes, ≤0.56-fold change (Table 2 ).

### *4.6.3 Correlation of miRNAs and biochemical parameters*

We performed Spearman's partial correlations and adjusted them for age, sex and BMI. The three miRNAs correlated positively which each other, r≥0.743, $p<0.001$, and the strongest correlation was between miR-30e-3p and miR-26-3p, r=0.967, $p<0.001$. All three miRNAs showed a negative correlation with waist circumference, r≤-0.398, $p\leq0.040$, but positively with post 2-hour glucose and HDL-cholesterol, r≥0.38, $p\leq0.05$ (Table 3).

### *4.6.4 Association between miRNAs and prediabetes or type 2 diabetes*

To investigate the association between high expression miR-1299, miR-30e-3p and miR-126-3p with prediabetes or T2D, we performed multivariate logistic regression analysis. miR-1299 and miR-30e-3p expression level values ($2^{-\Delta Ct}$) were too small, therefore, for interpretation purposes we converted the unit from 1 to 0.01 for these two miRNAs, meaning a 0.01 increase or decrease would represent a higher or lower odds of condition. All three miRNAs were significantly associated with prediabetes when compared to individuals with NGT in crude and adjusted models for age, sex, BMI or waist circumference, systolic blood pressure, HbA1c, triglycerides, HDL- and LDL-cholesterol, HbA1c, post 2-hour glucose, serum cotinine, odds ratio (OR) ≥1.26, 95% confidence interval (CI): ≥1.07-1.28, $p\leq0.007$). For T2D, only miR-126-3p showed an association when compared to NGT, OR ≥1.43 (1.21-

1.69, $p<0.001$), but only after adjustment of age, sex, BMI, SBP, triglycerides, HDL- and LDL-cholesterol (Table 4). When we assessed the association between the miRNAs and T2D using prediabetes individuals as the reference in multivariate logistic regression models, all three miRNAs were associated with a reduced risk of developing T2D, OR, ≤0.76 (0.59-0.99, $p \leq 0.042$). However, when the models were adjusted, only miR-30e-3p and miR-126-3p remained significant, OR, ≤0.73 (0.62-0.87, $p \leq 0.001$) (Table 5).

#### *4.6.5 Diagnostic specificity and sensitivity of the miRNAs for prediabetes and type 2 diabetes.*

Receiving operator characteristic (ROC) curves were drawn to investigate the diagnostic accuracy of the miRNAs as surrogate biomarkers for prediabetes and/or T2D versus the normotolerant individuals. Further, we investigated whether the miRNAs can serve as a surrogate marker between prediabetes and T2D . As shown in Fig. 3, best overall diagnostic accuracy was achieved for prediabetes versus NGT, particularly using miR-126-3p, area under the receiver operating characteristic curve (AUC) 0.76 (95% confidence interval [CI], 0.72-0.80, $p<0.0001$), (Fig. 3). All three miRNAs were of less value with regards to T2D compared to NGT (Fig. 3) whilst showed some value in differentiating between prediabetes and T2D versus normotolerant with miR-126-3p being the most sensitive, 0.67 (0.61-0.73, $p<0.0001$) (Fig. 3).

Compared with HbA1c, miR-126-3p had the same overall diagnostic accuracy for dysglycaemia (the combined outcome of prediabetes and T2D) with AUCs of 0.718 for miR-126-3p vs. 0.753 for HbA1c ($p=0.214$ for AUC comparison). For prediabetes diagnosis, miR-126-3p (AUC=0.760) outperformed HbA1c (AUC=0.695), $p=0.042$; while for diabetes diagnosis, HbA1c (AUC=0.861) largely outperformed miR-126-3p (AUC=0.574), $p<0.0001$. Measures of diagnostic performance of both markers to diagnoses dysglycaemia, prediabetes and diabetes at their respective sample-specific optimal thresholds are shown in Table 6. These performance measures were always better for miR-126-3p than HbA1c for the outcome of prediabetes, mostly overlapping across the two markers for dysglycaemia diagnosis, and largely better for HbA1c vs. miR-126-3p for the outcome of T2D .

### 4.7 Discussion

In this large sample of mixed-ancestry South Africans from Cape Town, selected miRNA including miR-1299, miR-30e-3p, miR-126-3p significantly correlated with each other, and with 2-hour post-OGTT glucose, but not with fasting glucose, HbA1c, fasting and 2-hour insulin after accounting for the effect of age, gender and BMI. In multivariable logistic regressions, the three miRNAs were consistently and continuously associated with prediabetes, while only miR-126-3p showed an association with T2D. ROC analyses confirmed that the three miRNA had a significant overall

predictive ability to diagnose prediabetes, diabetes and the combination of both (dysglycaemia), with AUC being always significantly higher for miR-126-3p across the three biomarkers, and always higher for prediabetes diagnosis across the three outcomes. Compared with HbA1c, miR-126-3p had better performance to diagnose prediabetes, while the two markers performed equally for dysglycaemia diagnosis, and HbA1c outperformed miR-126-3p for diabetes diagnosis. Altogether, our findings if confirmed, would tend to suggest that miR-126-3p has a potential to play a role in diabetes risk screening strategies, particularly in the detection of people with non-diabetic range dysglycaemia, and for whom lifestyle interventions can prevent the progression to the full stage of the disease.

Human studies have suggested that altered miRNA patterns probably precede or appear at the early stages of diabetes [6, 14-15]. For example, in the Bruneck study cohort, miR-126 was found to be reduced in the plasma of prevalent diabetes mellitus patients and this decrease of miR-126 preceded the manifestation of diabetes [6]. miR-126 is an endothelial cell-restricted miRNA which mediates vascular development and angiogenesis [16, 17]. Apart from its role in governing vascular integrity and wound repair [17, 18], miR-126 has also been reported to be associated with diabetes, specifically, micro/macrovascular complications [6, 19-23]. As a result, miR-126 has been touted as a promising marker which holds a potential for diagnosis and therapeutic management of hyperglycaemia [21-24]. In our study, miR-126 was significantly increased in both DM and prediabetes compared to normotolerant individuals, and significantly correlated positively with with 2-hour post-OGTT. These findings are similar to our previous genome-wide miRNA profiling in which miR-126-3p was 1.74- and 1.53-fold upregulated in IGT compared to normotolerant and screen-detected DM, respectively [9]. In contrast, lower circulating levels of miR-126 in prediabetes or diabetes versus normotolerant subjects have been reported [6-8]. The major difference between these studies and ours is that we extracted miRNA from whole blood, whilst others have used plasma or serum. Whole blood contains RNA from red blood cells, platelets and peripheral blood mononuclear cells (PBMCs). It has been demonstrated that the sample type affect the miRNA profile and/or expression levels [25, 26]. For example, the release of miRNAs from platelets and blood cells was thought to result in an increased concentration of miRNAs in serum compared to plasma [27]. Despite, these differences our data is in agreement that miR-126 is a potential biomarker for prediabetes and our data shows that its performance is superior to that of glycated haemoglobin in identifying individuals with prediabetes. HbA1c and fasting plasma glucose levels are commonly recognized as screening and diagnostic indices for diabetes and glucose intolerance. Although fasting glucose still remains a superior and standardised method, its use alone is not sufficient for the detection of all prediabetes individuals unless an oral glucose tolerance test (OGTT) is performed. Therefore, if our findings are proven and validated by others, the use of miR-126 might be of significant value in identifying these subjects in whom interventions that can prevent progression to overt diabetes can be initiated.

The other two miRNAs, miR-1299 and miR-30e-3p were also associated with prediabetes, however their diagnostic performance was inferior to that of miR-126-3p. miR-1299 has been shown to be linked with cancers such as prostate cancer, as well as hepatocellular carcinoma. Both are thought to be involved in tumour suppression, and significantly reduced expressions have been observed in cancer cells from these patients compared to controls [28, 29]. miR-1299 has also been seen to play a regulatory role in rheumatic heart disease (RHD), with significant upregulation in the RHD patients compared to healthy controls [30]. With respect to DM, there is limited information about miR-1299. In one study, the mononuclear cell miRNA profiles of Type 1 and Type 2 diabetes patients compared to healthy controls was evaluated. According to the target prediction and functional analysis, miR-1299 exhibited pathways related to T2DM, as well as pathways associated with diabetic complications (RET-HIF-1 signalling pathway) [31]. In our study, we observed a significant upregulation of miR-1299 in prediabetics versus NGT individuals. miR-30e family has been reported to play a vital role in inhibiting cell proliferation, and studies have linked downregulation of miR-30e with various cancers [32, 33]. It has also been shown to regulate renal function [1]. In their study, Wang and co-workers investigated the expression and clinical significance of plasma miRNAs in the pathogenesis and progression of diabetic nephropathy. They observed significantly low miR-30e plasma levels in diabetic patients at early stages of diabetic nephropathy versus healthy controls [1]. Hence our findings of marked expression of miR-30e-3p in individuals with prediabetes warrants future studies that can broaden our understanding of the functional role of the miR-30e family in dysglycaemia.

The strength of our study lies in the large number of participants selected from a community based cohort. The limitation of our study include absence of participants from a clinical setting for additional validation of our findings. The OGTT was not repeated as recommended [12]. The sample was skewed towards female participants, which is a common observation in community-based studies in our setting. Lastly, the data presented here was not corrected for multiple testing, thus our findings should be interpreted with caution. In conclusion, our study has revealed an association between miR-1299, -126-3p and -30e-3p and prediabetes and the ability of miR-126-3p to significantly discriminate prediabetes beyond the performance of HbA1c. This finding deserves validation in other populations and settings, to confirm if measuring miR-126-3p could play a role in diabetes risk screening strategies.

### 4.8 Author contributions

**CW:** wrote the first draft, experimental procedures, data analysis and interpretation. **DD:** experimental procedures, data analysis and interpretation. **SFGD:** recruitment and screening of cohort, statistical analysis and interpretation of data. **SR:** interpretation of data, editing and revising it for intellectual

content. **RTE:** conception, interpretation of the data, revising it for intellectual content and final approval of the version to be published. **APK:** conception, interpretation of the data, revising it for intellectual content and final approval of the version to be published. **GMD:** editing and revising it for intellectual content, final approval of the version to be published. **TEM:** conception and design of the study, analysis and interpretation of the data, revising it for intellectual content and final approval of the version to be published.

### 4.9 References


1. Wang J, Wang G, Liang Y, Zhou X. Expression profiling and clinical significance of plasma microRNAs in diabetic nephropathy. J Diabetes Res. 2019; 2019:5204394.

2. Kong L, Zhu J, Han W, Jiang X, Xu M, Zhao Y, Dong Q, Pang Z, Guan Q, Gao L, Zhao J, Zhao L. Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: A clinical study. Acta Diabetol 2011; 48:61–9.

3. de Candia P, Spinetti G, Specchia C, Sangalli E, La Sala L, Uccellatore A, Lupini S, Genovese S, Matarese G, Ceriello A. A unique plasma microRNA profile defines type 2 diabetes progression. PLoS One 2017; 12:e0188980.

4. International Diabetes Federation (2019). Available from: http://www.idf.org/about-diabetes/facts-figures. Accessed 04 April 2020

5. Diabetes Canada Clinical Practice Guidelines Expert Committee, Punthakee Z, Goldenberg R, Katz P. Definition, Classification and Diagnosis of Diabetes, Prediabetes and Metabolic Syndrome. Can J Diabetes 2018; 42:S10–5.

6. Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, Mayr A, Weger S, Oberhollenzer F, Bonora E, Shah A, Willeit J, Mayr M. Plasma MicroRNA profiling reveals loss of endothelial MiR-126 and other MicroRNAs in type 2 diabetes. Circ Res 2010; 107:810–7.

7. Liu Y, Gao G, Yang C, Zhou K, Shen B, Liang H, Jiang X. The role of circulating microRNA-126 (miR-126): A novel biomarker for screening prediabetes and newly diagnosed type 2 diabetes mellitus. Int J Mol Sci 2014; 15:10567–77.

8. Amr KS, Abdelmawgoud H, Ali ZY, Shehata S, Raslan HM. Potential value of circulating microRNA-126 and microRNA-210 as biomarkers for type 2 diabetes with coronary artery disease. Br J Biomed Sci 2018; 75:82–7.

9. Matsha TE, Kengne AP, Hector S, Mbu DL, Yako Y, Erasmus RT. MicroRNA profiling and their pathways in South African individuals with prediabetes and newly diagnosed type 2 diabetes mellitus. Oncotarget 2018; 9:30485–98.

10. Kengne AP, Erasmus RT, Levitt NS, Matsha TE. Alternative indices of glucose homeostasis as biochemical diagnostic tests for abnormal glucose tolerance in an African setting. Prim Care Diabetes 2017;11: 119-131.

11. Census 2011 Statistical release, Statistics South Africa. 2011.

12. Alberti KGMM, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus. Provisional report of a WHO consultation. Diabet Med 1998; 15:539–53.

13. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 2001; 25:402–8.

14. Tang X, Tang G, S. Özcan S. Role of MicroRNAs in Diabetes. Biochim Biophys Acta 2008; 1779 :697–701.

15. Erener S, Marwaha A, Tan R, Panagiotopoulos C, Kieffer TG. Profiling of circulating microRNAs in children with recent onset of type 1 diabetes. JCI Insight 2017; 2:1–13.

16. Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, Richardson JA, Bassel-Duby R, Olson EN. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Dev Cell 2008; 15:261–71.

17. Zhang D, Li Z, Wang Z, Zeng F, Xiao W, Yu A. MicroRNA-126: a promising biomarker for angiogenesis of diabetic wounds treated with negative pressure wound therapy. Diabetes Metab Syndr Obes 2019; 12:1685-1696.

18. Roy S, Sen CK. miRNA in wound inflammation and angiogenesis. Microcirculation 2012; 19:224–232.

19. Zhang T, Lv C, Li L, Chen S, Liu S, Wang C, Su B. Plasma miR-126 is a potential biomarker for early prediction of type 2 diabetes mellitus in susceptible individuals. Biomed Res Int 2013; 2013:761617.

20. Al-Kafaji G, Al-Mahroos G, Al-Muhtaresh HA, Skrypnyk C, Sabry MA, Ramadan AR. Decreased expression of circulating microRNA-126 in patients with type 2 diabetic nephropathy: A potential blood-based biomarker. Exp Ther Med 2016; 12:815–22.

21. Barutta F, Bellini S, Mastrocola R, Bruno G, Gruden G. MicroRNA and Microvascular Complications of Diabetes. Int J Endocrinol 2018; 2018: 6890501.

22. Qin L-L, An M-X, Liu Y-L, Xu H-C, Lu Z-Q. MicroRNA-126: a promising novel biomarker in peripheral blood for diabetic retinopathy. Int J Ophthalmol 2017; 10:530–4.

23. Rawal S, Munasinghe PE, Shindikar A, Paulin J, Cameron V, Manning P, Williams MJ, Jones GT, Bunton R, Galvin I, Katare R. Down-regulation of proangiogenic microRNA-126 and microRNA-132 are early modulators of diabetic cardiac microangiopathy. Cardiovasc Res 2017; 113:90–101.

24. Rezk NA, Sabbah NA, Saad MSS. Role of microRNA 126 in screening, diagnosis, and prognosis of diabetic patients in Egypt. IUBMB Life 2016; 68:452–8.

25. Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, Xiao T, Schafer J, Lee ML, Schmittgen TD, Nana-Sinkam SP, Jarjoura D, Marsh CB. Detection of microRNA Expression in Human Peripheral Blood Microvesicles. Plos One 2008; 3: e3694.

26. Felekkis K, Papaneophytou C. Challenges in Using Circulating Micro-RNAs as Biomarkers for Cardiovascular Diseases. Int. J. Mol. Sci 2020; 21:561.

27. Wang K, Yuan Y, Cho J-H, McClarty S, Baxter D, Galas, DJ. Comparing the MicroRNA spectrum between serum and plasma. PLoS ONE 2012; 7:e41561.

28. Zhang FB, Du Y, Tian Y, Ji ZG, Yang PQ. MiR-1299 functions as a tumor suppressor to inhibit the proliferation and metastasis of prostate cancer by targeting NEK2. Eur Rev Med Pharmacol Sci 2019; 23:530–8.

29. Zhu H, Wang G, Zhou X, Song X, Gao H, Ma C, Chang H, Li H, Liu FF, Lu J, Ma J. miR-1299 suppresses cell proliferation of hepatocellular carcinoma (HCC) by targeting CDK6. Biomed Pharmacother 2016; 83:792–7.

30. Li N, Lian J, Zhao S, Zheng D, Yang X, Huang X, Shi X, Sun L, Zhou Q, Shi H, Xu G, Incoom EK, Zhou J, Shao G. Detection of Differentially Expressed MicroRNAs in Rheumatic Heart Disease: MiR-1183 and miR-1299 as Potential Diagnostic Biomarkers. Biomed Res 2015; 2015:524519.

31. Souza AS, Sonon P, Paz MA, Tokplonou L, Lima THA, Porto IOP, Andrade HS, Silva NDSB, Veiga-Castelli LC, Oliveira MLG, Sadissou IA, Massaro JD, Moutairou KA, Donadi EA, Massougbodji A, Garcia A, Ibikounlé M, Meyer D, Sabbagh A, Mendes-Junior CT, Courtin D, Castelli EC. Post-transcriptional markers associated with clinical complications in Type 1 and Type 2 diabetes mellitus. Mol Cell Endocrinol 2019; 490:1–14.

32. Liu MM, Li Z, Han XD, Shi JH, Tu DY, Song W, Zhang J, Qiu XL, Ren Y, Zhen LL. MiR-30e inhibits tumor growth and chemoresistance via targeting IRS1 in Breast Cancer. Sci Rep 2017; 7:15929.

33. Bhattacharya S, Steele R, Shrivastava S, Chakraborty S, Di Bisceglie AM, Ray RB. Serum miR-30e and miR-223 as novel noninvasive biomarkers for hepatocellular Carcinoma. Am J Pathol 2016; 186:242–7.

**Table 1:** Characteristics of the study participants

| | NGT, n=972 | Prediabetes, n=207 | DM, n=94 | *p*-value |
|---|---|---|---|---|
| Age (years) | 45.2 ±15.3 | 55.1 ±13 | 58.4 ±10.6 | **<0.001** |
| Male, n (%) | 284 (29.3) | 42 (20.3) | 19 (20.2) | |
| Body mass index (kg/m$^2$) | 27.4 ±7.9 | 31.2 ±8.7 | 31.3 ±8 | **<0.001** |
| Waist circumference (cm) | 88.1 ±16.8 | 97 ±15.8 | 99.9 ±15.5 | **<0.001** |
| Hip circumference (cm) | 101.1 ±16.7 | 107.7 ±16.5 | 107.8 ±15.3 | **<0.001** |
| Waist to hip ratio | 0.9 ±0.1 | 0.9 ±0.1 | 0.9 ±0.1 | **<0.001** |
| Systolic blood pressure (mmHg) | 131 ±25 | 145 ±27.3 | 145.2 ±25.5 | **<0.001** |
| Diastolic blood pressure (mmHg) | 83.6 ±15.1 | 89.9 ±15.4 | 89.6 ±13.6 | **<0.001** |
| Fasting glucose (mmol/l) | 4.7±0.5 | 5.4±0.7 | 8.3±3.9 | **<0.001** |
| Post 2-hour glucose (mmol/l)* | 5.4 (4.5 ;6.3) | 8.6 (8 ;9.6) | 12.9 (11.6 ;16.8) | **<0.001** |
| HbA1c (%) | 5.6±0.5 | 5.9±0.5 | 7.4±2.1 | **<0.001** |
| HbA1c (mmol/mol | 37.7 | 41.0 | 57.4 | |
| Fasting insulin (mIU/l) | 7.6±7.2 | 11.3±11.3 | 14.4±29.5 | **<0.001** |
| Post 2-hour insulin (mIU/l)* | 30.5 (16 ;53.6) | 71.6 (42.3 ;113.2) | 50.5 (29.1 ;79.4) | **<0.001** |
| Triglycerides (mmol/l)* | 1.1 (0.8 ;1.5) | 1.4 (1 ;1.8) | 1.4 (1.1 ;2.4) | **<0.001** |
| HDL-cholesterol (mmol/l) | 1.4 ±0.4 | 1.4 ±0.4 | 1.3 ±0.5 | 0.640 |
| LDL-cholesterol (mmol/l) | 3.1 ±1 | 3.3 ±0.9 | 3.5 ±1.1 | **<0.001** |
| usCRP (mg/l) | 3.4 (1.3 ;7.7) | 5 (2.2 ;11.0) | 6.5 (3.3 ;13.1) | **<0.001** |
| Cotinine (ng/ml)* | 120.5 (10 ;285.3) | 10 (10 ;271.5) | 10 (10 ;183) | **<0.001** |
| GGT (IU/L) | 27 (19 ;42) | 31 (22 ;53) | 42 (25.5 ;76) | **<0.001** |
| Current smokers, n(%) | 540 (57.8) | 99 (48.3) | 29 (32.6) | **<0.001** |
| Current drinker, n(%) | 322 (33.3) | 56 (27.5) | 15 (16.1) | **0.002** |

*median (25$^{th}$, 75$^{th}$ percentile); NGT, normal glucose tolerance, HDL, high-density lipoprotein, LDL, low-density lipoprotein, usCRP, ultrasensitive C-reactive protein, GGT, γ-Glutamyltransferase

**Table 2.** Fold change analysis, $2^{-\Delta\Delta Ct}$ between the glucose tolerance groups

| | **Prediabetes vs NGT** | ***p*-value** | **DM vs NGT** | ***p*-value** | **DM vs prediabetes** | ***p*-value** |
|---|---|---|---|---|---|---|
| **RT-qPCR** | | | | | | |
| miR-1299 | 4.17±0.10 | **<0.001** | 1.99±0.13 | 0.332 | 0.48±0.06 | **0.02** |
| miR-30e-3p | 3.22±0.07 | **<0.001** | 1.32±0.17 | **<0.001** | 0.41±0.13 | 0.29 |
| miR-126-3p | 3.12±0.11 | **<0.001** | 1.75±0.03 | **<0.001** | 0.56±0.09 | **<0.001** |
| **NGS fold changes [9]*** | | | | | | |
| miR-1299 | 5.38±0.23 | **0.04** | 1±0.90 | 0.20 | 0.72±0.88 | 0.15 |
| miR-30e-3p | 2.40±0.03 | **0.007** | 1.78±0.1 | **0.007** | 0.51±1.15 | 0.24 |
| miR-126-3p | 1.74±0.10 | **0.03** | 1±1.21 | 0.28 | 1.53±0.05 | **0.06** |

NGT, normal glucose tolerance, DM, diabetes mellitus, NGS, next generation sequencing, *prediabetes only included individuals with impaired glucose tolerance

**Table 3.** Partial Spearman's correlation coefficients adjusted for age, sex and BMI

| | **miR-1299** | | **miR-30e-3p** | | **miR-126-3p** | |
|---|---|---|---|---|---|---|
| | **r** | ***p*-value** | **r** | ***p*-value** | **r** | ***p*-value** |
| miR-1299 | 1.000 | | 0.743 | **<0.001** | 0.747 | **<0.001** |
| miR-30e-3p | 0.743 | **<0.001** | 1.000 | | 0.967 | **<0.001** |
| miR-126-3p | 0.747 | **<0.001** | 0.967 | **<0.001** | 1.000 | |
| Waist circumference (cm) | -0.421 | **0.029** | -0.430 | **0.025** | -0.398 | **0.040** |
| Hip circumference (cm) | 0.161 | 0.424 | 0.072 | 0.723 | 0.082 | 0.683 |
| Waist hip ratio | -0.093 | 0.643 | -0.076 | 0.706 | -0.081 | 0.686 |
| Systolic blood pressure (mmHg) | 0.211 | 0.292 | 0.168 | 0.402 | 0.158 | 0.432 |
| Diastolic blood pressure (mmHg) | 0.199 | 0.319 | 0.124 | 0.537 | 0.096 | 0.635 |
| Fasting glucose (mmol/l) | 0.208 | 0.298 | 0.275 | 0.166 | 0.254 | 0.202 |
| Post 2-hour glucose (mmol/l) | 0.425 | **0.027** | 0.463 | **0.015** | 0.483 | **0.011** |
| HbA1c (mmol/mol) | 0.062 | 0.757 | 0.035 | 0.862 | 0.021 | 0.918 |
| Fasting insulin (mIU/l) | 0.320 | 0.104 | 0.362 | 0.064 | 0.379 | 0.052 |
| Post 2-hour insulin (mIU/l) | 0.278 | 0.161 | 0.321 | 0.103 | 0.351 | 0.072 |
| Triglycerides-S (mmol/l) | -0.019 | 0.925 | 0.038 | 0.849 | 0.092 | 0.648 |
| HDL-cholesterol (mmol/l) | 0.459 | **0.016** | 0.445 | **0.020** | 0.380 | 0.051 |
| LDL-cholesterol (mmol/l) | 0.103 | 0.610 | 0.056 | 0.781 | 0.057 | 0.777 |
| usCRP (mg/l) | 0.191 | 0.339 | 0.178 | 0.375 | 0.126 | 0.530 |
| Cotinine (ng/ml) | 0.431 | **0.025** | 0.364 | 0.062 | 0.326 | 0.097 |
| GGT (IU/L) | 0.113 | 0.576 | 0.086 | 0.670 | 0.069 | 0.733 |

HDL - high-density lipoprotein, LDL - low-density lipoprotein, usCRP - ultrasensitive C-reactive protein, GGT - γ-Glutamyltransferase

**Table 4:** Multivariate regression analysis of miRNAs for the presence of prediabetes and diabetes

| | Prediabetes | | | DM | | |
|---|---|---|---|---|---|---|
| | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** |
| **miR-1299*** | | | | | | |
| Model 1 | 1.38 | ( 1.21; 1.57) | **<0.001** | 1.14 | (0.93; 1.41) | 0.213 |
| Model 2 | 1.38 | ( 1.21; 1.57) | **<0.001** | 1.14 | (0.92; 1.42) | 0.225 |
| Model 3 | 1.42 | ( 1.23; 1.63) | **<0.001** | 1.17 | (0.94; 1.46) | 0.154 |
| Model 4 | 1.44 | ( 1.24; 1.68) | **<0.001** | 1.21 | (0.97; 1.52) | 0.094 |
| Model 5 | 1.26 | (1.07; 1.48) | **0.007** | 0.94 | (0.61; 1.47) | 0.800 |
| Model 6 | 1.32 | (1.09; 1.60) | **0.005** | 0.87 | (0.49; 1.57) | 0.651 |
| **miR-30e-3p*** | | | | | | |
| Model 1 | 2.11 | ( 1.79; 2.48) | **<0.001** | 1.18 | (0.89; 1.58) | 0.257 |
| Model 2 | 2.18 | ( 1.83; 2.59) | **<0.001** | 1.26 | (0.94; 1.68) | 0.123 |
| Model 3 | 2.17 | ( 1.82; 2.58) | **<0.001** | 1.25 | (0.93; 1.68) | 0.132 |
| Model4 | 2.16 | ( 1.81; 2.58) | **<0.001** | 1.22 | (0.89; 1.67) | 0.208 |
| Model 5 | 1.96 | (1.41; 2.74) | **<0.001** | 1.29 | (0.71; 2.33) | 0.402 |
| Model 6 | 2.08 | (1.42; 3.03) | **<0.001** | 1.35 | (0.70; 2.59) | 0.374 |
| **miR-126-3p**** | | | | | | |
| Model 1 | 2.08 | ( 1.85; 2.33) | **<0.001** | 1.44 | (1.24; 1.68) | **<0.001** |
| Model 2 | 2.15 | ( 1.9; 2.44) | **<0.001** | 1.52 | (1.3; 1.78) | **<0.001** |
| Model 3 | 2.13 | ( 1.88; 2.41) | **<0.001** | 1.5 | (1.28; 1.76) | **<0.001** |
| Model4 | 2.08 | ( 1.83; 2.36) | **<0.001** | 1.43 | (1.21; 1.69) | **<0.001** |
| Model 5 | 1.91 | (1.53; 2.38) | **<0.001** | 1.61 | (1.18; 2.20) | **0.003** |
| Model 6 | 2.08 | (1.60; 2.70) | **<0.001** | 1.83 | (1.28; 2.63) | **0.001** |

**Model 1:** Crude; **Model 2:** included age and sex; **Model 3**: included age, sex and BMI; **Model 4:** included age, sex, BMI, SBP, triglycerides, HDL- and LDL-cholesterol; **Model 5:** included age, sex, BMI, SBP, triglycerides, HbA1c, 2 glucose, cotinine, HDL- and LDL-cholesterol; **Model 6:** included age, sex, waist circumference, post 2-hour glucose, fasting insulin, HDL-cholesterol and cotinine
*calculated for 0.01-unit increase; ** calculated for 1-unit increase

**Table 5.** Multivariate regression analysis of miRNAs for diabetes using prediabetes as reference

| | Prediabetes | | | DM | | |
|---|---|---|---|---|---|---|
| | **OR** | **95% CI** | **p-value** | **OR** | **95% CI** | ***p*-value** |
| **miR-1299*** | | | | | | |
| Model 1 | - | - | - | 0.76 | (0.59; 0.99) | **0.042** |
| Model 2 | - | - | - | 0.77 | (0.59; 0.99) | **0.044** |
| Model 3 | - | - | - | 0.78 | (0.59; 1.03) | 0.075 |
| Model4 | - | - | - | 0.79 | (0.60; 1.04) | 0.087 |
| Model 5 | | | | 0.75 | (0.50; 1.13) | 0.173 |
| Model 6 | | | | 0.66 | (0.38; 1.15) | 0.145 |
| **miR-30e-3p*** | | | | | | |
| Model 1 | - | - | - | 0.53 | (0.39; 0.73) | **<0.001** |
| Model 2 | - | - | - | 0.54 | (0.40; 0.73) | **<0.001** |
| Model 3 | - | - | - | 0.54 | (0.37; 0.77) | **0.001** |
| Model4 | - | - | - | 0.51 | (0.35; 0.76) | **0.001** |
| Model 5 | | | | 0.66 | (0.40; 1.08) | 0.096 |
| Model 6 | | | | 0.65 | (0.38; 1.11) | 0.113 |
| **miR-126-3p**** | | | | | | |
| Model 1 | - | - | - | 0.70 | (0.60; 0.81) | **<0.001** |
| Model 2 | - | - | - | 0.70 | (0.60; 0.81) | **<0.001** |
| Model 3 | - | - | - | 0.74 | (0.63; 0.87) | **<0.001** |
| Model4 | - | - | - | 0.73 | (0.62; 0.87) | **<0.001** |
| Model 5 | | | | 0.84 | (0.67; 1.06) | 0.142 |
| Model 6 | | | | 0.88 | (0.69; 1.13) | 0.327 |

**Model 1:** Crude; **Model 2:** included age and sex; **Model 3:** included age, sex and BMI; **Model 4:** included age, sex, BMI, SBP, triglycerides, HDL- and LDL-cholesterol; **Model 5:** included age, sex, BMI, SBP, triglycerides, HbA1c, 2 glucose, cotinine, HDL- and LDL-cholesterol; **Model 6:** included age, sex, waist circumference, post 2-hour glucose, fasting insulin, HDL-cholesterol and cotinine
*calculated for 0.01-unit increase; ** calculated for 1-unit increase

**Table 6.** Performance of miR-126-3p and HbA1c to predict dysglycaemia

| Performance measure | dysglycemia | | Prediabetes | | Diabetes | |
|---|---|---|---|---|---|---|
| | **miR-126-3p** | **HbA1c** | **miR-126-3p** | **HbA1c** | **miR-126-3p** | **HbA1c** |
| AUC | 0.720 (0.682-0.755) | 0.753 (0.717-0.788) | 0.760 (0.719-0.802) | 0.695 (0.652-0.739) | 0.574 (0.513-0.634) | 0.861 (0.812-0.909) |
| Threshold | 1.41 (1.31-2.52) | 5.95 (5.75-6.05) | 1.78 (1.35-0.277) | 5.75 (5.75-9.95) | 1.31 (0.79-1.75) | 6.05 (6.05-6.45) |
| Sensitivity | 0.628 (0.429-0.689) | 0.591 (0.497-0.733) | 0.642 (0.466-0.740) | 0.598 (0.461-0.721) | 0.565 (0.413-0.772) | 0.761 (0.611-0.859) |
| Specificity | 0.740 (0.698-0.924) | 0.824 (0.670-0.893) | 0.798 (0.717-0.945) | 0.707 (0.643-0.846) | 0.638 (0.457-0.721) | 0.837 (0.807-0.959) |
| PPV | 0.434 (0.387-0.633) | 0.519 (0.410-0.616) | 0.403 (0.341-0.652) | 0.335 (0.234-0.430) | 0.108 (0.089-0.132) | 0.284 (0.239-0.566) |
| NPV | 0.862 (0.835-0.880) | 0.865 (0.846-0.891) | 0.910 (0.888-0.929) | 0.894 (0.875-0.916) | 0.947 (0.936-0.962) | 0.978 (0.968-0.986) |
| Accuracy | 0.714 (0.683-0.804) | 0.771 (0.685-0.810) | 0.767 (0.711-0.862) | 0.702 (0.647-0.789) | 0.631 (0.476-0.698) | 0.833 (0.805-0.938) |
| *p*-value for AUC comparison | 0.214 | | **0.042** | | **<0.0001** | |

AUC, area under the receiver operating characteristic curve; PPV, positive predictive value; NPV, negative predictive value

### 4.10 Figure legends

**Figure 1.** miR-16-5p Ct values distribution across glucose tolerance statuses. N, normotolerant, D, diabetes, P, prediabetes

**Figure 2.** Relative Expression of miR-1299, miR-30e-3p and miR-126-3p according to glycaemic status. The expression of the miRNAs was normalised to the relative expression of miR-16-5p. (A): miR-1299. (B): miR-30e-3p. (C): miR-126-3p. Data is shown as mean ± SD. N, normotolerant, D, diabetes, P, prediabetes

**Figure 3.** Receiver operating characteristic (ROC). ROCs were constructed for each miRNA to evaluate the diagnostic values for prediabetes, diabetes, dysglycaemia as positive cases and normotolerant as negative cases, as well as for diabetes as positive cases and prediabetes as negative cases. (A): Dysglycaemia vs normotolerant (B): Prediabetes vs normotolerant (C): Screen-detected diabetes vs normotolerant (D) Prediabetes vs screen-detected diabetes

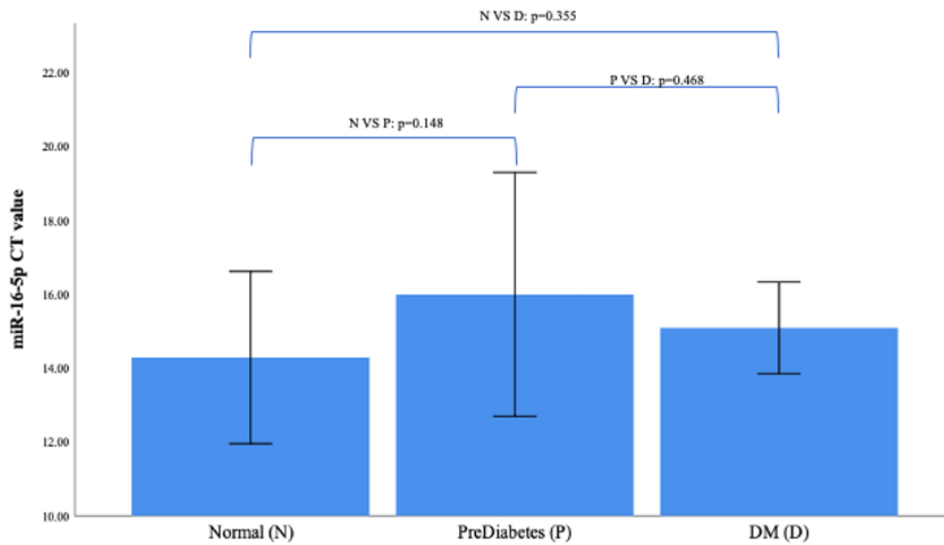



**Figure 1:** miR-16-5p Ct values distribution across glucose tolerance statuses. N, normotolerant, D, diabetes, P, prediabetes
338x190mm (54 x 54 DPI)

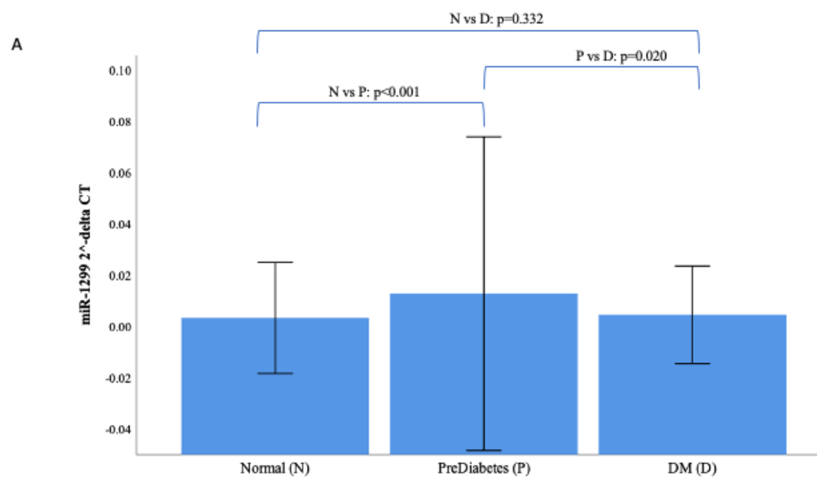
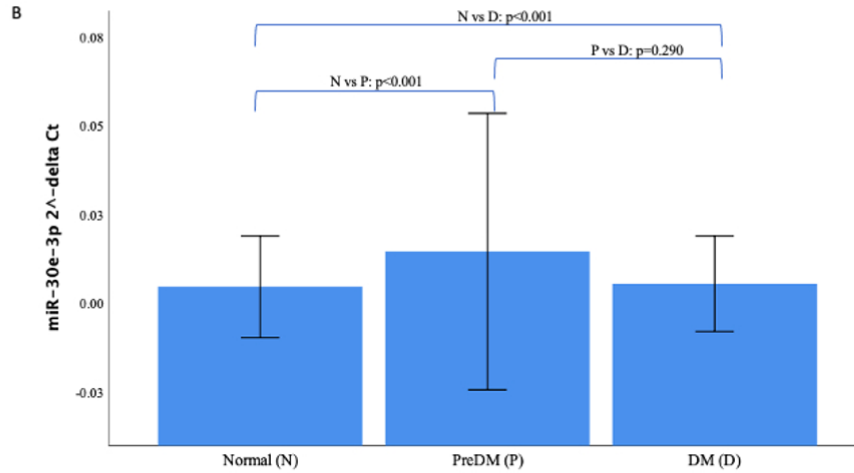



**Figure 2:** Relative Expression of miR-1299, miR-30e-3p and miR-126-3p according to glycaemic status. The expression of the miRNAs was normalised to the relative expression of miR-16-5p. **(A):** miR-1299. **(B):** miR-30e-3p. **(C):** miR-126-3p. Data is shown as mean ± SD. N, normotolerant, D, diabetes, P, prediabetes
338x190mm (54 x 54 DPI)

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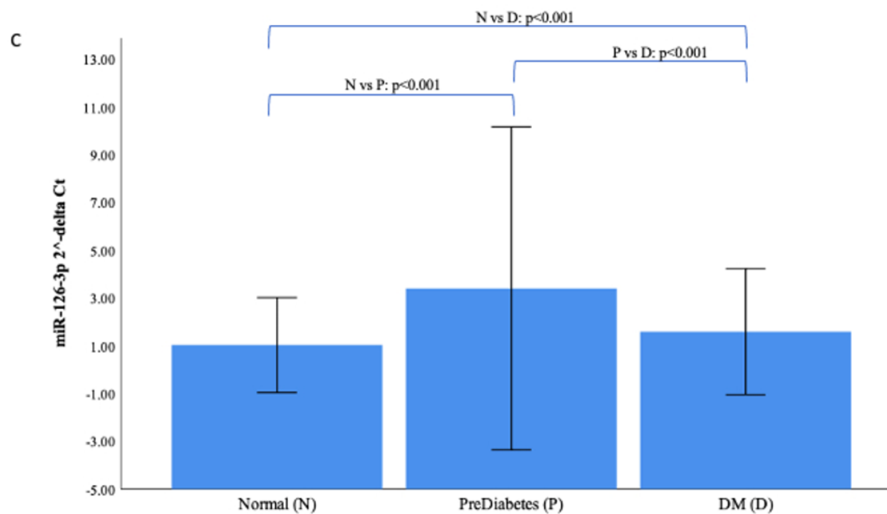
338x190mm (54 x 54 DPI)

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338x190mm (54 x 54 DPI)

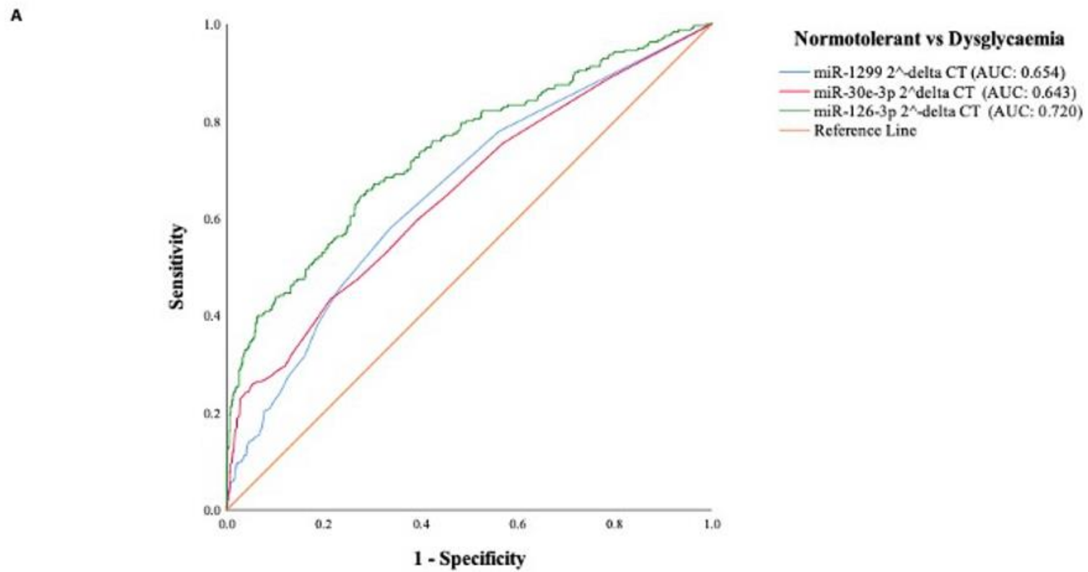


**Figure 3:** Receiver operating characteristic (ROC). ROCs were constructed for each miRNA to evaluate the diagnostic values for prediabetes, diabetes, dysglycaemia as positive cases and normotolerant as negative cases, as well as for diabetes as positive cases and prediabetes as negative cases. **(A):** Dysglycaemia vs normotolerant **(B):** Prediabetes vs normotolerant **(C):** Screen-detected diabetes vs normotolerant **(D):** Prediabetes vs screen-detected diabetes
338x190mm (54 x 54 DPI)

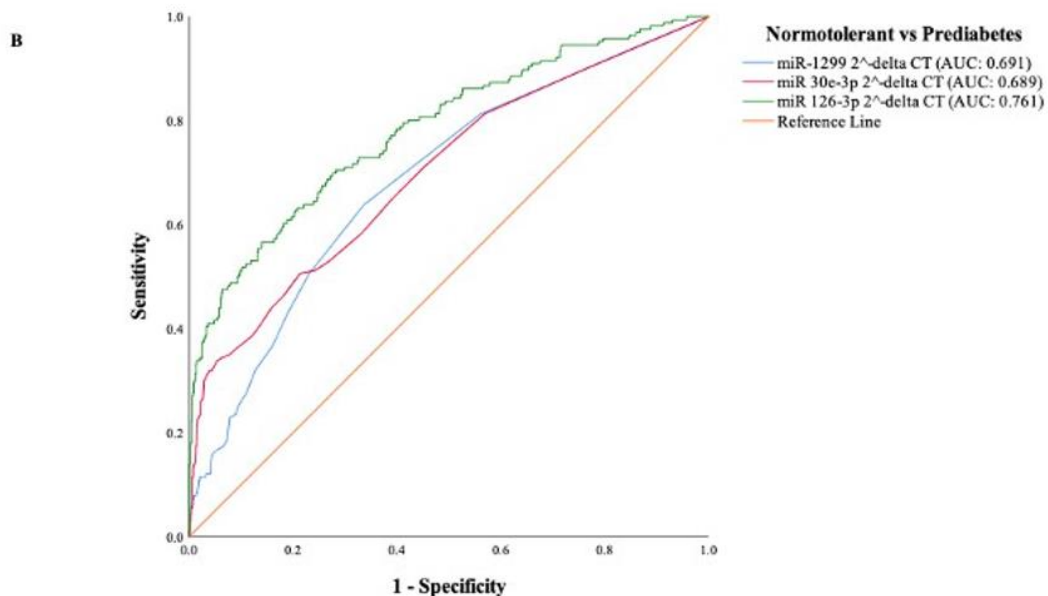


**Figure 3:** Receiver operating characteristic (ROC). ROCs were constructed for each miRNA to evaluate the diagnostic values for prediabetes, diabetes, dysglycaemia as positive cases and normotolerant as negative cases, as well as for diabetes as positive cases and prediabetes as negative cases. **(A):** Dysglycaemia vs normotolerant **(B):** Prediabetes vs normotolerant **(C):** Screen-detected diabetes vs normotolerant **(D):** Prediabetes vs screen-detected diabetes
338x190mm (54 x 54 DPI)

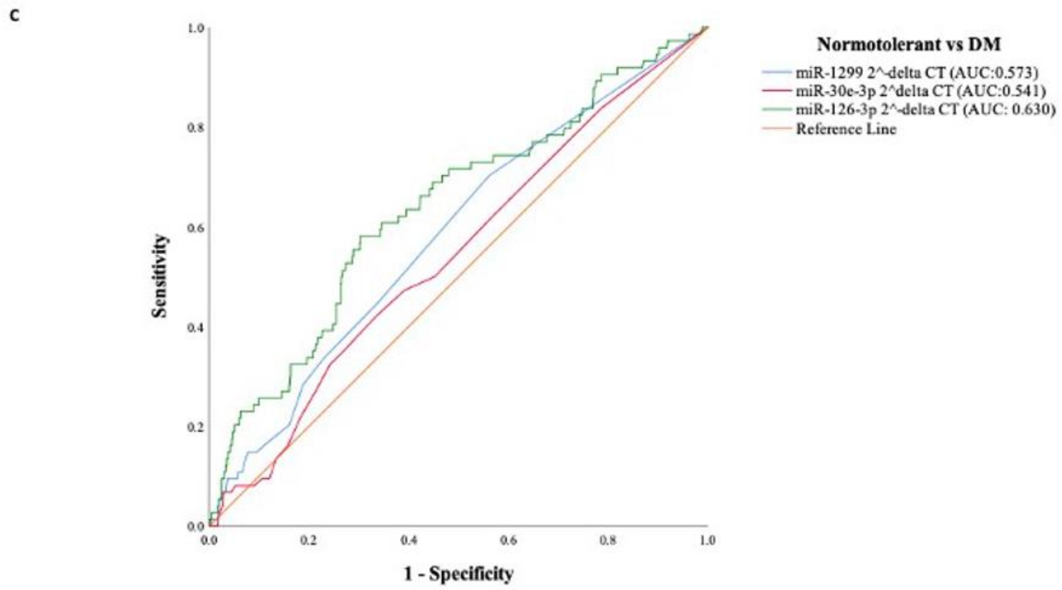


**Figure 3:** Receiver operating characteristic (ROC). ROCs were constructed for each miRNA to evaluate the diagnostic values for prediabetes, diabetes, dysglycaemia as positive cases and normotolerant as negative cases, as well as for diabetes as positive cases and prediabetes as negative cases. **(A):** Dysglycaemia vs normotolerant **(B):** Prediabetes vs normotolerant **(C):** Screen-detected diabetes vs normotolerant **(D):** Prediabetes vs screen-detected diabetes 338x190mm (54 x 54 DPI)

# CHAPTER 5 – RESULTS (MANUSCRIPT TWO)

**Circulating miR-30a-5p and miR-182-5p in prediabetes and screen-detected diabetes mellitus**

**Running title:** miRNAs and dysglycaemia

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## 5.1 Abstract

**Background:** microRNAs (miRNAs) have been touted as potential diagnostic and prognostic biomarkers for various diseases. The aim of the present study was to evaluate the diagnostic value of miR-30a-5p and miR-182-5p for prediabetes and screen-detected type 2 diabetes mellitus (T2DM).

**Methods:** The study included 1270 participants (207 prediabetes, 94 screen-detected diabetes and 969 normotolerant) from the Vascular and Metabolic Health (VMH) study. Whole blood levels of miR-30a-5p and miR-182-5p were quantitated by RT-qPCR. Multivariable logistic regressions were used to relate miRNAs with prediabetes or T2DM and receiver operating characteristic (ROC) curves were used to evaluate the ability of each miRNA to diagnose these conditions.

**Results:** Both miRNAs were significantly highly expressed in individuals with prediabetes or T2DM (both ≥3.2-fold, and $p<0.001$). We also observed significant under-expression in T2DM relative to prediabetes for miR-182-5p (0.49-fold, $p=0.001$). Age, sex and BMI-adjusted partial correlation coefficient analysis revealed a significant correlation between the two miRNAs across glucose tolerance statuses ($r \geq 0.932$, $p<0.001$). In normotolerant individuals, both miRNAs showed a negative correlation with waist circumference and positive correlation with HDL-cholesterol whilst in T2DM they correlated positively with hip circumference, 2-hour insulin, HDL- and LDL-cholesterol. Multivariable logistic regressions revealed both miRNAs to be consistently and continuously associated with prediabetes or T2DM (OR≥1.18, 95% 95% CI: 1.10–1.28, $p<0.001$), while only miR-182-5p associated with a reduced prevalence of T2DM relative to prediabetes (OR: 0.89, 95% CI: 0.83–0.96, $p=0.003$). In ROC analyses, miR-182-5p almost outperformed HbA1c in diagnosing prediabetes; area under the curve 0.74 vs 0.69.

**Conclusion:** Our findings demonstrate that miR-30a-5p and miR-182-5p are associated with dysglycaemia and could potentially predict prediabetes, particularly miR-182-5p.

**Keywords:** Africa, miR-30a-5p, miR-182-5p, diabetes, prediabetes

## 5.2 Background

Despite advances in the understanding of disease progression and related treatments, there has been a steady rise in diabetes mellitus (DM) incidence worldwide, with the global population of people with diabetes expected to increase to 700 million individuals by the year 2045.[1,2] Prediabetes is a state of intermediate hyperglycaemia comprising three sub-phenotypes: impaired fasting glucose (IFG), defined as a fasting plasma glucose of 6.1–6.9 mmol/L, impaired glucose tolerance (IGT), defined as a 2-hour plasma glucose of 7.8–11.0 mmol/L after oral glucose tolerance test (OGTT), or the combination of both.[3] Effective intervention for the prevention of progression from prediabetes to type 2 diabetes (T2DM) requires accurate diagnostic tools. Single-stranded noncoding microRNAs (miRNAs) in various metabolic disorders, especially T2DM, have drawn widespread attention as potential biomarkers.[4–7] These miRNAs are present in various tissues, including extracellular fluids such as plasma, saliva or urine, and are highly stable since they are protected from RNAse degradation.[8]

Since their discovery over twenty years ago, miRNAs have been proven to play a pivotal role in the pathophysiology of several diseases, including T2DM, and altered miRNA expression profiles are observed in a range of different diseases.[8] More recently, numerous additional miRNAs have been recognized as components of pathways triggered by, or contributing to, the pathology of both type 1 diabetes mellitus as well as T2DM.[9,10] Amongst these, miR-30a-5p and miR-182-5p have been shown to play a role in regulatory pathways involved in cardiovascular disease (CVD) and DM.[11–13] Both miRNAs have also displayed dysregulation in hyperglycaemic individuals.[14] Comprehensive analysis of specific miRNA signatures may shed light on the complex mechanisms involved with the progression of DM. Therefore, in this study, we aimed to investigate the expression of these two miRNAs across glucose tolerance status in a South African population previously reported to have a high prevalence of undiagnosed diabetes.[15]

## 5.3 Methods

### *5.3.1 Ethical approval of the study*

This study was based on the Cape Town Vascular and Metabolic Health (VMH) project, which has been approved by the Cape Peninsula University of Technology Research Ethics Committee, as well as the Stellenbosch University Research Ethics Committee (respectively, NHREC: REC – 230 408–014 and N14/01/ 003). For this sub-study, ethical clearance was also sought from, and granted by the CPUT Research Ethics Committee (CPUT/HW– REC 2019/H3). The investigation was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki 2013). All participants provided informed written consent.

### 5.3.2 Study population and study design

This was a cross-sectional study, in which quantitative methods were used to investigate the expression of miRNAs in a total of 1270 subjects: 207 prediabetes, 94 screen-detected diabetes and 969 normotolerant individuals. Participants in the VMH study were enrolled between 2014 and 2016, from Bellville South community in Cape Town, South Africa. Participants without prior diabetes underwent an OGTT, and diagnosed as either normotolerant, pre-diabetes, or screen-detected diabetes, in accordance with World Health Organization (WHO) guidelines.[16] Other study procedures which included anthropometric and blood pressure measurements were described in detail previously.[17] Blood glucose, HbA1c, insulin, lipids, ultra-sensitive C-reactive protein (us-CRP) and serum cotinine levels were acquired in a routine pathology laboratory.

### 5.3.3 MicroRNA quantification by Real-Time Quantitative-Polymerase Chain Reaction (RT q-PCR)

MicroRNA was isolated from whole blood that had been collected in a Tempus RNA tube (Applied Biosystems) using the MagMax Total RNA isolation kit (ThermoFisher Scientific) according to the manufacturer's instructions. This was then converted to cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies, USA) then diluted 1:10 before determining the expression levels by TaqMan miRNA Assay primers on the QuantStudio 7 Flex real-time PCR instrument (Life Technologies, USA) as per manufacturer's instructions. Data were obtained as Ct values and normalised to an endogenous control (miR-16 5p). The $2^{-\Delta Ct}$ method was used to assess the miRNA expression level in each sample whilst the $2-\Delta\Delta Ct$ value was used as the measure of the miRNA expression in each sample analysed compared with control.[18]

### 5.3.4 Statistical analysis

Analysis of data was performed using SPSS v.25 (IBM Corp, 2011). Count (and percentages), mean (and standard deviation) or median (25th-75th percentiles) were used to summarise variables. Baseline characteristics across glucose tolerance subgroups were compared using the chi square test, analysis of the variance (ANOVA), and Kruskal–Wallis test. Spearman's partial correlations adjusted for age, sex and body mass index (BMI) was performed to assess the relationship between the miRNAs and other variables. Multivariable logistic regression models were used to evaluate the association of the miRNAs with prediabetes or screen-detected DM, with crude or adjusted odds ratio (OR). The area under the receiver operating characteristic curve (AUC) was used to assess and compare the ability of each miRNA to predict the presence of prediabetes or DM. A *p*-value $<0.05$ was used to characterize statistically significant results.

## 5.4 Results

### *5.4.1 Descriptive characteristics of participants*

The basic characteristics according to sex are summarized in Table 1. In all, 924 (72.8%) of participants were women and were significantly older than men, mean age 49 versus 46 years, $p$=0.002. Women had significantly higher BMI, waist and hip circumferences compared to their men counterparts (all $p$<0.001). Fasting blood glucose, glucose 2-hour glucose, HbA1c, and insulin were all significantly higher in women (all of which, $p$≤0.001). Lipid variables (HDL and LDL cholesterol) were also significantly higher in women in comparison to men (both $p$<0.001). The expression levels of the miRNAs did not differ significantly by sex.

### *5.4.2 Relative expression of miR-30a-5p and miR-182-5p*

The relative expression of miRNAs 30a-5p and 182–5p is illustrated in Figure 1. The expression levels of both miRNAs were markedly elevated in prediabetes and diabetes when compared with normotolerants, as well as in prediabetes compared to diabetes. miR-30a-5p was significantly upregulated in prediabetes versus NGT by 3.5-fold ($p$<0.001), whilst miR-182-5p was upregulated by 3.2-fold, $p$<0.001. Both miRNAs showed decreased expression in diabetes when compared to prediabetes (both ≤0.58-fold), however this under-expression was significant for miR-182-5p (0.49-fold, $p$=0.001), but not for miR-30a-3p (0.58-fold, $p$=0.097).

### *5.4.3 Partial correlations between miR-30a-5p and miR-182-5p and biochemical characteristics according to glycaemic status*

Partial correlation coefficient analysis, adjusted for age, sex and BMI was performed to examine the link between miRNAs 30a–5p and 182–5p and other clinical variables. A significant positive correlation was observed between miR-30a-5p and miR-182-5p, across all glycaemic statuses (r≥0.932, $p$<0.001). In normotolerant individuals, miR-30a- 5p and miR-182-5p showed a negative correlation with the waist circumference (r>-0.470, $p$≤0.042) and positive correlation with HDL-cholesterol (r≥0.527, $p$≤0.020). In individuals with prediabetes, only miR-30a-5p showed a positive correlation with LDL-cholesterol (r=0.460, $p$=0.048), whilst in newly diagnosed diabetes, both miRNAs correlated positively with hip circumference, 2-hour insulin, HDL- and LDL-cholesterol. In addition, miR-30a-5p also showed positive correlations with systolic and diastolic blood pressures, and Gamma GT (Table 2).

### *5.4.4 Multivariable regression analysis*

When the normotolerant control was used as the reference group, it was observed that both miR-30a-5p and miR- 182-5p were significantly associated with prediabetes and diabetes. In the crude model, for prediabetes the odds ratio (OR) was 2.24 (95% CI: 1.77–2.83 ($p<0.001$)) in relation with miR-30a-5p and 1.33 (1.25–1.40, $p<0.001$) in relation with miR-182-5p. For diabetes, the crude OR were 1.87 (1.40–2.50) in relation with miR-30a-5p and 1.18 (1.10–1.28) in relation with miR-182-5p. These significant associations remained when the models were adjusted for age, sex, BMI, systolic blood pressure, HbA1c, triglycerides, HDL-cholesterol and LDL-cholesterol. When the models were repeated with prediabetes as reference, only miR- 182-5p was significantly associated with a reduced prevalence of diabetes (OR: 0.89, 95% CI: 0.83–0.96, $p=0.003$) in the crude model, and this association remained significant in multivariable models (all $p<0.05$; Table 3).

### *5.4.5 Diagnostic performance of the miRNAs for prediabetes and diabetes*

Figure 2A and B show that both miRNAs could acceptably discriminate prediabetes or dysglycaemia (combination of diabetes and prediabetes) from normal glucose tolerance with miR-182-5p outperforming HbA1c for prediabetes diagnosis, AUC of 0.74 for miR-182-5p, compared to 0.69 for HbA1c ($p=0.217$) which was also similar for miR-30a-5p. With regards to discriminating diabetes, the miRNAs performed poorly, AUC's ≤0.61 (Figure 2C). In addition, we examined whether the miRNAs could act as markers to potentially discriminate between prediabetes and diabetes and found that these performed poorly, AUCs ≤0.41 (Figure 2D).

## 5.5 Discussion

In this study, the profile of circulating miR-30a-5p and miR-182-5p was determined in 1270 peripheral blood mononuclear cell (PBMC) samples from individuals with prediabetes, screen-detected diabetes and normal glucose tolerance. Both miRNAs were markedly upregulated in prediabetes compared to normotolerant by more than 3-fold. Similarly, in screen-detected diabetes, the miRNAs were upregulated compared to normotolerants, but significantly downregulated versus prediabetes. The two miRNAs were significantly correlated with each other, as well as with HDL-cholesterol in age, sex and BMI adjusted analysis. In the prediabetic group, miR-30a-5p exhibited a significant positive correlation with LDL-cholesterol, and additionally, in newly diagnosed diabetes, both miRNAs showed positive correlations with LDL-cholesterol, as well as systolic and diastolic blood pressures. In multivariable logistic regression analysis both miRNAs were associated with prediabetes or screen-detected diabetes; however, only miR-182-5p was associated with reduced prevalent diabetes when prediabetes was used as a reference. Furthermore, miR-182-5p was superior to miR-30a-5p and HbA1c in discriminating between prediabetes and normal glucose tolerance, AUC, 0.74 and 0.69, respectively. Finally, the discrimination of regression models to predict diabetes and prediabetes was enhanced when miRNAs were added to models with covariates only.

Studies have established links between miRNAs 30a-5p and 182–5p and CVD.[11,12] An investigation into possible diagnostic biomarkers for unprotected left main coronary artery disease (uLMCAD) revealed overexpression of circulating miR-182 in uLMCAD patients, compared to patients without coronary artery disease (CAD).[19] In confluence, our study also illustrated similar associations with markers of CVD. Of all lipoproteins comprehensively studied to be involved in CAD risk, LDL-cholesterol has been the chief target for preventive strategies.[20] In our investigation, miR-30a-5p exhibited a significant positive correlation with LDL-cholesterol in the prediabetic group, and both miRNAs showed positive correlations with LDL-cholesterol in newly diagnosed diabetes. Additionally, in newly diagnosed diabetes both miRNAs displayed positive correlations with systolic and diastolic blood pressures. Microvascular rarefaction, which is the loss of terminal blood vessels, is consistent with hypertension.[21,22] The miR-30 family have been revealed to play a role in the regulation of arteriolar branching, and subsequent arteriole blood pressure.[22] Furthermore, the miR-30 family have been linked with CVDs and have been proposed to act as predictors for acute myocardial infarction (AMI).[11] Therefore, the correlations found between both miRNAs and CVD markers not only corroborate findings from other independent studies, but further promote a possible relationship with CVD risk assessment.

Human studies have suggested that altered miRNA patterns probably precede or appear at the early stages of diabetes.[23–25] For instance, in a cohort of children with different durations of T1DM, levels of miR-454-3p were significantly elevated in children diagnosed during the first 42 days compared to those with longer diagnosed duration of the disease.[24] In our study, both miR-30a-5p and miR- 182-5p were elevated in prediabetes and screen-detected diabetes, however, the increase was more pronounced in those with prediabetes. Similar to our findings, miR-182- 5p was shown was shown by Karolina and co-workers to be down-regulated in T2DM and slightly upregulated in subjects with impaired fasting glycaemia.[26] Karolina et al reported miR-182 to be a potential modulator of FOXO1, with upregulation displayed in impaired fasting glycaemia. Downregulation of the FOXO1 messenger RNA (mRNA) in impaired fasting glycaemia was observed, with an upregulation in T2DM.[26] FOXO proteins are major targets of insulin action, with FOXO1 mRNAs playing a key role in mediating the effects of hepatic insulin resistance (IR) signalling, by promoting glucose production.[27] These findings suggest potential hepatic gluconeogenesis to have a compensatory reduction in impaired fasting glycaemia, whilst increased in T2DM.[26] Additionally, high levels of miR-30a-5p were observed, and have been associated with a higher risk of T2DM development.[13] Similar to our findings, the CORPIOPREV longitudinal study described elevated levels of miR-30a-5p in prediabetic subjects compared to normotolerant individuals, and that levels rose several years before the development of diabetes.[13] In another study conducted by Kim et al, miR-30a-5p was shown to mediate beta-cell dysfunction induced glucotoxicity by suppressing Beta2/NeuroD gene expression in rats, and over-expression led to pancreatic beta-cell dysfunction.[28] Persistent exposure of pancreatic beta-cells to

elevated glucose levels results in the subsequent inhibition of glucose-induced insulin secretion, insulin gene expression impairment, as well as induced beta-cell death.[29] Consequently, the inhibition of miR-30a-5p in this animal model was shown to exhibit a protective effect on pancreatic islets and improved glucose tolerance.[28]

Despite the introduction of HbA1c in the diagnosis of prediabetes and diabetes, it has not improved the identification of these conditions due to various factors such as differences in ethnic thresholds, haemoglobinopathies and anaemia.[30] Thus, the development of new biomarkers that would allow early and confident identification of patients is desirable. In this regard, circulating miRNAs that are known to be robust and stable in human body fluids have led to their investigation as potential biomarkers in many pathologies including DM. In our study, we have shown that the increased circulating levels of miR-30a-5p and miR-182-5p in whole blood have the potential to predict these conditions. However, when comparing the AUC of the ROC curves, we observed that these miRNAs performed poorly in predicting diabetes, but miR- 182-5p exhibited greater potential to predict prediabetes. The AUC of miR-182-5p was higher than that of HbA1c for prediabetes, illustrating the diagnostic potential the miRNA possesses for intermediary glycaemic statuses. However, this diagnostic capability was inferior to that of HbA1c for screen- detected diabetes, and dysglycaemia as a whole. Nevertheless, our findings suggest that miR-182-5p has a potential use in clinical practice, as no known interferences with haemoglobinopathies or anaemia have been reported. Indeed, miR-30a-5p and miR-182-5p improved the predictive power of models based upon clinical or biochemical parameters for the outcome of prediabetes or diabetes.

A strength of this study is the large sample-size used compared to other studies, however, limited by the disproportionate representation of women versus men. Total RNA used for miRNA expression analysis was extracted from whole blood, while other studies used serum/plasma. Expression levels may vary between different tissue types, ultimately limiting accurate comparisons with studies that have used different tissues.

### 5.6 Conclusions

In conclusion, our study has revealed an important association between both miRNAs, more so miR-182-5p, and prediabetes and their potential ability to significantly discriminate prediabetes from normoglycaemia. Furthermore, our findings suggest that these miRNAs added to the predictive power of other known risk markers to predict diabetes or prediabetes. This discovery warrants validation in other study settings, to sanction the use of this miRNA in glucose tolerance screening, for risk estimation and classification.

## 5.7 Abbreviations

miRNAs, microRNAs; DM, diabetes mellitus; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; OGTT, oral glucose tolerance test; T2DM, type 2 diabetes; CVD, cardiovascular disease; VMH, Cape Town Vascular and Metabolic Health; WHO, World Health Organization; us-CRP, ultra-sensitive C-reactive protein; ANOVA, analysis of the variance; BMI, body mass index; OR, odds ratio; AUC, receiver operating characteristic curve; PBMC, peripheral blood mononuclear cell; uLMCAD, unprotected left main coronary artery disease; CAD, coronary artery disease; AMI, myocardial infarction; mRNA, messenger RNA; IR, insulin resistance.

## 5.8 Data Sharing Statement

The datasets generated and/or analysed during the current study are not publicly available due to the terms of consent to which participants agreed but are available from the principal investigator (TEM) of the main study on reasonable request.

## 5.9 Acknowledgments

We thank the Bellville South community and their community Health Forum for supporting the study.

## 5.10 Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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## 5.12 Disclosure

The authors declare no conflicts of interest.

### 5.13 References


1. Vaishya S, Sarwade RD, Seshadri V. MicroRNA proteins, and metabolites as novel biomarkers for prediabetes, diabetes, and related complications. *Front Endocrinol (Lausanne)*. 2018;9:180. doi:10.3389/fendo.2018.00180

2. International Diabetes Federation. *IDF Diabetes Atlas*. 9th ed. Brussels, Belgium: International Diabetes Federation; 2019.

3. World Health Organization (WHO) & International Diabetes Federation (IDF). *Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia: Report of a WHO/IDF Consultation*. 2006:1–50

4. Sayed AS, Xia K, Salma U, Yang T, Peng J. Diagnosis, prognosis and therapeutic role of circulating miRNAs in cardiovascular diseases. *Heart Lung Circ*. 2014;23(6):503–510. doi:10.1016/j.hlc.2014.01.001

5. Kumar M, Nath S, Prasad HK, Sharma GD, Li Y. MicroRNAs: a new ray of hope for diabetes mellitus. *Protein Cell*. 2012;3(10):726–738. doi:10.1007/s13238-012-2055-0

6. Feng J, Xing W, Xie L. Regulatory roles of microRNAs in diabetes. *Int J Mol Sci*. 2016;17(10):1729. doi:10.3390/ijms17101729

7. Pordzik J, Jakubik D, Jarosz-Popek J, et al. Significance of circulating microRNAs in diabetes mellitus type 2 and platelet reactivity: bioinformatic analysis and review. *Cardiovasc Diabetol*. 2019;18 (1):113. doi:10.1186/s12933-019-0918-x

8. Kong L, Zhu J, Han W, et al. Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study. *Acta Diabetol*. 2011;48(1):61–69. doi:10.1007/s00592-010-0226-0

9. Grieco GE, Cataldo D, Ceccarelli E, et al. Serum levels of miR-148a and miR-21-5p are increased in type 1 diabetic patients and correlated with markers of bone strength and metabolism. *Noncoding RNA*. 2018;4(4):37. doi:10.3390/ncrna4040037

10. Patoulias DI. Is miRNA-375 a promising biomarker for early detection and monitoring of patients with type 2 diabetes? *Arch Med Sci Atheroscler Dis*. 2018;3(1):e119–e122. doi:10.5114/ amsad.2018.78775

11. Maciejak A, Kostarska-Srokosz E, Gierlak W, et al. Circulating MIR-30a-5p as a prognostic biomarker of left ventricular dysfunction after acute myocardial infarction. *Sci Rep*. 2018;8(1):9883. doi:10.1038/s41598-018-28118-1

12. Zhang X, Dong S, Jia Q, et al. The microRNA in ventricular remodeling: the MIR-30 family. *Biosci Rep*. 2019;39(8):BSR20190788. doi:10.1042/BSR20190788

13. Jiménez-Lucena R, Camargo A, Alcalá-Diaz JF, et al. A plasma circulating miRNAs profile predicts type 2 diabetes mellitus and prediabetes: from the CORDIOPREV study. *Exp Mol Med*. 2018;50 (12):1–12. doi:10.1038/s12276-018-0194-y

14. Matsha TE, Kengne AP, Hector S, Mbu DL, Yako YY, Erasmus RT. MicroRNA profiling and their pathways in South African individuals with prediabetes and newly diagnosed type 2 diabetes mellitus. *Oncotarget*. 2018;9(55):30485–30498. doi:10.18632/oncotarget.25271

15. Erasmus RT, Soita DJ, Hassan MS, et al. High prevalence of diabetes mellitus and metabolic syndrome in a South African coloured population: baseline data of a study in Bellville, Cape Town. *S Afr Med J*. 2012;102(11 Pt 1):841–844. doi:10.7196/samj.5670

16. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus. Provisional report of a WHO consultation. *Diabet Med*. 1998;15(7):539–553. doi:10.1002/(SICI) 1096-9136(199807)15:7<539::AID-DIA668>3.0.CO;2-S

17. Kengne AP, Erasmus RT, Levitt NS, Matsha TE. Alternative indices of glucose homeostasis as biochemical diagnostic tests for abnormal glucose tolerance in an African setting. *Prim Care Diabetes*. 2017;11 (2):119–131. doi:10.1016/j.pcd.2017.01.004

18. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods*. 2001;25(4):402–408. doi:10.1006/meth.2001.1262

19. Zhu L, Chen T, Ye W, et al. Circulating miR-182-5p and miR-5187- 5p as biomarkers for the diagnosis of unprotected left main coronary artery disease. *J Thorac Dis*. 2019;11(5):1799–1808. doi:10.21037/ jtd.2019.05.24

20. Sharma SB, Garg S. Small dense LDL: risk factor for coronary artery disease (CAD) and its therapeutic modulation. *Indian J Biochem Biophys*. 2012;49(2):77–85.

21. Goligorsky MS. Microvascular rarefaction: the decline and fall of blood vessels. *Organogenesis*. 2010;6(1):1–10. doi:10.4161/org.6.1.10427

22. Jiang Q, Lagos-Quintana M, Liu D, et al. miR-30a regulates endothelial tip cell formation and arteriolar branching. *Hypertension*. 2013;62 (3):592–598. doi:10.1161/HYPERTENSIONAHA.113.01767

23. Tang X, Tang G, Ozcan S. Role of MicroRNAs in diabetes. *Biochim Biophys Acta*. 2008;1779(11):697–701. doi:10.1016/j. bbagrm.2008.06

24. Erener S, Marwaha A, Tan R, Panagiotopoulos C, Kieffer TJ. Profiling of circulating microRNAs in children with recent onset of type 1 diabetes. *JCI Insight*. 2017;2(4):e89656. doi:10.1172/jci. insight.89656

25. Arcidiacono B, Chiefari E, Foryst-Ludwig A, et al. Obesity-related hypoxia via miR-128 decreases insulin-receptor expression in human and mouse adipose tissue promoting systemic insulin resistance. *EBioMedicine*. 2020;59:102912. doi:10.1016/j.ebiom.2020.102912

26. Karolina DS, Armugam A, Tavintharan S, et al. MicroRNA 144 impairs insulin signaling by inhibiting the expression of insulin receptor substrate 1 in type 2 diabetes mellitus. *PLoS One*. 2011;6 (8):e22839. doi:10.1371/journal.pone.0022839

27. O-Sullivan I, Zhang W, Wasserman DH, et al. FoxO1 integrates direct and indirect effects of insulin on hepatic glucose production and glucose utilization. *Nat Commun*. 2015;6(1):7079. doi:10.1038/ ncomms8079

28. Kim JW, You YH, Jung S, et al. MiRNA-30a-5p-mediated silencing of Beta2/NeuroD expression is an important initial event of glucotoxicity-induced beta cell dysfunction in rodent models. *Diabetologia*. 2013;56(4):847–855. doi:10.1007/s00125-012-2812-x

29. Weir GC, Marselli L, Marchetti P, Katsuta H, Jung MH, Bonner- Weir S. Towards better understanding of the contributions of overwork and glucotoxicity to the beta-cell inadequacy of type 2 diabetes. *Diabetes Obes Metab*. 2009;11(Suppl 4):82–90. doi:10.1111/j.1463-1326.2009.01113.x

30. Florkowski C. HbA1c as a diagnostic test for diabetes mellitus - reviewing the evidence. *Clin Biochem Rev*. 2013;34(2):75–83.

**Table 1:** Characteristics of the study participants

| | Women, n=924 | Men, n=346 | *p*-value |
|---|---|---|---|
| Age (years) | 49 ±15 | 46 ±15 | **0.002** |
| Body mass index (kg/m$^2$) | 29.96 ±8.14 | 23.97 ±6.51 | **<0.001** |
| Waist circumference (cm) | 92.9 ±17.01 | 83.77 ±15.33 | **<0.001** |
| Hip circumference (cm) | 106.12 ±16.7 | 93.24 ±13 | **<0.001** |
| Waist to Hip ratio | 0.88 ±0.09 | 0.9 ±0.08 | **<0.001** |
| Systolic blood pressure (mmHg) | 134.58 ±25.66 | 133.82 ±27.06 | 0.645 |
| Diastolic blood pressure (mmHg) | 85.79 ±14.68 | 83.27 ±16.69 | **0.009** |
| Glucose Fasting Blood (mmol/L) | 4.9 (4.5 ;5.3) | 4.8 (4.35 ;5.2) | **0.001** |
| Glucose 2-hour (mmol/L) | 6.3 (5.2 ;7.7) | 5 (4.1 ;6.45) | **<0.001** |
| HbA1c (%) | 5.82 ±0.92 | 5.63 ±0.69 | **0.001** |
| HbA1c (mmol/mol) | 40.13 ±10.03 | 38.08 ±7.49 | **0.001** |
| Fasting Insulin (mIU/L) | 6.95 (4.6 ;10.8) | 4.5 (2.7 ;7.9) | **<0.001** |
| 2-hour Insulin (mIU/L) | 42.6 (23.55 ;77.48) | 21.4 (9.5 ;41.9) | **<0.001** |
| Glucose tolerance status | | | **0.009** |
| Normotolerant, n(%) | 684 (74.19) | 285 (82.37) | |
| Prediabetes, n(%) | 165 (17.90) | 42 (12.14) | |
| Screen-detected Diabetes, n(%) | 75 (7.92) | 19 (5.49) | |
| Triglycerides (mmol/L) | 1.16 (0.82 ;1.61) | 1.13 (0.85 ;1.71) | 0.746 |
| Cholesterol HDL (mmol/L) | 1.38 ±0.39 | 1.28 ±0.38 | **<0.001** |
| Cholesterol LDL (mmol/L) | 3.24 ±0.97 | 2.87 ±0.96 | **<0.001** |
| C-reactive Protein (mg/L) | 4.2 (1.79 ;9.07) | 2.79 (1.21 ;6.33) | **<0.001** |
| GGT (IU/L) | 28 (19 ;42) | 30 (22 ;49) | **0.002** |
| Cotinine (ng/mL) | 35.10 (10.00; 274.00) | 156.00 (10.00-290.50) | **0.005** |
| Currently smoking, n(%) | 451 (50.62) | 213 (63.96) | **<0.001** |
| Current drinker, n(%) | 245 (26.6) | 149 (43.44) | **<0.001** |
| miR-30a-5p ($2^{-\Delta Ct}$) | 0.0035 ±0.0083 | 0.0037 ±0.0075 | 0.684 |
| miR-182-5p ($2^{-\Delta Ct}$) | 0.2002 ±0.3198 | 0.1927 ±0.2571 | 0.697 |

**Table 2:** Partial Correlation coefficients adjusted for age, gender and body mass index for miR 30a-5p and miR-182-5p and other biochemical parameters.

| | NGT | | Prediabetes | | Diabetes | | NGT | | Prediabetes | | Diabetes | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | r | *p* | r | *p* | r | *p* | r | *p* | r | *p* | r | *p* |
| miR 30a-5p $2^{-\Delta Ct}$ | 1.000 | | 1.000 | | 1.000 | | 0.938 | **<0.001** | 0.932 | **<0.001** | 0.937 | **<0.001** |
| miR-182-5p $2^{-\Delta Ct}$ | 0.938 | **<0.001** | 0.932 | **<0.001** | 0.937 | **<0.001** | 1.000 | | 1.000 | | 1.000 | |
| Waist circumference (cm) | -0.470 | **0.042** | -0.207 | 0.395 | -0.261 | 0.281 | -0.409 | 0.082 | -0.491 | **0.033** | -0.242 | 0.319 |
| Hip circumference (cm) | -0.250 | 0.302 | 0.180 | 0.462 | 0.465 | **0.045** | -0.154 | 0.528 | -0.195 | 0.425 | 0.458 | **0.049** |
| Waist to Hip ratio | 0.086 | 0.727 | -0.014 | 0.955 | 0.016 | 0.948 | 0.049 | 0.841 | 0.050 | 0.838 | -0.058 | 0.813 |
| Systolic blood pressure (mmHg) | 0.213 | 0.382 | 0.287 | 0.234 | 0.566 | **0.012** | 0.200 | 0.413 | 0.197 | 0.420 | 0.454 | 0.051 |
| Diastolic blood pressure (mmHg) | 0.182 | 0.455 | 0.129 | 0.598 | 0.529 | **0.020** | 0.151 | 0.536 | 0.197 | 0.420 | 0.394 | 0.095 |
| Glucose Fasting Blood (mmol/L) | 0.188 | 0.441 | 0.273 | 0.258 | -0.010 | 0.967 | 0.237 | 0.328 | 0.176 | 0.472 | -0.017 | 0.945 |
| 2-hour glucose (mmol/L) | 0.085 | 0.730 | -0.144 | 0.556 | 0.320 | 0.182 | 0.326 | 0.173 | 0.114 | 0.642 | 0.205 | 0.400 |
| HbA1c (%) | -0.178 | 0.465 | -0.109 | 0.658 | -0.021 | 0.932 | -0.090 | 0.715 | -0.153 | 0.533 | -0.063 | 0.799 |
| Fasting Insulin (mIU/L) | 0.315 | 0.189 | 0.202 | 0.407 | 0.127 | 0.605 | 0.330 | 0.167 | 0.337 | 0.158 | 0.197 | 0.420 |
| 2-hour Insulin (mIU/L) | 0.110 | 0.653 | 0.072 | 0.770 | 0.503 | **0.028** | 0.265 | 0.272 | 0.149 | 0.543 | 0.463 | **0.046** |
| Triglycerides (mmol/L) | 0.033 | 0.893 | 0.208 | 0.393 | 0.243 | 0.317 | 0.099 | 0.687 | 0.045 | 0.855 | 0.128 | 0.602 |
| Cholesterol HDL (mmol/L) | 0.527 | **0.020** | 0.421 | 0.073 | 0.718 | **0.001** | 0.451 | 0.053 | 0.550 | **0.015** | 0.611 | **0.005** |
| Cholesterol LDL (mmol/L) | 0.148 | 0.546 | 0.460 | **0.048** | 0.636 | **0.003** | 0.159 | 0.516 | 0.142 | 0.561 | 0.513 | **0.025** |
| C-reactive protein (mg/L) | 0.121 | 0.621 | 0.016 | 0.948 | 0.232 | 0.339 | 0.107 | 0.662 | 0.142 | 0.561 | 0.220 | 0.366 |
| GGT (IU/L) | 0.123 | 0.616 | 0.086 | 0.726 | 0.517 | **0.023** | 0.135 | 0.581 | 0.169 | 0.490 | 0.433 | 0.064 |

**Table 3:** (A) Multivariable Regression Analysis of miRNAs for the Presence of Prediabetes and Diabetes (B) Multivariate Regression Analysis of miRNAs for the Development of T2DM

| | Prediabetes | | | | DM | | | |
|---|---|---|---|---|---|---|---|---|
| | | | c-Statistic | | | | c-Statistic | |
| | OR (95% CI) | *p*-value | Covariates Only | Covariates and miR | OR (95% CI) | *p*-value | Covariates Only | Covariates and miR |
| **(A)** | | | | | | | | |
| ***miR 30a-5p**** | | | | | | | | |
| Model 1 | 2.24 (1.77–2.83) | **<0.001** | NA | 0.692 (0.650–0.734) | 1.87 (1.40–2.50) | **<0.001** | NA | 0.611 (0.546–0.675) |
| Model 2 | 2.69 (2.08–3.49) | **<0.001** | 0.693 (0.657–0.728) | 0.768 (0.734–0.801) | 2.33 (1.71–3.17) | **<0.001** | 0.752 (0.711–0.792) | 0.768 (0.728–0.808) |
| Model 3 | 2.7 (2.07–3.52) | **<0.001** | 0.758 (0.722–0.798) | 0.808 (0.776–0.841) | 2.36 (1.72–3.23) | **<0.001** | 0.899 (0.859–0.939) | 0.906 (0.866–0.945) |
| Model 4 | 2.52 (1.94–3.29) | **<0.001** | 0.767 (0.732–0.802) | 0.814 (0.782–0.846) | 2.24 (1.63–3.09) | **<0.001** | 0.897 (0.858–0.937) | 0.902 (0.861–0.942) |
| ***miR 182–5p***** | | | | | | | | |
| Model 1 | 1.33 (1.25–1.40) | **<0.001** | NA | 0.735 (0.694–0.777) | 1.18 (1.10–1.28) | **<0.001** | NA | 0.579 (0.515–0.642) |
| Model 2 | 1.36 (1.28–1.44) | **<0.001** | 0.693 (0.657–0.728) | 0.800 (0.767–0.834) | 1.22 (1.12–1.32) | **<0.001** | 0.752 (0.711–0.792) | 0.769 (0.521–0.659) |
| Model 3 | 1.36 (1.28–1.44) | **<0.001** | 0.758 (0.722–0.798) | 0.829 (0.798–0.860) | 1.22 (1.13–1.33) | **<0.001** | 0.899 (0.859–0.939) | 0.903 (0.863–0.943) |
| Model 4 | 1.34 (1.26–1.43) | **<0.001** | 0.767 (0.732–0.802) | 0.835 (0.804–0.865) | 1.21 (1.11–1.31) | **<0.001** | 0.897 (0.858–0.937) | 0.900 (0.859–0.940) |
| **(B)** | | | | | | | | |
| ***miR 30a-5p**** | | | | | | | | |
| Model 1 | – | – | – | – | 0.84 (0.66;1.07) | 0.148 | NA | 0.584 (0.514–0.655) |
| Model 2 | – | – | – | – | 0.86 (0.68;1.10) | 0.233 | 0.578 (0.510–0.646) | 0.590 (0.521–0.659) |
| Model 3 | – | – | – | – | 0.94 (0.71;1.24) | 0.666 | 0.788 (0.726–0.850) | 0.793 (0.731–0.855) |
| Model 4 | – | – | – | – | 0.95 (0.72;1.25) | 0.700 | 0.772 (0.706–0.838) | 0.778 (0.713–0.843) |
| ***miR 182–5p***** | | | | | | | | |
| Model 1 | – | – | – | – | 0.89 (0.83;0.96) | **0.003** | NA | 0.668 (0.604–0.733) |
| Model 2 | – | – | – | – | 0.90 (0.83;0.97) | **0.04** | 0.578 (0.510–0.646) | 0.684 (0.619–0.748) |
| Model 3 | – | – | – | – | 0.91 (0.84;0.98) | **0.019** | 0.788 (0.726–0.850) | 0.810 (0.752–0.868) |
| Model 4 | – | – | – | – | 0.91 (0.84;0.99) | **0.023** | 0.772 (0.706–0.838) | 0.797 (0.737–0.858) |

**Notes: Model 1:** Crude; **Model 2:** included age and sex; **Model 3:** included age, sex, body mass index, systolic blood pressure, and HbA1c; **Model 4:** included age, sex, body mass index, systolic blood pressure, HbA1c, triglycerides, HDL- cholesterol and LDL-cholesterol; *Calculated for 0.1-unit increase; **Calculated for 0.01-unit increase.

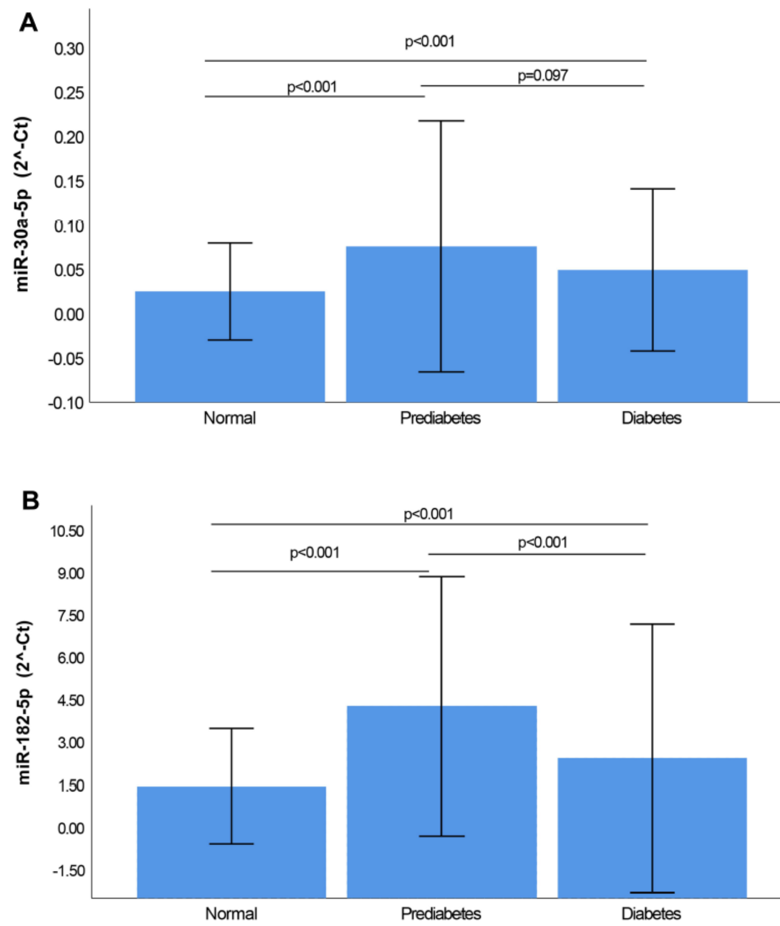


**Figure 1:** Relative Expression of miR-30a-5p and miR-182-5p according to glycaemic status. Normalization was relative to the expression of miR-16-5p. All Data is shown as mean ± standard deviation (SD)

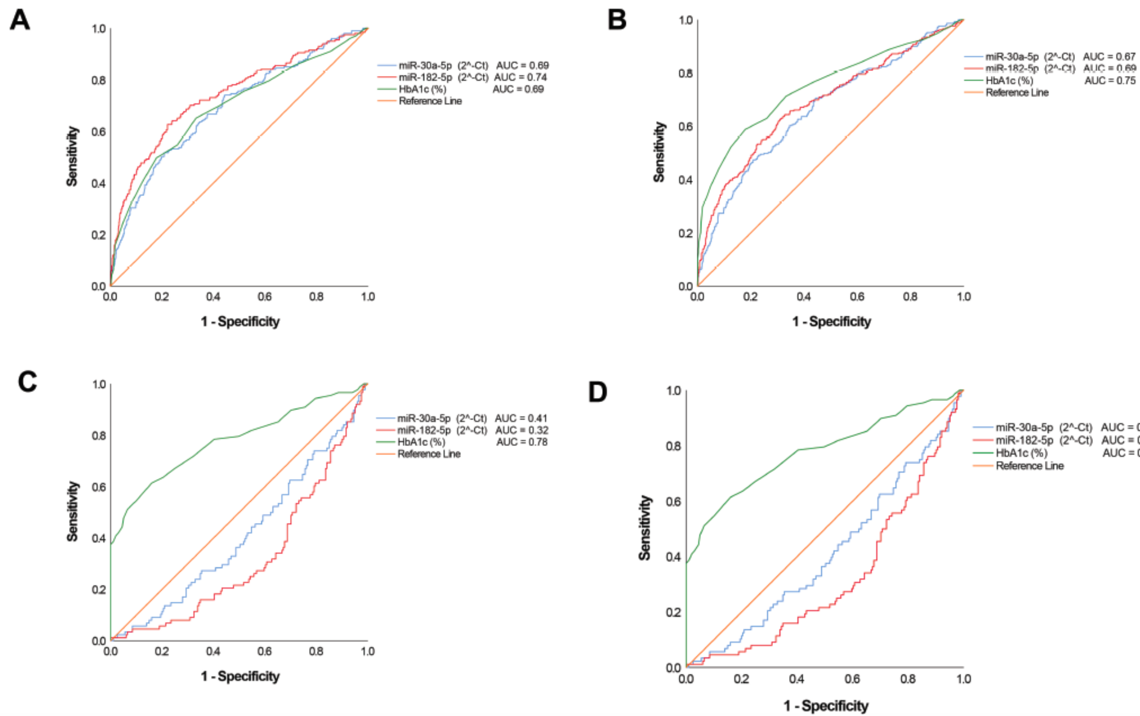


**Figure 2:** Receiver operating characteristic (ROC). ROCs were constructed for each miRNA and HbA1c to evaluate the diagnostic values for prediabetes, screen-detected diabetes, the combination of both (dysglycaemia) as positive cases and normotolerant as negative cases, as well as for diabetes as positive cases and prediabetes as negative cases. (**A**) Prediabetes versus normotolerant. (**B**) Dysglycaemia versus normotolerant. (**C**) newly diagnosed diabetes versus normotolerant. (**D**) screen-detected diabetes versus prediabetes. For this figure panel, the reverse levels of miRNA were used to account for their declining levels from normal glucose tolerance to diabetes, and accordingly obtaining ROC above the diagonal line of "no-discrimination

# CHAPTER 6 – RESULTS (MANUSCRIPT THREE)

**Expression profiles of circulating microRNAs in treated South African type 2 diabetic individuals**

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## 6.1 Abstract

**Aim:** The influence of disease duration and anti-diabetic treatment on epigenetic processes has been described, with limited focus on interactions with microRNAs (miRNAs). miRNAs have been found to play key roles in the regulation of pathways associated with type 2 diabetes mellitus (T2DM), and expression patterns in response to treatment may further promote their use as therapeutic targets in T2DM and its associated complications. We therefore aimed to investigate the expressions of circulating miRNAs (miR-30a-5p, -1299, -182-5p, -30e-3p and -126-3p) in newly diagnosed and known diabetic individuals on treatment.

**Methods:** 1254 participants with an average age of 53.8 years were included in the study and classified according to glycaemic status (974 normotolerant, 92 screen-detected diabetes and 188 known diabetes). Whole blood levels of miR-30a-5p, miR-1299, miR-182-5p, miR-30e-3p and miR-126-3p were quantitated using reverse transcriptase qPCR (RT-qPCR). Expression analysis was performed and compared across groups.

**Results:** All miRNAs were significantly overexpressed in subjects with known diabetes when compared to normotolerant individuals, as well as known diabetics versus screen-detected ($p<0.001$). Upon performing regression analysis, of all miRNAs, only miR-182-5p remained associated with the duration of the disease after adjustment for type of treatment, (OR: 0.127, CI: 0.018-0.236, p=0.023).

**Conclusions:** Our findings revealed important associations and altered expression-patterns of miR-30a-5p, -1299, -182-5p, -30e-3p and -126-3p in known diabetics on anti-diabetic treatment compared to newly diagnosed individuals. Additionally, miR-182-5p expression decreased with increasing duration of T2DM. Further studies are however recommended to shed light on the involvement of the miRNA in insulin signalling and glucose homeostasis, to endorse its use as a therapeutic target in DM and its associated complications.

**Keywords:** South Africa, miR-30a-5p, miR-1299, miR-182-5p, miR-126-3p, metformin, diabetes

## 6.2 Introduction

MicroRNAs (miRNAs) are a family of short, noncoding RNA molecules, averaging 22 nucleotides in length, responsible for regulating gene expression by repressing the translation of messenger RNA (mRNA) molecules, as well as by destabilization of the mRNA molecules [1,2]. Since their discovery, miRNAs have been found to play key roles in the regulation of pathways associated with various diseases including cancers [3,4], cardiovascular diseases (CVDs) [5,6] as well as diabetes mellitus (DM) [7-9]. These small non-coding transcripts have been shown to modulate insulin biosynthesis, pancreatic beta-cell development and survival, as well as glucose and lipid metabolism [10]. Investigations have illustrated altered expression levels of miRNAs such as miR-30a-5p and 126-3p across different glycaemic states, suggesting their potential use as novel biomarkers for early detection of diabetes [11,12]. With increased exploration of human miRNAs in the setting of disease, strategies for diagnosis have been the primary focus. However the focus has progressively extended towards assessing miRNA expression levels and treatment of disease [13].

Through activation of adenosine monophosphate activated protein kinase (AMPK), the widely used anti-diabetic drug metformin promotes insulin sensitivity by improving glucose utilization in target tissues such as the liver and skeletal muscle, as well as by inhibition of hepatic glucose output [14,15]. The interactions of medications such as metformin with epigenetic processes have been detailed, including influences in histone modifications, as well as the DNA methylation. DNA methylation-induced changes have been the most widely addressed, with reduced methylation due to metformin treatment reported at the insulin gene promoter in a beta-cell line cultured using high glucose concentrations [16]. Similarly, reduced methylation of transporter genes (SLC22A1, SLC22A3, and SLC47A1) was reported in the livers of diabetics on metformin therapy, compared to diabetics not receiving anti-diabetic medication [17]. Although not as widely investigated, key evidence surrounding the associations between DM therapies and miRNA expression also exists, with reports of metformin-induced alterations in miRNA expression in diabetic humans and mice, due to increased levels of DICER enzymes, which are essential in miRNA processing [18]. Additionally, plasma miR-222 has been linked with insulin action, and positive associations have been identified with type 2 diabetes mellitus (T2DM) [19]. In two separate studies, both insulin infusion as well as metformin treatment led to reduced circulating levels of the miRNA in patients with T2DM [19,20]. In the same way miRNA expressions aid in mediation of these processes in disease development and progression, changes in their expression in response to anti-diabetic treatment may pave the way for new therapeutic strategies. In view of this, we aimed to investigate the expression of a panel of miRNAs in a South African population with T2DM receiving either metformin treatment, insulin and/or both. Furthermore, we intended to assess the expression levels in association with the duration of T2DM since the date of first diagnosis. Our findings in addition to other investigations focused on miRNA dysregulation in

T2DM development and progression may lay the foundation for new targets for disease management and therapy.

## 6.3 Materials and methods

### *6.3.1 Ethical approval*

The study was approved by the Research Ethics Committees of the Cape Peninsula University of Technology and Stellenbosch University, (NHREC: REC—230 408–014 and N14/01/003 respectively). Ethical clearance for this sub-study was sought from and granted by the Cape Peninsula University of Technology Ethics Committee (CPUT/HW-REC 2019/H3). The study was conducted in accordance with the Declaration of Helsinki, and all participants voluntarily signed written informed consent after all the procedures were fully explained in their language of choice.

### *6.3.2 Study design*

This study was cross-sectional in design, and data was obtained from the ongoing Cape Town Vascular and Metabolic Health (VMH) study, as previously described [21]. Data collection took place between April 2014 and November 2016, involving only South Africans from Cape Town. In a total of 1989 participants enrolled in the VMH study, 1254 met the inclusion criteria for the study, including 92 screen-detected diabetes, 188 known diabetics on treatment and 974 normotolerant individuals. Participants with unknown glucose tolerance status underwent a 75g oral glucose tolerance test (OGTT) in accordance with World Health Organization (WHO) guidelines [22] and study procedures such as anthropometric and blood pressure measurements were also assessed.

Biochemical parameters were immediately analyzed at an ISO 15189 accredited Pathology practice (PathCare, Reference Laboratory, Cape Town, South Africa). Blood glucose levels (mmol/L) were determined with an enzymatic hexokinase method (Beckman AU, Beckman Coulter, South Africa) and HbA1c levels were determined with High Performance Liquid Chromatography (HPLC) (Biorad Variant Turbo, BioRad, South Africa). Serum insulin was by a paramagnetic particle chemiluminescence assay (Beckman DXI, Beckman Coulter, South Africa). High-density lipoprotein cholesterol (HDL-cholesterol) (mmol/L) was measured by enzymatic immune-inhibition – End Point (Beckman AU, Beckman Coulter, South Africa), low-density lipoprotein cholesterol (LDL-cholesterol) (mmol/L) by enzymatic selective protection – End Point (Beckman AU, Beckman Coulter, South Africa) and triglycerides (TG) (mmol/L) were estimated by glycerol phosphate oxidase-peroxidase, End Point (Beckman AU, Beckman Coulter, South Africa). Ultra-sensitive C-reactive protein (usCRP) was measured by Latex Particle immunoturbidimetry. γ-Glutamyltransferase (GGT) was measured using International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) standardized

reagents on a Beckman AU (Beckman Coulter, South Africa). Serum cotinine was by Competitive Chemiluminescent (Immulite 2000, Siemens, South Africa).

### *6.3.3 Total RNA Isolation*

Total RNA including miRNAs was isolated from whole blood which was collected in Tempus RNA tubes that were stored at stored at -80 °C before RNA isolation (Applied Biosystems). The MagMAX™ for Stabilized Blood Tubes RNA Isolation Kit was used to perform the RNA extraction, as per manufacturer's specifications (Life Technologies, South Africa). RNA purity and integrity was then evaluated using a nanodrop (Nanodrop Technologies, Wilmington, USA), and only samples with a concentration >15 ng/mL, and an OD (optical density) ratio $^{A260}/_{A280}$ >1.8 were deemed adequate for further processing.

### *6.3.4 cDNA conversion and reverse transcriptase qPCR (RT-qPCR)*

Subsequent RNA samples were then converted to cDNA, using the *TaqMan Advanced* miRNA cDNA Synthesis Kit, following manufacturer guidelines (Applied Biosystems, ThermoFisher Scientific, South Africa). This protocol converts RNA to cDNA in four separate reactions, namely: poly(A) tailing, adapter ligation, reverse transcription, and finally a miR-Amp reaction. Reaction mixtures were prepared as per manufacturer's specifications, and RNA samples were processed accordingly with incubations in between each reaction step, using a QuantStudio 7 Flex real-time PCR instrument (Life Technologies, USA). Prior to performing RT-qPCR, resultant cDNA samples were diluted 1:10 for optimum quantitative analysis. Thereafter, miRNA expression levels were evaluated using pre-designed *TaqMan Advanced* miRNA Assay primers, as per manufacturer instructions [23]. Data was obtained as Ct values, and normalised to an endogenous control (miR-16 5p). The $2^{-\Delta Ct}$ method was used to evaluate the miRNA expression level in each sample, whilst the $2^{-\Delta\Delta Ct}$ value was used as the measure of the miRNA expression in each sample analysed compared with the control sample [24].

### *6.3.5 Statistical analysis*

Analysis of data was performed using SPSS v.25 (IBM Corp, 2011). Count (and percentages), mean (and standard deviation) or median ($25^{th}$-$75^{th}$ percentiles) were used to summarise variables. Baseline characteristics across glucose tolerance subgroups were compared using the chi square test, analysis of the variance (ANOVA), and Kruskal-Wallis test. Spearman's partial correlations adjusted for age, sex and body mass index (BMI) was performed to assess the relationship between the miRNAs and other variables. A *p*-value <0.05 was used to characterize statistically significant results.

## 6.4 Results

### *6.4.1 Basic characteristics of participants*

Illustrated in Table 1, participants were 53.8 years old on average, with the majority being female (72.7%), and most normotolerant (n=974). As expected, glycaemic parameters (fasting blood glucose, 2 hour blood glucose and HbA1c) were significantly higher in the screen-detected and known diabetes groups versus the normotolerant group, ($p$<0.001). BMI, waist circumference and hip circumference were significantly higher in both screen-detected DM and known DM, compared to the normotolerant group ($p$<0.001). Additionally, lipid variables such as triglycerides and LDL-cholesterol increased significantly across the glycaemic groups ($p$<0.001), whilst HDL-cholesterol exhibited a significant reduction from the normotolerant group through to the known DM group ($p$=0.039). Both systolic blood pressure (SBP) and diastolic blood pressure (DBP) measurements were observed to increase significantly across the glycaemic groups (all of which $p$<0.001). Inflammatory markers C-reactive protein (CRP) and γ-Glutamyltransferase (gamma GT-S) were significantly higher in the diabetic groups in contrast to the normotolerant group, with reduced levels in known DM versus screen-detected. Of the known diabetics on treatment, 165 (87.8%) were on oral medication whilst 23 (12.2%) were on either both oral and/or insulin treatment.

### *6.4.2 Relative miRNA expression*

All miRNAs were significantly overexpressed in subjects with known diabetes when compared to normotolerant individuals ($p$<0.001) and additionally a significant elevation was observed in known diabetics versus screen-detected ($p$<0.001). miR-30a-5p, -1299, 182-5p, -30e-3p and -126-3p were all significantly upregulated in screen-detected DM compared to the normotolerant group ($p$≤0.013), with the exception of miR-30e-3p ($p$=0.145), and miR-30a-5p exhibited the most significant increase in expression ($p$=0.001) between the two groups (Figure 1).

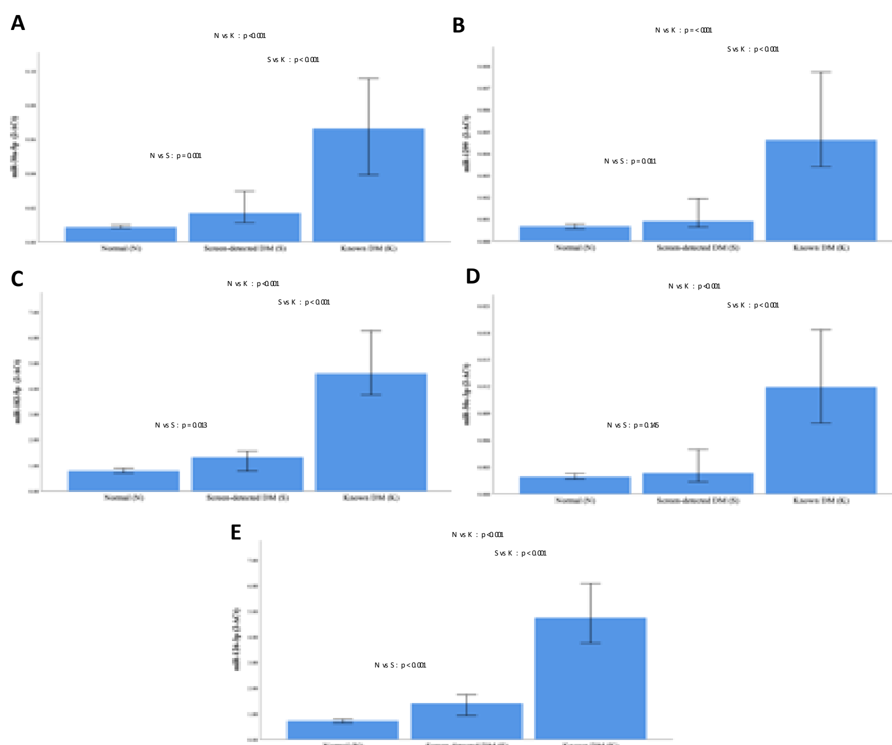


**Figure 1:** Relative expression of the miRNAs according to glycaemic status. Normalization was relative to the expression of miR-16-5p. (A): miR-30a-5p. (B): miR-1299. (C): miR-182-5p. (D): miR-30e-3p. E: miR-126-3p. Data is shown as median (25$^{th}$,75$^{th}$ percentile).

### *6.4.3 Comparisons between miRNA expression with treatment and duration of disease*

The median duration of condition in individuals with known diabetes and on anti-diabetic treatment was 8 years. Although not significant, all other miRNAs with the exception of miR-182-5p were increased in individuals who had had diabetes for over eight years with miR-30e-3p and -126-3p nearing significance, $p \leq 0.095$. miR-182-5p was significantly reduced in individuals who had had diabetes for over eight years, $p=0.033$ (Table 2). No significant differences were observed between the expression of miRNAs and the type of treatment, that is, oral or combination of oral and/or insulin, $p>0.05$ (Table 2).

### *6.4.4 Univariate regression analysis of miRNAs for the duration of diabetes*

The log miRNA $2^{-\Delta Ct}$ was used to perform univariate regression analysis to demonstrate the relationships between the miRNAs and the duration of T2DM (Table 3). miRNA-1299, 182-5p and -126-3p were associated with the duration of diabetes when the model was adjusted for age and sex, [odds ratio (OR)>0.076, 95% confidence interval (CI): 0.001–0.151, $p<0.046$] however after adjustment for type of treatment, only miR-182-5p remained associated with the duration of the disease, (OR: 0.127, CI: 0.018-0.236, $p=0.023$) Table 2.

## 6.5 Discussion

In this study, the expressions of circulating miRNAs (miR-30a-5p, -1299, -182-5p, -30e-3p and -126-3p) were investigated in newly diagnosed and known diabetic individuals on treatment. Our data shows that these miRNAs are differentially expressed in individuals with diabetes, as well as between newly diagnosed and those on treatment. We observed that the duration of the disease is the most determining factor in the expression of these miRNAs. For instance, miR-182-5p was significantly decreased in individuals who had had diabetes for over eight years, but no such differences were observed when anti-diabetic treatment was taken into account. Furthermore, miR-1299, -182-5p and -126-3p were significantly associated with T2DM in regression analysis adjusted for age, sex, but that association was lost for miR-1299 and -126-3p when medication was included in the model.

Advances in whole-genome sequencing, as well as epigenome profiling technologies have contributed towards accelerated growth in research aimed at unveiling the roles of epigenetics in human disease [25-28] particularly the roles of miRNAs in DM [7,11,29]. These small, non-coding RNA transcripts have not only been demonstrated to mediate pathways associated with the development of T2DM, but also pathways involved in long-term diabetes-related complications [30-32]. Upon comparing the expression levels of diabetes-related miRNAs miR-375 and miR-9 between normoglycaemic, prediabetic and known diabetics with a mean duration of disease of 15 years, Al-Muhtaresh *et al* reported marked overexpression of both miRNAs in prediabetics versus the control group, and more so in the diabetic participants. Similarly, we have found a significant decrease of miR-182-5p in subjects who had been diagnosed with diabetes for eight years or more [31]. miRNAs function as post-transcription gene regulators, targeting specific messenger RNA (mRNA) by either suppressing translation or degrading the transcripts, ultimately impeding protein synthesis [33]. Previous reports have described involvement of miR-182 in regulating glucose homeostasis, by targeting FOXO1 [29,34,35]. Mammalian cells express four FOXO variants, namely FOXO1, FOXO3, FOXO4 and FOXO6, of which FOXO1 is the most abundantly expressed in liver, adipose tissue as well as in pancreatic beta-cells [36]. FOXO1 is vital in regulating pancreatic beta-cell replication and differentiation, as well as maintenance in states of metabolic stress [37]. Furthermore, FOXO1 is involved in stimulating hepatic gluconeogenesis in states of hypoglycaemia via the phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) signalling pathway, whilst in hyperglycaemia, insulin signalling via insulin like growth factor-1 (IGF-1) and its receptor (IGF-1R), stimulate PI3K/Akt-dependent phosphorylation of FOXO1, leading to subsequent suppression of gluconeogenesis [38]. In this regard, increased levels in individuals with prediabetes diabetes compared to those with diabetes has been linked to the attempt to inhibit hepatic gluconeogenesis, whilst pathological downregulation in diabetes promoted gluconeogenesis [29].

Literary evidence has detailed the impact of anti-diabetic therapy on the miRNA profiles of T2D patients and in cancers [20,39-41]. Ortega *et al* longitudinally assessed miRNA expression profiles in 35 previously untreated T2D patients, and compared expressions in the same individuals after 3 months of metformin treatment to a placebo-group. The authors demonstrated altered expressions of miR-192, -140-5p and -222 in metformin-treated patients [20]. Similarly and more recently, in a study headed by Demirsoy and co-workers, the expression profiles of 13 plasma miRNAs were altered in T2D patients before treatment, and after 3 months of receiving metformin therapy [39]. In a study aimed at evaluating miRNAs connected with the anti-tumour effects of metformin human gastric cancer cells, upregulation of miR-182 was revealed in the cultured cells and cancer tissues treated with metformin when compared to untreated cells [40]. In our study, we did not observe any differences in the miRNAs and metformin and/or insulin, but marked differences in those individuals receiving anti-diabetic treatment.

This is the first study of its kind to be conducted in an African setting, and findings may contribute towards curbing the increasing burden of T2DM in Africa. The study was however limited by the disproportionate representation of normotolerant versus screen-detected diabetics and known diabetics on treatment. Furthermore, the cross-sectional nature of the study limits accurate evaluation of anti-diabetic induced epigenetic changes, hence longitudinal studies are advised. In conclusion, our study has revealed important associations and altered expression-patterns of miR-30a-5p, -1299, -182-5p, -30e-3p and -126-3p in individuals with diabetes on anti-diabetic treatment compared to newly diagnosed cases. Furthermore, we show that miR-182-5p in particular decreases with increasing duration of T2DM. Longitudinal and functional investigations are recommended to elucidate the involvement of the miRNA in insulin signalling and glucose homeostasis, to endorse its use as a therapeutic target in DM and its associated complications.

### 6.6 References


1. Zhou JY, Xu B, Li L. A New Role for an Old Drug: Metformin targets microRNAs in treating diabetes and cancer. *Drug Dev Res.* 2015;76(6):263-269. doi: 10.1002/ddr.21265.

2. O'Brien J, Hayder H, Zayed Y, Peng C. Overview of microRNA biogenesis, mechanisms of actions, and circulation. *Front Endocrinol (Lausanne).* 2018;9:402. doi: 10.3389/fendo.2018.00402.

3. Zhang FB, Du Y, Tian Y, Ji ZG, Yang PQ. 2019. MiR-1299 functions as a tumor suppressor to inhibit the proliferation and metastasis of prostate cancer by targeting NEK2. *Eur Rev Med Pharmacol Sci.* 2019;23(2):530-538. doi: 10.26355/eurrev_201901_16865.

4. Liu MM, Li Z, Han XD, Shi JH, Tu DY, Song W, Zhang J, Qiu XL, Ren Y, Zhen LL. MiR-30e inhibits tumor growth and chemoresistance via targeting IRS1 in Breast Cancer. *Sci Rep*. 2017;7(1): 1–10. doi: 10.1038/s41598-017-16175-x.

5. Maciejak A, Kostarska-Srokosz E, Gierlak W, Dluzniewski M, Kuch M, Marchel M, Opolski G, Kiliszek M, Matlak K, Dobrzycki S. Circulating miR-30a-5p as a prognostic biomarker of left ventricular dysfunction after acute myocardial infarction. *Sci Rep*. 2018;8(1):1-11. doi: 10.1038/s41598-018-28118-1.

6. Zhu L, Chen T, Ye W, Wang JY, Zhou JP, Li ZY, Li CC. 2019. Circulating miR-182-5p and miR-5187-5p as biomarkers for the diagnosis of unprotected left main coronary artery disease. *J Thorac Dis*. 2019;11(5):1799. doi: 10.21037/jtd.2019.05.24.

7. Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, Mayr A, Weger S, Oberhollenzer F, Bonora E, Shah A, Willeit J, Mayr M. Plasma microRNA profiling reveals loss of endothelial miR-126 and other MicroRNAs in type 2 diabetes. *Circ Res*. 2010;107(6):810-817. doi:10.1161/CIRCRESAHA.110.226357.

8. Massaro JD, Polli CD, Costa e Silva M, Alves CC, Passos GA, Sakamoto-Hojo ET, Rodrigues de Holanda Miranda W, Bispo Cezar NJ, Rassi DM, Crispim F, Dib SA, Foss-Freitas MC, Pinheiro DG, Donadi EA. Post-transcriptional markers associated with clinical complications in type 1 and type 2 diabetes mellitus. *Mol Cell Endocrinol*. 2019;490:1-14. doi: 10.1016/j.mce.2019.03.008.

9. Wang J, Wang G, Liang Y, Zhou X. Expression profiling and clinical significance of plasma microRNAs in diabetic nephropathy. *J Diabetes Res*. 2019;2019. doi: 10.1155/2019/5204394.

10. Mao Y, Mohan R, Zhang S. & Tang X. MicroRNAs as pharmacological targets in diabetes. *Pharmacol Res*. 2013;75:37–47. doi: 10.1016/j.phrs.2013.06.005.

11. Jiménez-Lucena R, Camargo A, Alcalá-Diaz JF, Romero-Baldonado C, Luque RM, Van Ommen B, Delgado-Lista J, Ordovás JM, Pérez-Martínez P, Rangel-Zúñiga OA. A plasma circulating miRNAs profile predicts type 2 diabetes mellitus and prediabetes: from the CORDIOPREV study. *Exp Mol Med*. 2018;50(12): 1–12.

12. Qin L-L, An M-X, Liu Y-L, Xu H-C, Lu Z-Q. MicroRNA-126: a promising novel biomarker in

peripheral blood for diabetic retinopathy. *Int J Ophthalmol*. 2017;10(4):530. doi: 10.18240/ijo.2017.04.05.

13. Walayat A, Yang M, Xiao D. Therapeutic implication of miRNA in human disease. *Antisense Ther*. 2018;(InTechOpen). doi: 10.5772/intechopen.82738.

14. Anedda A, Rial E, González-Barroso MM. Metformin induces oxidative stress in white adipocytes and raises uncoupling protein 2 levels. *J Endocrinol*. 2008;199(1):33-40. Available from: https://joe.bioscientifica.com/view/journals/joe/199/1/33/xml [Accessed 11 July 2020].

15. Song R. Mechanism of metformin: A tale of two sites. *Diabetes Care*. 2016;39(2):187-189. doi: 10.2337/dci15-0013.

16. Ishikawa K, Tsunekawa S, Ikeniwa M, Izumoto T, Iida A, Ogata H, Uenishi E, Seino Y, Ozaki N, Sugimura Y, Hamada Y, Kuroda A, Shinjo K, Kondo Y, Oiso Y. Long-term pancreatic beta cell exposure to high levels of glucose but not palmitate induces DNA methylation within the insulin gene promoter and represses transcriptional activity. *PLoS one*. 2015; 10(2): e0115350. doi: 10.1371/journal.pone.0115350

17. García-Calzón S, Perfilyev A, Männistö V, de Mello VD, Nilsson E, Pihlajamäki J, Ling C. Diabetes medication associates with DNA methylation of metformin transporter genes in the human liver. *Clin. Epigenet*. 2017;9(1): 102. doi: 10.1186/s13148-017-0400-0.

18. Noren Hooten N, Martin-Montalvo A, Dluzen DF, Zhang Y, Bernier M, Zonderman AB, Becker KG, Gorospe M, de Cabo R, Evans MK. Metformin-mediated increase in DICER1 regulates microRNA expression and cellular senescence. *Aging Cell*. 2016;15(3):572-581. doi: 10.1111/acel.12469.

19. Coleman CB, Lightell DJ, Moss SC, Bates M, Parrino PE, Woods TC. Elevation of miR-221 and -222 in the internal mammary arteries of diabetic subjects and normalization with metformin. *Mol Cell Endocrinol*. 2013;374(1-2):125-129. doi: 10.1016/j.mce.2013.04.019.

20. Ortega FJ, Mercader JM, Moreno-Navarrete JM, Rovira O, Guerra E, Esteve E, Xifra G, Martínez C, Ricart W, Rieusset J, Rome S, Karczewska-Kupczewska M, Straczkowski M, Fernández-Real JM. Profiling of circulating microRNAs reveals common microRNAs linked to type 2 diabetes that change with insulin sensitization. *Diabetes Care*. 2014;37(5):1375-1383. doi: 10.2337/dc13-1847.

21. Kengne AP, Erasmus RT, Levitt NS, Matsha TE. Alternative indices of glucose homeostasis as biochemical diagnostic tests for abnormal glucose tolerance in an African setting. *Prim Care Diabetes*. 2017;11(2): 119–131. doi:10.1016/j.pcd.2017.01.004.

22. Alberti KGMM, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus. Provisional report of a WHO consultation. *Diabet Med*. 1998;15(7):539-553. doi: 10.1002/(SICI)1096-9136(199807)15:7<539::AID-DIA668>3.0.CO;2-S.

23. Thermo Fisher Scientific. TaqMan® Advanced miRNA Assays User Guide (Single-tube Assays). 2016;(100027897):1-30. Available from: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/100027897_TaqManAdv_miRNA_Assays_UG.pdf [Accessed February 11, 2019].

24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods*. 2001;25(4): 402–408.

25. Hawkins D, Hon G, Ren B. Hawkins RD, Hon GC, Ren B. Next-generation genomics: an integrative approach. *Nat Rev Genet*. 2010;11(7): 476–486. doi: 10.1038/nrg2795.

26. Laird PW. Principles and challenges of genome-wide DNA methylation analysis. *Nat Rev Genet*. 2010;11(3):191-203.

27. Koperski Ł, Kotlarek M, Świerniak M, Kolanowska M, Kubiak A, Górnicka B, Jażdżewski K, Wójcicka A. Next-generation sequencing reveals microRNA markers of adrenocortical tumors malignancy. *Oncotarget*. 2017;8(30):49191-49200. doi: 10.18632/oncotarget.16788.

28. Afjeh, SSA, Ghaderian, SMH. The role of microRNAs in cardiovascular disease. *Int J Mol Cell Med*. 2013;2(2):50–57. Available from: https://pubmed.ncbi.nlm.nih.gov/24551791 [Accessed 3 September 2020].

29. Karolina DS, Armugam A, Tavintharan S, Wong MTK, Lim SC, Sum CF, Jeyaseelan K. MicroRNA 144 impairs insulin signaling by inhibiting the expression of insulin receptor substrate 1 in type 2 diabetes mellitus. *PloS one*. 2011;6(8): e22839.

30. Zhang T, Lv C, Li L, Chen S, Liu S, Wang C, Su B. 2013. Plasma miR-126 is a potential

biomarker for early prediction of type 2 diabetes mellitus in susceptible individuals. *Biomed Res Int*. 2013;2013.

31. Al-Muhtaresh HA, Al-Kafaji G. Evaluation of two-diabetes related microRNAs suitability as earlier blood biomarkers for detecting prediabetes and type 2 diabetes mellitus. *J Clin Med*. 2018;7(2):12.

32. Kato M, Castro NE, Natarajan R. MicroRNAs: potential mediators and biomarkers of diabetic complications. *Free Radic Biol Med*. 2013;64:85-94.

33. Tchurikov NA. Molecular mechanisms of epigenetics. *Biochem*. 2005;70(4):406-423.

34. Zhang D, Li Y, Yao X, Wang H, Zhao L, Jiang H, Yao X, Zhang S, Ye C, Liu W, Cao H, Yu S, Wang Y, Li Q, Jiang J, Liu Yi, Zhang L, Liu, Y, Iwai N, Wang H, Li J, Li J, Li X, Jin Z-B, Ying H. 2016. miR-182 regulates metabolic homeostasis by modulating glucose utilization in muscle. *Cell Rep*. 2016;16(3):757-768.

35. Zhou J, Meng Y, Tian S, Chen J, Liu M, Zhuo M, Zhang Y, Du H, Wang X. Comparative microRNA expression profiles of cynomolgus monkeys, rat, and human reveal that miR-182 is involved in T2D pathogenic processes. *J Diabetes Res*. 2014;2014(760397). doi: 10.1155/2014/760397.

36. Kitamura T, Nakae J, Kitamura Y, Kido Y, Biggs 3rd WH, Wright CVE, White MF, Arden KC, Accili D. The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. *J Clin Invest*. 2002;110(12): 1839–1847.

37. Kitamura T. The role of FOXO1 in β-cell failure and type 2 diabetes mellitus. *Nat Rev Endocrinol*. 2013;9(10):615. doi: 10.1038/nrendo.2013.157.

38. Tsuchiya K, Ogawa Y. Forkhead box class O family member proteins: The biology and pathophysiological roles in diabetes. *J Diabetes Investig*. 2017;8(6):726-734. doi: 10.1111/jdi.12651.

39. Demirsoy IH, Ertural DY, Balci Ş, ÇInkIr Ü, Sezer K, Tamer L, Aras N. Profiles of circulating miRNAs following metformin treatment in patients with type 2 diabetes. *J Med Biochem*. 2018;37(4): 499–506. doi: 10.2478/jomb-2018-0009.

40. Li, W, Yuan Y, Huang L, Qiao M, Zhang Y. Metformin alters the expression profiles of microRNAs in human pancreatic cancer cells. *Diabetes Res Clin*. 2012;96(2):187-195. doi: 10.1016/j.diabres.2011.12.028.

41. Kato K, Gong J, Iwama H, Kitanaka A, Tani J, Miyoshi H, Nomura K, Mimura S, Kobayashi M, Aritomo Y, Kobara H, Mori H, Himoto T, Okano K, Suzuki Y, Murao K, Masaki T. The antidiabetic drug metformin inhibits gastric cancer cell proliferation in vitro and in vivo. *Mol Cancer Ther*. 2012;11(3):549-560.

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## 6.8 Conflict(s) of Interest/Disclosure(s):

The authors declare no conflict of interests.

## 6.9 Acknowledgments

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## 6.10 Author contributions

**CJW:** wrote the first draft, experimental procedures, data analysis and interpretation. **DDM:** experimental procedures, data analysis and interpretation. **SFGD:** recruitment and screening of cohort, statistical analysis and interpretation of data. **SR:** interpretation of data, editing and revising it for intellectual content. **RTE:** conception, interpretation of the data, revising it for intellectual content and final approval of the version to be published. **APK:** conception, interpretation of the data, revising it for intellectual content and final approval of the version to be published. **GMD:** editing and revising it for intellectual content, final approval of the version to be published.**TEM:** conception and design of the study, analysis and interpretation of the data, revising it for intellectual content and final approval of the version to be published.

## 6.11 Data Availability Statement

The datasets generated and/or analysed during the current study are not publicly available due to the terms of consent to which participants agreed but are available from the principal investigator (TEM) of the main study on reasonable request.

**Table 1:** Characteristics of participants according to diabetic status

| | Normal n=974 | Screened DM n=92 | Known DM n=188 | *p*-value |
|---|---|---|---|---|
| miR-30a-5p( $2^{-\Delta Ct}$) | 0.0252±0.0549 | 0.0494±0.0916 | 0.1577±0.2512 | **<0.001** |
| miR-30a-5p ($2^{-\Delta Ct}$)* | 0.0086 (0.0023;0.0262) | 0.0169 (0.0041;0.0576) | 0.0664 (0.011;0.1858) | **<0.001** |
| miR-1299 ($2^{-\Delta Ct}$) | 0.0033±0.0108 | 0.0042±0.009 | 0.0264±0.062 | **<0.001** |
| miR-1299 ($2^{-\Delta Ct}$)* | 0.0007 (0.0001;0.0023) | 0.0009 (0.0003;0.0036) | 0.0046 (0.0012;0.0202) | **<0.001** |
| miR-182-5p ($2^{-\Delta Ct}$) | 1.457±2.0279 | 2.4347±4.742 | 8.979±11.1365 | **<0.001** |
| miR-182-5p ($2^{-\Delta Ct}$)* | 0.7994 (0.3065;1.7989) | 1.3223 (0.3966;2.4185) | 4.6079 (1.3219;13.0158) | **<0.001** |
| miR-30e-3p ($2^{-\Delta Ct}$) | 0.0047±0.0071 | 0.0056±0.0067 | 0.0291±0.0478 | **<0.001** |
| miR-30e-3p ($2^{-\Delta Ct}$)* | 0.0019 (0.0006;0.0062) | 0.0023 (0.0008;0.0079) | 0.0119 (0.0025;0.041) | **<0.001** |
| miR-126-3p ($2^{-\Delta Ct}$) | 1.0375±0.9964 | 1.6141±1.324 | 7.2226±7.1824 | **<0.001** |
| miR-126-3p ($2^{-\Delta Ct}$)* | 0.7251 (0.2917;1.5107) | 1.4188 (0.4404;2.4671) | 4.7558 (1.4091;10.7017) | **<0.001** |
| Age (years) | 45.22±15.3 | 58.15±10.62 | 57.88±11.97 | **<0.001** |
| **Sex, n (%)** | | | | |
| Female | 688 (70.6) | 73 (79.3) | 151 (80.3) | 0.008 |
| Male | 286 (29.4) | 19 (20.7) | 37 (19.7) | |
| Body mass index (kg/m$^2$) | 27.4±7.8 | 31.5±8.0 | 30.7±6.4 | **<0.001** |
| Waist circumference (cm) | 87.9±16.5 | 100.2±15.5 | 99.3±16.5 | **<0.001** |
| Hip circumference (cm) | 100.9±16.5 | 108.0±15.4 | 107.0±14.0 | **<0.001** |
| Systolic blood pressure (mmHg) | 131±25 | 146±26 | 148±26 | **<0.001** |
| Diastolic blood pressure (mmHg) | 84±15 | 90±14 | 88±15 | **<0.001** |
| Glucose Fasting Blood (mmol/L)* | 4.7 (4.4;5.1) | 7.3 (5.7;8.9) | 9.1 (6.6;13.3) | **<0.001** |
| Glucose 2-hour (mmol/L)* | 5.4 (4.5;6.3) | 12.8 (11.5;16.6) | 13.65 (7.6;0) | **<0.001** |
| HbA1c (%) | 5.6±0.5 | 7.3±1.9 | 8.9±2.4 | **<0.001** |
| HbA1c (mmol/mol) | 37.5±5.0 | 56.5±21.2 | 73.8±26.6 | **<0.001** |
| Insulin Fasting (mIU/L)* | 5.8 (3.7;9) | 9.4 (5.45;16.6) | 9.25 (5.48;15.43) | **<0.001** |
| Insulin 2-hour (mIU/L)* | 30.5 (15.9;53.6) | 51.2 (29.2;80.4) | 39.3 (14.3;0) | **<0.001** |
| Triglycerides (mmol/L)* | 1.1 (0.8;1.5) | 1.4 (1.1;2.4) | 1.6 (1.2;2.2) | **<0.001** |
| Cholesterol HDL (mmol/L) | 1.4±0.4 | 1.3±0.5 | 1.3±0.3 | **0.039** |
| Cholesterol LDL (mmol/L) | 3.1±1,0 | 3.5±1.1 | 3.2±1.1 | **0.001** |
| Cholesterol-S (mmol/L) | 5.0±1.2 | 5.7±1.3 | 5.3±1.2 | **<0.001** |
| CRP(mg/L)* | 3.4 (1.3;7.8) | 6.5 (3.2;13.1) | 4.9 (2.3;10.3) | **<0.001** |
| GGT * (IU/L)* | 27.0 (19.0;42.0) | 42.5 (26.3;76.0) | 33.0 (20,0;61.0) | **<0.001** |
| S-Creatinine (μmol/L)* | 59.0 (52.0;68.0) | 61.0 (52.0;77.0) | 60.5 (52.0;75.0) | 0.127 |
| MDRD eGFR (ml/min) | 107.3±30.0 | 93.2±30.7 | 93.9±32.5 | **<0.001** |
| CKD-EPI eGFR (ml/min) | 106.3±20.6 | 90.3±23.1 | 90.8±24.1 | **<0.001** |
| **Education level, n (%)** | | | | |
| <7 years | 271 (28) | 39 (43.3) | 75 (40.3) | **<0.001** |
| ≥7 years | 698 (72) | 51 (56.7) | 111 (59.7) | |
| **Tobacco use, n (%)** | | | | |
| Non-smoker | 393 (42.1) | 58 (66.7) | 128 (70.3) | **<0.001** |
| Current smoker | 540 (57.9) | 29 (33.3) | 54 (29.7) | |
| **Alcohol use, n (%)** | | | | |
| Non-drinker | 645 (66.5) | 76 (83.5) | 161 (86.6) | **<0.001** |
| Current drinker | 325 (33.5) | 15 (16.5) | 25 (13.4) | |

** Cholesterol HDL – high-density lipoprotein cholesterol, Cholesterol LDL – low-density lipoprotein cholesterol, Cholesterol-S - serum cholesterol, S-creatinine – serum creatinine, CRP – C-reactive protein, GGT – γ-Glutamyltransferase, MDRD eGFR - Modification of Diet in Renal Disease estimated glomerular filtration rate, CKD-EPI eGFR - Chronic Kidney Disease Epidemiology Collaboration estimated glomerular filtration rate

**Table 2**: Comparisons between miRNA expression with treatment type and duration of disease

| | Types of medication | | | Duration of disease | | |
|---|---|---|---|---|---|---|
| | Both/Insulin | Oral | *p*-value | <8 years | ≥8 years | *p*-value |
| **miR-30a-5p ($2^{-\Delta Ct}$)** | 0.0641 (0.0292; 0.2787) | 0.0664 (0.0098; 0.184) | 0.280 | 0.0581 (0.0069; 0.1787) | 0.0607 (0.0182; 0.1906) | 0.567 |
| **miR-1299 ($2^{-\Delta Ct}$)** | 0.0048 (0.0015; 0.0233) | 0.0045 (0.0011; 0.0196) | 0.665 | 0.0041 (0.0006; 0.0207) | 0.0075 (0.0015; 0.0243) | 0.155 |
| **miR-182-5p ($2^{-\Delta Ct}$)** | 7.4965 (2.183; 14.1241) | 4.5773 (1.1516; 12.9642) | 0.427 | 3.2669 (1.0255; 10.3437) | 6.4257 (2.2623; 13.4441) | **0.033** |
| **miR-30e-3p ($2^{-\Delta Ct}$)** | 0.0098 (0.003; 0.0375) | 0.0128 (0.0025; 0.0431) | 0.765 | 0.0073 (0.0019; 0.0295) | 0.0128 (0.0034; 0.0437) | 0.065 |
| **miR-126-3p ($2^{-\Delta Ct}$)** | 4.1712 (1.765; 9.1078) | 4.8205 (1.3649; 10.9543) | 0.873 | 3.8398 (1.3521; 8.4545) | 5.749 (2.0933; 11.674) | 0.095 |

**Table 3:** Univariate regression analysis of miRNAs for the duration of diabetes

| | B | Std error | Upper | Lower | *p*-value |
|---|---|---|---|---|---|
| **Log miR-30a-5p ($2^{-\Delta Ct}$)** | | | | | |
| Model 1 | -0.021 | 0.037 | -0.094 | 0.053 | 0.581 |
| Model 2 | 0.023 | 0.036 | -0.047 | 0.094 | 0.511 |
| Model 3 | 0.029 | 0.040 | -0.049 | 0.108 | 0.464 |
| **Log miR-1299 ($2^{-\Delta Ct}$)** | | | | | |
| Model 1 | 0.058 | 0.040 | -0.022 | 0.138 | 0.151 |
| Model 2 | 0.076 | 0.038 | 0.001 | 0.151 | **0.046** |
| Model 3 | 0.064 | 0.027 | -0.010 | 0.138 | <u>0.088</u> |
| **Log miR-182-5p ($2^{-\Delta Ct}$)** | | | | | |
| Model 1 | 0.078 | 0.040 | 0.000 | 0.156 | <u>0.051</u> |
| Model 2 | 0.101 | 0.037 | 0.029 | 0.174 | **0.007** |
| Model 3 | 0.127 | 0.055 | 0.018 | 0.236 | **0.023** |
| **Log miR-30e-3p ($2^{-\Delta Ct}$)** | | | | | |
| Model 1 | 0.040 | 0.045 | -0.050 | 0.130 | 0.384 |
| Model 2 | 0.070 | 0.042 | -0.013 | 0.153 | <u>0.097</u> |
| Model 3 | 0.086 | 0.047 | -0.008 | 0.179 | <u>0.072</u> |
| **Log miR-126-3p ($2^{-\Delta Ct}$)** | | | | | |
| Model 1 | 0.083 | 0.043 | -0.012 | 0.177 | <u>0.086</u> |
| Model 2 | 0.102 | 0.044 | 0.014 | 0.189 | **0.023** |
| Model 3 | 0.119 | 0.062 | -0.003 | 0.241 | <u>0.056</u> |

**Model 1:** Crude; **Model 2:** included age and sex; **Model 3:** included age, sex, type of medication

# CHAPTER 7 – CONCLUSIONS AND PERSPECTIVES

Herein details a summary of the novel findings from the results chapters and how these observations covered the aims and objectives of the research project. Moreover, proposed implications in diabetes risk assessment and management strategies, as well as recommendations for futures studies.

### 7.1 Summary of novel insights from this study

The genome-wide sequencing results portrayed in the foundation chapter showed altered expressions of microRNAs (miRNAs) in a small sample of individuals with prediabetes, newly diagnosed diabetes and known diabetes on anti-diabetic therapy, in relation to individuals with normal glucose tolerance. Observations revealed miRNAs previously reported in other population groups, as well as those unique to this particular population and the subsequent findings formed the basis for the miRNAs that were further investigated in this project.

Findings presented in chapter four validated the genome-wide miRNAs in a much larger cohort and illustrated compelling associations between the investigated miRNAs and prediabetes as well as screen-detected diabetes. More notably miR-126-3p demonstrated greater diagnostic capacity in discriminating prediabetes than the HbA1c, suggesting possible use a biomarker in early detection of dysglycaemia.

Similarly, the second results chapter further presented dysregulation and significant relations between miRNAs and dysglycaemia, with miR-182-5p found to be associated with reduced prevalent diabetes when prediabetes was used as a reference. Novelty was demonstrated by the superiority of miR-182-5p over the HbA1c, in differentiating prediabetics from normotolerant individuals, implicating potential incorporation of the miRNA in diabetes screening programmes.

The last results chapter portrayed differences in miRNA expression between screen-detected diabetics and known diabetics on treatment. Furthermore, miR-182-5p expression in particular, was shown to be impacted more by disease duration than anti-diabetic treatment in participants with long-term diabetes, eluding to prospects of inclusion of miR-182-5p as a therapeutic target for diabetes.

### 7.2 Implications of miRNAs in the management of diabetes mellitus (DM)

Type 2 diabetes mellitus (T2DM) accounts for about 90 to 95% of all diabetes cases worldwide, and is fast becoming a major public health concern, as well as a huge economic burden in both developed and developing nations (International Diabetes Federation, 2019; Kumar *et al.*, 2012). In South Africa particularly, rapid westernization may be attributed to the observed rise in T2DM prevalence, with Erasmus *et al* reporting a prevalence of 28.2% in the population group involved in this study (Erasmus

*et al*., 2012). Hence the need for innovative management strategies to curb the increased burden the disease imposes on the public health sector.

Prediabetes, which is an intermediate state of hyperglycaemia is a strong determinant for the development of overt DM. Early identification of individuals with prediabetes is an important step in curbing the rising rates of DM, since at this stage interventions may prevent progression. However, this is limited by the need to conduct an oral glucose tolerance test (OGTT) to cover all spectrums of prediabetes, or the use of HbA1c which has been shown to have limitations, especially anaemia and haemoglobinopathies which are both prevalent in Africa. Therefore in light of the advancements in whole-genome sequencing and epigenome profiling technologies, evidence published in previous literature as well as findings from this study clearly demonstrate the potential of miRNAs as new diagnostic and therapeutic agents. However, these would need standardisation and harmonisation.

Additionally, an emerging field has been the use of miRNAs as targets of anti-diabetic therapy, with altered expression of some miRNAs resulting in poor insulin release and resistance in target tissues, and restoring the expression of these miRNAs to normal levels has been proposed to rectify these imbalances. Such techniques involve the use of oligonucleotide templates mimicking the sequence of the underexpressed miRNA of interest, as well as viral and reagent-based transfection (Chen *et al*., 2014). Interventions such as these may provide potential therapeutic benefits in normalizing miRNAs that exhibit underexpression in disease-states, such as miR-182-5p which we found, as an alternative to conventional anti-diabetic treatments such as insulin and metformin therapy.

### 7.3 Limitations of the study and future recommendations

This is the first study of its kind to be conducted in an African setting, and findings may contribute towards curbing the increasing burden of T2DM in South Africa, and Africa as a whole. A strength of our study lies in the large number of participants selected; however, the sample was skewed towards female participants, which is a common observation seen in community-based studies with similar settings as ours. Additionally, there was disproportionate representation of normotolerant versus screen-detected diabetics and known diabetics on treatment. The cross-sectional nature of the study limits accurate evaluation of anti-diabetic induced epigenetic change, therefore longitudinal studies, with proportionate numbers of participants in each sub-group are recommended for future investigations. Total RNA used for miRNA expression analysis was extracted from whole blood, while other studies used serum/plasma, with expression levels sometimes varying between different tissue types. Important cell types such as leucocytes and platelets are reservoirs for functional miRNAs, with red blood cell-derived miRNAs representing the majority of miRNAs present in whole blood (Sun *et al*., 2020). Release of these miRNAs during coagulation may affect the repository of extracellular

circulating miRNAs in blood, ultimately limiting accurate comparisons with studies using serum, and those using whole blood, such as ours. Caution should therefore be taken when comparing miRNA expressions from different tissue types, and further research should be aimed at identifying the most optimal tissue types for miRNA expression profiling investigations.

In conclusion, our study has revealed associations between miR-1299, -126-3p, -30a-5p, -30e-3p and -182-5p and prediabetes, of note the abilities of miR-126-3p and -182-5p in significantly discriminating prediabetes beyond the performance of HbA1c. These findings deserve validation in other populations and settings, to confirm if measuring miR-126-3p and -182-5p could play a role in diabetes risk screening strategies. Furthermore, important associations and expression patterns were revealed between the evaluated miRNAs and duration of T2DM, prompting necessary investigations to clarify their use as therapeutic targets in DM and its associated complications. Although the comprehension of the interplay between miRNA expression and DM is at infant stages, with advancements their roles may ultimately provide novel strategies to anticipate, diagnose, as well as provide prognostic predictions, for DM in the near future.

### 7.4 References


Chen, H., Lan, H.-Y., Roukos, D.H. & Cho, W.C. 2014. Application of microRNAs in diabetes mellitus. *J Endocrinol*, 222(1): R1–R10.

Erasmus, R.T., Soita, D.J., Hassan, M.S., Blanco-Blanco, E., Vergotine, Z., Kengne, A.P. & Matsha, T.E. 2012. High prevalence of diabetes mellitus and metabolic syndrome in a South African coloured population: Baseline data of a study in Bellville, Cape Town. *South African Medical Journal*, 102(11): 841–844.

International Diabetes Federation. 2019. *IDF Diabetes Atlas*. 9th ed. Brussels, Belgium. https://www.diabetesatlas.org [5 January 2020].

Kumar, M., Nath, S., Prasad, H.K., Sharma, G.D. & Li, Y. 2012. MicroRNAs: a new ray of hope for diabetes mellitus. *Protein & Cell*, 3(10): 726–738.

Sun, L., Yu, Y., Niu, B. & Wang, D. 2020. Red Blood Cells as Potential Repositories of MicroRNAs in the Circulatory System. *Frontiers in genetics*, 11: 442.

# APPENDICES

## Appendix 1 – Ethical approval

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

Registration Number NHREC: REC- 230408-014

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18 January 2019

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***REC Approval Reference No:***
***CPUT/HW-REC 2019/H3***

Dear Mr Cecil Jack Weale

**RE: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE**

Approval was granted by the Health and Wellness Sciences-REC to Mr Weale for ethical clearance. This approval is for research activities related to student research in the Department of Biomedical Sciences at this Institution.

TITLE: **MICRORNA PROFILING AND VALIDATION IN MIXED ANCESTRY INDIVIDUALS IN SOUTH AFRICA SUPERVISOR: PROF T MATSHA AND PROF G DAVISON**

**Comment**:

**Approval will not extend beyond 19 January 2020.** An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

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

Kind Regards

*Dr. Navindhra Naidoo*

**CHAIRPERSON – RESEARCH ETHICS COMMITTEE**

Faculty of Health and Wellness Sciences

## Appendix 2 – Ethical approval (renewal)

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

Registration Number NHREC: REC- 230408-014

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18 January 2019

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***REC Approval Reference No:***
***CPUT/HW-REC 2019/H3***

Dear Mr Cecil Jack Weale

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TITLE: **MICRORNA PROFILING AND VALIDATION IN MIXED ANCESTRY INDIVIDUALS IN SOUTH AFRICA SUPERVISOR: PROF T MATSHA AND PROF G DAVISON**

**Comment**:

**Approval will not extend beyond 19 January 2020.** An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

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

Kind Regards

*Dr. Navindhra Naidoo*

**CHAIRPERSON – RESEARCH ETHICS COMMITTEE**

Faculty of Health and Wellness Sciences