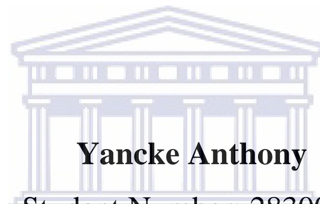
UNIVERSITY of the
WESTERN CAPE

Identification and validation of microRNAs for diagnosing type 2 diabetes: an *in silico* and molecular approach



Yancke Anthony
Student Number: 2830072

UNIVERSITY of the
WESTERN CAPE

A thesis submitted in partial fulfillment of the requirements for the degree of Magister Scientiae, in the Department of Biotechnology, University of the Western Cape.

Supervisor: Dr Ashley Pretorius

8 May 2015

Abstract

Type 2 diabetes mellitus (T2DM), a metabolic disease characterized by chronic hyperglycemia, is the most prevalent form of diabetes globally, affecting approximately 95 % of the total number of people with diabetes i.e. approximately 366 million. Furthermore, it is also the most prevalent form in South Africa (SA), affecting approximately 3.5 million individuals. This disease and its adverse complications can be delayed or prevented if detected early. Standardized diagnostic tests for T2DM have a few limitations which include the inability to predict the future risk of normal glucose tolerance individuals developing T2DM, they are dependent on blood glucose concentration, its invasiveness, and they cannot specify between T1DM and T2DM. Therefore, there is a need for biomarkers which could be used as a tool for the early and specific detection of T2DM.

MicroRNAs are small non-coding RNA molecules which play a key role in controlling gene expression and certain biological processes. Studies show that dysregulation of microRNAs may lead to various diseases including T2DM, and thus, may be useful biomarkers for disease detection. Therefore, identifying biomarkers like microRNAs as a tool for the early and specific detection of T2DM, have great potential for diagnostic purposes.

The main focus of this investigation, therefore, is the early detection of T2DM by the identification and validation of novel biomarkers. Furthermore, based on previous studies, the aim of the investigation was to identify differentially expressed miRNAs as well as identify their potential target genes associated with the onset and progression of T2DM.

UNIVERSITY of the
WESTERN CAPE

An *in silico* approach was used to identify miRNAs found to be differentially expressed in the serum/plasma of T2DM individuals. Three publically available target prediction software were used for target gene prediction of the identified miRNA. The target genes were subjected to functional analysis using a web-based software, namely DAVID. Functions which were clustered with an enrichment score > 1.3 were considered significant. The ranked target genes mostly had gene ontologies linked with “transcription regulation”, “neuron signalling, and “metal ion binding”. The ranked target genes were then split into two lists – an up-regulated (*ur*) miRNA targeted gene list and a down-regulated (*dr*) miRNA targeted gene list. The *in silico* method used in this investigation produced a final total of 4 miRNAs: miR-*dr*-1, miR-*ur*-1, miR-*ur*-2, and miR-*ur*-3. Based on the bioinformatics results, miR-*dr*-1 and its target genes LDLR, PPARA and CAMTA1, seemed the most promising miRNA for biomarker validation, due to the function of the target genes being associated with T2DM onset and progression.

UNIVERSITY of the
WESTERN CAPE

The expression levels of the miRNAs were then profiled in kidney tissue of male Wistar rats that were on a high fat diet (HFD), streptozotocin (STZ)-induced T1DM, and non-diabetic control rats via qRT-PCR analysis. The hypothesis was that similar miRNA expression would be found in the HFD kidney samples compared to serum expression levels of the miRNA obtained from the two databases, since kidneys are involved in cleansing the blood from impurities. This hypothesis proved to be true for all miRNAs except for miR-*ur*-2. Additionally, miR-*ur*-1 seemed the most significant miRNA due to it having different expression ratios for T1DM and T2DM (i.e. -7.65 and 4.2 fold, respectively).

Future work, therefore, include validation of the predicted target genes to the miRNAs of interest i.e. miR-*dr*-1: PPARA and LDLR and miR-*ur*-1: CACNB2, using molecular approaches such as the luciferase assays and western blots.

Keywords: T2DM, pre-diabetes, early diagnosis, novel biomarkers, *in silico*, biomarker validation, miRNA expression profiling

Declaration

I declare that *Identification and validation of microRNAs for diagnosing type 2 diabetes: an in-silico and molecular approach* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name................................... Date.................................

Signed..

UNIVERSITY of the
WESTERN CAPE

Dedication

For my parents, Priscilla and Herman Anthony.

UNIVERSITY *of the*
WESTERN CAPE

Acknowledgements

I would like to acknowledge and express my appreciation and gratitude to those that have contributed to the success of my project.

Firstly, I am grateful to God for constantly blessing me with the wisdom, courage and wellbeing needed to complete this project.

Secondly, to Mommy and Daddy, thank you for always being there for me and for all the encouragement. I would not have been finishing my MSc degree, if you never showed your constant support in my studies.

Thirdly, to my supervisor, Dr Ashley Pretorius, thank you for always having your door open and sharing your valuable expertise. I especially thank you for the talk you had with my mom. I could not have asked for a better supervisor. I would also like to thank the entire BRG group – especially Habeeb Bankole and Firdous Khan – for giving me helpful advice when I was lost in the lab. And to Faghri February, for helping me with qRT-PCR analysis.

I would like to thank my friends Fatima Janodien, Kyle Phillips, Yo-Neal Bless and Arun Gokul for all the laughs and being a welcome distraction when I needed a break from my work. I really appreciate our daily "missions" around campus.

Lastly, I would like to thank the National Research Foundation for funding me and my research as well as Prof. Meyer and the NIC for allowing me to use the laboratory equipment and reagents.

UNIVERSITY of the
WESTERN CAPE

Abstract i

Declaration iv

Dedication v

Acknowledgements vi

List of Tables xii

List of Figures xiv

List of Abbreviations xvi

Chapter 1: Literature Review 1

1. Introduction 1

1.1. Diabetes mellitus: A clinical definition 1

1.2. Epidemiology of T2DM 3

1.2.1. The prevalence of T2DM in Africa and South Africa 3

1.3. Type 2 diabetes mellitus 5

1.4. Glucose metabolism and regulation 6

1.4.1. Normal glucose regulation 7

1.5. Pathogenesis and pathophysiology of T2DM 9

1.5.1. Insulin resistance 9

1.5.2. β-cell dysfunction 10

1.6. Complications linked to T2DM 11

1.7. Current methods used for diagnosing T2DM 13

UNIVERSITY of the
WESTERN CAPE

1.7.1. Random plasma glucose (RPG) test 14

1.7.2. Fasting plasma glucose (FPG) test 14

1.7.3. The oral glucose tolerance test 14

1.7.4. The haemoglobin A1c (HbA1c) test 15

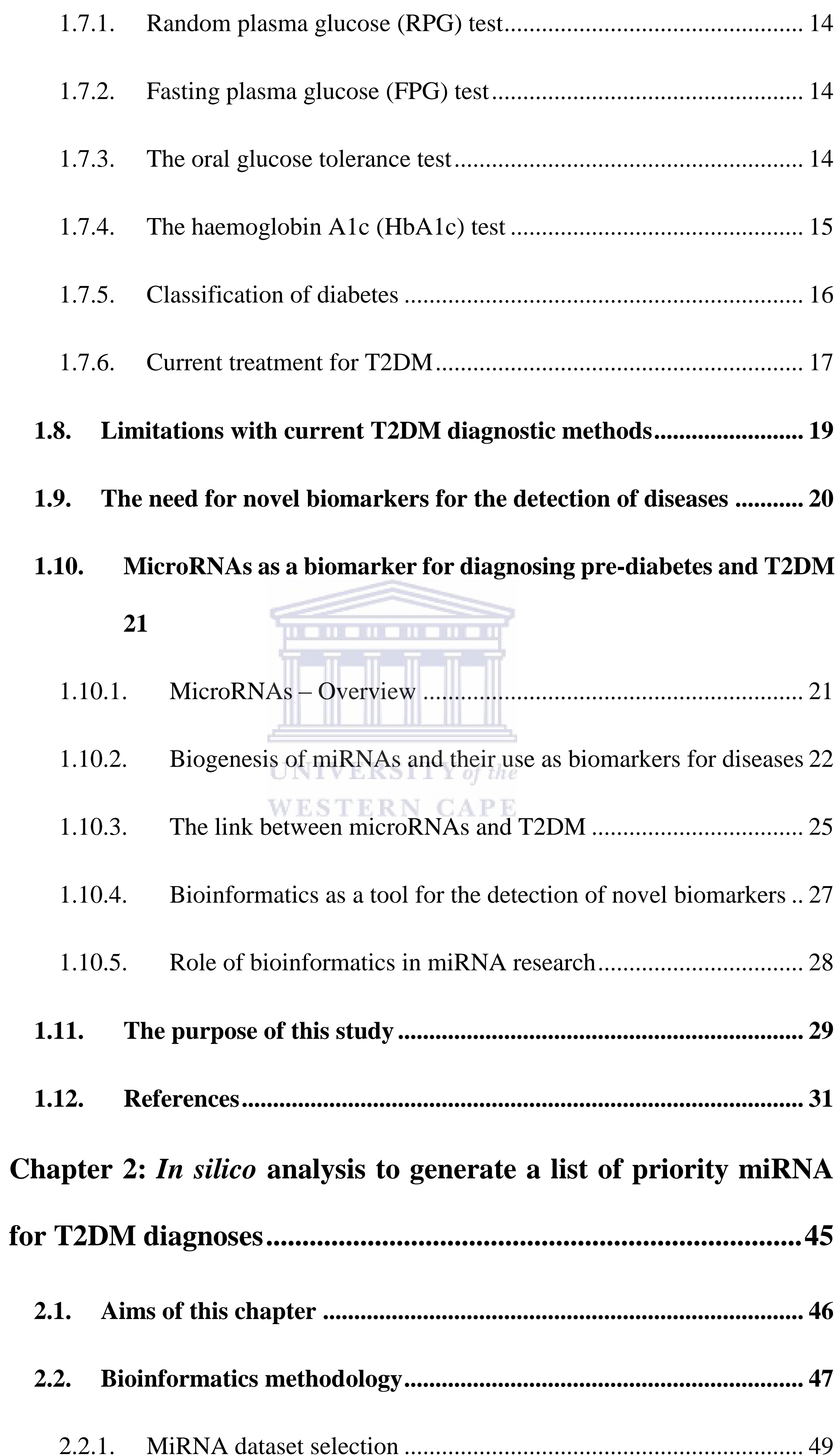
1.7.5. Classification of diabetes 16

1.7.6. Current treatment for T2DM 17

1.8. Limitations with current T2DM diagnostic methods 19

1.9. The need for novel biomarkers for the detection of diseases 20

1.10. MicroRNAs as a biomarker for diagnosing pre-diabetes and T2DM 21



UNIVERSITY of the
WESTERN CAPE

1.10.1. MicroRNAs – Overview 21

1.10.2. Biogenesis of miRNAs and their use as biomarkers for diseases 22

1.10.3. The link between microRNAs and T2DM 25

1.10.4. Bioinformatics as a tool for the detection of novel biomarkers .. 27

1.10.5. Role of bioinformatics in miRNA research 28

1.11. The purpose of this study 29

1.12. References 31

Chapter 2: *In silico* analysis to generate a list of priority miRNA for T2DM diagnoses 45

2.1. Aims of this chapter 46

2.2. Bioinformatics methodology 47

2.2.1. MiRNA dataset selection 49

2.2.1.1. miRNA prioritisation *49*

2.2.2. Target prediction 50

2.2.2.1. TargetScan Human *50*

2.2.2.2. Diana Micro-T *52*

2.2.2.3. PITA *52*

2.2.2.4. Target Prediction procedure *53*

2.3.3. Functional annotation of predicted target genes and miRNAs 55

2.3.4. Network analysis and pathway enrichment analysis 56

2.4. Results and Discussion 56

2.4.1. MiRNA dataset selection 56

2.4.2. Target gene prediction 57

2.4.2.1. LDLR *59*

2.4.2.2. PPARA *59*

2.4.2.3. CAMTA1 .... *60*

2.4.2.4. IGF2BP2 .... *61*

2.4.2.5. ANK2 .... *62*

2.4.3. Functional annotation of predicted target genes and miRNAs 62

2.4.3.1. Target genes *62*

2.4.3.2. MicroRNA functional analysis *66*

2.4.3.3. The ubiquitin proteasome system *66*

2.4.4. Network analysis and pathway enrichment analysis 71

UNIVERSITY of the
WESTERN CAPE

2.5. Summary 73

2.5.1. MiRNA identification and target prediction 74

2.6. References 75

Chapter 3: Molecular analysis - validation of the miRNAs of interest 83

3.1. Introduction 83

i. Microarrays (Hybridzation-based)... 83

ii. Next generation sequencing (NGS)... 84

iii. qRT-PCR 85

3.2. Aims 88

3.3. Molecular materials and methods 89

3.3.1 Materials and suppliers: 89

3.3.2. General Stocks and Solutions 90

3.3.3. List of equipment 90

3.3.4. Decontamination of glass- and plastic-ware 90

3.3.5. Sample collection 91

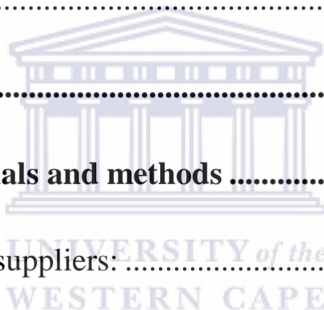
3.3.6. Sample processing and miRNA extraction 91

3.3.6.1. Qubit® quantitation 92

3.3.7. Primer synthesis 93

3.3.8. cDNA synthesis 95

3.3.9. Conventional PCR 96


UNIVERSITY of the
WESTERN CAPE

3.3.10. Quantitative real-time PCR 97

3.4. Results 99

3.4.1. MiRNA extraction 99

3.4.2. CDNA synthesis 99

3.4.3. Conventional PCR 99

3.4.2. qRT-PCR analysis 100

3.4.2.1. Standardisation of qRT-PCR 100

3.4.2.2. Melting curve analysis 101

3.4.2.3. Reference genes: statistical analysis 102

3.4.2.4. Expression analysis of the 4 miRNAs of interest in HFD (T2DM) and TIDM tissues. 104

3.4.2.5. Summary 109

3.5. References 112

Chapter 4: General discussion and Future work 115

4.1. Future work 118

References 121

Chapter 5: Appendix - List of susceptibility genes from T2DGAD 123

Appendix B: Primer design example (adopted from Kramer, 2011) 125

Appendix C: Melting and amplification plots for all miRNAs 126

UNIVERSITY of the
WESTERN CAPE

List of Tables

Chapter 1: Literature Review

Table 1.1: An overview of the different forms of diabetes

Table 1.2: Diagnostic criteria for T2DM

Table 1.3: Target drugs available for the management of T2DM

Table 1.4: MiRNAs, their validated target genes and their function in regulating glucose homeostasis

Chapter 2: *In silico* analysis to generate a list of priority miRNA for T2DM diagnoses

Table 2.1: List of databases used for the bioinformatics methodology

Table 2.2: An overview of the recommended cut-offs for the target gene selection from the three target prediction algorithms used in this study

Table 2.3: Summary of miRNA prioritisation

Table 2.4: Raw total of target genes obtained after target prediction before elimination of duplicates and functional analysis

Table 2.5: Functional annotation clustering of down-regulated miRNA predicted target genes for biological process characterisation

Table 2.6: Functional annotation clustering of up-regulated miRNA target genes for biological process characterisation

UNIVERSITY of the
WESTERN CAPE

Table 2.7: Functional annotation clustering of down-regulated genes for molecular function characterisation

Table 2.8: Final target gene lists after functional annotation

Chapter 3: Molecular analysis – validation for the miRNAs of interest

Table 3.1: Designed primer sequences for reference genes miR-191a and miR-17

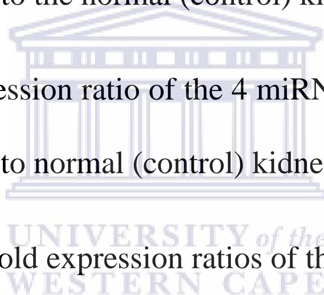
Table 3.2: Parameters used for conventional PCR reactions

Table 3.3: Parameters used for qRT-PCR reactions

Table 3.4: Relative expression ratio of the 4 miRNAs of interest in HFD kidney tissue samples compared to the normal (control) kidney samples

Table 3.5: Relative expression ratio of the 4 miRNAs of interest in T1DM kidney tissue samples compared to normal (control) kidney samples

Table 3.6: Summary of fold expression ratios of the 4 miRNAs of interest in HFD and T1DM rat kidney tissue samples


UNIVERSITY of the
WESTERN CAPE

List of Figures

Chapter 1: Literature review

Figure 1.1: The bi-hormonal model of glucose homeostasis by insulin and glucagon hormones in normal individuals

Figure 1.2: The biogenesis of miRNAs

Figure 1.3: Schematic overview of miRNAs involved in the regulation of pancreatic β-cells function and insulin target tissues in the context of T2DM

Chapter 2: *In silico* analysis to generate a list of priority miRNA for T2DM diagnoses

Figure 2.1: Schematic overview of the bioinformatics workflow followed to obtain a reduced list of both miRNAs and target genes

Figure 2.2: Comparison of known susceptible genes linked with T2DM (blue) to the predicted up- (green) and down-regulated (yellow) gene lists before functional annotation

Figure 2.3: Functional annotation of A) up-regulated miRNAs and B) down-regulated miRNAs using Diana mir-Path. Functions with $p < 0.001$ were considered as significant

Figure 2.4: Comparison of known susceptible genes linked with T2DM (blue) to the predicted up- (green) and down-regulated (yellow) gene lists after functional annotation

Figure 2.5: Graphical display of signalling pathways predicted by STRING for the down-regulated predicted target genes

UNIVERSITY of the
WESTERN CAPE

Figure 2.6: STRING analysis of pathway enrichment and interaction in the A) down-regualated and B) up-regulated predicted miRNA target genes

Chapter 3: Molecular analysis – validation for the miRNAs of interest

Figure 3.1: Schematic representation of stem-loop qRT-PCR for profiling miRNA differential expression using A) the hydrolysis probe assay and B) SYBR green I assay

Figure 3.2: PCR amplification of the control and test miRNAs

Figure 3.3: Amplification plots for the reference gene, GADPH, and miR-*dr*-1

Figure 3.4: Melting curve analysis ranging from 65 ˚C to 95 ˚C for A) the reference gene, GADPH, and B) miR-*dr*-1

Figure 3.5: Expression levels of the reference genes in control tissue sample compared to HFD and T1DM samples

Figure 3.6: Relative expression ratio plot of the 4 miRNAs in normal (control) kidney tissue compared to HFD kidney tissue

Figure 3.7: Relative expression ratio plot of the 4 miRNAs in normal (control) kidney tissue compared to T1DM kidney tissue

UNIVERSITY of the
WESTERN CAPE

List of Abbreviations

ADA	American Diabetes Association
ANK2	Ankyrin 2
BMI	Body mass index
BP	Biological processes
CACNB2	Calcium channel, voltage-dependent, beta 2
CAMTA1	Calmodulin-binding transcription activator 1
CVD	Cardiovascular disease
dr	Down-regulated
FPG	Fasting plasma glucose
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDM	Gestational diabetes mellitus
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide 1
GO	Gene ontologies
HbA1c	Haemoglobin A1c
HcG	Human chorionic gonadotrophin
HDL	High-density lipoprotein
HFD	High fat diet
HMDD	Human miRNA disease database

UNIVERSITY of the
WESTERN CAPE

IDF	International diabetes federation
IFG	Impaired fasting glucose
IGF2	Insulin-like growth factor 2
IGF2BP2	Insulin-like growth factor 2 mRNA-binding protein 2
IGT	Impaired glucose tolerance
IRS-1 and -2	Insulin receptor substrate-1 and -2
IP3	Inositol 4,4,5-triphosphate
KEGG	Kyoto Encyclopedia of Genes and Genomes
LADA	Late-onset autoimmune diabetes of the adult
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
MF	Molecular function
mitG	Minimum energy of potential
MODY	Maturity-onset diabetes of the young
MTPN	Myotrophin
NGS	Next generation sequencing
NGT	Normal glucose tolerance
OGTT	Oral glucose tolerance test
PCR	Polymerase chain reaction
P_{CT}	Probability of conserved targeting

UNIVERSITY of the
WESTERN CAPE

PITA	Probability of Interaction by Target Accessibility
PPARA	Peroxisome proliferator-activated receptor-alpha
PPI	Protein-protein interaction
PSA	Prostate-specific antigen
qRT-PCR	real-time quantitative reverse transcription - PCR
SA	South Africa
SGLT2	Sodium-glucose co-transporter 2
SNR	Signal-to-noise ratio
STRING	Search Tool for the Retrieval of Interacting genes
T1DM	Type 1 Diabetes Mellitus
T2DGAD	Type 2 Diabetes Genetic Association Database
T2DM	Type 2 Diabetes Mellitus
TGF	Transforming growth factor
ur	Up-regulated
UPS	Ubiquitin proteasome system
USD	US Dollars
UTR	Untranslated region
WHO	World Health Organization

UNIVERSITY of the
WESTERN CAPE

Chapter 1: Literature Review

1. Introduction

1.1. Diabetes mellitus: A clinical definition

Diabetes mellitus is a complex metabolic disease, characterised by chronic high blood glucose levels (Lin and Sun, 2010). Over time, diabetes has rapidly become a worldwide epidemic (Stumvoll et al, 2005; IDF, 2014) – the annual increasing incidence of this disease is predicted to make it the leading cause of global morbidity and mortality (Sebastiani, 2011). There are several types of diabetes, caused by the interplay between environmental and genetic factors and is characterised according to its aetiology (Sebastiani, 2011). The most common forms of diabetes are Type 1 Diabetes Mellitus (T1DM), Type 2 Diabetes Mellitus (T2DM) (Lin and Sun, 2010) and Gestational Diabetes Mellitus (GDM) (IDF, 2014). Other less common forms of diabetes include maturity-onset diabetes of the young (MODY) and late-onset autoimmune diabetes of the adult (LADA) (Stumvoll et al, 2005). Table 1.1 gives a brief description of each form of diabetes. This thesis will focus solely on T2DM, as it is the most common form of diabetes worldwide – affecting, approximately, 95 % of the world's population (IDF, 2014).

UNIVERSITY of the
WESTERN CAPE

Table 1.1: An overview of the different forms of diabetes

TYPE	DESCRIPTION
T1DM	An autoimmune disease whereby the immune system attacks the β-cells within the pancreas, therefore, lowering/stops the secretion of insulin. It mainly occurs in children and young adults (Gilespie, 2006)
T2DM	A heterogenous disorder which is characterised by the poor action and/or secretion of insulin. This mainly affects people between 20 – 70 years of age (Ahmad and Crandall, 2010).
GDM	Occurs in pregnant women due to hormonal changes. Women who had GDM have a greater chance of developing T2DM later in life (IDF, 2014).
MODY	A monogenetic and autosomal-domininant form of diabetes. The action of the gene mutations involved results in the dysfunctioning of β-cells in the pancreas and is often mistaken for T2DM. It mainly occurs in young individuals (Nyunt et al, 2009)
LADA	Type 1 diabetes develops in individuals over the age of 30 years. These individuals are said to produce autoantibodies against β-cells, therefore, affecting the secretion of insulin (as seen in T2DM) (Malecki and Skupien, 2008)

UNIVERSITY of the
WESTERN CAPE

1.2. Epidemiology of T2DM

Diabetes mellitus is considered a global epidemic, estimated to have affected approximately 382 million people in 2013 (IDF, 2014). Furthermore, of that number, 90 to 95 % were estimated to have type 2 diabetes (T2DM) (IDF, 2014). The remaining 5 to 10 % is accounted for by monogenic forms (Stumvoll et al, 2005) i.e. T1DM, MODY and LADA. The epidemic is on the constant rise due to rapid urbanisation, nutrition transition, and an increase in sedentary lifestyle (Hu, 2011). Furthermore, it was found that T2DM is particularly common in low- to middle-income communities in all countries (Hu, 2011; IDF, 2014). High development and wealth is correlated with lower early mortality rates caused by diabetes, thus, clarifying why socially disadvantaged communities are more vulnerable to T2DM (IDF, 2014). Pilkington et al (2011) states that due to the lack of money and nearby healthcare facilities, managing diabetic complications is more challenging for individuals from low- to middle-income communities, hence the higher mortality rates within those communities.

UNIVERSITY of the
WESTERN CAPE

1.2.1. The prevalence of T2DM in Africa and South Africa

Africa is said to have 20 million people living with diabetes – however, the number could be higher as it was further estimated that approximately 62 % of the population is undiagnosed (IDF, 2014). In South Africa (SA), the prevalence of T2DM is unknown (Jacovides et al, 2014) as epidemiological data for this disease is scarce (Motala et al, 2003). However, it is estimated that approximately 3.5 million South Africans are affected (Ottermann, 2013). The South African government has to pay a large amount of money for the management of short- and long-term complications associated with diabetes (Distiller, 2004). In 2013 it was estimated that the African region paid 4 billion US dollars (USD) on diabetes health

care (IDF, 2014). The IDF does not specify how much money SA spent in 2013, but an epidemiological study done by Zhang et al (2010) estimated that SA spent between 865,000 and 1.54 million USD for treating diabetic adults between the ages of 20 to 70 in 2010. They further predicted that in the year 2030, SA diabetic health expenditure will increase between 1.08 million and 1.97 million USD (Zhang et al, 2010). Most of the money is thought to be spent on treating the complications linked with T2DM complications (Ottermann, 2012).

The prevalence of diabetes varies according to population and ethnic groups. Pima Indians from North America is an example which further proves that the influence of environmental factors plays an equally important role in the development of T2DM as genetic susceptibility does (Soita, 2009). This group is reported to have the world's highest prevalence of T2DM. It is said that urbanization could be the main cause of this i.e. ethnic groups from rural communities, accustomed to labour intensive activities and high fibre diets, adapt to the western lifestyle and eat diets rich in fat accompanied by a sedentary lifestyle (Soita, 2009). Population based studies conducted in SA in 2005 have reported varying diabetes prevalence rates amongst different racial groups, with the highest prevalence being observed in the Indian community with 8.5 % and 11.5 % for men and women, respectively (Soita, 2009). This is closely followed by the coloured community with the diabetes prevalence percentage of 3.1 % and 5.8 % for men and women, respectively (Soita, 2009).

UNIVERSITY of the
WESTERN CAPE

The high percentage of South Africans undiagnosed with T2DM and the amount of money that is being spent on healthcare for diabetes-linked complications is worrying and unnecessary. Especially since this disease, as well as its associated short- and long-term complications, can be prevented (or delayed) if diagnosed

early and if certain lifestyle changes and eating habits are implemented (OECD, 2013).

1.3. Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is a heterogenous metabolic disorder, characterised by three metabolic defects: (i) impaired insulin secretion from the pancreas, (ii) cells that are resistant to insulin action (Ahmad and Crandall, 2010), and (iii) abnormal glucose uptake in the splanchnic area (DeFronzo, 2004). These defects ultimately result in metabolic malfunctions which include: hyperglycaemia, hyperinsulinaemia – due to the cells being less sensitive to insulin, causing a build-up of insulin in the blood (Nicolaides and Jones, 2002), and hyperlipidaemia (Novosyadly et al, 2010). This disease is usually preceded by impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) – also referred to as pre-diabetes. Pre-diabetes is defined as the state where blood glucose levels are higher than the normal, but not yet high enough for the diagnosis of T2DM (Soita, 2009).

UNIVERSITY of the
WESTERN CAPE

The symptoms associated with hyperglycaemia include: excessive urination, increased hunger, increased thirst, tiredness, and weight loss (Soita, 2009). Over time, the symptoms may include blurry vision, reoccurring infections, and slow wound healing (Clark et al, 2007). However, these symptoms are not specific for only type 2 diabetes (Clark et al, 2007). Additionally, individuals may be asymptomatic i.e. they do not experience any of the abovementioned symptoms, therefore, impairing the early diagnosis of diabetes (Levitt, 2009; Rao et al, 2009; Soita, 2009; Valdez, 2009) and these individuals are usually diagnosed with T2DM when irreversible complications linked with T2DM presents itself (ADA, 2014).

1.4. Glucose metabolism and regulation

Glucose is the primary source for energy production in cells and is maintained within a narrow and well-balanced range (Aronoff et al, 2004; Soita, 2009; Triplitt, 2012) i.e. between 70 to 110 mg/dL (Grayson et al, 2012). Glucose in the circulatory system is acquired in the following ways: (1) absorption through the gastrointestinal tract after ingestion, (2) breakdown of glycogen in the liver (glycogenolysis), (3) breakdown of fat and protein during fasting (gluconeogenesis) (Aronoff et al, 2004, Giugliano et al, 2008, Triplitt, 2012), and (4) renal reabsorption in the kidney (Gerich, 2009; Triplitt, 2012). Most glucose consumption (approximately 50 %) takes place in the brain (which is not dependent on insulin), 25 % gets consumed by insulin-dependent tissues (i.e. adipose tissue and skeletal muscle) and the remaining 25 % gets used by the splanchnic area (liver and gastrointestinal tissue) (DeFronzo, 2004; Triplitt, 2012). However, Gerich (2009) describes that the kidney utilizes approximately 10 % of ingested glucose while approximately 45 % of glucose gets converted to glycogen in the liver, ~ 30 % is taken up by the skeletal muscle (whereby excess is later converted to glycogen), ~ 15 % is taken up by the brain, and the remaining 5 % is taken up by adipose tissue.

UNIVERSITY of the
WESTERN CAPE

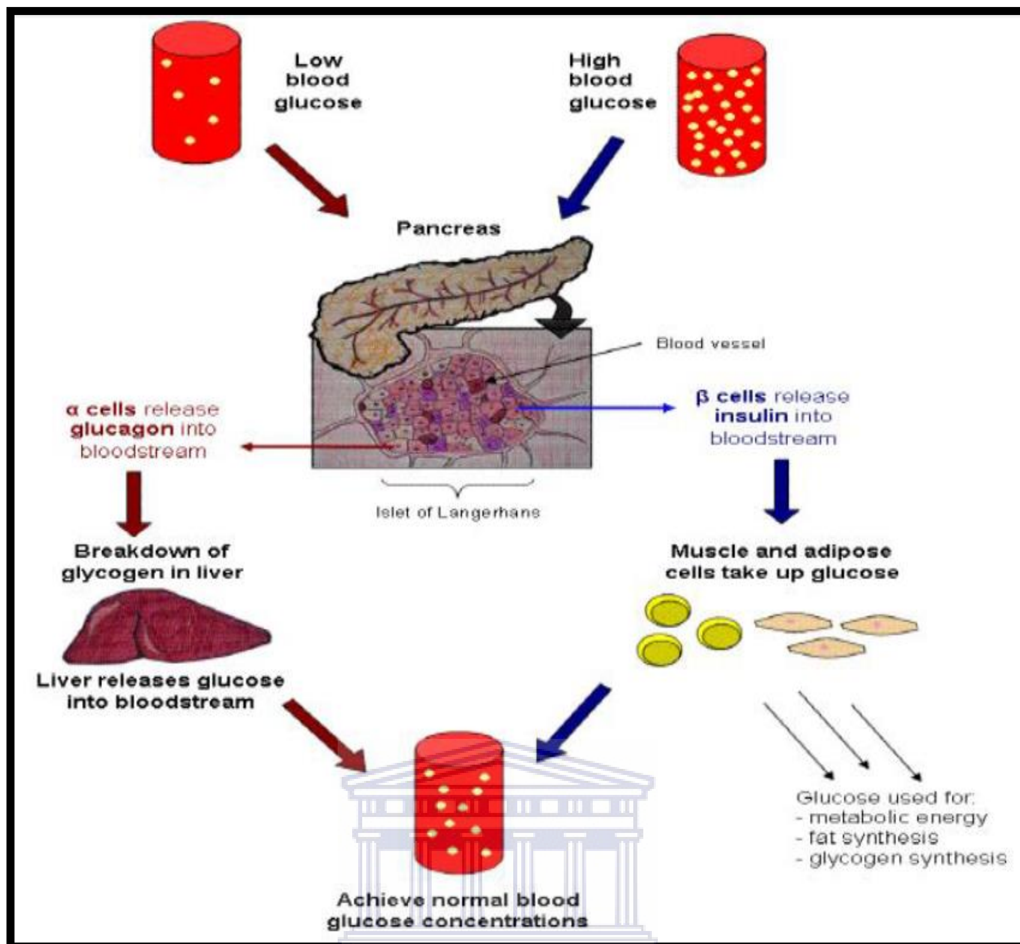
Glucose regulation and homeostasis is driven mainly by insulin and glucagon hormones (Aronoff et al, 2004; Triplitt, 2012). The role that these two hormones play in the homeostasis and regulation of blood glucose will be briefly explained in the following section.

1.4.1. Normal glucose regulation

After ingestion, carbohydrates are broken down to simple sugars such as glucose and fructose in the intestinal tract (Schaefer et al, 2009). This results in a spike in blood glucose levels, stimulating the beta cells (β-cells) within the pancreatic islets of Langerhans to secrete insulin (Soita, 2009). The main metabolic role of insulin is to increase the rate at which glucose is transported into the skeletal muscle and adipose tissue (Maitra, 2012), thus aiding in decreasing blood glucose levels. Additionally, insulin aids in converting excess glucose to glycogen in the liver. Simultaneously, due to the presence of insulin, endogenous glucose production (gluconeogenesis and glycogenolysis) is inhibited due to glucagon being suppressed due to the paracrine effect (Aronoff et al, 2004).

Alternatively, during the first 8 to 12 hours of fasting, the primary mechanism of glucose appearance is made available by glycogenolysis. Hence, low blood glucose levels stimulates the alpha cells (α-cells) within the pancreas to secrete glucagon. Glucagon converts glycogen in the liver back to glucose (Aronoff et al, 2004). This process, therefore, raises blood glucose levels back within the normal range, as illustrated in Figure 1.1. The liver is the main site of gluconeogenesis and only occurs under extreme starvation periods (Aronoff et al, 2004). It should be noted that may also occur within the kidneys (Triplitt, 2012). Gluconeogenesis is the metabolic process where glucose gets produced from non-carbohydrate substrates such as pyruvate, lactate, glycerol, and glucogenic amino acids (Soita, 2009)

UNIVERSITY of the
WESTERN CAPE



UNIVERSITY of the
WESTERN CAPE

Figure 1.1: The bi-hormonal model of glucose homeostasis by insulin and glucagon hormones in normal individuals. Insulin is involved in glucose disappearance, whereas glucagon is involved in glucose appearance (Aronoff et al, 2004). (Adopted from Soita, 2009)

Despite insulin and glucagon being the main glucoregulatory hormones involved in glucose homeostasis, there are other hormones involved such as: amylin, glucagon-like peptide 1 (GLP-1), and glucose-dependent insulinotropic peptide (GIP) (Aronoff et al, 2004). Both GLP-1 and GIP are incretin hormones, which are secreted from the small intestine to the pancreas for the production of insulin after eating a meal (Knop et al, 2009). Furthermore, Amylin, is a neuroendocrine

hormone which is secreted simultaneously with insulin from the β-cells in the pancreas. Its functions include inhibiting glucagon secretion after eating a meal, slowing down the emptying of the stomach, and increasing satiety (Aronoff et al, 2012).

1.5. Pathogenesis and pathophysiology of T2DM

The pathogenesis of T2DM is multifactorial; it involves the combination of both genetic and environmental risk factors such as obesity, sedentary lifestyle, smoking, and age (Kaku, 2010). There are three main defects which play a role in the development of hyperglycaemia in T2DM: increased hepatic glucose production, diminished insulin secretion, and impaired insulin action (Lin and Sun, 2010). Hence, the two main metabolic defects which plays a central role in the pathophysiology of T2DM are insulin resistance and β-cell dysfunction (D'Adamo and Caprio, 2011; Guay et al, 2011; Maitra, 2012), which will be briefly discussed in the sections below.

UNIVERSITY of the WESTERN CAPE

1.5.1. Insulin resistance

Insulin resistance refers to the condition where insulin-sensitive tissues fail to respond to physiological concentrations of insulin, resulting in poor glucose uptake, reduced glycolysis and fatty acid oxidation in the liver, and the inability to suppress gluconeogenesis (Gastaldelli, 2011; Maitra, 2012). Evidence has demonstrated that insulin resistance occurs approximately 10 to 20 years before the onset of the disease and is the best predictor of whether or not the individual would eventually develop T2DM (D'Adamo and Caprio, 2011). There are many defects linked with insulin resistance, with obesity being one of the major factors involved in the

development of insulin resistance (Soita, 2009; D'Adamo and Caprio, 2011; Day and Bailey, 2011; Maitra, 2012) – this usually precedes the presence of hyperglycaemia in obese individuals (Kasuga, 2006; Maitra, 2012). An individual is classified as obese if their body mass index (BMI) exceeds 30 kg/m^2 (Soita, 2009). BMI is calculated as weight in kilograms divided by the individual's height in metres squared (Flegal et al, 2013). Further evidence has shown that the important contributing factor of insulin resistance is fat partitioning (D'Adamo and Caprio, 2011), particularly visceral fat accumulation (which is reflected as increased abdominal girth) has been shown to be linked with insulin resistance and T2DM (D'Adamo and Caprio, 2011; Eckel et al, 2011).

There are three main metabolic stresses that link obesity to T2DM: (i) increased levels of adipokines/cytokines (e.g. tumor necrosis factor-α, resistin, retinol binding protein 4) and decreased levels of adiponectin, (ii) ectopic fat deposition in the liver, muscles and dysmetabolic sequelae, and (iii) mitochondrial dysfunction, which was shown to not only decrease insulin sensitivity, but also compromises β-cell function (Kim et al, 2011; Eckel et al, 2011).

UNIVERSITY of the
WESTERN CAPE

1.5.2. β-cell dysfunction

β-cell dysfunction and the reduction of β-cell mass was shown to play important roles in the transition from normal glucose tolerance (NGT) to hyperglycaemia and the pathogenesis of T2DM (Gastaldelli, 2011; Meier and Bonadonna, 2013). The progression of NGT to T2DM usually involves increased insulin secretion rates due to the body trying to compensate for the insulin-dependent tissues becoming increasingly more insulin resistant (D'Adamo and Caprio, 2011; Gastaldelli, 2011; Eckel et al, 2011). Furthermore, studies have shown that increased insulin secretion rates are dependent on increased insulin resistance i.e. secretion rates are

approximately two times higher in obese individuals compared to healthy individuals (Gastaldelli, 2011). It should be noted, however, that approximately 20 % of obese individuals do not develop T2DM (Meier and Bonadonna, 2013). Their blood sugar levels are within the normal range due to increased β-cell mass and function (Eckel et al, 2011). T2DM eventually develops due to the inability of the β-cells' secretory capacity to overcome the insulin resistance of tissues (Gastaldelli, 2011).

There are many additional factors besides insulin resistance which influence the function of pancreatic β-cells, which include hyperglycaemia/glucotoxicity, autoimmunity, inflammation, adipokines, islet amyloid, incretins (Cernea and Dobreanu, 2013), and lipotoxicity (D'Adamo and Caprio, 2011; Cernea and Dobreanu, 2013). Other factors that may be involved in the deterioration of β-cell mass and function could be due to genetic/epigenetic (Eckel et al, 2011) and environmental factors (D'Adamo and Caprio, 2011).

UNIVERSITY of the
WESTERN CAPE

Despite all the current findings that link β-cell dysfunction to the eventual development of T2DM, it is still unclear whether the root cause lies in β-cell dysfunction as an initiator of β-cell loss, or whether increasing secretory demand causes ongoing loss of β-cells (Gastaldelli, 2011).

1.6. Complications linked to T2DM

Diabetes mellitus has the potential to increase the prevalence of already burdensome diseases (Piotie, 2013). It is, therefore, vitally important that diabetic patients maintain their blood sugar levels within the normal range. The long-term effects of hyperglycaemia, if not properly managed, is damaging to both small and

large blood vessels (Forbes and Cooper, 2013), hence the complications is characterised by macro- and microvascular complications (Fowler, 2008; Soita, 2009; Piotie, 2013). Macrovascular complications include coronary artery disease and strokes. Whereas microvascular complications include diabetic nephropathy (kidney diseases), neuropathy (nerve damage), and retinopathy (eye damage) (Fowler, 2008; Forbes and Cooper, 2013), with a high probability of vision loss (ADA, 2012a).

In Africa, the leading cause of death due to T2DM is cardiovascular diseases (CVDs) (Piotie, 2013). Diabetic patients are three to four times more likely to present with a stroke than a non-diabetic individual. Furthermore, diabetic patients are two to three times more likely to die of CVDs such as strokes or heart attacks (Piotie, 2013).

According to previous studies, the major risk factor for the development of CVDs (and other macrovascular diseases) in T2DM patients is impaired kidney function (Forbes and Cooper, 2013; Wang et al, 2014). A study done by Rodriguez-Poncelas et al (2014) found that the decrease in estimated glomerular filtration rate (eGFR) and the increase in urine albumin creatinine ratio (UACR), independently, are risk factors that increases the risk of CVDs in T2DM patients. Therefore, it is recommended that greater attention be paid to the development of nephropathy during the early stage of disease in order to manage it correctly (Forbes and Cooper, 2013).

Foot amputations, caused by peripheral neuropathy, are also a common occurrence amongst diabetics (Clayton and Elasy, 2009). It is estimated that approximately 80 % of all amputations occur after the occurrence of foot injuries or ulcerations

UNIVERSITY of the
WESTERN CAPE

(Fowler, 2008). Neuropathy is defined as damage of the nerves and, in T2DM patients, is caused by prolonged exposure of the nerves to high blood glucose levels (Clayton and Elasy, 2009). With regard to peripheral neuropathy, individuals usually lose sense of feeling in their lower and upper extremities (Fecko, 2012). T2DM patients are recommended to frequently check for foot wounds (such as ulcerations under the foot) that do not heal fast enough. Foot ulcerations that are not properly cared for would eventually lead to the amputation of that limb (Ibbald et al, 2012). Other types of neuropathy includes autonomic neuropathy (which could cause problems such as erectile dysfunction, incontinence, gastroparesis), radiculoplexus neuropathy (affects nerves in the thighs, hips, and legs), and mononeuropathy (this causes damage to one specific nerve) (Tanenberg, 2009).

1.7. Current methods used for diagnosing T2DM

UNIVERSITY of the
WESTERN CAPE

In order to prevent the continuously increasing morbidity and mortality rates associated with the abovementioned complications caused by T2DM, it is very important for the disease to be diagnosed early. T2DM (and its irreversible complications) can be prevented, or delayed, if detected early (Maynard et al, 2007). Current screening methods used depends solely on glucose levels in the blood, and are invasive as they all require blood samples. The American Diabetes Association (ADA) recommends that asymptomatic individuals be screened for T2DM by one of the following tests: the oral glucose tolerance test (OGTT), fasting plasma glucose (FPG) test, and the Haemoglobin A1c (HbA1c) test (ADA, 2012b; ADA, 2014), which was recently approved by the American Diabetes Association (ADA) in 2010 and The World Health Organisation (WHO) in 2011. Another test includes the random plasma blood test, however, this is not as reliable as the

aforementioned diagnostic methods. A brief summary for each diagnostic test is briefly explained in the subsequent sections.

1.7.1. Random plasma glucose (RPG) test

Random plasma glucose tests can be done at any time of the day, without regard of the time since the last meal (SEMDSA, 2012). Patients with a blood glucose levels of ≥ 11.1 mmol/L and displaying classic symptoms of diabetes are diagnosed as diabetic (Patel and Macerollo, 2010). However, this test is not as sensitive as other tests due to more definitive tests being required when blood glucose levels are between 7.8 to 10.0 mmol/L (Patel and Macerollo, 2010).

1.7.2. Fasting plasma glucose (FPG) test

The FPG test is one of the preferred methods for the diagnosing diabetes due to its convenience, ease of use, and reduced costs compared to other tests according to the American Diabetes Association (SEMDSA, 2012). The process involves the patients having to fast 8 hours before blood is drawn. A positive diagnosis is given when the patient has a blood glucose levels of ≥ 7.0 mmol/L (Patel and Macerollo, 2010) (Table 1.2).

UNIVERSITY of the
WESTERN CAPE

1.7.3. The oral glucose tolerance test

This test is considered as a first-line diagnostic test (Patel and Macerollo, 2010) and involves the determination of how efficiently glucose is removed from the blood. The individual is asked to fast overnight for ten to 14 hours. Thereafter, a 75 g glucose solution must be orally ingested. Blood samples are drawn before and two hours after glucose intake. These two samples then give the physician fasting glucose and two-hour post glucose intake results, respectively (ADA, 2012b).

Normal fasting glucose levels usually lies in the range between 4.0 to 5.5 mmol/L, whereas blood glucose levels higher than 11.1 mmol/L will classify the individual as being diabetic (Table 1.2).

1.7.4. The haemoglobin A1c (HbA1c) test

HbA1c refers to glycated haemoglobin i.e. glucose in the system which naturally binds to haemoglobin. Measuring HbA1c levels allow clinicians to get an overall picture of an individual's average glucose intake over a period of one week to three months (Goldenberg et al, 2011; Diabetes.co.uk, 2015). This is possible because red blood cells in the human body can survive for up to eight to 12 weeks before renewal (Diabetes.co.uk, 2015). In healthy individuals HbA1c levels range from approximately 4 to 5.9 %. Alternatively, an individual is diagnosed as a diabetic if HbA1c levels are ≥ 6.5 % (ADA, 2012b; Diabetes.co.uk, 2015). HbA1c levels between 5.7 to 6.4 % signifies that the individual is pre-diabetic and has an increased risk for developing diabetes (ADA, 2012b) (Table 1.2).

UNIVERSITY of the
WESTERN CAPE

In contrast to the OGTT test, the HbA1c test is more practical due to it not requiring the individual to fast before having a blood sample taken, and can thus be done at any time of the day (Goldenberg et al, 2011). Furthermore, no glucose solution has to be taken and it reflects the average blood glucose levels over a two to three month period (Goldenberg et al, 2011).

There are situations where the HbA1c test is not appropriate and would give unreliable results (falsely high or falsely low) for the diagnosis of diabetes. This includes individuals with rapid red cell turnover as found in patients suffering from sickle cell anaemia or other haemoglobinopathies, some medications may affect the HbA1c readings, inconsistencies of average HbA1c readings in certain ethnicities

(Patel and Macerello, 2010; WHO, 2011; ADA, 2012b) and its high costs, which limits this screening method to developed countries only (ADA, 2012b; Patel and Macerollo, 2010; Goldenberg et al, 2011). Age is another factor that has to be taken into consideration, due to older individuals tending to have higher HbA1c levels (International Expert Committee, 2009). Lastly, patients suspected to have T1DM, patients who are newly diagnosed with T2DM (less than two months), women who are pregnant, and patients who are very sick (Diabetes.org.uk, 2015a) cannot be diagnosed using the HbA1c test.

Table 1.2: Diagnostic criteria for T2DM

Diagnosis	HbA1c (%)	Fasting Plasma Glucose (mmol/L)	Oral Glucose Tolerance Test (mmol/L)
Diabetic	> 6,5	> 7	> 11,1
Pre-diabetic	5,7 – 6,4	5,56 – 7	7,77 – 11
Healthy	< 5,6	3,89 – 5,5	< 7,72

UNIVERSITY of the WESTERN CAPE

1.7.5. Classification of diabetes

Distinguishing between the types of diabetes after being diagnosed is important for the management of the disease (Goldenberg and Punthakee, 2013). The C-peptide test is one such test which can specify whether an individual has either T1DM, T2DM, or insulin resistance (Diabetes.org.uk, 2015b). This test is also useful for the management of diabetes, specifically those individuals who are using insulin (Jones and Hattersly, 2013) C-peptide is made in equal amounts to insulin and is considered the best method to measure endogenous insulin secretion (Jones and Hattersly, 2013). The C-peptide assay can be measured in blood samples from individuals who are either in a fasting or non-fasting state as well as in a formal stimulation test (which requires glucagon to be injected into the blood stream to

stimulate insulin secretion) (Diabetes.org.uk, 2015b, Goldenberg and Punthakee, 2013; Jones and Hattersly, 2013).

The presence of autoimmune markers such as anti-glutamic acid decarboxylase and anti-islet cell antibody, is another method which could be used to distinguish between the various types of diabetes (Goldenberg and Punthakee, 2013).

1.7.6. Current treatment for T2DM

Once an individual has been diagnosed with overt T2DM, it is important that their glycaemic levels are properly managed. Diabetic patients are first recommended to begin treatment by incorporating a healthier lifestyle, by eating healthily and exercising regularly (Fowler, 2007), in order to keep blood glucose levels as close to normal as possible (Sena et al, 2010). However, if glycaemic levels do not decrease after exercising, diabetics would then be advised to use anti-hyperglycaemic drugs simultaneously (Sena et al, 2010). T2DM patients have a wide variety of anti-diabetic drugs to choose from; these drugs are categorized into five major classes i.e. insulin secretagogues, insulin sensitizers, alpha glucosidase inhibitors, insulin (Sena et al, 2010; Skugor, 2014), and sodium-glucose co-transporter 2 (SGLT2) inhibitors (Thynne and Doongue, 2014; Skugor, 2014) as shown in Table 1.3. Drugs used for treating T2DM aims to either control blood glucose levels in the fasting state or postpandrially, and can therefore be used either on their own or in combination with each other (Sena et al, 2010). However, over time, current drug therapies become less effective due to the progressive loss of β-cell function and mass (Sena et al, 2014). Hence, detecting diabetes early is important so that the appropriate treatment can be administered in order to bypass a variety of complications linked with this disease.

UNIVERSITY of the WESTERN CAPE

Table 1.3: Target drugs available for the management of T2DM

Drug type	Mechanism of action	Example/s
Insulin secretagogues	Agents which increases the amount of insulin secreted by the pancreas (Bösenberg and van Zyl, 2008)	Sulfonylureas (e.g. glimepiride) Incretin mimetics
Insulin sensitizers	Agents which increase the sensitivity of target organs - e.g. muscles or adipose tissue (Bösenberg and van Zyl, 2008)	Metformin Thiazolidinediones (e.g. Rosiglitazone)
Alpha glucosidase inhibitors	Agents which decrease the rate at which glucose is absorbed from the gastrointestinal tract (Bösenberg and van Zyl, 2008)	Acarbose
Sodium-glucose co-transporter 2 (SGLT2) inhibitors	Lowers plasma glucose concentrations by increasing renal excretion of glucose (Thynne and Doongue, 2014)	Canagliflozin
Insulin	Stimulates peripheral glucose uptake and inhibits glucose production and release by the liver	

UNIVERSITY of the WESTERN CAPE

1.8. Limitations with current T2DM diagnostic methods

Diagnosis of diabetes is usually too late due to some individuals being asymptomatic for up to four to seven years (Rao et al, 2009; Levitt, 2009) and in some cases symptoms are not that obvious (Levitt, 2009). Additionally, late presentation is also due to individuals having limited access to health facilities (Kengne, 2005). Thus, resulting in the patient ending up with irreversible complications and being dependent on anti-diabetic medications for the rest of their lives, since diabetes is considered a chronic disease. If detected earlier, in the pre-diabetic, asymptomatic phase the individual can take precautions from developing overt diabetes i.e. by adopting a healthier and active lifestyle (Soita, 2009).

Additionally, tests like the HbA1c test, is costly and has specific criteria (as explained in section 1.7.4) that an individual has to meet to achieve accurate results (International Expert Committee, 2009). The tests are also time-consuming for both the health-care professionals and patients, as is the case of the FPG and OGTT tests, requiring patients to fast for up to ten to 14 hours before blood samples can be collected (Vora and Evans, 2012). Furthermore, there are instances where the diagnosis for T1DM and T2DM is uncertain, requiring further tests (e.g. auto-immune antibodies) for classification purposes (Vora and Evans, 2012).

UNIVERSITY of the
WESTERN CAPE

Finally, all the above-mentioned diagnostic methods are invasive as they require blood samples. This could be a major problem with individuals who have a fear of needles and who cannot stand the sight of blood or the pain after being pricked (Zhang et al, 2009; Soita, 2009) and find the diagnostic tests a daunting process (Rao et al, 2009).

1.9. The need for novel biomarkers for the detection of diseases

Based on the aforementioned limitations, there is an urgent need for novel biomarkers which could aid in the early and specific detection for T2DM which could possibly act as a supplementary test to current diagnostic methods. Biomarkers are biological molecules (e.g. genes, proteins, and metabolites) in biological fluids (e.g. serum, urine, saliva) or tissue, which could aid in indicating normal or abnormal processes in the body, or the condition of a specific disease (Yousef et al, 2014). Furthermore, they are also important for the early detection of diseases (Yousef et al, 2014). Despite various research identifying a large amount of biomarkers, a large fraction of them have not gone beyond clinical trials due to lack of sensitivity, specificity, and reproducibility (Ngcoza, 2013). Therefore, it is important that biomarkers to be properly validated first before sending it for clinical trials.

UNIVERSITY of the
WESTERN CAPE

There are three types of biomarkers used in a clinical setting: (i) diagnostic (for disease identification), (ii) prognostic (for predicted outcome or progression of a disease), and (iii) theranostic (for the identification of appropriate treatment) (McCeough and Bjourson, 2012). Examples of current biomarkers clinically approved include human chorionic gonadotropin (hCG), which is an important biomarker found in urine for the detection of pregnancy (Butler et al, 2001) and prostate-specific antigen (PSA), which aids in the diagnosis of prostate cancer (Prensner et al, 2012). Despite the various biomarker breakthroughs, there is still a limited availability of theranostic biomarkers (McGeough and Bjourson, 2012). It is suggested that these types of biomarkers could be very useful for indicating the correct dosage, predict response to a particular treatment, could maximise drug

efficacy, and minimise drug toxicity for each individual (McGeough and Bjourson, 2012).

The discovery of biomarkers is a lengthy and challenging process. Good biomarkers has to be sensitive, specific, and it must be standardised and reproducible (Yousef et al, 2014). Many strategies have been used over the years for the identification of new biomarkers (Rodrigues and Kluskens, 2011). Computational biology (or bioinformatics) has played an instrumental role in the discovery of new biomarkers as well as the validation of potential biomarkers (Rodrigues and Kluskens, 2011; Yousef et al, 2014).

1.10. MicroRNAs as a biomarker for diagnosing pre-diabetes and T2DM

1.10.1. MicroRNAs – Overview

UNIVERSITY of the
WESTERN CAPE

MicroRNAs (miRNAs) are non-coding, short single-stranded RNA molecules, approximately 18 to 24 nucleotides in length (Saikumar et al, 2012). The mode of function of miRNAs relies on their binding to the 3'-untranslated region (3'-UTR) of their specific mRNA target genes. Once bound, they inhibit the expression of their target gene products by blocking initiation and elongation of translation (Saikumar et al, 2012; Molitoris and Molitoris, 2011) or through deadenylation of the mRNA transcript (Molitoris and Molitoris, 2011). It was found that miRNAs are highly conserved across all species (Molitoris and Molitoris, 2011). However, unlike plants, miRNAs in humans and most animals do not have to be entirely complimentary to their target mRNA (Chen and Zeller, 2014), as the seed region (the anti-sense strand of the miRNA duplex, which is two to eight nucleotides long

(Yilmazel et al, 2014) is the most important part for binding to and regulating their mRNA target (Kume et al, 2014). Hence, one miRNA can target many mRNA transcripts and one mRNA transcript can be repressed by many miRNAs (Molitoris and Molitoris, 2011).

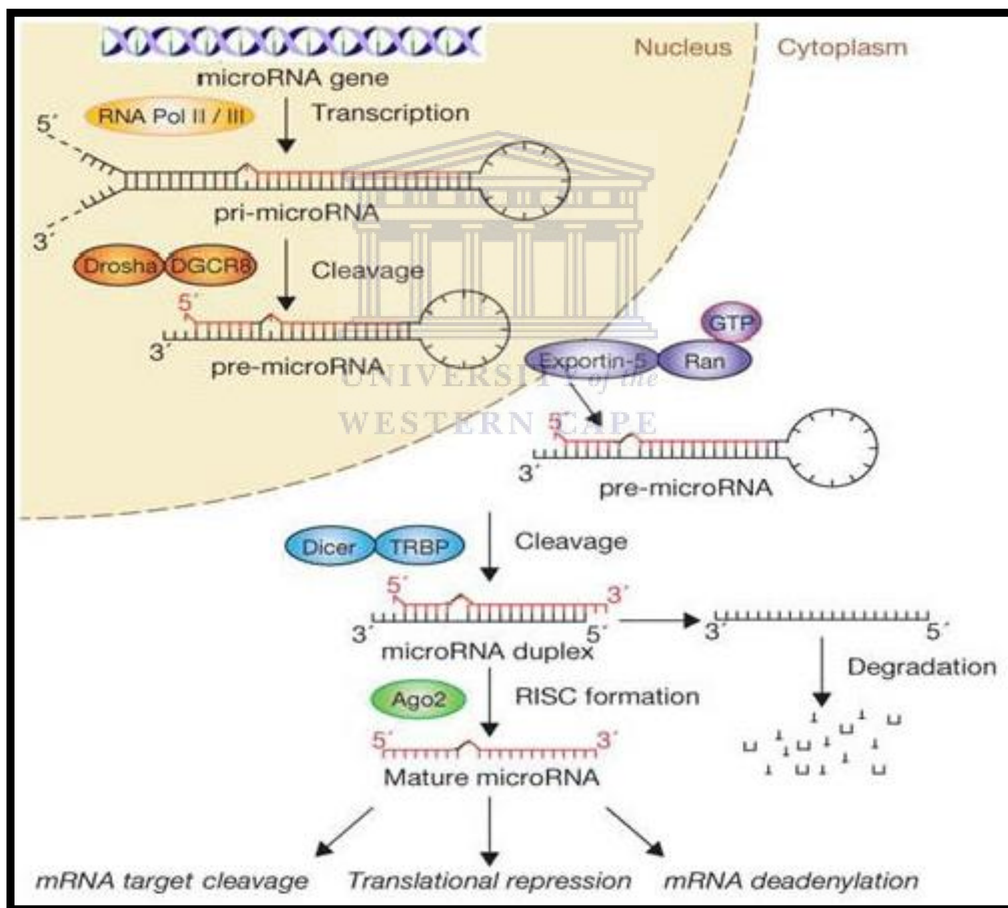
MiRNAs have been identified to play a substantial role in many regulatory pathways i.e. modulating signals by participating in negative or positive feedback loops (Tomasetti et al, 2014). Moreover, they have been found to be involved in a number of normal physiological and developmental processes (Molitoris and Molitoris, 2011; Tomasetti et al, 2014) such as the cell cycle, cell growth, apoptosis, cell differentiation and stress response, and in fine-tuning regulation of gene expression by targeting multiple molecules (Tomasetti et al, 2014). Therefore, dysregulation of miRNAs within either tissues or body fluids in response to intrinsic or extrinsic factors could be linked to abnormal gene expression (Tomasetti et al, 2014) and as a result be linked to several diseases, which include T2DM (Karolina et al, 2011; Molitoris and Molitoris, 2011; Saikumar et al, 2012).

UNIVERSITY of the
WESTERN CAPE

1.10.2. Biogenesis of miRNAs and their use as biomarkers for diseases

Biogenesis of miRNAs is tightly regulated at the transcriptional and posttranscriptional levels, as shown in Figure 2. MiRNAs are encoded in the genome and undergo transcription in a similar process to protein coding genes i.e. by both RNA polymerase II or RNA polymerase III (Molitoris and Molitoris, 2011; Velu et al, 2012), forming a stem-looped molecule known as the primary miRNA (pri-miRNA) transcript within the nucleus. The pri-miRNA is then cleaved by Drosha (an RNase III enzyme) and the DGCR8/Pasha protein complex, resulting in a ~70 nucleotide hairpin miRNA precursor (pre-miRNA) structure (Bushati and Cohen, 2007; Guay et al, 2011), which is transported into the cytoplasm by

Exportin-5 (Krol et al, 2004; Bushati and Cohen, 2007). The pre-miRNA is cleaved a second time by another RNase III enzyme, Dicer, to yield a miRNA duplex structure (Guay et al, 2011; Molitoris and Molitoris, 2011), approximately 22 nucleotides in length (Guay et al, 2004). The duplex then separates into two single miRNA strands which binds to an Argonaute-2 (Ago2) protein and is incorporated into the RNA-induced silencing complex (RISC) structure; this helps the miRNA to identify and bind to the complementary sites of its target mRNA (Guay et al, 2011) where it will inhibit gene expression.



UNIVERSITY of the
WESTERN CAPE

Figure 1.2: The biogenesis of miRNAs (Adopted from Velu et al, 2012)

The regulation of miRNAs has been linked to many important physiological and pathological processes (Fendler, 2011). In cancer research, studies have shown that the application of specific differentially expressed miRNAs could clearly differentiate between cancerous tissues and healthy tissue, could identify tumours from an unknown origin, could discriminate various tumour subtypes, and could characterise poorly differentiated tumours (Carlsson et al, 2011; Fendler, 2011; Zhu et al, 2014). Therefore, miRNAs have shown promise as ideal tools for diagnostic, prognostic, or monitoring therapeutic success of diseases (Fendler, 2011; Saikumar et al, 2012).

The advantage of using miRNAs over other biological markers (e.g. proteins or mRNA), is that they are stable and can be expressed in degraded RNA samples from human tissues (Jung et al, 2010; Fendler, 2012). Furthermore, circulatory miRNAs, which have been detected in body fluids such as blood, urine and saliva (Karolina et al, 2011), are found to be extremely stable under harsh conditions such as boiling temperatures, low or high pH, long-time storage at room temperature and even multiple freeze-thaw cycles (Creemers et al, 2012). Circulatory miRNAs is of particular interest for highly-sensitive non-invasive diagnostic purposes for the early detection of many diseases (Fendler, 2011; Ali Sheikh et al, 2015).

UNIVERSITY of the
WESTERN CAPE

Research has found that circulatory miRNAs are released through "secretory machinery", such as microvesicles and exosomes, which contains cellular gene products i.e. miRNA, mRNA, and proteins which can be transferred to recipient cells to carry out a specific function (Karolina et al, 2011). MiRNAs are also present in body fluids due to circulating tumour cells or Ago2 proteins (Fendler, 2011). Studies have shown that there is a distinct dysregulation of circulatory miRNAs after myocardial and liver injuries (Molitoris and Molitoris, 2011; Saikumar et al,

2012). Additionally, other studies have found that miR-21 and miR-141 may be used as non-invasive biomarkers in prostate cancer (Fendler, 2011). Lastly, several miRNAs have been linked to both glucose metabolism, metabolic disorders, and T2DM (Karolina et al, 2011).

1.10.3. The link between microRNAs and T2DM

Several reports have identified the critical role of specific miRNAs in the regulation of insulin production and secretion. Furthermore, multiple miRNAs are able to control, or are involved in, glucose metabolism by regulating a network of genes in the liver, the peripheral tissues (Fernandez-Hernando et al, 2013) and kidneys (Tang et al, 2008). The specific miRNA involved will be determined by the tissue and its metabolic state (Fernandez-Hernando et al, 2013).

Previous studies have found that miR-375 is one of the most abundant miRNAs located in the pancreas and it plays a role in negatively regulating insulin secretion (Karolina et al, 2011; Fernandez-Hernando, 2013) when bound to its myotrophin (*Mtpn*) target (Karolina et al, 2011), a gene involved in the depolymerisation of actin and transport of vesicles (Fernandez-Hernando et al, 2013). Whilst in a knockout study, mice that had the miR-375 gene deleted, displayed normal levels of insulin secretion, however, they were also hyperglycaemic and glucose intolerant (Fernandez-Hernando et al, 2013). Furthermore, these miR-375 *null* mice, showed a significant increase in the number of α-cells and glucagon levels (during both fasting and fed states) (Fernandez-Hernando et al, 2013). Therefore, a lack of miR-375 would result in reducing insulin exocytosis (Fernandez-Hernando et al, 2013). Table 1.4 gives a brief summary of several additional miRNAs and their validated gene targets.

UNIVERSITY of the
WESTERN CAPE

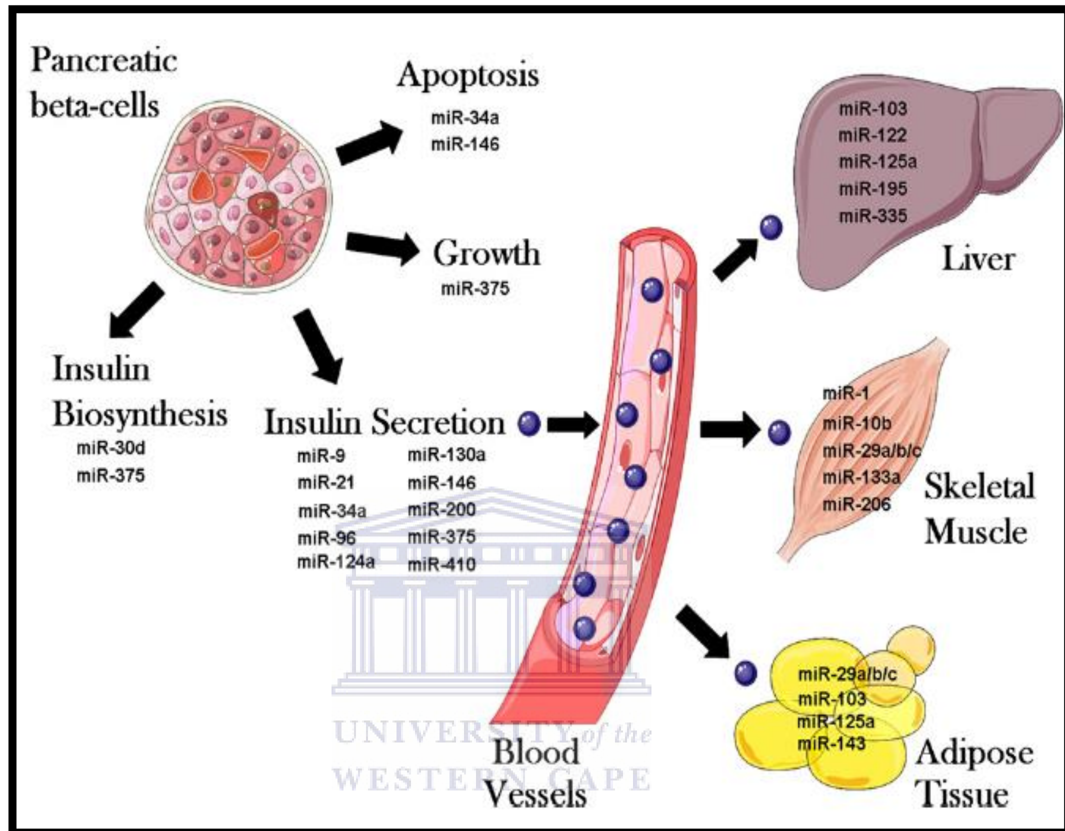
Table 1.4: MiRNAs, their validated target genes and their function in regulating glucose homeostasis (Adapted from Tang et al, 2008; Farr et al, 2013)

MiRNA	Target tissue	Function	Target gene/s
miR-375	Pancreas	Insulin secretion, pancreatic islet development	Mtpn, Usp1, Jak2, Adipor2
miR-124a	Pancreas	Pancreatic islet development	FoxA2, Rab27
miR-9	Pancreas	Insulin secretion	Onecut2
miR-29a, b	Muscle, adipose, liver	Glucose transport	Insig1, Cav2
miR-143	Adipose	Adipocyte differentiation	ERK5/BMK1/MAPK7
miR-145	Colon	Cell proliferation	IRS1
miR-133	Heart	Long QT syndrome, cardiac hypertrophy	HERG, RhoA, Cdc42, Nelf-A/WHSC2
miR-133a	Skeletal muscle	Glucose homeostasis	KLF15
miR-1	Heart	Heart development and physiology	KCNJ2, GJA1
miR-192	Kidney	Kidney and diabetic nephropathy development	SIP1

UNIVERSITY of the
WESTERN CAPE

Even though miRNA research in T2DM is very recent, research has proven that there are clear links with them playing a role in both the regulation of glucose and a pathogenic role in T2DM (shown in Figure 1.3 below). Other miRNAs that influence insulin secretion and signalling include miR-124a, miR-9 and miR-33

(Farr et al, 2013). Due to miRNAs displaying differential expression in blood and in various tissues involved in glucose homeostasis of T2DM patients, makes them ideal candidates for potential biomarkers.



UNIVERSITY of the WESTERN CAPE

Figure 1.3: Schematic overview of miRNAs involved in the regulation of pancreatic β-cells function and insulin target tissues in the context of T2DM (Adopted from Guay et al, 2011)

1.10.4. Bioinformatics as a tool for the detection of novel biomarkers

Over the past years, major advances has been accomplished in the field of molecular biology linked with advances in high throughput technologies such as genomics, transcriptomics, proteomics (Emmett et al, 2014) and metabolomics (Bartel et al, 2013). Moreover, these technologies have brought forward an explosive amount of biological information which has led to the need for computerised databases to

store, organise, and analyse the data (Martone et al, 2004; Kemkar and Dahikar, 2012).

Hence, the field of bioinformatics, or systems biology, which is the merging of the computational and biological science disciplines, has been an important tool for the organisation and analysis of the vast amount of biological data (Lewis, 2008). The main aim of bioinformatics is to find key biological information hidden amongst a mass of raw data to identify important trends and patterns which would eventually lead to novel biomarker discovery for both diagnostic and therapeutic purposes (Ngcoza, 2013). Additionally, bioinformatics allows for *in silico* simulations of complex disease physiologies, such as interactions between components, on their molecular level (Calvert-Joshua, 2013). Bioinformatics has presented ways in which data mining approaches can be used to filter valuable targets (miRNA, genes, or proteins) for the discovery of possible novel biomarkers for diseases (Sommer et al, 2010).

UNIVERSITY of the
WESTERN CAPE

1.10.5. Role of bioinformatics in miRNA research

Identifying miRNAs, their target genes, and their respective regulatory function are important for understanding normal biological processes as well as understanding their various roles in disease development (Zhang and Verbeek, 2010; Liu et al, 2012; Fujiwara and Yada, 2013). Bioinformatics facilitates experimental validation of miRNAs and their target genes by producing statistically significant hypotheses from biological data that has been stored in databases, based on other biological experimental data (Liu et al, 2012). Potential target identification is based on the software's algorithm. There are several miRNA-target prediction software that is publically available (Fujiwara and Yada, 2013) which include TargetScan, PITA and Diana Micro-T (Witkos et al, 2011). Possible targets are predicted based on the

software's prediction algorithm, which is categorized into three groups: (i) sequence-based, (ii) energy-based, and (iii) machine learning-based (Zhang and Verbeek, 2010). Target prediction and the various software used in this study will be elaborated on in Chapter 2, section 2.2.2.

1.11. The purpose of this study

Non-invasive diagnostic methods have been shown a lot of interest. Specifically, diagnostic methods which are quick, painless, cost-effective, and sensitive as well as being specific for a particular disease. Most of the current diagnostic tests for most diseases usually involve painful or uncomfortable methods for retrieving samples e.g. tissue samples from biopsies (Vlastos and Verkooijen, 2007). Particular interest is also focussed on diagnostic methods which can detect diseases in its early stages.

UNIVERSITY of the
WESTERN CAPE

If diabetes were to be diagnosed in its early stages (i.e. pre-diabetes), the development of overt T2DM, and its long-term complications, can be avoided. MiRNAs have shown remarkable promise as potential biomarkers for the early (and specific) detection for T2DM, as well as for other diseases, due to its stability compared to other biomolecules such as mRNA and proteins. The aim of this project, therefore, was to identify and validate differentially expressed miRNAs in T2DM as well as to identify their potential target genes. The objectives of this thesis included:

- Identifying differentially expressed miRNA in serum and plasma involved in the pathogenesis of T2D using *in silico* methods

- Generating a target gene list for the identified miRNA and understand the mechanism of action of these target genes as it relates to T2DM
- Correlating the target genes involved in T2DM back to those regulating miRNAs
- Generating a final list of miRNAs for molecular validation
- Expression analysis of miRNAs shown through *in silico* work, to be linked with T2DM using rat kidney tissue

UNIVERSITY of the
WESTERN CAPE

1.12. References

- ADA, see American Diabetes Association. 2012a. Diagnosis and classification of diabetes mellitus. *Diabetes Care*, 35(S1): S64 – S71
- ADA. 2012b. Executive summary: standards of medical care in diabetes – 2012. *Diabetes Care*, 35(1): S4 – S10
- ADA. 2014. Executive summary: standards of medical care in diabetes – 2014. *Diabetes Care*, 37(S1): S5 – S13
- Ahmad, L.A. and Crandall, J.P. 2010. Type 2 diabetes prevention: a review. *Clinical Diabetes*, 28(2): 53 – 59
- Ali Sheikh, M., Xia, K., Li, F., Deng, X., Salma, U., Deng, H., Wei Wei, L., Yang, T. and Peng, J. 2015. Circulating miR-765 and miR-149: Potential noninvasive diagnostic biomarkers for geriatric coronary artery disease patients. *BioMed Research International*, 2015: 1 – 8
- Aronoff, S.L., Berkowitz, K., Shreiner, B., Want, L. 2004. Glucose metabolism and regulation: beyond insulin and glucagon. *Diabetes Spectrum*, 17(3): 183 – 190
- Bartel, J., Krumsiek, J. and Theis, F. 2013. Statistical methods for the analysis of high-throughput metabolomics data. *Computational and Structural Biotechnology Journal*, 4(5): 1 – 9
- Bosenberg, L.H. and van Zyl, D. G. 2008. The mechanism of action of oral antidiabetic drugs: A review of recent literature. *Journal of Endocrinology, Metabolism and Diabetes of South Africa*, 13(3): 80 – 88
- Bushati, N. and Cohen, S. 2007. microRNA functions. *Annual Review of Cell and Developmental Biology*, 23(1): 175 – 205

UNIVERSITY of the
WESTERN CAPE

- Butler, S.A., Khanlian, S.A., Cole, L.A. Detection of early pregnancy forms of human chorionic Gonadotropin by home pregnancy test devices. *Clinical Chemistry,* 47(12): 2131 – 2136
- Calvert-Joshua, T. 2013. *An in silico identification of putative biomarkers in cervical cancer*. Hons Thesis, University of the Western Cape
- Carlsson, J., Davidsson, S., Helenius, G., Karlsson, M., Lubovac, Z., Andrén, O., Olsson, B. and Klinga-Levan, K. 2011. A miRNA expression signature that separates between normal and malignant prostate tissues. *Cancer Cell International*, 11(1): 14
- Cernea, S. and Dobreanu, M. 2013. Diabetes and beta cell function: from mechanisms to evaluation and clinical implications. *Biochemical Medicine*, 23(3): 266 – 280
- Chen, J.S. and Zeller, R. 2014. Using engineered microRNAs as vectors for animal RNA interference: promises and challenges. *Advances in Bioscience and Biotechnology*, 5(4): 301 – 310
- Clark, N., Fox, K. and Grandy, S. 2007. Symptoms of diabetes and their association with the risk and presence of diabetes: findings from the study to help improve early evaluation and management of risk factors leading to diabetes (SHIELD). *Diabetes Care*, 30(11): 2868 – 2873
- Clayton, W. and Elasy, T. (2009). A Review of the Pathophysiology, Classification, and Treatment of Foot Ulcers in Diabetic Patients. *Clinical Diabetes*, 27(2): 52 – 58
- Creemers, E.E., Tijsen, A.J. and Pinto, Y.M. 2012. Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease. *Circulation Research*, 110: 483 - 495

UNIVERSITY of the
WESTERN CAPE

- D'Adamo, E. and Caprio, S. 2011. Type 2 diabete in youth: Epidemiology and pathophysiology. *Diabetes Care*, 34(S2): S161 – S165
- Day, C. and Bailey, C.J. 2011. Obesity in the pathogenesis of type 2 diabetes. *British Journal of Diabetes & Vascular Disease*, 11(2): 55 - 61
- DeFronzo, R.A. 2004. Pathogenesis of type 2 diabetes mellitus. *Medical Clinics of North America*, 88: 787 – 835
- Diabetes.co.uk. 2015. *What is HbA1c? – Definition, Units, Conversion, Testing & Control*. [online] Available at: http://www.diabetes.co.uk/what-is-hba1c.html [Accessed 19 Jan. 2015]
- Diabetes.org.uk. 2015a. Diagnostic criteria for diabetes – Diabetes UK. [online] Available at: http://www.diabetes.org.uk/About_us/What-we-say/Diagnosis-prevention/New_diagnostic_criteria_for_diabetes/ [Accessed 20 Jan. 2015]
- Diabetes.co.uk, 2015b. *C-Peptide Test*. [online] Available at: http://www.diabetes.co.uk/c-peptide-test.html [Accessed 31 Mar. 2015].
- Distiller, L.A. 2004. Improved diabetes management in South Africa: the case for a capitation model. Available at: https://www.idf.org/sites/default/files/attachments/article_279_en.pdf [Accessed 16 March 2015]
- Eckel, R.H., Kahn, S.E., Ferrannini, E., Goldfine, A.B., Nathan, D.M., Schwartz, M.W., Smith, R.J. and Smith, S.R. 2011. Obesity and type 2 diabetes: What can be unified and what needs to be individualized? *Diabetes Care*, 34(6): 1424 – 1430

UNIVERSITY of the WESTERN CAPE

- Emmett, M., Kroes, R., Moskal, J., Conrad, C., Priebe, W., Laezza, F., Meyer-Baese, A. and Nilsson, C. 2013. Integrative biological analysis for neuropsychopharmacology. *Neuropsychopharmacology*, 39(1): 5 – 23
- Farr, R., Taylor, C.J., Satoor, S.N., Williams, M.D. and Joglekar, M.V. 2013. From cradle to the grave: tissue-specific microRNA signatures in detecting clinical progression of diabetes. *Non-coding RNAs in Endocrinology*, 1(1): 16 – 27
- Fecko, A. 2012. Diabetes-Related Amputations: Understanding Secondary Conditions and How They Affect Your Practice. *The Academy Today*, 8(1) [online] Available at: http://www.oandp.org/AcademyTODAY/2012Mar/2.asp
- Fendler, A. 2011. *MiRNAs and prostate cancer: Identification, functional characterization and their potential use in medical practice*. PhD Thesis, Free University of Berlin
- Fernandez-Hernando, C., Ramirez, C.M., Goedeke, L., and Suarez, Y. 2013. MicroRNAs in metabolic disease. *Arteriosclerosis, Thrombosis and Vascular Biology*, 33(2): 178 – 185
- Flegal, K.M., Kit, B.K., Orpana, H. and Graubard, B.I. 2013. Association of all-cause mortality with overweight and obesity using standard body mass index catergories. A systematic review and meta-analysis. *Journal of the American Medical Association*, 309(1): 71 – 82
- Forbes, J.M. and Cooper, M.E. 2013. Mechanisms of diabetic complications. *Physiological Reviews*, 93(1): 137 – 188
- Fowler, M.J. 2008. Microvascular and macrovascular complications of diabetes. *Clinical Diabetes*, 26(2): 77 – 82

UNIVERSITY of the
WESTERN CAPE

- Fujiwara, T. and Yada, T. 2013. miRNA-target prediction based on transcriptional regulation. *BMC Genomics*, 14(S 2): S3
- Gastaldelli, A. 2011. Role of beta-cell dysfunction, ectopic fat accumulation and insulin resistance in the pathogenesis of type 2 diabetes mellitus. *Diabetes Research and Clinical Practice*, 93S: S60 – S65
- Gerich, J.E. 2010. Role of the kidney in normal glucose homeostasis and in the hyperglycaemia of diabetes mellitus: therapeutic implications. *Diabetic Medicine*, 27(2): 136 – 142
- Gilespie, K.M. 2006. Type 1 diabetes: pathogenesis and prevention. *Canadian Medical Association Journal,* 175(2): 165 - 170
- Giugliano, D., Ceriello, A., and Esposito, K. 2008. Glucose metabolism and hyperglycemia. *The American Journal of Clinical Nutrition*, 87 (Suppl. 1): 217S – 222S
- Goldenberg, R.M., Cheng, A.Y.Y. and Punthakee, Z. and Clement, M. 2011. Use of glycated hemo-globin (A1C) in the diagnosis of type 2 diabetes mellitus in adults. *Canadian Journal of Diabetes*, 35(3): 247 – 249
- Goldenberg, R. and Punthakee, Z. 2013. Definition, Classification and Diagnosis of Diabetes, Prediabetes and Metabolic Syndrome. *Canadian Journal of Diabetes*, 37 (Suppl. 1): S8 – S11
- Grayson, B.E., Seeley, R.J., and Sandoval, D.A. 2013, Wired on sugar: the role of the CNS in the regulation of glucose homeostasis. *Nature Reviews*, 24(1): 24 – 37
- Hu, F.B. 2011. The role of diet, lifestyle, and genes. *Diabetes Care*, 34: 1249 – 1257

UNIVERSITY of the
WESTERN CAPE

- Guay, C., Roggli, E., Nesca, V., Jacovetti, C., Regazzi, R. 2011. Diabetes mellitus, a microRNA-related disease? *Translational Research*, 157(4): 253 – 264
- IDF, see International Diabetes Federation. 2014. The IDF diabetes atlas, 6th Ed. [online] Available at: http://www.idf.org/sites/default/files/EN_6E_Atlas_Full_0.pdf [Accessed 17 March 2015]
- Ibbald, R.G., Ayello, E.A., Alavi, A., Ostrow, B., Lowe, J., Botros, M., Goodman, L., Woo, K. and Smart, H. 2012. Screening for the high-risk diabetic foot: a 60-second tool (2012) ©. *Advances in Skin & Wound Care: The Journal for Prevention and Healing*, 25(10): 465 – 476
- Jacovides, A., Bogoshi, M., Distiller, L.A., Mahgoub, E.Y., Omar, M.K., Tarek, I.A. and Wajsbrot, D.B. 2014. An epidemiological study to assess the prevalence of diabetic peripheral neuropathic pain among adults with diabetes attending private and institutional outpatient clinics in South Africa. *The International Journal of Medical Research*, 42(2): 1018 – 1028
- Jones, A. and Hattersley, A. 2013. The clinical utility of C-peptide measurement in the care of patients with diabetes. *Diabetic Medicine*, 30(7): 803 – 817
- Jung, M., Schaefer, A., Steiner, I., Kempkensteffen, C., Stephan, C., Erbersdobler, A. and Jung, K. 2010. Robust MicroRNA Stability in Degraded RNA Preparations from Human Tissue and Cell Samples. *Clinical Chemistry*, 56(6): 998 – 1006
- Kaku, K. 2010. Pathophysiology of type 2 diabetes and its treatment policy. *Japan Medical Association Journal*, 53(1): 41 – 46

UNIVERSITY of the
WESTERN CAPE

- Karolina, D.S., Armugam, A., Tavintharan, S., Wong, M.T.K., Lim, S.C., Sum, C.F., and Jeyaselan, K. 2011. MicroRNA 144 impairs insulin signalling by inhibiting the expression of insulin receptor substrate 1 in type 2 diabetes mellitus. *PLoS ONE*, 6(8): e22839
- Kasuga, M. 2006. Insulin resistance and pancreatic β cell failure. *The Journal of Clinical Investigation*, 116(7): 1756 – 1760
- Kemkar, O.S. and Dahikar, P.B. 2012. Bioinformatics: A revolutionary way of using computer technology for evidence based medicine. *International Journal of Advanced Research in Computer and Communication Engineering*, 1(9): 622 – 626
- Kengne, A. 2005. Cardiovascular Complications of Diabetes Mellitus in Sub-Saharan Africa. *Circulation*, 112(23): 3592 – 3601
- Kim, J., Wei, Y. and Sowers, J. 2008. Role of Mitochondrial Dysfunction in Insulin Resistance. *Circulation Research*, 102(4): 401 – 414
- Knop, F.K., Vilsboll, T., Holst, J.J. 2009. Incretin-based therapy of type 2 diabetes mellitus. *Current Protein and Peptide Science*, 10(1): 46 – 55
- Krol, J., Sobczak, K., Wilczynska, U., Drath, M., Jasinska, A., Kaczyncka, D. and Krzyzosiak, W.J. 2004. Structural features of microRNA (miRNA) precursors and their relevance to miRNA biogenesis and small interfering RNA/short hairpin RNA design. *The Journal of Biological Chemistry*, 279(40): 42230 – 42239
- Kume, H., Hino, K., Galipon, J. and Ui-Tei, K. 2014. A-to-I editing in the miRNA seed region regulates target mRNA selection and silencing efficiency. *Nucleic Acids Research*, 42(15): 10050 – 10060

UNIVERSITY of the
WESTERN CAPE

- Levitt, A.S. 2009. Saliva test to catch early diabetes. [online] Available at: http://thefutureofthings.com/news/6481/saliva-test-to-catch-early-diabetes.html. [Accessed 16 July 2012]
- Lewis, J. 2008. *Computing genomic science: bioinformatics and standardisation in proteomics*. PhD Thesis, Cardiff University
- Lin, Y. and Sun, Z. 2010. Current views on type 2 diabetes. *Journal of Endocrinology*, 204: 1 – 11
- Liu, B., Li, J. and Cairns, M. 2012. Identifying miRNAs, targets and functions. *Briefings in Bioinformatics*, 15(1): 1 – 19
- Maitra, A. 2012. "Endocrine system", in Kumar, V., Abbas, A.K., and Aster, J.C. (eds), *Robbins Basic Pathology*, pp. 739-743, W.B. Saunders Company, Philadelphia
- Malecki, M. and Skupien, J. 2008. Problems in differential diagnosis of diabetes types. *Polskie Archiwum Medycyny Wewnetrznej*, 118(7-8): 435 – 440
- Martone, M., Gupta, A. and Ellisman, M. 2004. e-Neuroscience: challenges and triumphs in integrating distributed data from molecules to brains. *Nature Neuroscience*, 7(5): 467 – 472
- Maynard, J.D., Rohrscheib, M., Way, J.F., Nguyen, C.M., and Ediger, M.N. 2007. Noninvasive type 2 diabetes screening: superior sensitivity to fasting plasma glucose and glycosylated haemoglobin. *Diabetes Care*, 30: 1120 – 1124
- McGeough, C and Bjourson, A.J. 2012. Diagnostic, prognostic and theranostic biomarkers for rheumatoid arthritis. *Journal of Clinical & Cellular Immunology*, S6: 1 – 5

UNIVERSITY of the WESTERN CAPE

- Meier, J.J. and Bonadonna, R.C. 2013. Role of reduced β-cell mass versus impaired β-cell function in the pathogenis of type 2 diabetes. *Diabetes Care*, 36(S2): S113 – S119
- Molitoris, J.K. and Molitoris, B.A. 2011. Circulating micro-RNAs in acute kidney injury: early observations. *Clinical Journal of the American Society of Nephrology*, 6(7): 1517 – 1519
- Motala, A.A., Omar, M.A. and Pirie, F.J. 2003. Diabetes in Africa: epidemiology of type 1 and type 2 diabetes in Africa. *Journal of Cardiovascular Risk*, 10: 77 – 83
- Ngcoza, N. 2013. *Identification of biomarkers in breast cancer as potential diagnostic and therapeutic agents using a combined in-silico and molecular approach*. MSc Thesis, University of the Western Cape
- Nicolaides, E. and Jones, C.J.H. 2002. Type 2 diabetes – implications for macrovascular mechanics and disease. *The British Journal of Diabetes & Vascular Disease*, 2: 9 – 12
- Novosyadly, R., Lann, D.E., Vijayakumar, A., Rowzee, A., Lazzarino, D.A., Fierz, Y., Carboni, J.M., Gottardis, M.M., Pennisi, P.A., Molinolo, A.A., Kurshan, N., Mejia, W., Santopietro, S., Yakar, S., Wood, T.L. and LeRoith D. 2010. Insulin-mediated acceleration of breast cancer development and progression in a non-obese model of type 2 diabetes. *Cancer Research*, 70(2): 741 – 751
- Nyunt, O., Wu, J.Y., McGown, I.N., Harris, M., Huynh, T., Leong, G.M., Cowley, D.M. and Cotteril, A.M. 2009. Investigating maturity onset diabetes of the young. *The Clinical Biochemist Reviews*, 30(2): 67 – 74

UNIVERSITY of the
WESTERN CAPE

- OECD, see Organisation for Economic Co-operation and Development. 2013. *Health at a glance 2013: OECD Indicators*. OECD Publishers, Paris
- Ottermann, B. 2012. *Prevalence of diabetes in South Africa*. [online] Available at: http://www.health24.com/Medical/Diabetes/About-diabetes/Diabetes-tsunami-hits-South-Africa-20130210 [Accessed 17 March 2015]
- Patel, P. and Macerollo, A. 2010. Diabetes mellitus: diagnosis and screening. *American Family Physician*, 81(7): 863 - 870
- Pilkington, F.B., Daiski, I., Lines, E., Bryant, T., Raphael, D., Dinca-Panaitescu, M., and Dinca-Panaitescu, S. 2011. Type 2 diabetes in vulnerable populations: community healthcare providers' perspectives on health service needs and policy implications. *Canadian Journal of Diabetes*, 35(5): 503 – 511
- Piotie, P.N. 2013. *Diabetes epidemic in Africa: Now is the time to act*. [Online] Available at: http://www.consultancyafrica.com/index.php?option=com_content&view=article&id=1320:diabetes-epidemic-in-africa-now-is-the-time-to-act-part-1&catid=61:hiv-aids-discussion-papers&Itemid=268. [Accessed: 12 November 2015]
- Prensner, J., Rubin, M., Wei, J. and Chinnaiyan, A. 2012. Beyond PSA: The Next Generation of Prostate Cancer Biomarkers. *Science Translational Medicine*, 4(127): 127rv3
- Rao, P.V., Reddy, A.P., Lu, X., Dasari, S., Krishnaprasad, A., Biggs, E., Roberts, Jr. C.T. and Nagalla, S.R. 2009. Proteomic identification of salivary biomarkers of type-2 diabetes. *Journal of Proteome Research*, 8: 239 – 245

UNIVERSITY of the WESTERN CAPE

- Rodrigues, L.R. and Kluskens, L.D. 2011. 'Synthetic biology & bioinformatics: prospects in the cancer arena' in Lopes, H.S. and Cruz, L.M (eds.), *Computational Biology and Applied Bioinformatics*, Rejieka, Croatia: InTech: 159 - 186
- Rodriquez-Poncelas, A., Coll-De Tuero, G., Turró-Garriga, O., Barrot-de la Puente, J., France-Nadal, J., Mundet-Tuduri, X., and RedGDPS Study Group. 2014. Impact of chronic kidney disease on the prevalence of cardiovascular disease in patients with type 2 diabetes in Spain: PERCEDIME2 study. *BMC Nephrology*, 15(150): 1 – 8
- Saikumar, J., Hoffmann, D., Kim, T., Ramirez Gonzalez, V., Zhang, Q., Goering, P.L., Brown, R.P., Bijol, V.,Park, P.J., Waikar, S.S., and Vaidya, V.S. 2012. Expression, circulation, and excretion profile of microNRA-21, -155, and -18a following acute kidney injury. *Toxicological Sciences*, 129(2): 256 – 267

UNIVERSITY of the
WESTERN CAPE

- Schaefer, E., Gleason, J. and Dansinger, M. 2009. Dietary fructose and glucose differentially affect lipid and glucose homeostasis. *Journal of Nutrition*, 139(6): 1257S – 1262S
- Sebastiani, G. 2011. *MicroRNA expression profiling reveals tissue-specific and disease-associated signatures in diabetes mellitus*. PhD Thesis, University of Siena
- SEMDSA, see Society for Endocrinology, Metabolism and Diabetes of South Africa. 2012. The 2012 SEMDSA guideline for the management of type 2 diabetes (Revised). *Journal of Endocrinology, Metabolism and Diabetes of South Africa*, 17(2): S1 – S95

- Sena, C., Bento, C., Pereira, P. and Seiça, R. 2010. Diabetes mellitus: new challenges and innovative therapies. *The EPMA Journal*, 1(1): 138 – 163
- Skugor, M. 2014. *Diabetes mellitus treatment*. [online] Available at: http://www.clevelandclinicmeded.com/medicalpubs/diseasemanagement/endocrinology/diabetes-mellitus-treatment/Default.htm [Accessed 8 Apr. 2015]
- Smaldone, A. 2008. Glycemic Control and Hemoglobinopathy: When A1C may not be reliable. *Diabetes Spectrum*, 21(1): 46 – 49
- Soita, D. 2009. *The prevalence of impaired glucose tolerance and diabetes amongst the middle aged population of Bellville South Community, Cape Town, South Africa*. MTech Thesis, Cape Peninsula University of Technology
- Sommer, B., Tiys, E.S., Kormeier, B., Hippe, K., Janowski, S.J., Ivanisenko, S.J., Bragin, A.O., Arrigo, P., Demenkov, P.S., Kochetov, A.V., Ivanisenko, V.A., Kolchanov, N.A. and Hofestädt, R. 2010. Visualization and analysis of a cardio vascular disease and MUPP1-related biological network combining text mining and data warehouse approaches. *Journal of Integrative Bioinformatics*, 7(1): 148
- Stumvoll, M. Goldstein, B.J., van Haeften, T.W. 2005. Type 2 diabetes: principles of pathogenesis and therapy. *Lancet*, 365: 1333 – 1346
- Tanenberg, R.J. 2009. Diabetic peripheral neuropathy: Painful or painless, Clinical review article. *Hospital Physician*, 2009: 1 – 8
- Tang, X., Tang, G., Özcan, S. 2008. Role of microRNAs in diabetes. *Biochimica et Biophysica Acta*, 1779(11): 697 -701
- Targetscan.org, 2015. *TargetScanHuman 6.2*. [online] Available at: http://www.targetscan.org/vert_61/ [Accessed 17 March 2015]

UNIVERSITY of the
WESTERN CAPE

- Thynne, T. and Doogue, M. 2014. Sodium-glucose co-transporter inhibitors Mechanisms of action. *Australian Prescriber*, 37(1): 14 – 16
- Tomasetti, M., Neuzil, J. and Dong, L. 2014. MicroRNAs as regulators of mitochondrial function: role in cancer suppression. *Biochimica et Biophysica Acta – General Subjects*, 1840(4): 1441 – 1453
- Triplitt, C.L. 2012. Examining the mechanisms of glucose regulation. *American Journal of Managed Care*, 18(Suppl. 1): S4 – S10
- Valdez, R. 2009. Detecting undiagnosed type 2 diabetes: family history as a risk factor and screening tool. *Journal of Diabetes Science and Technology*, 3(4): 722 – 726
- Velu, V.K., Ramesh, A., and Srinivasan, A.R. 2012. Circulating microRNAs as biomarkers in health and disease. *Journal of Clinical and Diagnostic Research*, 6(10): 1791 – 1795
- Vlastos, G., and Verkooijen, H.M. 2007. Minimally invasive approaches for diagnosis and treatment of early stage breast cancer. *The Oncologist*, 12(1): 1 – 10
- Vora, J. and Evans, M. 2012. *Managing diabetes*. Springer Healthcare Communications, London
- Wang, Y., Katzmarzyk, P.T., Horswell, R., Zhao, W., Johnson, J. and Hu, G. 2014. Kidney function and the risk of cardiovascular disease in patients with type 2 diabetes. *Kidney International*, 85: 1192 – 1199
- WHO, see The World Health Organisation. 2011. Use of glycated haemoglobin (HbA1c) in the diagnosis of diabetes mellitus. [online] Available at: http://www.who.int/diabetes/publications/report-hba1c_2011.pdf [Accessed 17 March 2015]

UNIVERSITY of the
WESTERN CAPE

- Witkos, T.M., Koscianska, E. and Krzyzosiak, W.J. 2015. Practical aspects of microRNA target prediction. *Current Molecular Medicine*, 11(2): 93 – 109
- Yilmazel, B., Hu, Y., Sigoillot, F., Smith, J., Shamu, C., Perrimon, N. and Mohr, S. 2014. Online GESS: prediction of miRNA-like off-target effects in large-scale RNAi screen data by seed region analysis. *BMC Bioinformatics*, 15(1): 192
- Yousef, M., Najami, N., Abedallah, L. and Khalifa, W. 2014. Computational approaches for biomarker discovery. *Journal of Intelligent Learning Systems and Applications*, 6(4): 153 – 161
- Zhang, P., Dalton, C. and Jullien, G.A. 2009. Design and fabrication of MEMS-based microneedle arrays for medical applications. *Microsystem Technologies*, 15: 1073-1082
- Zhang, A., Sun, H., Yan, G., Wang, P. and Wang, X. 2014. Metabolomics for biomarker discovery: moving to the clinic. *BioMed Research Internation,* 2015: 1 – 6
- Zhang, P., Zhang, X., Brown, J., Vistisen, D., Sicree, R., Shaw, J. and Nichols, G. 2010. Global healthcare expenditure on diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice,* 87(3): 293-301
- Zhang, Y. and Verbeek, F.J. 2010. Comparison and Integration of target prediction algorithms for microRNA studies. *Journal of Integrative Bioinformatics*, 7(3): 1 – 13
- Zhu, J., Zheng, Z., Wang, J., Sun, J., Wang, P., Cheng, X., Fu, L., Zhang, L., Wang, Z. and Li, Z. 2014. Different miRNA expression profiles between human breast cancer tumors and serum. *Frontiers in Genetics,* 5: 149

UNIVERSITY of the
WESTERN CAPE

Chapter 2: *In silico* analysis to generate a list of priority miRNA for T2DM diagnoses

MiRNAs inhibit gene expression by binding to the 3'-UTR site of its target mRNA, resulting in the inhibition or degradation of that particular mRNA. Furthermore, while one miRNA can have many mRNA targets, similarly, one mRNA can be targeted by multiple miRNAs (Fendler, 2011). Despite the increased number of identified miRNAs using experimental approaches, the identification process has its limitations since all current methods are time-consuming, laborious, and expensive (Radfar, 2014). Experimental target prediction approaches are also unable to provide a genome-wide prediction of miRNA targeting (Radfar, 2014).

Target prediction using bioinformatics tools has become very important for identifying potential (and novel) binding targets of specific miRNAs and other types of biomarkers as it allows for fast, less laborious, and cheaper means of creating a priority list of potential biomarkers. Many target prediction algorithms exist for this exact reason; each having their own criteria for binding predictions which include: perfect complementarity of the seed sequence, evolutionary conservation, free energy of the miRNA:mRNA duplex, and proximity of different binding sites of the same miRNA (Lekprasert, 2012; Schmitz et al, 2014).

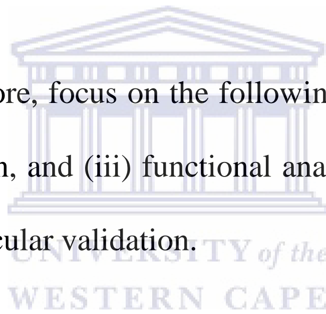
However, despite the availability of many target prediction programs, there are currently challenges regarding standardized methodologies for miRNA target recognition. Therefore, the predicted target genes of specific miRNAs can only be

UNIVERSITY of the WESTERN CAPE

validated using molecular methods such as qRT-PCR, luciferase assays or western blots (Kuhn et al, 2008).

2.1. Aims of this chapter

The purpose of the work described in this chapter was to identify miRNAs associated with T2DM and their target genes. The identified target genes will be further evaluated using several bioinformatics tools to determine their role in T2DM onset or progression. Those target genes shown to play a vital role in T2DM will be correlated back to their miRNAs to generate a priority list of miRNAs for molecular validation.


UNIVERSITY of the
WESTERN CAPE

This chapter will, therefore, focus on the following: (i) miRNA dataset selection, (ii) target gene prediction, and (iii) functional analysis – for generating a priority list of miRNAs for molecular validation.

2.2. Bioinformatics methodology

Table 2.1: List of Databases used for the bioinformatics methodology

Procedure	Database	Website
miRNA selection	miR2Disease	http://www.mir2disease.org/
	HMDD v2.0	http://202.38.126.151/hmdd/tools/hmdd2.html
Target prediction	TargetScan Human 6.2	http://www.targetscan.org/vert_61/
	PITA	http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html
	Diana Micro-T v.3.0	http://diana.cslab.ece.ntua.gr/microT/
miRNA sequences	miRBase	http://www.mirbase.org/
Alignment of sequences	ClustalW2	http://www.ebi.ac.uk/Tools/msa/clustalw2/
Functional annotation of target genes	DAVID v6.7	http://david.abcc.ncifcrf.gov/
Functional annotation of miRNAs	Diana mir-Path v.2.0	http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=mirpath/index
Network and pathway enrichment	STRING 10	http://string-db.org/

UNIVERSITY of the WESTERN CAPE

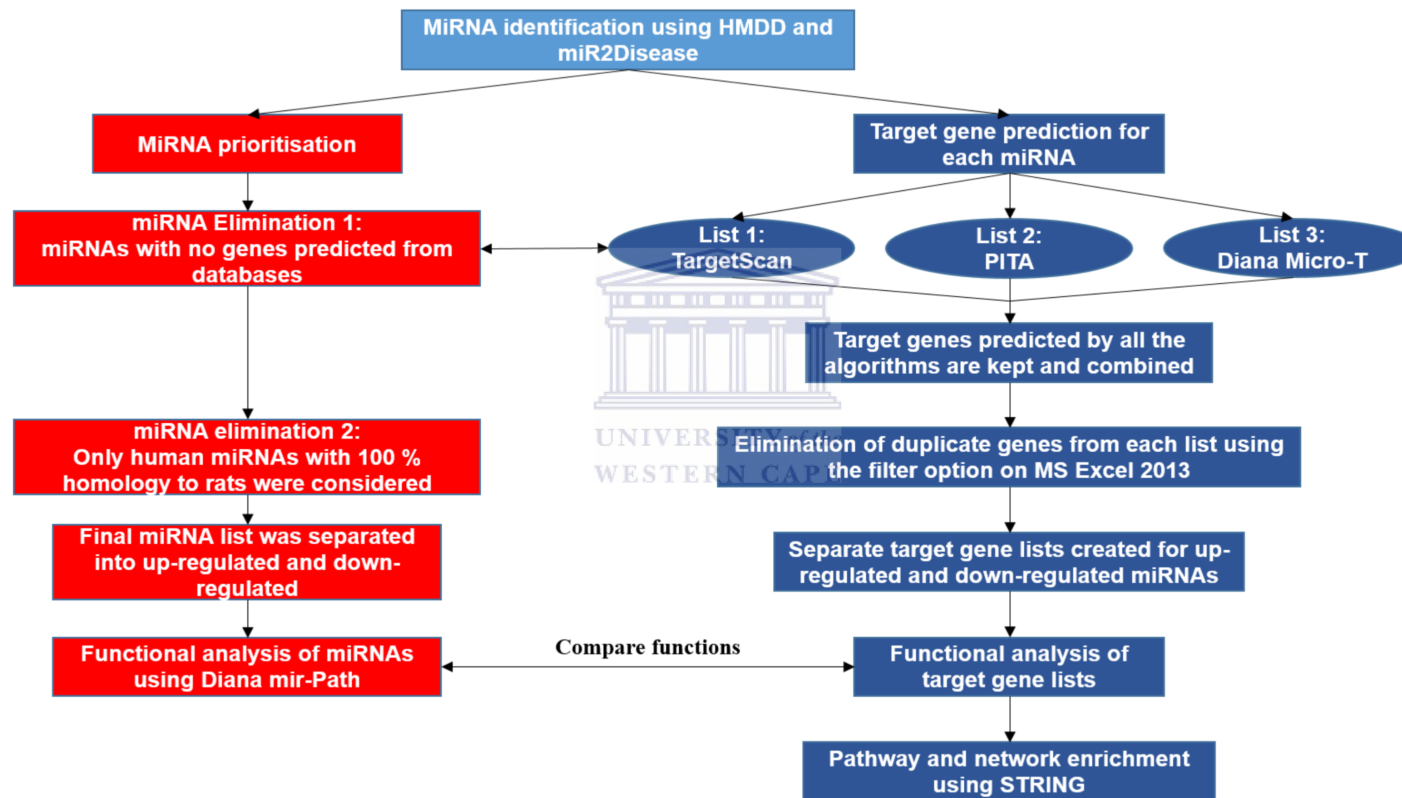


Figure 2.1: Schematic overview of bioinformatics workflow followed to obtain a reduced list of both miRNAs and target genes

2.2.1. MiRNA dataset selection

Experimentally validated microRNAs (miRNAs), which were shown to be differentially expressed in type 2 diabetes (T2DM), particularly in the blood/plasma/serum, was selected from publically available databases i.e. the Human MicroRNA Disease Database v.2.0 (HMDD) (http://202.38.126.151/hmdd/tools/hmdd2.html) (Li et al, 2013) and miR2disease (http://www.mir2disease.org/) (Jiang et al, 2009).

The miR2Disease database is manually curated and provides a comprehensive repository of differentially expressed miRNA in various human diseases (Jiang et al, 2009). The input queries used to obtain the miRNA list for this database was <diabetes mellitus> and <pre-diabetes>, respectively.

The HMDD is a collection of experimentally validated human miRNAs and disease associations (da Silva Santos, 2014). A list of miRNAs were obtained from the database by running a "fuzzy search" using <diabetes mellitus> as the input query (Li et al, 2013).

Thereafter, the two miRNA lists obtained were combined and duplicates were eliminated in MicroSoft (MS) Excel 2013 by selecting the column containing the list and then using the elimination shortcut "**Alt+A+M**". The miRNA list was saved in Excel 2013 for further analysis.

2.2.1.1. miRNA prioritisation

A shortened list of miRNAs were obtained (i) after target prediction and (ii) if human miRNAs were not 100 % homologous to rats. In the case of elimination of miRNAs after target prediction, miRNAs were eliminated if it did not generate a

UNIVERSITY of the
WESTERN CAPE

predicted target gene list, or if the target prediction software did not generate a statistically significant gene list using the cut-off criteria of the three algorithms (see section 2.2.2). Lastly, 100 % homology of the mature human miRNA sequences to rats were only considered since molecular validation (see Chapter 3) was carried out using rat kidney tissue to compare differential expression levels of the putative T2DM miRNAs to those already validated in serum/plasma obtained from the two databases (see Figure 2.1).

Mature miRNA sequences of interest were obtained for both humans and rats from miRBase 20 (http://www.mirbase.org/) (Kozomara and Griffiths-Jones, 2011). The sequences of each specific miRNA from rats and humans were pairwisely aligned using the ClustalW tool (Larkin et al, 2007). ClustalW is a global sequence alignment tool (Holland et al, 2013) used for aligning nucleotide sequences of interest (Larkin et al, 2007). Only sequences with a score of 100 % was considered for further analysis in this investigation. A list of miRNAs were generated after applying the above-mentioned criteria.

UNIVERSITY of the
WESTERN CAPE

2.2.2. Target prediction

Target prediction was performed for each of the miRNAs obtained, as described in the previous section. The target prediction programs used were (i) TargetScan Human, (ii) PITA, and (iii) Diana Micro-T. A brief description is given for each of the target prediction program is given below.

2.2.2.1. TargetScan Human

The algorithm in TargetScan Human 6.2 bases its scoring of the miRNA target gene depending on the level of conserved 8-mer and 7-mer sites that match the seed

region of each miRNA (Li et al, 2014). The specificity of the target site when using TargetScan is based on the following seven determinants:

i. Type site contribution, which determines the score for both 7- and 8-mer motifs. 8-mer motifs are allotted a higher score as they are more down-regulated than those with 7-mer motifs (Radfar, 2014)
ii. Conserved complementary sites at the 3' end (Fendler, 2011; Radfar, 2014), as it improves down-regulation (Radfar, 2014)
iii. AU content 30 nucleotides up- and downstream of the predicted site, flanking the seed region (Fendler, 2011; Witkos et al, 2011; Reyes~Herrera and Ficarra, 2012; Radfar, 2014)
iv. Target site position contribution i.e. the further away the target site is positioned away from the centre of the 3'-UTR, the more favourable the score (Radfar, 2014)
v. Target-site abundance: miRNAs with target sites enriched in many mRNA target genes are considered as weak regulators and would weaken the effect of its proposed target gene (Reyes~Herrera and Ficarra, 2012; Radfar, 2014)
vi. Seed pairing stability (Reyes~Herrera and Ficarra, 2012; Radfar, 2014)
vii. Conservation branch length for each site (Radfar, 2014)

UNIVERSITY of the
WESTERN CAPE

All determinants, except for conservation score, are scored individually and then tallied up to achieve the final context score (Radfar, 2014). It should be noted that a more negative context score is associated with a more favourable binding target site (Garcia et al, 2011). TargetScan also ranks the target genes according to their probability of conserved targeting (P_{CT}). P_{CT} values with a lower probabilistic value

would decrease the integrity of conservation for the predicted binding target site across multiple species (Carroll et al, 2012).

2.2.2.2. Diana Micro-T

Diana Micro-T makes use of an algorithm based on several parameters calculated individually for each miRNA (Migliore, 2010). It combines conserved and non-conserved miRNA recognition elements into a final prediction score (Migliore, 2010). A minimum of seven nucleotides is required by this prediction program to select a potential target (Vejnar, 2012). The significance of a predicted target gene is based on the conservation score which is attributed to every possible target site if found at the same position across multiple species following sequence alignment (Vejnar, 2012), a signal-to-noise ratio (SNR) (Migliore, 2010; Vejnar, 2012), which determines the ratio between the conservation score and the score of randomized miRNA (Vejnar, 2012), and the final gene list output is ranked according to its minimum energy of potential (mitG) (Witkos et al, 2011). Lastly, this software program also allows for the identification of putative miRNAs for specific target genes (Abdullayev, 2010).

UNIVERSITY of the
WESTERN CAPE

2.2.2.3. PITA

The algorithm used in PITA, focuses primarily on the target accessibility that is connected to the secondary RNA structure of the transcript (Hinske, 2009; Witkos et al, 2011). It is assumed that the mRNA structure plays a role in target recognition by thermodynamically promoting or disfavouring binding interaction (Witkos et al, 2011). Therefore, the algorithm first identifies potential matches by aligning the seed region to the 3'-UTR of its potential target gene. Thereafter, it calculates and combines thermodynamic scores for each potential binding site of the miRNA in order to obtain a unique score for a miRNA target interaction (Hinske, 2009; Witkos

et al, 2011). Unlike TargetScan and Diana Micro-T, it does not require cross-species conservation scores (Hinske, 2009).

2.2.2.4. Target Prediction procedure

Target prediction was based on a modified methodology described by Masotti and Alisi (2013). Target sites of miRNAs were predicted using the three abovementioned, publically available miRNA target prediction software: (1) TargetScanHuman 6.2 (http://www.targetscan.org/vert_61/) (Grimson et al, 2007; Friedman et al, 2009), (2) Diana Micro-T v3.0 (http://diana.cslab.ece.ntua.gr/microT/) (Maragkakis et al, 2009a; Maragkakis et al, 2009b), and (3) PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html) (Kertesz et al, 2007). Thus, for each miRNA, three gene lists were obtained. Furthermore, separate gene lists were obtained i.e. for miRNAs that were up-regulated and down-regulated, respectively (Masotti and Alisi, 2013). Highly reliable target genes for each miRNA were selected based on the following criteria:

UNIVERSITY of the
WESTERN CAPE

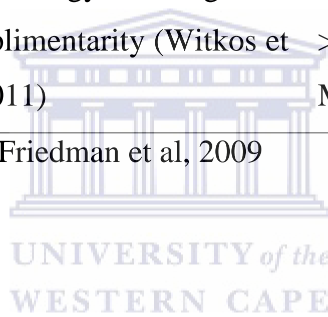
i. Genes were only considered from each respective prediction programme if they fell above a certain recommended cut-off (see Table 2.2 below)

ii. Genes were considered only if predicted by all three algorithms

Table 2.2: An overview of the recommended cut-offs for the target gene selection from the three target prediction algorithms used in this study (Adapted from Witkos et al, 2011)

Program	Brief description	Data selection criteria
TargetScan Human v. 6.2	Seed match, 3'-UTR complementarity local AU content and position contribution (Witkos et al, 2011; Van Rooij, 2011)	Predictions with a context score* ≤ 0.4 and percentile > 85 or an aggregate P_{CT**} value of 0.8 (Lu and Clark, 2012)
PITA	Target site accessibility energy (Witkos et al, 2011)	Predictions with a score < -10 (Meunier et al, 2013)
Diana micro-T v. 3.0	Free energy binding and complimentarity (Witkos et al, 2011)	Predictions with mitG score > 20 (Raghavan and Manasa, 2012)

*Grimson et al, 2007; **Friedman et al, 2009



Thereafter, the genes obtained were combined and checked for duplicates and eliminated (Shinde et al, 2013) in MS Excel 2013 by selecting the column containing the list and then using the elimination shortcut "**Alt+A+M**". The remaining list of ranked genes were saved in an .xls format. Thereafter, the gene list was sorted into two lists i.e. up-regulated miRNA target genes and down-regulated miRNA target genes (Masotti and Alisi, 2013).

Before functional annotation, the ranked genes were compared against 532 known genes involved in T2DM, obtained from the Type 2 Diabetes genetic association database (T2DGAD) (http://t2db.khu.ac.kr:8080/) (last updated in 2010) (Lim et al, 2010) to investigate whether the predicted target genes were previously linked to T2DM. The VENNY tool v. 2.0 (http://bioinfogp.cnb.csic.es/tools/venny/)

(Oliveros, 2007) was used to create Venn diagrams. The full 532 gene list for the T2DM susceptibility genes can be found in Appendix A.

2.3.3. Functional annotation of predicted target genes and miRNAs

Functional annotation was done separately for each gene list using the clustering tool available on DAVID (The Database for Annotation, Visualization and Integrated Discovery) (http://david.abcc.ncifcrf.gov/) (Huang et al, 2009a; Huang et al, 2009b). The clustering tool grouped genes that may be linked biologically i.e. similar, redundant and heterogeneous genes will be grouped in the same cluster (Huang et al, 2014). Default parameters were used for functional annotation i.e. classification parameters were set to "Medium" and raw p-values were used (Huang et al, 2014). Moreover, gene clusters with an enrichment score of > 1.3 (Huang et al, 2014) for biological processes ("GO-TERM-BP") and molecular functions ("GO-TERM-MF") were chosen. This process prioritised the target gene lists even further. The shortened gene lists were once again subjected to the VENNY tool (Oliveros, 2007) and compared to the list of 532 T2DM susceptible genes. The genes that overlapped were especially considered as significant and will be validated for future studies.

UNIVERSITY of the
WESTERN CAPE

In a parallel approach, Diana-mirPath v.2.0 (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=mirpath/index) (Vlachos et al, 2012) was used to determine putative functions of the final up-regulated and down-regulated miRNAs, respectively. The possible functions for the one down-regulated miRNA (miR-*dr*-1) and the three up-regulated miRNAs (miR-*ur*-1, miR-*ur*-2, and miR-*ur*-3), were queried separately using default settings. Predicted functions were considered significant if p > 0.001 (Hu et al, 2011). Functions annotated for the miRNAs obtained from Diana-mirPath was then compared to functions annotated

to the predicted target genes using DAVID (Hu et al, 2011) to observe if there were any overlaps.

2.3.4. Network analysis and pathway enrichment analysis

The STRING version 10 (Search Tool for the Retrieval of Interacting Genes) (http://string-db.org/) database (Szklarczyk et al, 2014) was used to identify possible protein interactions between the predicted target genes. This database provides both experimental and predicted interaction information (Liu et al, 2012) collected from high-throughput experiments, co-expression analyses, genomics, and published literature (Calvert-Joshua, 2013). Separate queries of the differentially expressed target gene lists were mapped to STRING for KEGG pathway and gene network enrichment, respectively. The genes were cut from Excel and pasted into the query window, under the “multiple names” tab in STRING. For pathway analyses $p < 0.05$ were considered as significant. All interaction analyses were carried out using default settings i.e. interactions with a confidence score > 0.4.

UNIVERSITY of the
WESTERN CAPE

2.4. Results and Discussion

2.4.1. MiRNA dataset selection

Lists of 11 and ten miRNAs from HMDD and miR2disease, respectively, were obtained (Table 2.3). The two lists were combined and duplicate miRNAs were eliminated, which resulted in a final list of 20 miRNAs in total. Thereafter, 13 miRNAs were eliminated because the three target prediction programs used in this study failed to predict targets, producing a list of seven miRNAs. This list was shortened even further if the mature miRNA sequences were not 100 %

homologous to those in rats (see section 2.2.1.1), thus, resulting in the elimination of three miRNAs. A total of four miRNAs was identified as significant for further analysis in this investigation i.e. one down-regulated (*dr*) miRNA i.e. miR-*dr*-1 and three up-regulated (*ur*) i.e. miR-*ur*-1, miR-*ur*-2, and miR-*ur*-3. The nomenclature for each of the four miRNAs used throughout this thesis will be referred to as miR-*dr*-1, miR-*ur*-1, miR-*ur*-2, and miR-*ur*-3.

Table 2.3: Summary of miRNA prioritisation

Procedure	Number of miRNAs eliminated	Total miRNAs
miRNA collection from databases	0	21
Elimination of duplicates	1	20
Target prediction	13	7
Homology	3	4*

*MicroRNAs of interest

UNIVERSITY of the WESTERN CAPE

2.4.2. Target gene prediction

A total of 10, 312 genes were obtained after integrating the three aforementioned target prediction software programs, shown in Table 2.4 below. Furthermore, duplicates were removed and the remaining genes were only retained if they appeared in all three prediction algorithms, bringing the putative target gene list to 104 genes in total. It should be noted that target prediction was done separately for up- and down-regulated miRNAs. Hence, 46 and 58 genes for the down- and up-regulated miRNAs, respectively, were obtained after gene ranking. Three genes (PPARA, LDLR, CAMTA1) and two genes (IGF2BP2 and ANK2) were found to

overlap with known genes linked to T2DM, obtained from T2DGAD (Lim et al, 2010), for the down-regulated miRNA and up-regulated miRNAs, respectively (see Figure 2.2). A brief description is given for each of these genes below.

Table 2.4: Raw total of target genes obtained after target prediction before elimination of duplicates and functional analysis

Database	Number of genes
Target Scan	6036
PITA	3336
Diana Micro-T	940

UNIVERSITY of the
WESTERN CAPE

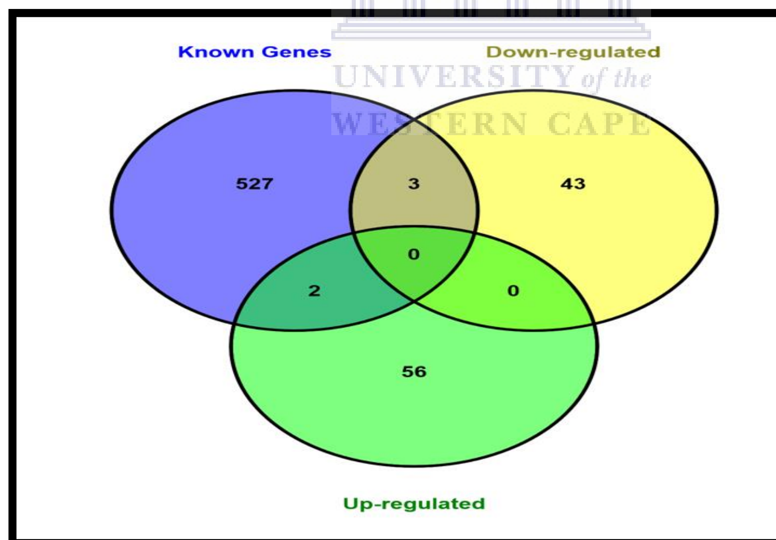


Figure 2.2: Comparison of known susceptible genes linked with T2DM (blue) to the predicted up- (green) and down-regulated (yellow) gene lists before functional annotation. There are three genes (PPARA, LDLR, CAMTA1) and two genes (IGF2BP2 and ANK2) overlapping from down- and up-regulated, respectively.

2.4.2.1. LDLR

Insulin resistance and T2DM are linked with clustering of interrelated plasma and lipoprotein abnormalities (Krauss, 2004). This includes reduced levels in high-density lipoproteins (HDL) ("good cholesterol") and elevated levels of low-density lipoproteins (LDL) ("unhealthy cholesterol") and triglyceride levels (Krauss, 2004).

Low-density lipoprotein receptor (LDLR) play a key role in regulating cholesterol metabolism by removing excess LDL cholesterol from the blood (Ye et al, 2014). Mutations of genes involved in lipoprotein and lipid metabolism, plays a crucial role in the susceptibility of developing many cardiovascular diseases (Krauss, 2004; Ye et al, 2014) such as coronary heart disease (Ye et al, 2014). This is due to the LDLR playing a pathogenic role in familial hypercholesterolemia, and therefore high levels of LDLR in the blood can cause abnormal cholesterol metabolism (Ye et al, 2014). Moreover, past research has found that polymorphisms of LDLR were linked with T2DM and hypertension (Ye et al, 2014).

UNIVERSITY of the
WESTERN CAPE

2.4.2.2. PPARA

The peroxisome proliferator-activated receptor (PPAR) subfamily was shown to be valuable pharmacological targets, whereby its activation can normalize metabolic dysfunctions and somewhat reduce the risk of the development of cardiovascular diseases linked with T2DM (Gross and Staels, 2007). PPAR-alpha (PPARA) agonists, if used as a therapeutic target, has shown to correct dyslipidaemia (Gross and Staels, 2007). In humans, if this gene is activated, it causes a decrease of triglycerides in plasma as well as an enhancement of HDL cholesterol (Gross and Staels, 2007).

PPARA is mainly expressed in tissues with high levels of fatty acid catabolism, such as the liver, heart, kidney and skeletal muscle (Gross and Staels, 2007). Its main role is to regulate the expression of genes involved in lipid and lipoprotein metabolism (Gross and Staels, 2007). Moreover, PPARA plays an important role in glucose metabolism, the inflammatory response, and energy homeostasis (Lacquemant et al, 2007).

A study by Lacqemant et al (2007) failed to show a link between PPARA variants and diabetes, suggesting that it does not have a major role in the eventual development of diabetes. Despite their findings, they stated that PPARA could not be totally excluded, as they could play a minor role in the risk of diabetes development through the variation of atherogenic plasma lipids (Lacquemant et al, 2007).

Alternatively, a study by Bernal-Mizrachi et al (2003) identified hepatic activation of PPARA as a mechanism involved in glucocorticoid-induced insulin resistance. The study was performed by treating mice deficient in LDLR (Ldlr -/-) and with Ppara +/+, or without Ppara (-/-), with dexamethasone (glucocorticoid treatment). Only Ppara +/+ developed hyperglycaemia, hyperinsulinaemia, and hypertension – which are common side effects of glucocorticoid treatment (Bernal-Mizrachi, 2003). Furthermore, it was found that that hepatic gluconeogenic expression was increased, thus, causing the suppression of insulin-mediated endogenous glucose production to be less effective in Ppara +/+ mice (Bernal-Mizrachi, 2003).

2.4.2.3. CAMTA1

Calmodulin-binding transcription activator 1 (CAMTA1) has been examined as a risk factor for the development for T2DM (Miller et al, 2011). A study by Cauchi

et al (2008) was conducted on the French population in which a risk analysis was done on the loci susceptible for the development of T2DM. CAMTA1 was one of the genes mentioned in this study, however, the results obtained were not significant enough. It was explained that this was possibly due to the minor role that the gene could play in the development of diabetes (Cauchi et al, 2008). Currently, not much research has been done on this gene with regards to its role in T2DM pathogenesis.

2.4.2.4. IGF2BP2

The insulin-like growth factor 2 mRNA-binding protein 2 (IGF2BP2) gene is involved in insulin secretion, and is especially important for glucose metabolism (Kommoju et al, 2013). Moreover, IGF2BP2 found in pancreatic and adipose tissues can down-regulate IGF2 (insulin-like growth factor 2), a growth factor which plays an important role in controlling adipogenesis and pancreatic development (Wu et al, 2014). Therefore, the formulation of the hypothesis that IGF2BP2 may contribute to the pathogenesis of T2DM through impaired β-cell function or alterations in adipose tissue was made (Wu et al, 2014).

UNIVERSITY of the
WESTERN CAPE

Wu et al (2014) conducted a study where they analysed the interaction of obesity and IGF2BP2 variant (rs4402960) in T2DM susceptibility. It was found that obese individuals had an elevated risk for the development of T2DM. Therefore, it was speculated that there is some interaction between obesity and IGF2BP2. The exact method for this interaction is not yet elucidated (Wu et al, 2014). Statistically, however, the link between obesity and IGF2BP2 was not significant due to the small sample size (Wu et al, 2014).

Other studies have been done on this gene and T2DM, and all studies reported conflicting results regarding the nature of how the gene links up to T2DM

susceptibility (Kommoju et al, 2013). Kommoju et al (2013) explains that the possible reasons for the inconsistencies in these studies, could be due to sample size and ethnic variability.

2.4.2.5. ANK2

It was found that ankyrin 2 (ANK2) is needed for cholinergic augmentation of insulin release and acts through stabilization of Inositol 1,4,5-trisphosphate (IP3) receptors within pancreatic β-cells (Bennett, 2010). A study by Healy et al (2010) found that ANK2-deficient islets resulted in impaired insulin secretion. Additionally, it was found that the mutation of R1788W of ANK2 is a potential risk factor for developing T2DM in 1 % of Caucasian and Hispanic adults (Bennett, 2010).

2.4.3. Functional annotation of predicted target genes and miRNAs

2.4.3.1. Target genes

UNIVERSITY of the

With the aid of the clustering tool in DAVID, clusters with an enrichment score > 1.3 for biological processes and molecular functions (done separately for each gene list) were only considered. The results in Table 2.5 and Table 2.6, respectively, shows that the down- and up-regulated miRNA gene lists had two enriched clusters after the characterisation of biological processes. Most of the down-regulated genes were involved in biological processes such as regulation of transcription and neuromuscular processes. Whereas, the up-regulated genes were involved in camera eye-type development and nerve transmission, which was considered significant as it is well known that nerve damage and retinopathy are two of the irreversible complications linked with T2DM as mentioned in Chapter 1. The results for molecular function categorization (Table 2.7) in the down-regulated list was consistent with the biological process assigned for the predicted targets, as

cluster 2 was predicted to participate in transcription. Moreover, it was enriched in the metal/cation binding group.

UNIVERSITY of the
WESTERN CAPE

Table 2.5: Functional annotation clustering of down-regulated miRNA predicted target genes for biological process characterisation

Annotation Cluster 1	**Enrichment Score: 1.71**		
Category	**Term**	**Count**	**PValue**
GOTERM_BP_FAT	GO:0045944~positive regulation of transcription from RNA polymerase II promoter	6	0.0032
GOTERM_BP_FAT	GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	7	0.0064
GOTERM_BP_FAT	GO:0051173~positive regulation of nitrogen compound metabolic process	7	0.0075
GOTERM_BP_FAT	GO:0010604~positive regulation of macromolecule metabolic process	8	0.0077
GOTERM_BP_FAT	GO:0010557~positive regulation of macromolecule biosynthetic process	7	0.0081
GOTERM_BP_FAT	GO:0045893~positive regulation of transcription, DNA-dependent	6	0.0092
GOTERM_BP_FAT	GO:0051254~positive regulation of RNA metabolic process	6	0.0095
GOTERM_BP_FAT	GO:0031328~positive regulation of cellular biosynthetic process	7	0.0100
GOTERM_BP_FAT	GO:0009891~positive regulation of biosynthetic process	7	0.0107
GOTERM_BP_FAT	GO:0045941~positive regulation of transcription	6	0.0180
GOTERM_BP_FAT	GO:0010628~positive regulation of gene expression	6	0.0201
GOTERM_BP_FAT	GO:0006355~regulation of transcription, DNA-dependent	10	0.0456
GOTERM_BP_FAT	GO:0006357~regulation of transcription from RNA polymerase II promoter	6	0.0464
Annotation Cluster 2	**Enrichment Score: 1.37**		
Category	**Term**	**Count**	**PValue**
GOTERM_BP_FAT	GO:0050885~neuromuscular process controlling balance	3	0.0040
GOTERM_BP_FAT	GO:0050905~neuromuscular process	3	0.0124
GOTERM_BP_FAT	GO:0030534~adult behavior	3	0.0230

UNIVERSITY of the
WESTERN CAPE

Table 2.6: Functional annotation clustering of up-regulated miRNA target genes for biological process characterisation

Annotation Cluster 1	Enrichment Score: 1.72		
Category	Term	Count	PValue
GOTERM_BP_FAT	GO:0043010~camera-type eye development	4	0.0044
Annotation Cluster 2	**Enrichment Score: 1.31**		
Category	**Term**	**Count**	**PValue**
GOTERM_BP_FAT	GO:0007268~synaptic transmission	5	0.0134
GOTERM_BP_FAT	GO:0019226~transmission of nerve impulse	5	0.0228

UNIVERSITY of the
WESTERN CAPE

2.4.3.2. MicroRNA functional analysis

Similarly, based on the Diana-mirPath algorithm, miR-*dr*-1 was significantly enriched in pathways that are related to "neurotrophin signalling" and "ubiquitin mediated proteolysis", "hepatitis C", and "chronic myeloid leukemia" (Figure 2.3 B). Alternatively, the up-regulated gene list was not able to calculate molecular function clusters that were > 1.3. However, based on the functional analysis of miR-*ur*-1, miR-*ur*-2 and miR-*ur*-3, using Diana mirPath, possible molecular pathways which links it to T2DM were identified, which included: PI3K-Akt signalling pathway, MAPK signalling pathway, and the neurotrophin signalling pathway.

This study further justified that there is a link between the functions of the four miRNAs to the functions of their target genes (see section 2.4.3.2) i.e. the target genes identified are also involved in nerve signalling and transcription. Previous studies have shown that there is a link between transcription and the ubiquitin proteasome system (UPS) (Geng et al, 2012).

UNIVERSITY of the
WESTERN CAPE

2.4.3.3. The ubiquitin proteasome system

The UPS is involved in many gene regulation mechanisms (Geng et al, 2012; Gao et al, 2014) and serves as a mechanism to modify cellular and protein functions such as cell signalling, DNA repair, cell cycle progression and apoptosis (Gao et al, 2014). Furthermore, both proteolytic and nonproteolytic activities of UPS impacts the transcriptional process (Geng et al, 2012). Ubiquitin and ubiquitin-like modifications has been recognised as one of the important regulatory events in the development of several diseases (Balasubramanyam et al, 2005; Gao et al, 2014). Since the UPS is linked with protein degradation, dysregulation of this system would lead to inappropriate degradation of specific proteins, resulting in the development of various diseases (Balasubramanyam et al, 2005). For example,

studies have identified that inappropriate degradation of insulin signalling molecules i.e. insulin receptor substrate (IRS) -1 and -2, in the presence of up-regulated cytokine signalling suppressors, has been observed in diabetic experiments (Balasubramanyam et al, 2005). Therefore, it is assumed that UPS may be linked to insulin resistance (Balasubramanyam et al, 2014).

Other studies have shown members associated with the UPS i.e. cullin-1, cullin-3, and the 11S proteasome regulators – PA28-β and PA28-γ – are up-regulated in the renal capillaries of mice with diabetic nephropathy. Diabetic nephropathy is one of the common irreversible microvascular complications linked with T2DM (see section 1.5) (Gao et al, 2014).

The lists obtained from the molecular function and biological process categorization (in the case of the down-regulated gene list) was combined in DAVID and saved for further analysis in an .xls format. Whereas, only the biological processes target gene list for the up-regulated miRNA, were saved in an .xls format (due to it not retrieving a statistically significant molecular functions in DAVID).

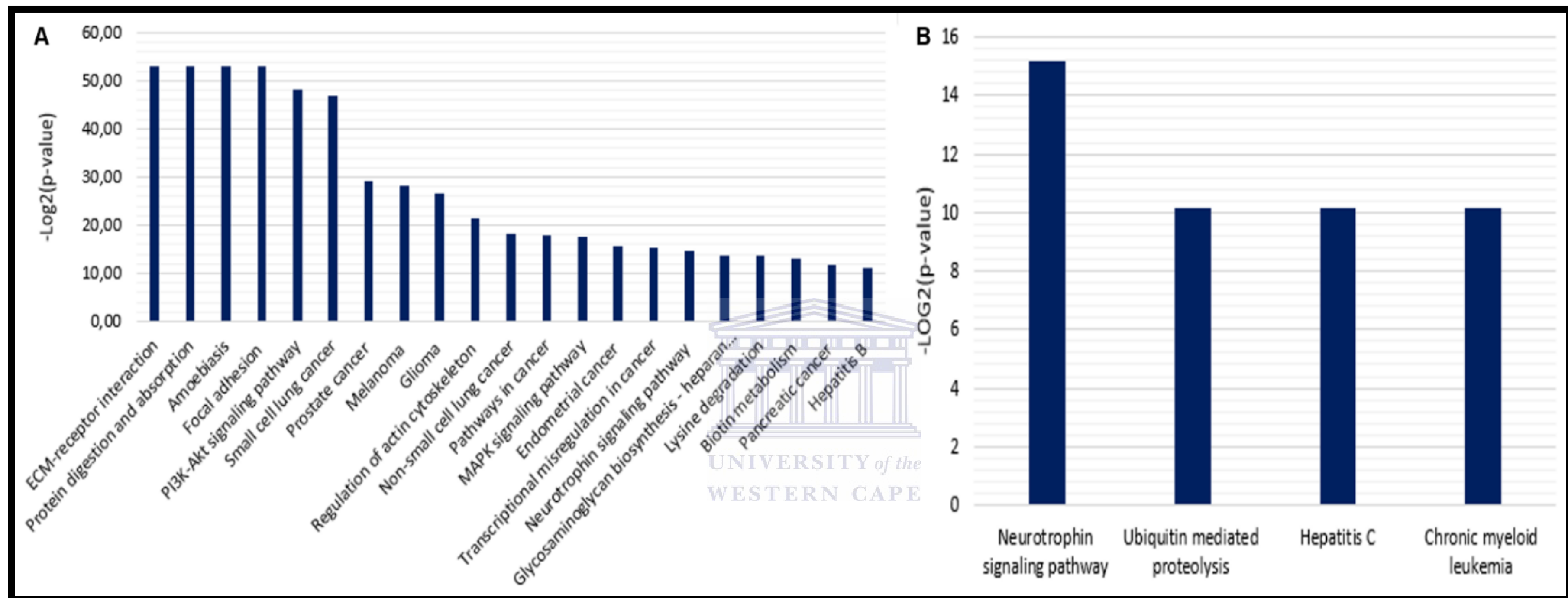
The final gene list for both up- and down-regulated genes is provided in Table 2.8. More specifically, miR-*ur*-1 is predicted to target FOXP2, SIX5 and CACNB2; miR-*ur*-2 is predicted to target COL2A1, ATXN1 and KLF4; and miR-*ur*-3 is predicted to target PVRL1, SCN2B and SYT1. The three genes from the down-regulated gene list were still present after functional annotation, whereas, no up-regulated genes were found to overlap with the known genes involved in T2DM (Figure 2.4).

UNIVERSITY of the WESTERN CAPE

Table 2.7: Functional annotation clustering of down-regulated genes for molecular function characterisation

Annotation Cluster 1	Enrichment Score: 1.57		
Category	**Term**	**Count**	**PValue**
GOTERM_MF_FAT	GO:0046872~metal ion binding	20	0.0107
GOTERM_MF_FAT	GO:0043169~cation binding	20	0.0119
GOTERM_MF_FAT	GO:0043167~ion binding	20	0.0141
GOTERM_MF_FAT	GO:0046914~transition metal ion binding	14	0.0389
Annotation Cluster 2	**Enrichment Score: 1.41**		
Category	**Term**	**Count**	**PValue**
GOTERM_MF_FAT	GO:0003700~transcription factor activity	8	0.0186
GOTERM_MF_FAT	GO:0030528~transcription regulator activity	10	0.0233

UNIVERSITY of the WESTERN CAPE



UNIVERSITY of the
WESTERN CAPE

Figure 2.3: Functional annotation of **A)** up-regulated miRNAs and **B)** down-regulated miRNAs using Diana-mirPath. Functions with $p < 0.001$ were considered as significant

Table 2.8: Final target gene lists after functional annotation

DOWN-REGULATED GENES		UP-REGULATED GENES	
Gene Symbol	Gene name	Gene Symbol	Gene name
E2F5	E2F transcription factor 5, p130-binding	COL2A1	collagen, type II, alpha 1
MAP3K2	mitogen-activated protein kinase kinase kinase 2	PVRL1	poliovirus receptor-related 1 (herpesvirus entry mediator C)
PRRG1	proline rich Gla (G-carboxyglutamic acid) 1	SCN2B	sodium channel, voltage-gated, type II, beta
NR4A3	nuclear receptor subfamily 4, group A, member 3	ATXN1	ataxin 1
TNKS2	tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase 2	FOXP2	forkhead box P2
APP	amyloid beta (A4) precursor protein	SYT1	synaptotagmin I
ZNFX1	zinc finger, NFX1-type containing 1	SIX5	SIX homeobox 5
BMPR2	bone morphogenetic protein receptor, type II (serine/threonine kinase)	CACNB2	calcium channel, voltage-dependent, beta 2 subunit
YOD1	YOD1 OTU deubiquinating enzyme 1 homolog (S. cerevisiae)	KLF4	Kruppel-like factor 4 (gut)
PPARA	peroxisome proliferator-activated receptor alpha		
METAP1	methionyl aminopeptidase 1		
E2F1	E2F transcription factor 1		
TSHZ3	teashirt zinc finger homeobox 3		
CYBRD1	cytochrome b reductase 1		
PKD2	polycystic kidney disease 2 (autosomal dominant)		
KCNMA1	potassium large conductance calcium-activated channel, subfamily M, alpha member 1		
CAMTA1	calmodulin binding transcription activator 1		
MTMR3	myotubularin related protein 3		
NEUROG2	neurogenin 2		
NPAS2	neuronal PAS domain protein 2		
CYP26B1	cytochrome P450, family 26, subfamily B, polypeptide 1		
PDLIM5	PDZ and LIM domain 5		
LDLR	low density lipoprotein receptor		
RNF38	ring finger protein 38		
HLF	hepatic leukemia factor		
TRPV6	transient receptor potential cation channel, subfamily V, member 6		
ZFYVE26	zinc finger, FYVE domain containing 26		

UNIVERSITY of the
WESTERN CAPE

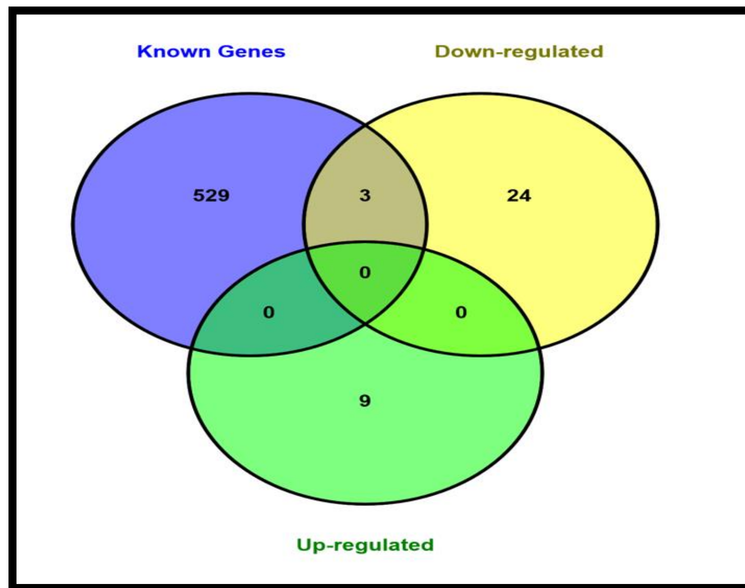


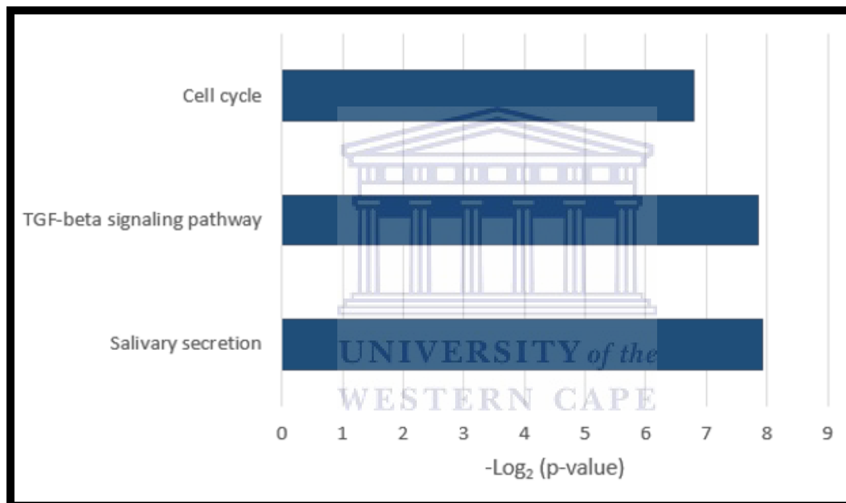
Figure 2.4: Comparison of known susceptible genes linked with T2DM (blue) to the predicted up- (green) and down-regulated (yellow) gene lists after functional annotation. The three genes (PPARA, LDLR, CAMTA1) were observed to still overlap the known genes associated with T2DM. No genes were from the up-regulated gene list overlapped.

UNIVERSITY of the
WESTERN CAPE

2.4.4. Network analysis and pathway enrichment analysis

STRING is a web-based tool to explore gene ontology (GO) annotations, protein-protein interactions (PPI) and KEGG pathway. Therefore, using STRING, KEGG pathway enrichment analysis was performed separately on the up- and down-regulated target gene lists. Based on the results, most of the down-regulated gene variants were projected to be involved in the cell cycle, transforming growth factor (TGF)-β signalling, and salivary secretion pathways (Figure 2.5). Previous studies has identified that TGF-β/Smad3 signalling is involved in insulin gene transcription in the pancreatic β-cells, with Smad3 mediating the expression of TGF-β and that they are involved in the pathogenesis of both obesity and T2DM (Tan et al, 2012).

No significant pathways were identified for the up-regulated gene list, as all the pathways produced p > 0.05. Moreover, STRING analysis of PPI interactions revealed five interactions observed for down-regulated miRNA target genes (Figure 2.6 A). Interestingly, the proteins of the two genes shown for T2DM susceptibility (LDLR and PPARA) are shown to interact with each other. In contrast, only one interaction was observed for the up-regulated miRNA targeted genes (Figure 2.6 B). However, SIX5 and ATXN1 does not seem to play a pathogenic role in T2DM.



UNIVERSITY of the
WESTERN CAPE

Figure 2.5: Graphical display of signalling pathways predicted by STRING for the down-regulated predicted target genes. Analysis of KEGG pathway enrichment of the predicted genes was performed by STRING analysis. Pathways with a p-value of < 0.05 were selected.

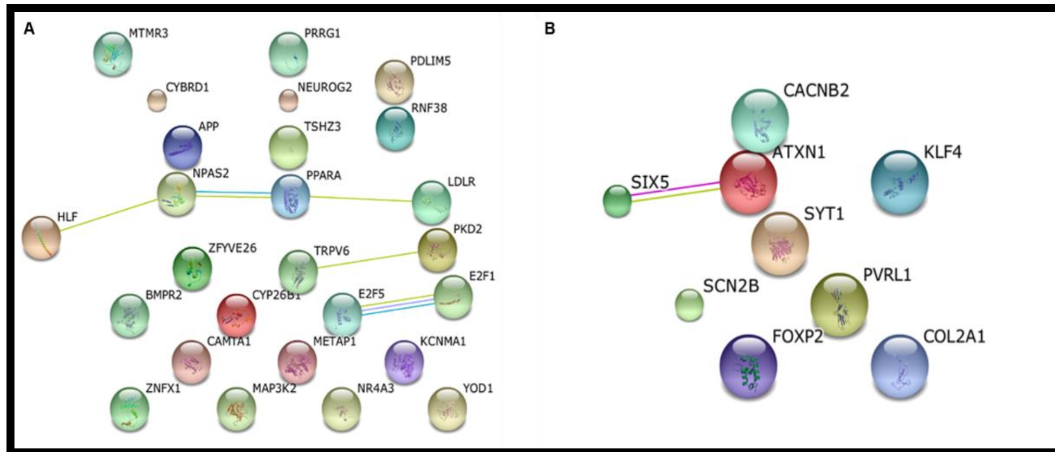


Figure 2.6: STRING analysis of pathway enrichment and interaction in the A) down-regulated and B) up-regulated predicted miRNA target genes. Five protein interactions were observed for the down-regulated target genes, whereas, only one interaction was observed for the up-regulated target genes.

2.5. Summary

UNIVERSITY of the
WESTERN CAPE

In this study, a list of miRNAs found to have differential expression in the serum/plasma of T2DM were obtained, as well as determining their functional relevance by performing *in silico* target analysis.

To date, there have been many publications based on miRNA target prediction. However, there is still uncertainty about which method gives the best results with the least amount of false-positive target genes (Leitner, 2009). It has been suggested that the main cause of this uncertainty is due to the small size and binding behaviour of the mature miRNA in animal genomes (Williamson, 2012). Despite this disadvantage, the main reason for target prediction is to determine the possible functions and pathways of miRNA (Fendler, 2010; Keller et al, 2014).

2.5.1. MiRNA identification and target prediction

Masotti and Alisi (2014) incorporated stringent parameters in order for the researcher to obtain both a shorter miRNA list and target gene list. Moreover, compared to most methods for miRNA and target gene prioritization, this method was more simplified. Using a modified methodology for this investigation, a number of four miRNAs i.e. miR-*dr*-1, miR-*ur*-1, miR-*ur*-2, and miR-*ur*-3 was identified. All four of these miRNAs were found to be involved in the insulin signalling pathway. Furthermore, the target gene list obtained from Diana Micro-T, PITA, and TargetScan was shortened from ~ 10, 000 genes to 46 genes and 58 genes for the down-regulated miRNA and up-regulated miRNAs, respectively. The down-regulated miRNA target gene list had three known genes found to play a role in T2DM: PPARA, LDLR and CAMTA1. Whereas, the up-regulated miRNA target gene list had two known genes: IGF2BP2 and ANK2.

UNIVERSITY of the
WESTERN CAPE

The miRNA of particular interest was miR-*dr*-1, due it being the only one linking the predicted target genes, obtained in this section, to possible molecular functions and biological processes that might be involved in the pathogenesis of T2DM. Furthermore, the target genes LDLR and PPARA seems to be promising targets for miR-*dr*-1, as they seem to be somehow linked to each other, based on the study done by Bernal-Mizrachi (see section 2.4.2.2) and based on STRING analysis in section 2.4.1.

2.6. References

- Abdullayev, I. 2010. *MiR-155 target prediction and validation in nasopharyngeal carcinoma*. MSc Thesis, KTH Computer Science and Communication
- Balasubramanyam, M., Sampathkumar, R. and Mohan, V. 2005. Is insulin signaling molecules misguided in diabetes for ubiquitin–proteasome mediated degradation?. *Mollecular and Cellular Biochemistry,* 275(1-2): 117 – 125
- Bennett, V. 2010. *Ankyrins and Functional Organization of Membrane-Spanning Proteins in Vertebrate Tissues*. [online] Available at: http://www.hhmi.org/research/ankyrins-and-functional-organization-membrane-spanning-proteins-vertebrate-tissues [Accessed 20 April 2015]
- Bernal-Mizrachi, C., Weng, S., Feng, C., Finck, B., Knutsen, R., Leone, T., Coleman, T., Mecham, R., Kelly, D. and Semenkovich, C. 2003. Dexamethasone induction of hypertension and diabetes is PPAR-α dependent in LDL receptor–null mice. *Nature Medicine*, 9(8): 1069 – 1075
- Carroll, A.P., Tran, N., Tooney, P.A. and Cairns, M.J. 2012. Alternative mRNA fates identified in microRNA-associated transcriptome analysis. *BMC Genomics*, 13: 561
- Cauchi, S., Proença, C., Choquet, H., Gaget, S., De Graeve, F., Marre, M., Balkau, B., Tichet, J., Meyre, D., Vaxillaire, M. and Froguel, P. 2008. Analysis of novel risk loci for type 2 diabetes in a general French population: the D.E.S.I.R. study. *Journal of Molecular Medicine (Berlin, Germany*, 86(3): 341 – 348

UNIVERSITY of the
WESTERN CAPE

- da Silva Santos, B.F. 2014. *Immunohistochemistry detection of putative miR-200c and miR-203 targets in breast cancer patients*. MSc Thesis, Universidade Nove de Lisboa
- Fendler, A. 2011. *MiRNAs and prostate cancer: Identification, functional characterization and their potential use in medical practice*. PhD Thesis, Free University of Berlin
- Friedman, R., Farh, K., Burge, C. and Bartel, D. 2008. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Research*, 19(1): 92 – 105
- Gao, C., Huang, W., Kanasaki, K. and Xu, Y. 2014. The role of ubiquitination and sumoylation in diabetic nephropathy. *BioMed Research International*, 2014: 1 – 11
- Garcia, D.M., Baek, D., Shin, C., Bell, G.W., Grimson, A. and Bartel, D.P. 2011. Weak seed-pairing stability and high target-site abundance decrease the proficiency of *lsy-6* and other miRNAs. *Nature Structural & Molecular Biology*, 18(10): 1139 – 1146
- Geng, F., Wenzel, S. and Tansey, W.P. 2012. Ubiquitin and proteasomes in transcription. *Annual Review of Biochemistry*, 81(1): 177 – 201
- Grimson, A., Farh, K., Johnston, W., Garrett-Engele, P., Lim, L. and Bartel, D. 2007. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Molecular Cell*, 27(1): 91 – 105
- Gross, B. and Staels, B. 2007. PPAR agonists: multimodal drugs for the treatment of type-2 diabetes. *Best Practice & Research Clinical Endocrinology & Metabolism*, 21(4): 687 – 710

UNIVERSITY of the
WESTERN CAPE

- Healy, J.A. 2009. *Defining ankyrin-B syndrome: characterization of ankyrin-B variants in mice and men and the discovery of a role for ankyrin-B in parasympathetic control of insulin resistance*. PhD Thesis, Duke University
- Hinske, L. C.G. 2009. *Silencing the host – the role of intronic microRNAs*. MSc Thesis, Massachusetts Institute of Technology
- Hu, H., Guo, S., Xi, J., Yan, Z., Fu, N., Zhang, X., Menzel, C., Liang, H., Yang, H., Zhao, M., Zeng, R., Chen, W., Pääbo, S. and Khaitovich, P. 2011. MicroRNA expression and regulation in human, chimpanzee, and macaque brains. *PLoS Genetics*, 7(10), p.e1002327
- Huang, C., Xiao, X., Chintagari, N., Breshears, M., Wang, Y. and Liu, L. 2014. MicroRNA and mRNA expression profiling in rat acute respiratory distress syndrome. *BMC Medical Genomics*, 7: 46
- Huang, D.W., Sherman, B.T., Lempicki, R.A. 2009a. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. *Nature Protocols*, 4(1): 44 – 57
- Huang, D.W., Sherman, B.T. and Lempicki, R.A. 2009b. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Research*, 37(1): 1 – 13
- Jiang Q., Wang Y., Hao Y., Juan L., Teng M., Zhang X., Li M., Wang G., Liu Y. 2009. miR2Disease: a manually curated database for microRNA deregulation in human disease. *Nucleic Acids Research*, 37(Databases): D98 – D104

UNIVERSITY of the
WESTERN CAPE

- Kertesz, M., Iovino, N., Unnerstall, U., Gaul, U. and Segal, E. 2007. The role of site accessibility in microRNA in microRNA target recognition. *Nature Genetics*, 39(10): 1278 – 1284
- Kozomara, A. and Griffiths-Jones, S. 2011. miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Research*, 39(Database): D152 – D157
- Kuhn, D., Martin, M., Feldman, D., Terry, A., Nuovo, G. and Elton, T. 2008. Experimental validation of miRNA targets. *Methods*, 44(1): 47 – 54
- Kommoju, U., Maruda, J., Kadarkarai, S., Irgam, K., Kotla, J., Velaga, L. and Mohan Reddy, B. 2013. No detectable association of IGF2BP2 and SLC30A8 genes with type 2 diabetes in the population of Hyderabad, India. *Meta Gene*, 1: 15 – 23
- Lacquemant, C., Lepretre, F., Pineda Torra, I., Manraj, M., Charpentier, G., Ruiz, J., Staels, B. and Froguel, P. 2000. Mutation screening of the PPAR a gene in type 2 diabetes associated with coronary heart disease. *Diabetes & Metabolism*, 26(5): 393
- Leitner, A. 2009. *MicroRNA target prediction*. BSc Thesis, Graz University of Technology
- Lekprasert, P. 2012. *MicroRNA target prediction via duplex formation features and direct binding evidence*. PhD Thesis, Duke University
- Li, X., Sun, R., Chen, W., Lu, B., Li, X., Wang, Z. and Bao, J. 2014. A systematic *in silico* mining of the mechanistic implications and therapeutic potentials of estrogen receptor (ER)-α in breast cancer. *PLoS ONE*, 9(3): 91894

UNIVERSITY of the
WESTERN CAPE

- Li, Y., Qiu, C., Tu, J., Geng, B., Yang, J., Jiang, T. and Cui, Q. 2013. HMDD v2.0: a database for experimentally supported human microRNA and disease associations. *Nucleic Acids Research*, 42(Database): D1070 – D1074
- Lim, J., Hong, K., Jin, H., Kim, Y., Park, H. and Oh, B. 2010. Type 2 diabetes genetic association database manually curated for the study design and odds ratio. *BMC Medical Informatics & Decision Making*, 10(1): 76
- Liu, D., Wu, J., Wang, J., Liu, Q., Gao, Z., Liu, Y. 2012. MicroRNA expression profile analysis reveals diagnostic biomarker for human prostate cancer. *Asian Pacific Journal of Cancer Prevention*, 13: 3313 – 3317
- Lu, J. and Clark, G. 2012. Impact of microRNA regulation on variation in human gene expression. *Genome Research*, 22: 1243 – 1254
- Maragkakis, M., Alexiou, P., Papadopoulos, G., Reczko, M., Dalamagas, T., Giannopoulos, G., Goumas, G., Koukis, E., Kourtis, K., Simossis, V., Sethupathy, P., Vergoulis, T., Koziris, N., Sellis, T., Tsanakas, P. and Hatzigeorgiou, A. 2009a. Accurate microRNA target prediction correlates with protein repression levels. *BMC Bioinformatics*, 10(1): 295
- Maragkakis, M., Alexiou, P., Papadopoulos, G., Reczko, M., Dalamagas, T., Giannopoulos, G., Goumas, G., Koukis, E., Kourtis, K., Simossis, V., Sethupathy, P., Vergoulis, T., Koziris, N., Sellis, T., Tsanakas, P. and Hatzigeorgiou, A. 2009b. Accurate microRNA target prediction correlates with protein repression levels. *BMC Bioinformatics*, 10(1): 295
- Masotti, A. and Alisi, A. 2012. Integrated bioinformatics analysis of microRNA expression profiles for an in-depth understanding of pathogenic

UNIVERSITY of the
WESTERN CAPE

mechanisms in non-alcoholic fatty liver disease. *Journal of Gastroenterology and Hepatology*, 27: 331 – 340

- Meunier, J., Lemoine, F., Soumillon, M., Liechti, A., Weier, M., Guschanski, K., Hu, H., Khaitovich, P. and Kaessmann, H. 2013. Birth and expression evolution of mammalian microRNA genes. *Genome Research*, 23(1): 34 – 45
- Migliore, C.M. 2010. *RNA-sequencing based identification of microRNA-204 targets*. PhD Thesis, University of Trieste
- Miller, L.A., Gunstad, J., Spitznagel, M.B., Mccafferey, J., Mcgeary, J., Poppas, A., Paul, R.H., Sweet, L.H. and Cohen, R.A. (2011). CAMTA1 T polymorphism is associated with neuropsychological test performance in older adults with cardiovascular disease. *Psychogeriatrics*, 11(3): 135 – 140
- Oliveros, J.C., 2007. *VENNY. An interactive tool for comparing lists with Venn Diagrams*. [online] Available at: http://bioinfogp.cnb.csic.es/tools/venny/index.html [Accessed on the 30 June 2014]
- Radfar, H. 2014. *Computational prediction of target genes of microRNAs*. PhD Thesis, University of Toronto
- Raghavan, V. and Manasa, D.B. 2012. Computational analysis of human microRNA and its disease target network. *International Proceedings of Chemical, Biological & Environmental*, 20: 154 – 158
- Reyes~Herrera, P. and Ficarra, E. 2012. One Decade of Development and Evolution of MicroRNA Target Prediction Algorithms. *Genomics, Proteomics & Bioinformatics*, 10(5): 254 – 263

UNIVERSITY of the
WESTERN CAPE

- Schmitz, U., Lai, X., Winter, F., Wolkenhauer, O., Vera, J. and Gupta, S. 2014. Cooperative gene regulation by microRNA pairs and their identification using a computational workflow. *Nucleic Acids Research*, 42(12): 7539 – 7552
- Shinde, S., Arora, N. and Bhadra, U. 2013. A complex network of microRNAs expressed in brain and genes associated with Amyotrophic Lateral Sclerosis. *International Journal of Genomics*, 2013: 1 – 12
- Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., Simonovic, M., Roth, A., Santos, A., Tsafou, K., Kuhn, M., Bork, P., Jensen, L. and von Mering, C. 2014. STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Research*, 43(Database): D447 – D452
- Takahashi, P., Xavier, D.J., Evangelista, A.F., Manoel-Caetano, F.S., Macedo, C., Collares, C.V.A., Foss-Freitas, M.C., Foss, M.C., Rassi, D.M., Donadi, E.A., Passos, G.A. and Sakamoto-Hojo, E. 2014. MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus. *Genes*, 539: 213 – 223
- Tan, C.K., Chong, H.C., Tan, E.H.P. and Tan, N.S. Getting 'Smad" about obesity and diabetes. *Nutrition and Diabetes*, 2(3): e29
- Van Rooij, E. 2011. The art of microRNA research. *Circulation Research*, 108: 219 – 234
- Vejnar, C. 2012. *Computational prediction of microRNA targets: thermodynamic, probabilistic and evolutionary models parameterized by genome-scale experimental data*. PhD Thesis, University of Geneva

UNIVERSITY of the
WESTERN CAPE

- Vlachos, I., Kostoulas, N., Vergoulis, T., Georgakilas, G., Reczko, M., Maragkakis, M., Paraskevopoulou, M., Prionidis, K., Dalamagas, T. and Hatzigeorgiou, A. 2012. DIANA miRPath v.2.0: investigating the combinatorial effect of microRNAs in pathways. *Nucleic Acids Research*, 40(Web Server Issue): W498 – W504
- Williamson, V. 2012. *Using next generation sequencing (NGS) to identify and predict microRNAs (miRNAs) potentially affecting schizophrenia and bipolar disorder*. PhD Thesis, Virginia Commonwealth University
- Witkos, T.M., Koscianska, E. and Krzyzosiak, W.J. 2011. Practical aspects of microRNA target prediction. *Current Molecular Medicine*, 11: 93 – 109
- Wu, H., Liu, N., Yang, Z., Tao, X., Du, Y., Wang, X., Lu, B., Zhang, Z., Hu, R. and Wen, J. 2014. IGF2BP2 and obesity interaction analysis for type 2 diabetes mellitus in Chinese Han population. *European Journal of Medical Research*: 19(1): 40

UNIVERSITY of the
WESTERN CAPE

- Ye, H., Zhao, Q., Huang, Y., Wang, L., Liu, H., Wang, C., Dai, D., Xu, L., Ye, M. and Duan, S. 2014. Meta-analysis of low density lipoprotein receptor (LDLR) rs2228671 polymorphism and coronary heart disease. *BioMed Research International*, 2014: 1 – 6
- Yuan, Z., Sun, X., Jiang, D., Ding, Y., Lu, Z., Gong, L., Liu, H. and Xie, J. 2010. Origin and evolution of a placental-specific microRNA family in the human genome. *BMC Evolutionary Biology*, 10(1): 346

Chapter 3: Molecular analysis - validation of the miRNAs of interest

3.1. Introduction

MiRNAs are approximately 18 – 24 nucleotides long (Wu et al, 2007), thus, making them too short for standard and quantitative PCR methods, which require a template that is at least double the size of the forward or reverse primers (i.e. approximately 20 nucleotides long) (Kramer, 2011). Hence, the shortest length the target strand could be is approximately ≥ 40 nucleotides (Kramer, 2011).

Efficient and reliable profile expression methods of miRNAs are an important step in order to understand their roles in specific tissues and cells (Wu et al, 2007). Currently, there are three commonly used high-throughput methods for identifying expression levels of miRNA: (i) microarrays, (ii) next-generation sequencing (NGS) technologies, and (iii) real-time quantitative PCR (qRT-PCR) (Rooij, 2011; Martinez-Sanchez and Murphy, 2013; Dedeoğlu, 2014). A brief description for each of these methods is given below.

UNIVERSITY of the
WESTERN CAPE

i. Microarrays (Hybridzation-based)

Microarrays is a popular method for the identification of biomarkers and therapeutic targets (Dedeoğlu, 2014). Data obtained using this method also aids in the identification of predicting function(s) of specific miRNA targets by comparing the miRNA expression to known mRNA targets and protein profiles (Dedeoğlu, 2014). The steps required for miRNA microarray analysis involve: (i) purification of mature miRNAs from cells or tissues, (ii) enrichment and labelling with a dye

using T4 RNA ligase to attach two flourophore-labelled nucleotides to the 3'-end of the miRNA, and (iii) hybridization to arrays with the appropriate probes (produced from synthetic oligonucleotides or cDNA fragments) specific for the mature miRNA of interest, resulting in the easy detection of the double-stranded fragments (van Rooij, 2011).

It should be noted, however, that this method is not used to obtain quantitative results, but rather to determine the relative change in expression between two states e.g. diseased compared to non-diseased or used to detect the presence of a miRNA of interest (van Rooij, 2011). This is mainly due to the differing binding affinities among miRNAs to their targets (van Rooij, 2011). For this reason, data obtained using this tool for miRNA profiling should be used as a guide and should be validated using other detection methods (e.g. qRT-PCR) (van Rooij, 2011).

ii. Next generation sequencing (NGS)

UNIVERSITY of the WESTERN CAPE

NGS platforms are available for sequencing small RNA molecules, including miRNAs. Unlike microarray analysis, this tool for miRNA expression profiling uses vast parallel sequencing, resulting in the generation of millions of miRNA sequence reads i.e. approximately 3 Gbp of sequence data, from given samples. Furthermore, NGS is more sensitive than microarray analysis and can, thus, measure absolute abundance and allows for the discovery of novel miRNA (van Rooij, 2011; Dedeoğlu, 2014). There are specified criteria involved for defining whether the miRNA sequence of interest is a putative novel miRNA. This includes for the miRNA sequence: (i) to be 22 nucleotides in length that maps precisely to the genome of interest, (ii) to be phylogenetically conserved, and (iii) to form a hairpin structure without large internal loops or bulges (van Rooij, 2011). The main limitation of using this method for miRNA expression profiling is mainly due to a

vast amount of bioinformatics challenges when trying to analyse the 3 Gbp of sequence data appropriately (van Rooij, 2011).

iii. qRT-PCR

Quantitative real-time PCR is considered the gold standard for miRNA expression profiling as it serves as a platform for single reverse PCR amplification studies and for a large number of miRNAs in parallel (van Rooij, 2011; Dedeoğlu, 2014; Stokowy et al, 2014; Usó et al, 2014), by multiplexing and plate-based arrays (Dedeoğlu, 2014). Some of the main advantages using this method includes its high sensitivity, its speed, and it does not require a large volume of RNA (Usó et al., 2014). Additionally, this method is less time-consuming compared to microarrays and NGS and analysis of results do not require to be analysed/processed by biostaticians (Usó et al, 2014).

UNIVERSITY of the
WESTERN CAPE

The principle of qRT-PCR is based on the real-time detection of a reporter molecule where fluorescence intensity exactly correlates to the amount of DNA present in each amplification cycle (Benes and Castoldi, 2010; Usó et al, 2014). TaqMan probes and SYBR Green I is the only fluorescent technologies suitable for miRNA detection when performing qRT-PCR (Benes and Castoldi, 2010; Usó et al., 2014).

Dual-labelled hydrolysis probes (e.g. Taqman probes) are designed to hybridize to an internal stretch of the amplicon. Taqman probes consist of a fluorescent reporter on the 5'-end and a quencher molecule on the 3'-end (Benes and Castoldi, 2010; Usó et al, 2014) (see Figure 3.1 A). If the reporter and quencher are in close proximity to each other, no fluorescence would be emitted (Benes and Castoldi, 2010; Usó et al, 2014). During PCR, the probe and the primers anneal to the target sequence, allowing the Taq polymerase to extend the primer upstream of the probe

(Usó et al, 2014). If the probe is bound to the appropriate target, the Taq polymerase hydrolyses the probe, causing an increase in fluorescence being emitted, which is proportional to the amount of product generated during the PCR process (Usó et al, 2014). The main limitation of using this technology is that Taqman probes are costly, especially when screening for a large number of miRNAs (Wu et al, 2007).

Alternatively, the SYBR Green I assay is more cost-effective. SYBR Green is an intercalating fluorescent dye that binds to all double-stranded DNAs (dsDNAs) (see Figure 3.1 B), as well as to non-specific products such as primer dimers (Usó et al, 2014). Non-specific binding limits the accuracy of this detection method, thus, performing a melting point analysis (or dissociation curve analysis) is very important when using SYBR Green (Benes and Castoldi, 2010; Usó et al, 2014). The purpose for melting point analysis, is to monitor the homogeneity of the PCR products (Benes and Castoldi, 2010). This procedure involves the recording of fluorescence intensity, which is emitted by the SYBR Green intercalated into the PCR products, at different temperatures i.e. from 65 ˚C to 95 ˚C (Benes and Castoldi, 2010).

UNIVERSITY of the
WESTERN CAPE

The increasing temperature eventually denatures the dsDNA, resulting in a reduction of the fluorescent signal. When the strands separate completely, it appears as a sharp drop in signal intensity (Benes and Castoldi, 2010). The number of points of inflection in the melting points indicates the number of PCR products, including primer dimers, produced (Benes and Castoldi, 2010). An acceptable dissociation curve has a single peak, whereas, multiple peaks indicates the presence of primer-dimers or non-specific amplification products (Benes and Castoldi, 2010). Furthermore, stem-loop qRT-PCR enhances sensitivity and specificity of qRT-PCR array methods and enables researchers to analyse and detect a larger number of

mature, processed miRNAs in a single experiment (Czimmerer et al, 2013; Usó et al, 2014).

Stem-loop qRT-PCR assay involves two steps (Figure 3.1 A and B): (i) miRNA-specific stem-loop primer-based reverse transcription (RT) and (ii) quantification of RT products using the SYBR Green I assay or probe assay (Czimmerer et al, 2013). Designing stem-loop primers is more complex and the following components are required (Benes and Castoldi, 2010; Kramer, 2011):

i. A reverse transcription (RT) stem-loop primer, which contains the highly stable stem-loop structure that lengthens the target cDNA from ~22 to > 60 nucleotides.

ii. A forward primer gives the target cDNA extra nucleotide length and increases the melting temperature (Tm) and, thus, enhances the specificity of the assay.

iii. A universal reverse primer, which eventually disrupts the stem-loop as it is complimentary to a sequence within the RT stem-loop primer.

UNIVERSITY of the
WESTERN CAPE

This investigation makes use of the SYBR green assay for profiling miRNA differential expression. An example of how to design the RT stem-loop primer, forward and reverse primers is given in Appendix B.

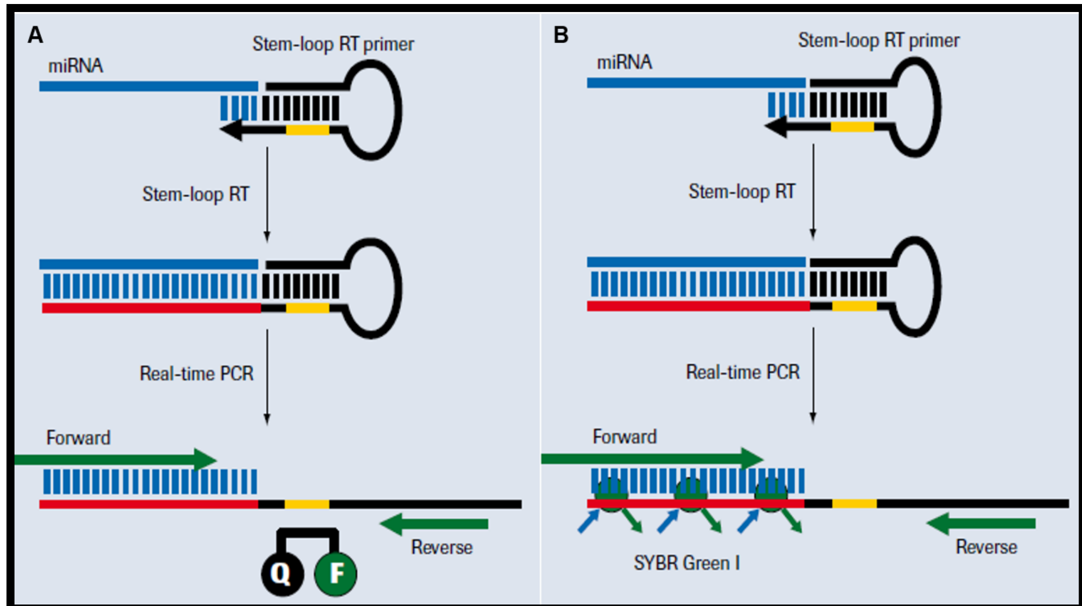
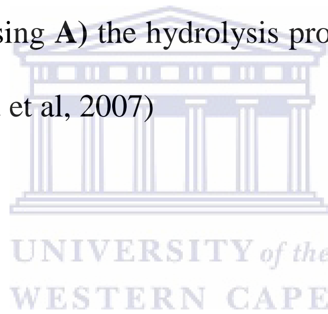


Figure 3.1: Schematic representation of stem-loop qRT-PCR for profiling miRNA differential expression using **A)** the hydrolysis probe assay and **B)** SYBR Green I assay (Adopted from Wu et al, 2007)


UNIVERSITY of the
WESTERN CAPE

3.2. Aims

In Chapter 2, four miRNAs of interest, shown to be differentially expressed in serum/plasma of T2DM individuals, were identified using *in silico* methods. In this chapter, the miRNAs of interest identified in the previous chapter were analysed using qRT-PCR to determine its expression in the kidney tissue of male Wistar rats. The hypothesis was that due to the kidneys playing an important role in cleansing the blood from impurities, it would exhibit similar miRNA expression patterns as found in serum/plasma. The aim for this section, therefore, is to identify and validate differential miRNA expression in rat kidney tissue.

3.3. Molecular materials and methods

3.3.1 Materials and suppliers:

Material	Supplier
SeaKem® LE Agarose	Lasec
Boric acid	Merck
Dimethyl percarbonate (DEPC)	Sigma
EDTA	Saarchem
Gel Loading Dye (6X)	Fermentas
GelRed	Biotium
High-Pure miRNA Isolation Kit	Roche
KAPA SYBR FAST qPCR Kit, Optimised for LightCycler 480	KAPA Biosystems
KAPATaq EXtra HotStart ReadyMix and dye	KAPA Biosystems
Transcriptor First Strand Synthesis Kit	Roche
Tris [Hydroxymethyl] aminoethane (Tris)	Merck
Nuclease-free water	Fermentas
O'GeneRuler™ 50 bp DNA Ladder, Ready-to-use	Thermo Scientific
PCR plates for LightCycler®	Lasec
Platemax sealing film	Lasec
Primers	Inqaba Biotech

UNIVERSITY of the WESTERN CAPE

Qubit® RNA Broad Range Assay Kit	Life Technologies

3.3.2. General Stocks and Solutions

10X TBE:	1.2 M Tris, 1.2 M Boric, 2 mM EDTA
DEPC treated water:	1 ml DEPC dissolved in 1 L of distilled water, incubated at 37 ˚C overnight and autoclaved

3.3.3. List of equipment

LightCycler® 480	Roche
BioSpectrum® Imaging System	UVP
Eppendorf Centrifuge 5417R, F45-30-11 rotor	Sigma-Aldrich
GeneAmp® PCR System 2700	Applied Biosystems
Thermo Scientific NanoDrop ND-1000 Spectrophotometer	Thermo Fisher
Qubit® 2.0 Fluorometer	Life Technologies

UNIVERSITY of the
WESTERN CAPE

3.3.4. Decontamination of glass- and plastic-ware

All glass bottles used were treated with 1 % DEPC-treated water overnight and autoclaved at 120 ˚C for 20 minutes. Mortar and pestles were first washed with dish-washing liquid, then allowed to soak in 0.5 M EDTA overnight, and finally rinsed with water (DEPC-treated (1:100) and autoclaved). The 0.6 ml, 1.5 ml, and

2 ml tubes were designated for RNA only and used straight from the bag. Lastly, pre-packed 10 µl, 20 µl, 200 µl and 1 ml filter tips were used to avoid contamination.

3.3.5. Sample collection

Rat kidney tissue from male Wistar rats was donated from Professor Daneel Dietrich from the Medical Bioscience Department, University of the Western Cape. For this investigation healthy, streptozotocin-induced type 1 diabetic, and high fat diet (HFD) rats' kidney tissue were used. It should be noted that the HFD diet rats served as the T2DM sample. Wistar rats are rendered hypertensive if left on a HFD over a period of time (Huisamen et al, 2013). Furthermore, studies have suggested that there is a link between T2DM and hypertension (Lago et al, 2007).

At the time of tissue harvesting, tissues were flash-frozen in liquid nitrogen and then stored in – 80 ˚C for later usage.

3.3.6. Sample processing and miRNA extraction

UNIVERSITY of the
WESTERN CAPE

Tissue disruption and homogenization was done according to the High Pure miRNA Isolation Kit (Roche). Approximately 30 mg of kidney tissue was first disrupted using a sterilized mortar and pestle by grinding each of the samples in liquid nitrogen to a fine powder. The sample was then transferred into a sterile, RNase-free tube which contained 400 µl of 20 % Binding Buffer (i.e. for one sample 80 µl of Binding Buffer and 320 µl nuclease free water, or Elution Buffer, was mixed together in a sterile, RNase-free tube). The lysate was homogenized by passing it through a sterile syringe and 20-gauge (ø 0.9 mm) needle until a homogenous lysate was achieved. The lysate was centrifuged for two minutes at maximum speed, thereafter the supernatant was collected into a new sterile tube for miRNA isolation.

For miRNA extraction from tissue, the two-column protocol for small RNA isolation was used. All centrifugation steps were done at 4˚ C. A volume of 150 µl lysate was added into a sterile tube along with 312 µl Binding Buffer. The lysate was then vortexed for five seconds three times. Thereafter, the mixed lysate was transferred into the upper reservoir of a High Pure filter tube combined to a collection tube then centrifuged for 30 seconds at 13,000 g for one minute and the flow-through was discarded thereafter.

Thereafter, 500 µl Wash Buffer was added to the column and centrifuged at 13,000 g for 30 seconds, and the flow-through discarded. An additional 300 µl of Wash buffer was added to the column and centrifuged for 30 seconds at 13, 000 g and the the flow-through discarded. The column's filter was then dried by centrifuging for an additional one minute at 13, 000 g.

The filter tube was then placed into a sterile 1.5 ml Eppendorf tube into which 50 µl of Elution Buffer was added and allowed to incubate for one minute at room temperature (i.e. 15˚ C to 25˚C). Thereafter, the tube was centrifuged at 13, 000 g for one minute and the eluted sample was stored at -80˚ C. The filter was placed into a second sterile 1.5 ml Eppendorf tube for a second elution i.e. 50 µl of Elution Buffer was added to the filter tube and then centrifuged for one minute at 13, 000 g. The concentration of each elute was quantified using the Qubit® 2.0 Fluorometer, according to the manufacturers' protocol (Life Technologies, South Africa), and was stored at -80˚ C.

3.3.6.1. Qubit® quantitation

The Qubit® RNA Broad Range (BR) Assay Kit was used for the determination of miRNA concentration. This assay is fluorescence-based and is highly selective for

UNIVERSITY of the WESTERN CAPE

RNA and will not quantitate DNA, protein, or free nucleotides. Moreover, it provides an assay range from 20 – 1000 ng, and the RNA starting concentration can be 1 ng/µl – 1 µg/µl.

Quantitation using this assay was performed at room temperature. The Working Solution was prepared in a sterile 2 ml Eppendorf tube containing BR reagent diluted 1:200 in BR buffer and briefly mixed by vortexing. The 2 Qubit® standards were prepared by adding 190 µl of working solution into 0.6 ml clear-walled, sterile tubes followed by 10 µl of the standard solutions. The standards were vortexed briefly and left to incubate at room temperature for two minutes.

The miRNA samples were prepared by diluting 2 µl of the sample with 198 µl of working solution (i.e. 1:100 dilution) in 0.6 ml clear-walled, sterile tubes. The sample was briefly vortexed and left to incubate at room temperature for two minutes.

UNIVERSITY of the
WESTERN CAPE

Thereafter, the concentration of the standards were first read in order to calibrate the Qubit® 2.0 Fluorometer. Thereafter, the concentrations of the miRNA samples were determined. The Qubit® 2.0 Fluorometer has the option were it calculates the concentration using the following equation:

$$Concentration\ of\ unkwown\ sample = QF\ x\ \frac{200}{x}$$

Where QF = the value given by the Qubit® 2.0 Fluorometer, and x = volume (µl) of sample.

3.3.7. Primer synthesis

Mature miRNA sequences for each of the four miRNAs of interest and reference miRNAs were obtained from miRBase (http://www.mirbase.org/), as described in

section 2.2.1.1. Reference miRNAs used in this investigation shown to be stably expressed in kidney tissue was miR-17 and miRNA-191a (Eskildsen et al, 2013). Moreover, these reference miRNAs were found to be 100 % homologous to rats using the ClustalW sequence alignment tool (see description in section 2.2.1.1). GAPDH was used as a third reference gene, due to it displaying stable expression within kidney tissues as well (Ji et al, 2013).

The miRNA primers used for stem-loop RT-PCR was designed according to Kramer (2011). An example for miRNA primer design is given in Appendix B. Firstly, once the mature miRNA sequence of interest was obtained from miRBase, the RT stem-loop primer was designed. Designing the RT stem-loop primer combined the 44 nucleotide stem-loop sequence designed by Chen et al (2005) i.e. 5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC -3′ region to an additional six nucleotides, complimentary to the 3' nucleotide from the mature miRNA sequence. Secondly, designing the forward primer involved taking the first 12 to 17 nucleotides of the 5' end of the mature miRNA sequence and adding another three to seven random nucleotides to the 5' end in order to increase the melting temperature (Tm) to aproximately 60 ˚C (± 1 ˚C) (Kramer, 2011). The OligoAnalyzer 3.1 tool (https://eu.idtdna.com/calc/analyzer) (Owczarzy et al, 2008) was used to calculate the estimated melting temperature for each forward primer. The reverse primer is universal since all RT stem-loop primers uses the same 44 nucleotide sequence. The sequence of the universal primer as recommended by Kramer (2011) is: 5′-CCA GTG CAG GGT CCG AGG TA-3′.

UNIVERSITY of the
WESTERN CAPE

Reference genes used for normalization in kidney tissue was miR-191a, miR-17 (Eskildsen et al, 2013) and GAPDH (Ji et al, 2013). Primer sequences for reference miRNAs are given in Table 3.1. Primer sequences for GAPDH are as follows:

forward primer – TGATGACATCAAGAAGGTGGTGAAG; reverse primer: TCCTTGGAGGCCATGTGGGCCAT.

Table 3.1: Designed primer sequences for reference genes miR-191a and miR-17

MiRNA name	Primer type	Sequence
rno-MiR-17-5p	Stem-loop RT primer	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TAC CT
	Forward primer	CCG TCA CCA AAG TGC TTA CAG TGC
	Reverse primer	CCA GTG CAG GGT CCG AGG TA
rno-MiR-191a-5p	Stem-loop RT primer	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC AGC TG
	Forward primer	GAC GCT CAA CGG AAT CCC AAA AG
	Reverse primer	CCA GTG CAG GGT CCG AGG TA

All primer oligonucleotide sequences were synthesised by Inqaba biotech (http://www.inqababiotec.co.za/). A 100 µM of stock solution of each primer was prepared in 1X TE buffer (10 mM Tris, pH 7.5 to 8.0, 1 mM EDTA). The concentrated stock oligonucleotide solutions and working stock solutions were stored at -20 ˚C.

UNIVERSITY of the
WESTERN CAPE

3.3.8. cDNA synthesis

The Transcriptor First Strand cDNA Synthesis Kit protocol was slightly modified for cDNA synthesis from miRNA. A cocktail of miRNA-specific stem-loop (SL) primers was prepared (10 µM/primer, six primers in total). In sterile, nuclease-free PCR tubes, 3 µl template miRNA (~200 ng of miRNA), 2 µl SL cocktail primer, and PCR-grade water was added to make up a final volume of 13 µl. The primer mix was briefly centrifuged and then incubated in the GeneAmp® PCR System 2700 thermal block cycler at 65˚ C for five minutes, then placed on ice for two minutes, thereafter (Varkonyi-Gasic et al, 2007). After incubation, 4 µl Transcriptor Reverse Transcriptase Reaction Buffer (1X), 0.5 µl Protector RNase Inhibitor (20

U), 2 µl Deoxynucleotide mix (1 mM each), and 0.5 µl Transcriptor Reverse Transcriptase (10 U) was added to the primer mixture and gently mixed by finger tapping. The tubes were briefly centrifuged to bring all the contents down to the bottom of the tube and then placed in the thermal block cycler to perform pulse RT-PCR at the following parameters according to Varkonyi-Gasic (2007): 30˚ C for 30 seconds, 42˚ C for 30 seconds, and 50˚ C for one second for 60 cycles. The synthesised cDNA was stored at -20˚ C until needed for further analysis by conventional PCR and qRT-PCR.

3.3.9. Conventional PCR

The quality of miRNAs after extraction and cDNA synthesis was checked by performing conventional PCR, to observe if the primers do in fact bind to, and amplified, the cDNA template.

In sterile, nuclease-free PCR tubes, 12.5 µl KAPATaq EXtra HotStart ReadyMix and dye (1X), 1 µl of RT product (~ 100 ng), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM) was added and nuclease-free PCR-grade water to make up the reaction to a final volume of 25 µl. The reactions were placed on the thermal heating block and amplified according to the parameters shown in Table 3.2:

UNIVERSITY of the
WESTERN CAPE

Table 3.2: Parameters used for conventional PCR reactions

Step	Temperature (˚C)	Time	Cycle/s
Initial denaturation	94	2 minutes	1
Denaturation	94	15 seconds	40
Annealing*	65 – 66.6	30 seconds	
Elongation	60	1 minute	
Final elongation	75	5 minutes	1
Cooling	4	∞	1

*Annealing temperature is primer dependent

Thereafter, 10 µl of the amplified products was electrophoresed on 4 % agarose gel, stained with GelRed (stock 10, 000 X, diluted to 0.3 X when added to the molten agarose), at 90 V for 80 minutes, in 1X TBE running buffer (10X TBE stock: 1.2 M Tris, 1.2 M Boric acid, and 2 mM EDTA). Visualization was done with the BioSpectrum® Imaging System.

UNIVERSITY of the
WESTERN CAPE

3.3.10. Quantitative real-time PCR

Real-time PCR (qRT-PCR) was performed using the SYBR Green miRNA assay according to the KAPA SYBR ® FAST qPCR kit protocol Optimized for LightCycler® 480. Reactions including the standards were carried out in 96-well, clear plates.

Each reaction was made up to a final volume of 10 µl with the following components: ~200 ng cDNA, 5 µl of 2X SYBR qPCR master mix, 1 µl of both forward and reverse primers at a concentration of 10 µM each, and nuclease-free, PCR-grade water. The LightCycler®480 was used to quantify differential expression according to the parameters shown in Table 3.3.

Table 3.3: Parameters used for qRT-PCR reactions

Program	Step	Temperature (˚C)	Hold	Cycle	Fluorescence Acquisition
Pre-incubation		95	5 minutes	1	None
PCR	Denaturation	95	10 seconds	45	None
	Annealing*	64	20 seconds		None
	Extension	72	1 second		Single
Melting Curve	Denaturation	95	5 seconds	1	None
	Annealing	65	1 minute		None
	Extension	97	5 – 10 acquisitions/˚C		Continuous
Cooling			30 seconds	1	None

*Annealing temperature is primer dependent

Thereafter, data on expression levels of the reference genes were determined in the form of crossing points/cycle threshold (Cp/C_t). And the PCR efficiencies were calculated using the Relative Expression Software Tool (REST®) (Pfaffl, 2002). All Ct values were taken into consideration using the following equation:

UNIVERSITY of the WESTERN CAPE

$$E = 10^{[-\frac{1}{Slope}]}$$

Lastly, the expression levels of all four miRNAs were determined relative to the reference genes, using the following equation (Pfaffl, 2001):

$$R = \frac{(E\ target)^{\Delta Cp}.\ target^{(control\ sample)}}{(E\ ref)^{\Delta Cp}.\ ref^{(control\ sample)}}$$

3.4. Results

3.4.1. MiRNA extraction

Tissue from four HFD (T2DM), three T1DM, and three normal (control) were extracted from male Wistar rats as described in section 3.3.6. Due to the small size of miRNAs, agarose gel electrophoresis was not carried out. However, all isolated miRNA samples were quantified using the Qubit® Fluorometer 2.0 as described in section 3.3.6.1.

3.4.2. CDNA synthesis

The stem-loop reverse transcription method was used to synthesise cDNA for all the miRNA samples as described in section 3.3.8. The resulting cDNA samples were then quantified using the Thermo Scientific NanoDrop ND-1000 Spectrophotometer.

UNIVERSITY of the

3.4.3. Conventional PCR

A conventional PCR was performed using cDNA from the four miRNAs of interest (mir-*dr*-1, miR-*ur*-1, miR-*ur*-2, and miR-*ur*-3) and the reference miRNAs: miR-191a and miR-17, as described in section 3.3.9. This was done to observe if the primers bound and aided in amplifying the targets of interest. It should be noted that a cDNA sample from a normal kidney tissue was used to test whether miRNAs of interest amplified.

From the results shown in Figure 3.2, expected bands of approximately 70 bp was observed for all miRNAs after agarose electrophoresis. Moreover, some bands were more intense than others, this could be due to these miRNAs having different expression levels within the control sample.

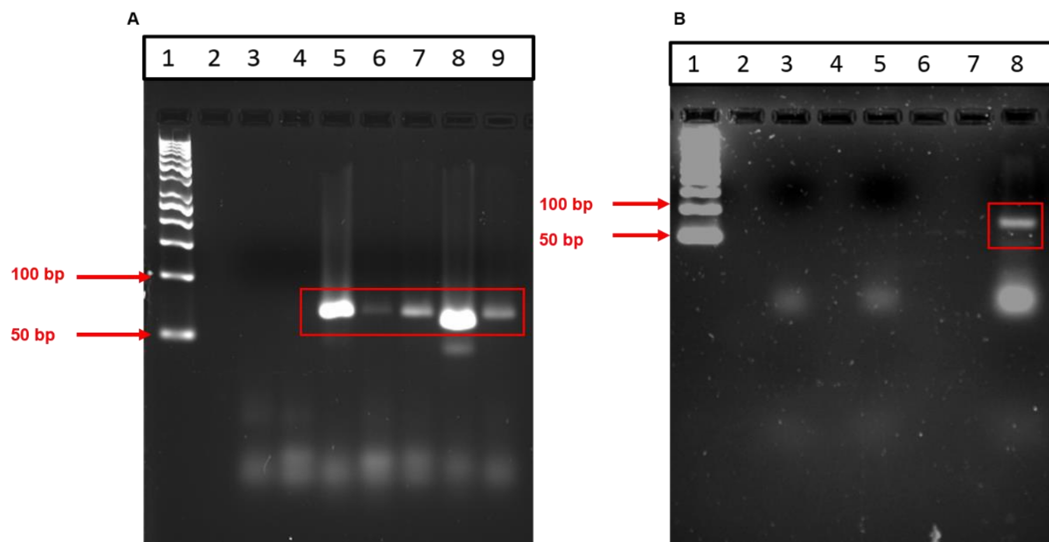


Figure 3.2: PCR amplification of the control and test miRNAs. A) LANE 1: 50 bp DNA ladder, LANE 2: empty, LANE 3 and LANE 4: control containing no template, LANE 5: normalisation miRNA mir-17, LANE 6: miR-*dr*-1, LANE 7: miR-*ur*-2, LANE 8: mir-*ur*-3, and LANE 9: normalization miRNA mir-191. B) LANE 1: 50 bp DNA ladder, LANE 3 and LANE 5: control containing no template, and LANE 8: miR-*ur*-1.

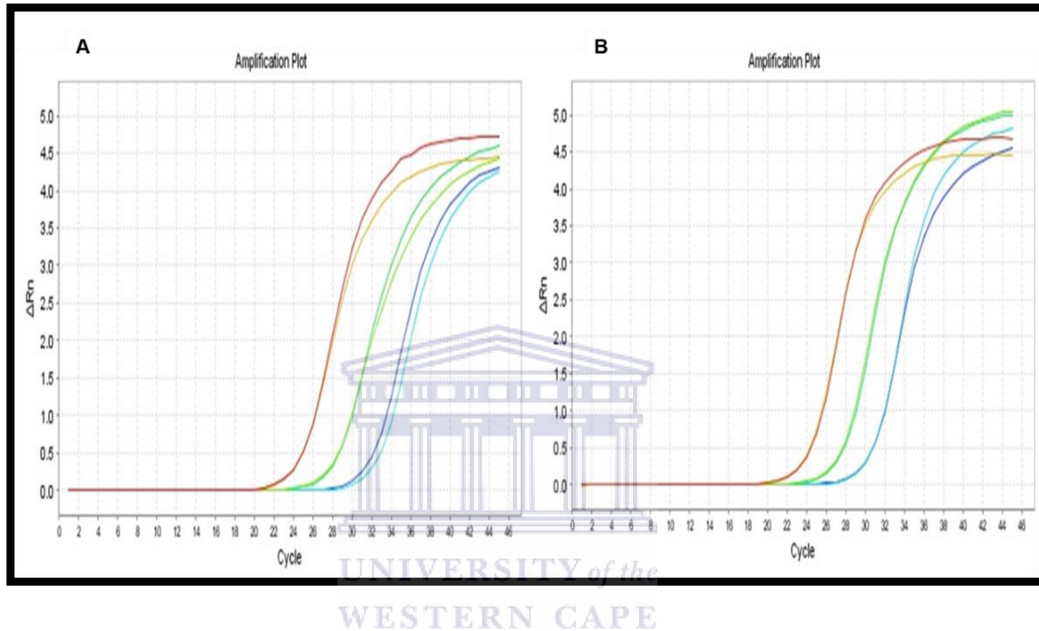
UNIVERSITY of the
WESTERN CAPE

3.4.2. qRT-PCR analysis

3.4.2.1. Standardisation of qRT-PCR

The sets of crossing points for the reference genes were imported into REST® (Relative Expression Software Tool) in order to normalize the relative quantification of the microRNAs (miRNAs) to the reference genes. A randomization test was performed to determine the factor of regulation and level of significance of each miRNA expression across the different tissue tested. The amplification plot for the reference gene GAPDH is shown in Figure 3.3 A. GAPDH is a suitable housekeeping gene and has been found to be stable in the different tissues tested. Figure 3.3 B shows the amplification plot for the miR-*dr*-1. In both Figure 3.3 A and B, evenly spread out slopes were observed. During the

qRT-PCR assays performed amplification efficiencies between 3.35 and 3.4 were obtained for the respective miRNAs, indicating that optimized qRT-PCR reactions were performed. The amplification curves for the rest of the test and other control miRNAs are given in Appendix C.



UNIVERSITY of the
WESTERN CAPE

Figure 3.3 (A): **Amplification plot for the reference gene, GAPDH, and miR-*dr*-1.** The red, green, and blue slopes indicate the amplification of GAPDH at the 1:10, 1:100, and 1:1000 dilutions, respectively. **(B)** The red, green, and blue slopes indicate the amplification of miR-*dr*-1 at the 1:10, 1:100, and 1:1000 dilutions, respectively.

3.4.2.2. Melting curve analysis

A melting curve ranging from 60°C to 95°C was constructed for every reference and target gene/miRNA. The obtained melting curves were used to determine whether any contamination, mis-priming (referring to the annealing of primers to complementary sequences on non-target DNA), primer-dimers (primers annealing

to themselves), or other problems occurred. The melting curve for the reference gene GAPDH and miR-*dr*-1 are shown in Figure 3.4 A and B.

As the peaks in both figures are similar, it suggests that no contamination, mis-priming or primer-dimers are present. Only one peak is observed (one T_m) per gene, indicating that only one amplicon was amplified. Therefore, no non-specific amplification occurred and it is evident that accurate quantification of the genes of interest has been achieved through optimized qRT-PCR. The melting curve plots for the rest of the test and control miRNAs are given in Appendix C.

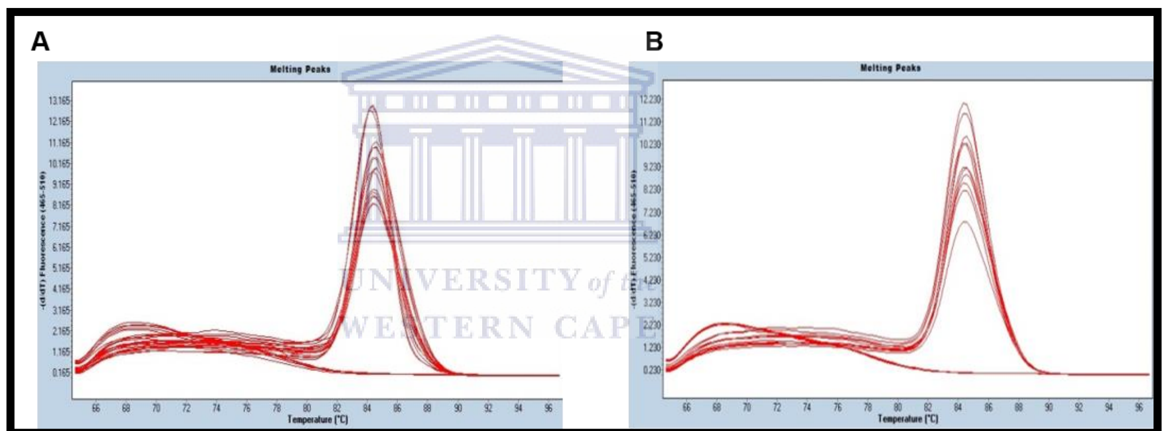


Figure 3.4: Melting curve analysis ranging from 65 ˚C to 95 ˚C for A) the reference gene, GAPDH, and B) miR-*dr*-1.

3.4.2.3. Reference genes: statistical analysis

The accuracy of qRT-PCR is heavily dependent on the proper normalization of expression data, therefore, inappropriate normalization of qRT-PCR data can lead to incorrect conclusions (Peltier and Latham, 2008, Roberts et al, 2014). Thus, the main aim of reference genes is to remove variations between groups – the variations in this instance is caused by the disease (Peltier and Latham, 2008) or by experimental error. Ideally, a reference gene is a single nucleic acid that exhibits

constant expression across all samples, is expressed along with the target of interest within cells, and shows equivalent storage, stability, extraction, and quantification efficiency as the specific target of interest (Pfaffl, 2001; Peltier and Latham, 2008). However, a reference gene such as this does not exist in reality (Peltier and Latham, 2008).

MiRNAs, particularly, poses a significant challenge for normalization. This is thought to be due to miRNAs only representing 0.01 % of the mass amount of total RNA and this fraction can have significant variation across different samples (Peltier and Latham, 2008). Despite these challenges, however, there are three normalization strategies which aid in expression profiling of miRNAs which include (i) average of all the quantification cycles values (Cq) from the experiments, (ii) stably expressed endogenous reference miRNAs, and (iii) external spike-in synthetic oligonucleotides. In this investigation, miR-17 (Eskildsen et al, 2013), miR-191a (Eskildsen et al, 2013) and GADPH (Ji et al, 2013) were used as reference genes for normalization, based on previous studies which identified them to be stably expressed in kidney tissue. The expression of these reference genes were profiled in the different kidney tissue samples to test whether they had the same expression i.e. in the normal, HFD (T2DM), and T1DM kidney tissue samples. In this investigation, miR-17 was used for normalization due it exhibiting the same expression in all sample types (shown in Figure 3.5).

UNIVERSITY of the
WESTERN CAPE

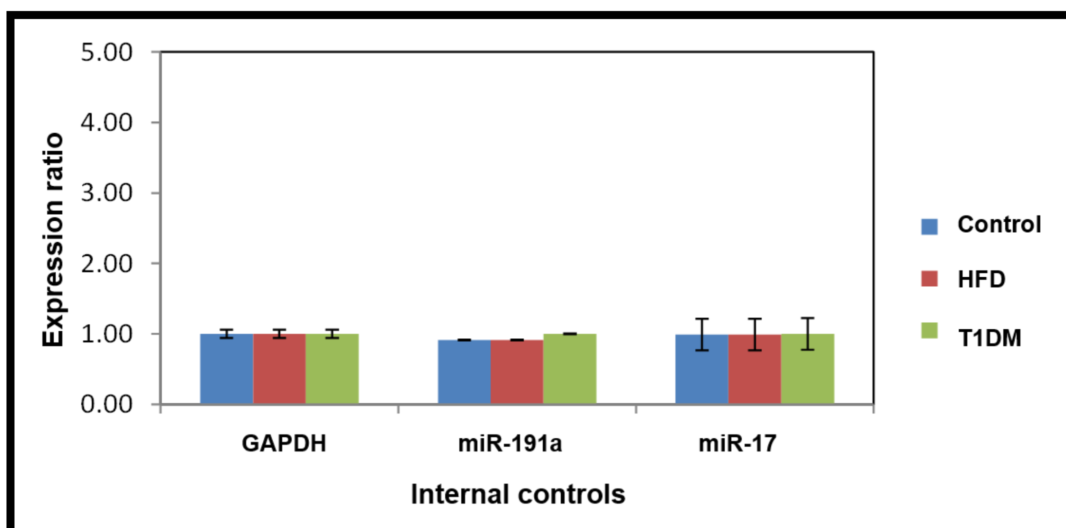


Figure 3.5: Expression levels of the reference genes in control tissue sample compared to HFD (T2DM) and T1DM samples

UNIVERSITY of the
WESTERN CAPE

3.4.2.4. Expression analysis of the 4 miRNAs of interest in HFD (T2DM) and TIDM tissues.

For the expression analysis performed for the 4 miRNAs of interest in normal, T1DM, and HFD (T2DM) kidney tissue samples, ~ 200 ng of miRNA was extracted from the tissues, as described in section 3.3.6, and reverse transcribed to cDNA as described in section 3.3.8. A serial dilution ranging from 1:10 to 1:10 000 was performed using the cDNA obtained after reverse transcription. The cDNA was used in qRT-PCR reactions as described in section 3.3.10. The expression levels of the target miRNA were normalized against the expression of the reference gene (GADPH) and the two control miRNAs (miR-17). Furthermore, the expression ratios were determined using the REST® software package. The values obtained from the software package were exported to an Excel spreadsheet and a table was created containing all the descriptive statistics as indicated in Table 3.4.

From the results obtained for Table 3.4 it is shown that three of the miRNAs, miR-*ur*-1, miR-*dr*-1 and miR-*ur*-2, showed differential expression in the HFD (T2DM) samples when compared to the control samples. MiR-*ur*-1 was up-regulated in the HFD (T2DM) samples with a factor of 4.2 and a p-value of 0.05. MiR-*dr*-1 and miR-*ur*-2 were down-regulated in the HFD (T2DM) samples with a factor of 2.96 and 3.6 respectively, with p-values of 0.02 and 0.001. MiR-*ur*-3 was slightly up-regulated with a factor of 1.2 but this was not statistically significant, due to it having a p-value of 0.55.

In Figure 3.6, the expression ratios of all 4 miRNAs of interest are shown relative to the expression level in the control samples, where the control samples are given as an arbitrary value of one which indicates no variation of regulation of the four miRNAs.

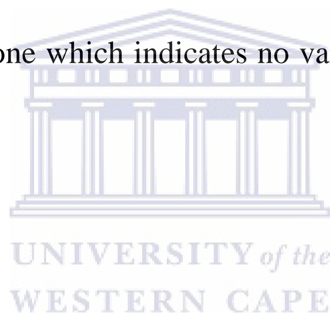


Table 3.4: Relative expression ratio of the four miRNAs of interest in HFD (T2DM) kidney tissue samples compared to normal (control) kidney samples.

Gene	Sample	Mean C_T	Std. error	Fold change	p-value
miR*-ur-*1	Control	34.93	0.07	4.2	0.05
	HFD	32.70	0.11		
miR*-dr-*1	Control	34.90	0.10	-2.96	0.02
	HFD	36.33	0.16		
miR*-ur-*2	Control	34.17	0.15	-3.6	0.001
	HFD	35.87	0.13		
miR*-ur-*3	Control	32.20	0.13	1.2	0.55
	HFD	31.82	0.12		
HKG	Control	28.19	0.04	1.098	0.209
	HFD	28.05	0.11		

*HKG = Housekeeping gene

UNIVERSITY of the
WESTERN CAPE

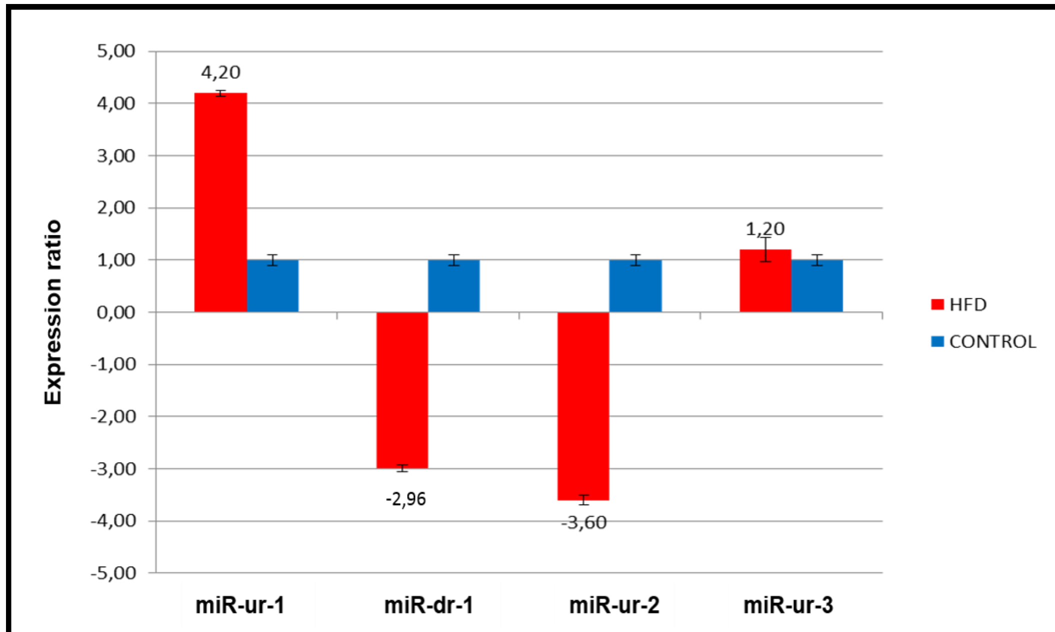


Figure 3.6: Relative expression ratio plot of the four miRNAs in normal kidney tissue compared to HFD (T2DM) kidney tissue.

Table 3.5 represents the descriptive statistics for the expression of the 4 miRNAs in T1DM samples. From the results obtained in Table 3.5 it is shown that all of the miRNAs showed differential expression in T1DM when compared to the control samples. Three miRNAs, i.e. miR-*ur*-1, mir-*dr*-1, and miR-*dr*-2, were found to be down-regulated in T1DM with a factor of 7.65, 3.25 and 2.77 respectively, with p-values of 0.001. However, miR-*ur*-3 was slightly up-regulated with a factor of 1.86 with a p-value of 0.05

In Figure 3.7, the expression ratios of all 4 miRNAs in T1DM are shown relative to the expression level in the control samples, where the control samples are a given an arbitrary value of one which indicates no variation of regulation of the miRNAs of interest.

Table 3.5: Relative expression ratio of the four miRNAs of interest in T1DM kidney tissue samples compared to normal (control) kidney samples.

Gene	Sample	Mean C_T	Std. error	Fold change	p-value
miR*-ur-*1	Control	34.36	0.08		
	TID	37.17	0.11	-7.65	0.001
miR*-dr-*1	Control	32.53	0.21		
	TID	34.10	0.10	-3.25	0.001
miR*-ur-*2	Control	34.87	0.13		
	TID	36.2	0.12	-2.77	0.001
miR*-ur-*3	Control	32.13	0.07		
	TID	31.06	0.15	1.864	0.05
***HKG**	Control	28.19	0.04		
	TID	28.05	0.11	1.098	0.209

*HKG = Housekeeping gene

UNIVERSITY of the
WESTERN CAPE

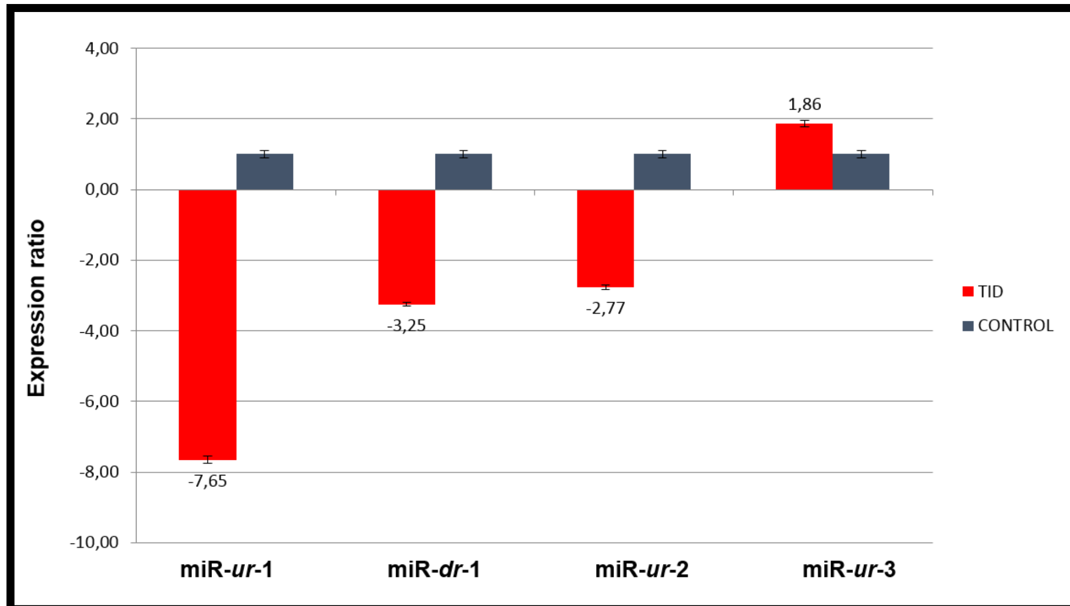


Figure 3.7: Relative expression ratio plot of the four miRNAs in normal kidney tissue compared to T1DM kidney tissue.

UNIVERSITY of the
WESTERN CAPE

3.4.2.5. Summary

Validation of biomarkers is an important step in biomarker discovery, as discussed in Chapter 1. Additionally, qRT-PCR is the golden standard for validation studies, and was, therefore, used in this investigation.

In this study, we evaluated the expression of four of miRNAs found to have differential expression in the serum/plasma of T2DM, as shown from the data obtained from various databases in section 2.2.1, and compared it to the expression in HFD (T2DM), T1DM and normal rat kidney tissues using qRT-PCR. The results indicate that three of these miRNA, i.e. miR-*ur*-1, miR-*dr*-1 and miR-*ur*-2, show significant differential expression in HFD (T2DM) rat kidney tissue, as shown in Table 3.2 and Figure 3.3. Whereas, all four miRNAs have significant differential expression in T1DM. The results obtained, therefore, indicates that these miRNA could potentially be used as predictive biomarkers in HFD (T2DM) and TIDM.

The miRNA which showed the most significant expression between T1DM and the HFD (T2DM) tissue samples, is miR-*ur*-1. This would be an ideal biomarker because it would clearly differentiate between T1DM and T2DM due to it being significantly up-regulated in HFD (T2DM) tissue samples, have a fold expression ratio of 4.2, and significantly down-regulated in T1DM tissue samples, as it had a fold expression ratio of -7.65 (see Table 3.6 below). The target genes that were predicted for miR-*ur*-1 in Chapter 2 were FOXP2, SIX5, and CACNB2.

Of these three genes, CACNB2 (calcium channel, voltage-dependent, beta 2) would be a target gene of interest. A study done by Lin et al (2011) found that variations of this gene is linked with hypertension. Furthermore, diabetic nephropathy (kidney failure) is the most common cause of hypertension (Lago et al, 2007). Furthermore, hypertension is also commonly linked with central obesity; which is one of the risk factors for T2DM (Lago et al, 2007).

UNIVERSITY of the
WESTERN CAPE

Table 3.6: Summary of fold expression ratios of the 4 miRNAs of interest in HFD (T2DM) and T1DM rat kidney tissue samples

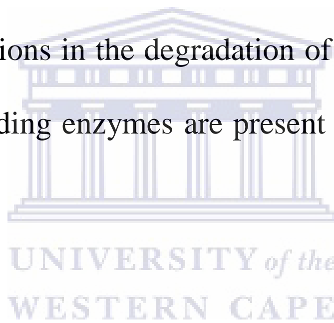
Sample Type	miR-*dr*-1	miR-*ur*-1	miR-*ur*-2	miR-*ur*-3
HFD	**-2.96***	**4.2***	**-3.6***	1.2
T1DM	**-3.25***	**-7.65***	**-2.77***	1.864

*Significant fold expression ratios are in red, bold font.

Furthermore, in the beginning of this chapter, a hypothesis was made that similar expression would be found in HFD (T2DM) rat kidney tissues as compared to

serum/plasma of T2DM, due to kidneys playing an important role in cleansing the blood from impurities.

Based on the results only three miRNAs i.e. miR-*dr*-1, miR-*ur*-1 and miR-*ur*-3 showed similar expression in rat kidneys as found in serum/plasma (which was obtained from the two databases as described in section 2.2.1) after qRT-PCR analysis (Table 3.5). However, miR-*ur*-2 was the only one which never showed similarity in expression compared to serum/plasma samples, and displayed significantly down-regulated expression in the HFD (T2DM) kidney tissue sample. A possible explanation for the contrasting expression in tissue and blood could be due to the alterations in the degradation of the miRNAs in circulation because more RNA degrading enzymes are present in circulation (Saikumar et al, 2012).


UNIVERSITY of the
WESTERN CAPE

3.5. References

- Czimmerer, Z., Hulvely, J., Simandi, Z., Varallyay, E., Havelda, Z., Szabo, E., Varga, A., Deszo, B., Balogh, M., Horvath, A., Domokos, B., Torok, Z., Nagy, L. and Balint, B.L. 2013. A versatile method to design stem-loop primer-based quantitative PCR assays for detecting small regulatory RNA molecules. *PloS ONE*, 8(1): e55168
- Dedeoğlu, B.G. 2014. High-throughput approaches for microRNA expresson analysis. *Methods in Molecular Biology*, 1107(2014): 91 – 103
- Eskildsen, T., Jeppesen, P., Schneider, M., Nossent, A., Sandberg, M., Hansen, P., Jensen, C., Hansen, M., Marcussen, N., Rasmussen, L., Bie, P., Andersen, D. and Sheikh, S. 2013. Angiotensin II regulates microRNA-132/-212 in hypertensive rats and humans. *International Journal of Molecular Sciences*, 14(6): 11190 – 11207
- Huisamen, B., George, C., Dietrich, D. and Genade, S. 2013. Cardioprotective and anti-hypertensive effects of Prosopis glandulosa in rat models of pre-diabetes: cardiovascular topics. *Cardiovascular Journal of Africa*, 24(2): 10 – 16
- Ji, H., Wang, J., Liu, J., Guo, J., Wang, Z., Zhang, X., Guo, L. and Yang, H. 2013. Selection of Reliable Reference Genes for Real-time qRT-PCR Analysis of Zi Geese (*Anser anser domestica*) Gene Expression. *Asian-Australasian Journal of Animal Sciences*, 26(3): 423 – 432
- Kramer MF. 2011. Stem-loop RT-qPCR for miRNAs. *Current Protocols in Molecular Biology*, 15:1 – 5

UNIVERSITY of the
WESTERN CAPE

- Lago, R.M., Singh, P.P. and Nesto, R.W. 2007. Diabetes and hypertension. *Nature Clinical Practice Endocrinology & Metabolism*, 3(10): 667
- Lin, Y., Lai, X., Chen, B., Xu, Y., Huang, B., Chen, Z., Zhu, S., Yao, J., Jiang, Q., Huang, H., Wen, J. and Chen, G. 2011. Genetic variations in CYP17A1, CACNB2 and PLEKHA7 are associated with blood pressure and/or hypertension in She ethnic minority of China. *Atherosclerosis*, 219(2): 709 – 714
- Owczarzy, R., Tataurov, A., Wu, Y., Manthey, J., McQuisten, K., Almabrazi, H., Pedersen, K., Lin, Y., Garretson, J., McEntaggart, N., Sailor, C., Dawson, R. and Peek, A. 2008. IDT SciTools: a suite for analysis and design of nucleic acid oligomers. *Nucleic Acids Research*, 36(Web Server): W163 – W169
- Peltier, H. and Latham, G. 2008. Normalization of microRNA expression levels in quantitative RT-PCR assays: Identification of suitable reference RNA targets in normal and cancerous human solid tissues. *RNA*, 14(5): 844 – 852
- Pfaffl, M. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, 29(9): e45
- Pfaffl, M., Horgan, G.W. and Dempfle, L. 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research*, 30(9): e36.

UNIVERSITY of the
WESTERN CAPE

- Saikumar, J., Hoffmann, D., Kim, T., Gonzalez, V., Zhang, Q., Goering, P., Brown, R., Bijol, V., Park, P., Waikar, S. and Vaidya, V. 2012. Expression, circulation, and excretion Profile of microRNA-21, -155, and -18a following acute kidney injury. *Toxicological Sciences*, 129(2): 256 – 267
- Stokowy, T., Eszlinger, M., Świerniak, M., Fujarewicz, K., Jarząb, B., Paschke, R. and Krohn, K. 2014. Analysis options for high-throughput sequencing in miRNA expression profiling. *BMC Research Notes*, 7(1): 144.
- Usó, M., Jantus-Lewintre, E., Sirera, R., Bremnes, R. and Camps, C. 2014. miRNA detection methods and clinical implications in lung cancer. *Future Oncology*, 10(14): 2279 – 2292
- van Rooij, E. 2011. The art of microRNA research. *Circulation Research*, 108: 219 – 234
- Varkonyi-Gasic, E., Wu, R., Wood, M., Walton, E.F., and Hellens, R.P. 2007. Protocol: a highly sensitive RT-PCR method for the detection and quantification of microRNAs. *Plant Methods*, 3: 12
- Wu, R., Wood, M., Thrush, A., Walton, E.F. and Varkonyi-Gasic, E. 2007. Real-time PCR quantification of plant miRNAs using universal ProbeLibrary technology. *Biochemica*, 2: 12 – 15

UNIVERSITY of the
WESTERN CAPE

Chapter 4: General discussion and Future work

Diabetes mellitus has made a significant impact on the non-communicable disease burden in South Africa (Volmink et al, 2014). Additionally, it has contributed considerably to mortality rates, causing 3.3 % of total deaths recorded in 2008 (Volmink et al, 2014). This mortality percentage has most likely increased since then.

The amount of individuals with T2DM, in particular, has increased rapidly over the years, as discussed in Chapter 1. However, unlike the other forms of diabetes, T2DM is the only type which can be prevented if diagnosed in its early stages i.e. pre-diabetes, by incorporating proper lifestyle habits, such as eating healthily and exercising regularly. Alternatively, it could also prevent the irreversible micro- and macrovascular complications linked with T2DM from developing. However, early diagnosis of T2DM rarely occurs and there are quite a number of individuals who are not even aware that they have the disease.

UNIVERSITY of the
WESTERN CAPE

Despite current methods such as the OGTT and FPG tests being the most commonly used methods for screening diabetes in a clinical setting, there are limitations linked with them such as it being invasive as all tests require a blood sample, patients are required to fast beforehand, some tests are time-consuming for both the patient and health professional, and tests like the HbA1c tests are expensive. Lastly, these tests can not differentiate between the types of diabetes

the individual may have. There is, therefore, a need for biomarkers which can detect pre-diabetes and T2DM without having the aforementioned limitations.

MiRNAs has shown promise as ideal biomarkers for diagnostic, prognostic and therapeutic purposes for many diseases, as well as T2DM. Thus, the main purpose of this study was to identify potential miRNAs as new biomarkers for the early detection of T2DM. This study was split into two methodologies i.e. *in silico* and molecular identification.

The objectives for the *in silico* study involved identifying miRNAs found to be differentially expressed in the serum/plasma of T2DM individuals, identifying their target genes and their mechanism of action using several web-based *in silico* tools. This study successfully identified four miRNAs (miR-*dr*-1, miR-*ur*-1, miR-*ur*-2, and miR-*ur*-3) as well as their target genes, and thus may be used as biomarkers for the early detection of T2DM. All the miRNAs of interest play a role in the insulin signalling pathway. Furthermore, due to the fact that the *in silico* methodology by Masotti and Alisi (2013) identified known genes linked with T2DM (LDLR, PPARA, CAMTA1, IGF2BP2, and ANK2) suggests that using this methodology, can be considered credible. However, one should bear in mind that *in silico* prediction of biomarkers should be validated using appropriate *in vitro* laboratory techniques (Ngcoza, 2013).

UNIVERSITY of the
WESTERN CAPE

In this study, miR-*dr*-1 and its two target genes, LDLR and PPARA, was found to be of particular interest, seeing as STRING analysis found an interaction between the two genes. Additionally, as described in section 2.4.2.2, the study conducted by Bernal-Mizrachi et al (2003) found that LDLR deficient mice, with

PPARA (+/+), eventually developed insulin resistance when continuously treated with glucocorticoids. Therefore, it would be worth validating miR-*dr*-1 to these target genes.

Furthermore, miR-*dr*-1 was predicted to target APP (amyloid precursor protein); a protein playing a main role in the pathology of Alzheimer's disease (AD) (Zhao and Townsend, 2009). Evidence showing a link between AD and T2DM has grown (Janson et al, 2004; Dandona et al, 2011). Recent studies have shown that glucose metabolism is severely impaired in the cerebral cortex of AD patients. More specifically, disruptions of the APP gene was shown to affect glucose metabolism and tolerance (Zhao and Townsend, 2009). Therefore, despite it not being discussed in Chapter 2, APP could be another target gene of interest that could be validated in future studies.

UNIVERSITY of the
WESTERN CAPE

It should be noted that the identification of putative biomarkers using high-throughput *in silico* methods instead of traditional laboratory techniques could lead to the identification of a large number of novel biomarkers in a shorter period of time. Therefore, a molecular approach was employed to compare the differential expression of the 4 miRNAs found to be differentially expressed in serum/plasma of T2DM individuals (obtained from the databases in Chapter 2) to analyse expression levels in kidney tissue from HFD (T2DM), T1DM and normal male Wistar rats. The hypothesis was made that similar miRNA expression was expected for the HFD (T2DM) kidney tissue samples compared to serum miRNA expression levels found in T2DM (as shown in the databases),

due to the fact that kidneys play an important role in purifying the blood from impurities.

All expression profiles for the miRNAs were similar to expression in serum/plasma of T2DM, except for miR-*ur*-2. As discussed in the summary of Chapter 3, the possible reason for this inconsistency could be due to the alterations in degradation of miRNAs in circulation because there is a higher level of RNA degrading enzymes in circulation (Saikumar et al, 2012).

Based on the molecular analysis, miR-*dr*-1 had significant differential expression, thus, suggesting that this miRNA should be validated in future as a potential biomarker for T2DM. Interestingly, miR-*ur*-1 showed vast differences in expression between T1DM and T2DM samples, see section 3.4.2.4; it was significantly up-regulated in the HFD (T2DM) samples and significantly down-regulated in T1DM samples. Thus, this miRNA could possibly differentiate between the two types of diabetes. Due to the results obtained, it should be validated in the future.

UNIVERSITY of the
WESTERN CAPE

4.1. Future work

Future work would include testing expression levels of the miRNAs of interest in T2DM induced rats, using the same methodology as described in sections 3.3.5 to 3.3.10. One of the shortcomings of this thesis was that there was not a large enough sample population. Therefore, more rat kidney samples i.e. normal, HFD, T1DM and T2DM, will be included for future studies. Once a miRNA which can

specifically and consistently discriminate between the normal, T1DM and T2DM rat kidney samples, comparison studies would be done in human blood and urine samples to determine if similar expression levels will be obtained as compared to the results achieved from this study.

Furthermore, urine and blood samples would be used in place of kidney samples when doing human studies as it would be invasive.

Furthermore, binding studies will be done for miR-*dr*-1 and its predicted target genes i.e. LDLR and PPARA, and miR-*ur*-1 and its predicted target gene CACNB2. A common method used for binding studies is the luciferase reporter gene assay; which is used to test the effect of miRNA-mediated, post-transcriptional regulation on the target genes (Jin et al, 2013). This is made possible by engineering a luciferase gene construct which contains the predicted miRNA target gene (Jin et al, 2013).

UNIVERSITY of the
WESTERN CAPE

Lastly, if the target gene is predicted to be the true target of the miRNA of interest, an experiment will be done to test the effect of the miRNA on the specific target gene (Kuhn et al, 2008). A typical approach for this would be to transiently over-express the miRNA/s of interest (miRNA mimics would be used) within a cell type which is known to express the predicted target protein. The effect of the miRNA/s on the target gene would then be analysed either by Western blotting or ELISA (Kuhn et al, 2008). It is expected that if the miRNA of interest is overexpressed, the target gene will decrease in expression (Kuhn et al, 2008).

This study may greatly contribute to current research to identify biomarkers for alternative, non-invasive diagnostic methods for T2DM.

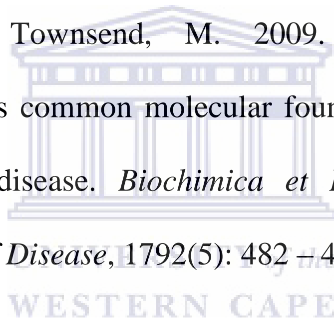
UNIVERSITY of the
WESTERN CAPE

References

- Bernal-Mizrachi, C., Weng, S., Feng, C., Finck, B., Knutsen, R., Leone, T., Coleman, T., Mecham, R., Kelly, D. and Semenkovich, C. 2003. Dexamethasone induction of hypertension and diabetes is PPAR-α dependent in LDL receptor–null mice. *Nature Medicine*, 9(8): 1069 – 1075
- Dandona, P., Mohamed, I., Ghanim, H., Sia, C., Dhindsa, S., Dandona, S., Makdissi, A. and Chaudhuri, A. 2011. Insulin suppresses the expression of amyloid precursor protein, presenilins, and glycogen synthase kinase-3β in peripheral blood mononuclear cells. *The Journal of Clinical Endocrinology & Metabolism*, 96(6): 1783 – 1788
- Janson, J., Laedtke, T., Parisi, J., O'Brien, P., Petersen, R. and Butler, P. 2004. Increased risk of type 2 diabetes in Alzheimer disease. *Diabetes*, 53(2): 474 – 481
- Jin, Y., Chen, Z., Liu, X. and Zhou, X. 2013. Evaluating the microRNA targeting sites by luciferase reporter gene assay. *Methods in Molecular Biology*, 936: 117 – 127
- Kuhn, D., Martin, M., Feldman, D., Terry, A., Nuovo, G. and Elton, T. 2008. Experimental validation of miRNA targets. *Methods*, 44(1): 47 – 54
- Ngcoza, N. 2013. *Identification of biomarkers in breast cancer as potential diagnostic and therapeutic agents using a combined in-silico and molecular approach*. MSc Thesis, University of the Western Cape

UNIVERSITY of the
WESTERN CAPE

- Saikumar, J., Hoffmann, D., Kim, T., Gonzalez, V., Zhang, Q., Goering, P., Brown, R., Bijol, V., Park, P., Waikar, S. and Vaidya, V. 2012. Expression, circulation, and excretion Profile of microRNA-21, -155, and -18a following acute kidney injury. *Toxicological Sciences*, 129(2): 256 – 267
- Volmink, H.C., Bertram, M.Y., Jina, R., Wade, A.N. and Hofman, K.J. 2014. Applying a private sector capitation model to the management of type 2 diabetes in the South African public sector: a cost-effectiveness analysis. *BMC Health Services Research*, 14: 444
- Zhao, W. and Townsend, M. 2009. Insulin resistance and amyloidogenesis as common molecular foundation for type 2 diabetes and Alzheimer's disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1792(5): 482 – 496



UNIVERSITY of the
WESTERN CAPE

Chapter 5: Appendix - List of susceptibility genes from T2DGAD

TCF7L2	GCK	CASQ1	GCGR	KLF16	FAM129A	UTS2R	SHBG	NKX6-1
PPARG	APOE	PDE3B	NAMPT	MCF2L2	GSTT1	10q21.1	SULF2	PHLDB1
KCNJ11	KCNQ1	PTEN	VEGFA	NR1H3	GSTM1	1p31.1	WAC	PTPN22
IGF2BP2	HNF1B	STX1A	CDKN2A	PLAGL1	IL1RN	5p14.3	11p15.1	SLC15A4
HHEX	SOD2	FOXO1	HHEX	RBM18	KLF6	ADCY10	20p12.1	TGIF2
CDKAL1	IAPP	HIF1A	IDE	SLC5A7	MMP12	ARHGEF12	5q35.3	XRCC1
SLC30A8	JAZF1	ANXA1	INSR	12q24.22	NUCB2	DDIT3	AVPR1A	11q22.3
FTO	ADAMTS9	CCL2	PPARGC1B	2q32.2	SLC9A9	FHIT	DLGAP4	6q14.1
HNF4A	PPP1R3A	MC3R	RPL21P7	8p12	TRPS1	IMMT	HMBOX1	C11ORF41
ADIPOQ	PON1	AHSG	SORBS1	AKT1	14q12	KRT4	MTHFSD	EGR2
CAPN10	GNB3	PLIN	APOC3	CSE1L	3p26.1	MT1B	PAX4	GC
ENPP1	BTC	13q21.1	GSTT1	ESRRG	9q34.13	OPRM1	PPP3CA	MYL9
CDKN2A	APOA5	CSN3	POMC	ICAM1	CX3CR1	RNLS	SAMD12	PCK2
CDKN2B	RBP4	PYY	NPY	KL	EYA2	UBR1	SPDEF	SF1
ACE	TSPAN18	AGT	CAMTA1	LTA	IFT52	16q21	18q12.1	STAT4
PPARGC1A	LGR5	LDLR	11q14.2	PIGT	KLF12	4p14	4q24	10q21.3
RETN	THADA	KLF7	20p13	PTPRT	NQO1	CD47	ACTN2	1p34.3
HNF1A	11p12	MMP26	6p25.2	SLC25A27	PKLR	CYP27B1	APOM	5q21.3
EXT2	SLC2A10	A2BP1	ADRA2B	THBS2	RALGPS2	FABP3	CDK11A	ADCY3
MTHFR	LEPR	MC4R	BCL11A	11q23.2	ANGPTL1	GSTK1	DBC1	ARNT
TNF	MTTP	CRP	CHI3L1	20q13.12	12q21.2	KLF2	FAM60A	CDKN1C
LOC387761	PBX1	ESR1	G6PC2	7p12.3	2q14.3	MGEA5	GYPC	DIO2
ABCC8	UCP3	GFPT1	ISL1	AGER	7q31.1	NR3C1	IL4	FOXA2
WFS1	CDC123	KCTD12	PRKAB2	C20orf24	CPNE4	RBM19	NUDT12	L3MBTL
ADIPOR2	CAMK1D	20q12	SDF2L1	CNTN1	EPHX2	SLC6A2	PON2	MT1E
IL6	SLC2A2	CLPS	SPRY1	HPSE2	GFM1	TRPA1	RICH2	OR13D1
UCP2	ABCA1	LIPC	ANKRD50	KCNJ10	HTR4	2q36.1	SMAD7	RORA
PDX1	IL10	PRKCZ	10q11.21	LMX1A	ADRB2	8q11.23	TSC22D1	SOD3
NOS3	IDE	VDR	1p21.1	NAP5	LRGUK	ANGPT4	14q21.1	17p12
PCK1	NOTCH2	UCP1	5p14.1	SGK2	NKX2-2	CAT	3q13.13	4p15.33
HFE	C4orf32	GSTM1	CDK5	VWF	PECAM1	EXOC4	AP2M1	APOB
IRS1	GCKR	NRF1	DCD	11p14.3	SLC13A1	GHSR	CCR5	CD96
INS	USF1	13q21.33	HADH	1q43	WRN	KLF1	CXCL5	CYP3A4
ADRB3	PKN2	AGTR1	KLF9	5q34	11q21	MAPK8	F3	FABP4
UTS2	ADRB2	CACNA1E	MT1A	ATP5O	20q11.23	PIK3C2G	GPC5	IL1B
GHRL	RAGE	BCHE	RNF34	DLGAP2	6q13	SLC27A5	IGF1	KLF3
PTPN1	MTNR1B	CETP	SOD1	INSRR	ADRB1	TLR4	KLF13	MIF
SREBF1	LMNA	LEP	UBQLNL/OR52H1	NTRK1	CHRM3	11q24.1	NR0B2	PNPLA2

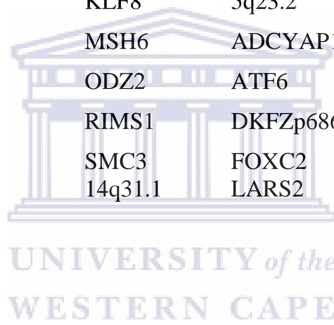
UNIVERSITY of the
WESTERN CAPE

ADIPOR1	KLF11	PRKAA2	15q24.1
CDKN2B	LPL	SPINK1	3q26.31
NEUROD1	DUSP12	DBI	AACS
IRS2	ARHGEF11	3q26.1	APOA2
PPARA	FFAR1	CD14	CD36
NEUROG3	OGG1	CXCR4	CYP11B2
IL6R	PPARD	AKR1B1	GRIK1

LCORL	DYRK2	20q13.13	RAPGEF1	SLC8A1
NCAPG	IFNG	7p13	TNFRSF4	TRPM6
PARL	GAL3ST1	COL13A1	12q21.31	3p12.3
RUNX2	ITGB3	ENSA	2q21.2	8q21.3
SOS1	LINGO2	HSD11B1	7q31.33	ANK2
18p11.31	MYBL2	NDRG3	AKAP10	CCDC138
4q13.3	PRKCB	PDE4B	CARTPT	CTLA4

IDE	APOA1	PANK4
HHEX	F7	17q24.3
KIF11	GRB10	4q13.1
KLF10	KLF14	ABCC9
MC2R	MC5R	CDC123
NPPB	NR1H2	CYP3A5
PIK3CB	PLA2G4A	FABP6
SLC2A1	RASGEF1A	IL1RAP
TMCO7	SLC44A3	KLF5
12q13.11	TOP1	MMP1
21q21.3	12q22	NRP1
7p21.3	2q22.1	REG1A
COX7A1	7q32.3	SLC9A8
EPB41L3	CASP9	TRPM7
GDAP1L1	CRTC2	13q31.1
HSPA1B	ESRRA	3p24.3
KCNJ9	GFPT2	8q23.3
LPIN2	ICA1	ANKRD50
PDK4	NXPH1	CCDC60
PTGIS	KIAA0319L	CWC22
SIDT1	LRP6	EXT2
TAS2R16	PHLPP	ALX4
11p15.4	PTPRS	GLP1R
20p12.3	SLC24A3	IFNG
6p12.3	ZPBP	MC2R
DNAJC19	11q23.1	MC5R
FXN	20q13.11	PIK3R1
HMOX1	6q24.1	RALGPS2
PAX6	C12orf75	TMEFF2
SCD	CLVS1	12q15
UTRN	CHD7	2p13.2
18q21.2	EIF2AK4	7q21.11
4q28.3	HNRPUL1	AHI1

ADAM30	KCND2	CPLX2
NOTCH2	PCLO	EPHB3
ARFGEF2	PRKG2	GDNF
CDK4	SGK1	EGFLAM
FBXL17	STK11	HTR2C
GYS1	1q24.2	KCNMB1
KLF8	5q23.2	PTGS2
MSH6	ADCYAP1	SLA2
ODZ2	ATF6	TCEB1
RIMS1	DKFZp686O24166	WNT5B
SMC3	FOXC2	A2M
14q31.1	LARS2	MT2A


UNIVERSITY of the
WESTERN CAPE

Appendix B: Primer design example (adopted from Kramer, 2011)

1. Quantification Standard—This is the precise sequence of the miRNA of interest. Here, miR-H1, expressed by HSV-1 (Cui, 2006; Umbach, 2008; Jurak, 2010), will be used as an example.

The 20 nt RNA sequence of miR-H1 is the Quantification Standard:

5′-UGG AAG GAC GGG AAG UGG AA-3′

2. Stem-Loop (SL) RT primer—This combines 44 nt of the stem-loop sequence of Chen et al., 2005, 5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC -3′ with the complement of the six 3′ nt of the mature miRNA sequence (boxed)*This is the stem-loop RT primer for miR-H1:*

5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC TTC CAC -3′

3. Forward qPCR primer—Together the Forward qPCR Primer and the Probe should maximally cover the mature miRNA sequence. Take the first 12 to 17 nt of the 5′ end of the mature miRNA, and 3 to 7 additional 5′ nt selected for getting the Tm to 60°C. Start with the first 12 nt, as in this example below, so as to maximize probe coverage of the remaining miRNA sequence.

a. Take the first twelve 5′ nt from the mature miRNA sequence (transformed to DNA, boxed)

5′- TGG AAG GAC GGG -3′

b. Add six nt to the 5′ end. The sequence of the six nt should be selected to confer upon the final oligo a Tm of 60 +/- 1 °C. An online calculator such as http:www.idtdna.com/analyzer/Applications/OligoAnalyzer can be useful. This is the miR-H1 Forward primer:

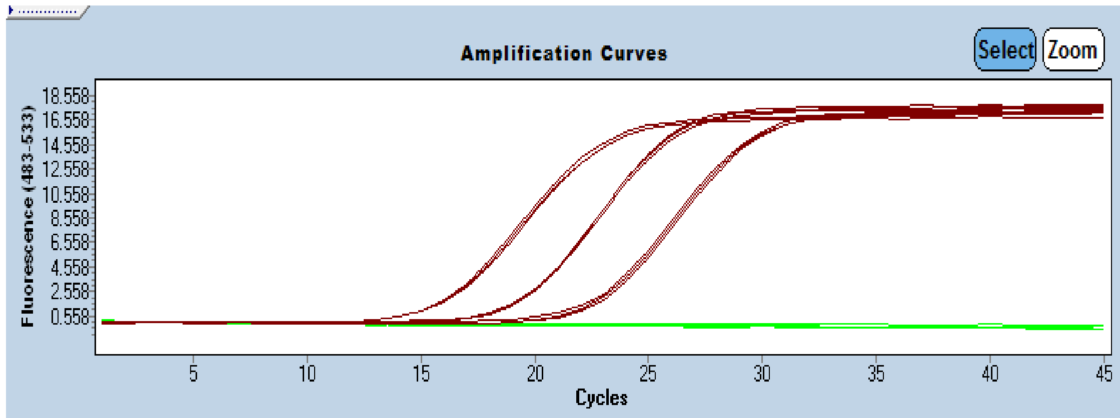
5′- CAC GCA TGG AAG GAC GGG -3′

4. Reverse qPCR Primer—By using the same 44 nt stem-loop sequence for all RT primers, a universal primer can be derived from sequences within the stem-loop. We recommend this Reverse Primer:

5′-CCA GTG CAG GGT CCG AGG TA-3′

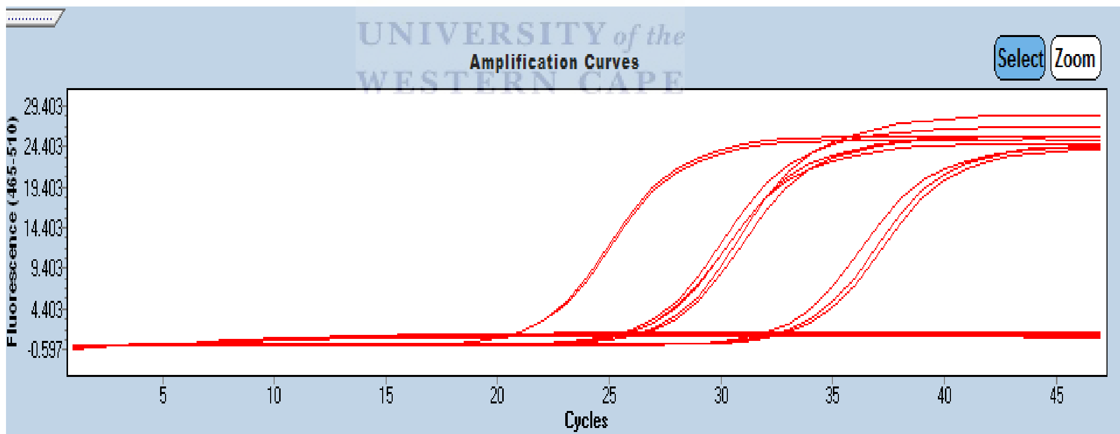
The reverse primer sequence reported by Chen et al., 2005, *is: 5′- GTG CAG GGT CCG AGG T -3′. We found improved sensitivity with the longer primer.*

Appendix C: Melting and amplification plots for all miRNAs

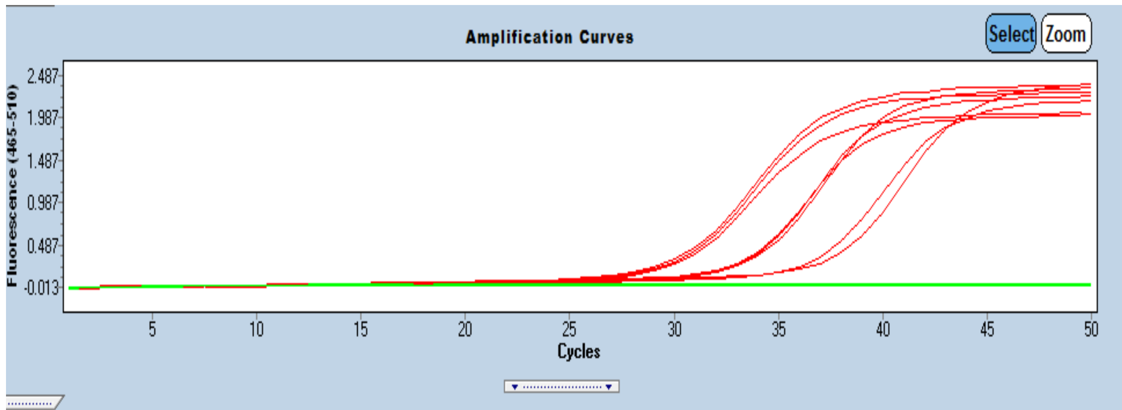


MiRNA 17A – Amplification curve

UNIVERSITY of the
WESTERN CAPE

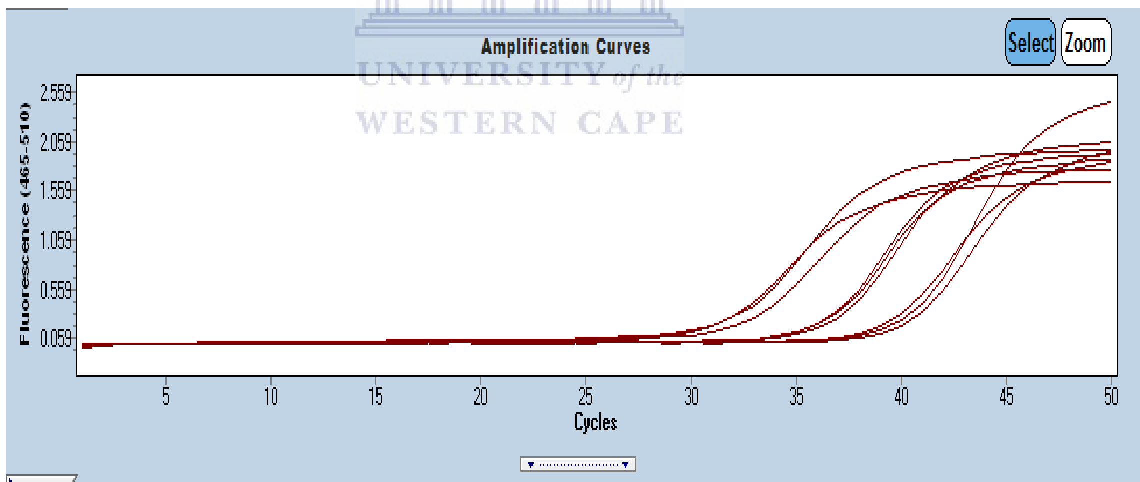


MiRNA 191A – Amplification curve

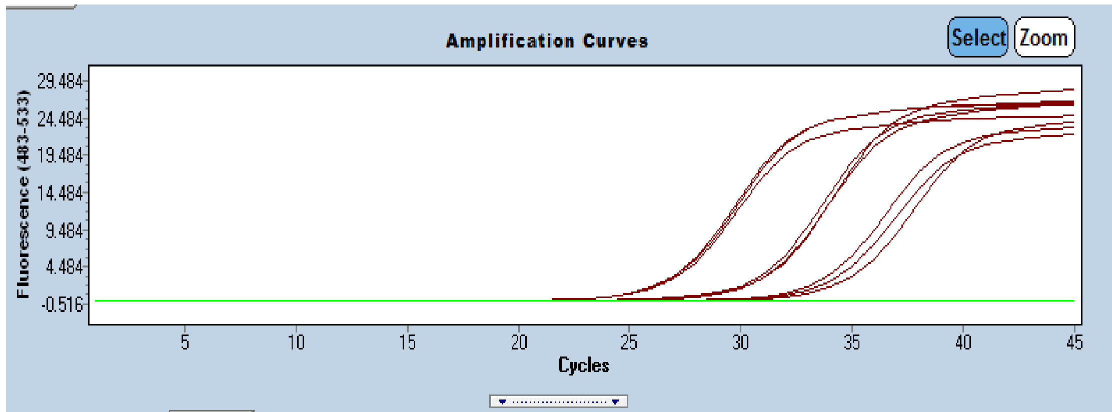


Mir-ur-1 Amplification curve

UNIVERSITY of the
WESTERN CAPE



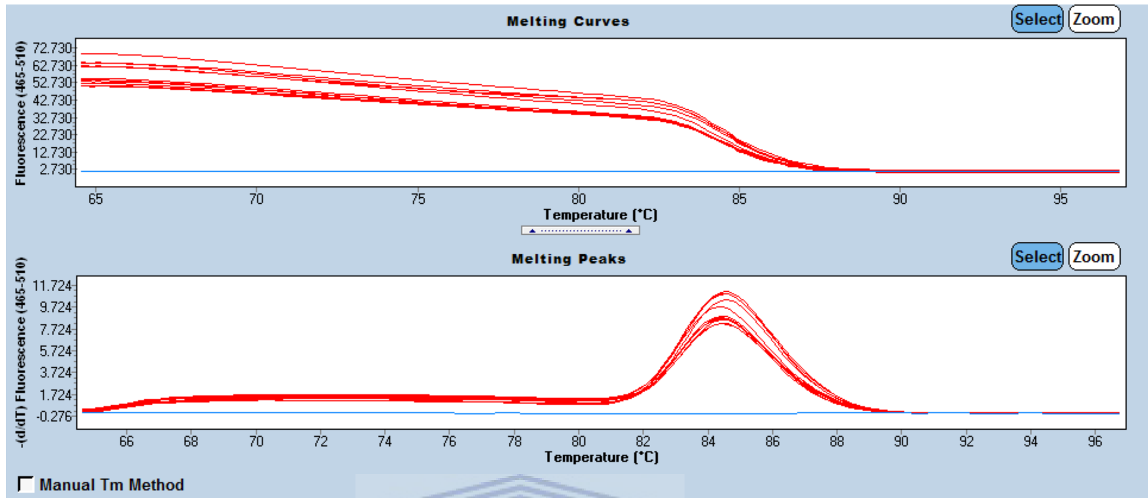
Mir-ur-2 amplification curve



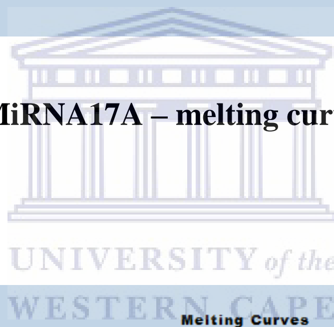
Mir-ur-3 amplification curve

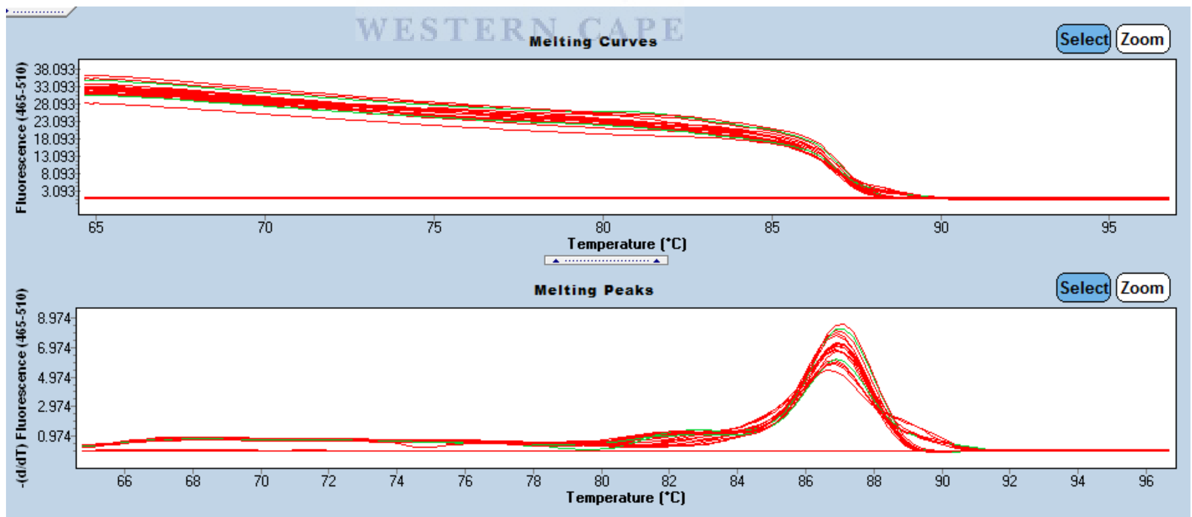
UNIVERSITY of the
WESTERN CAPE

Melting Curves:

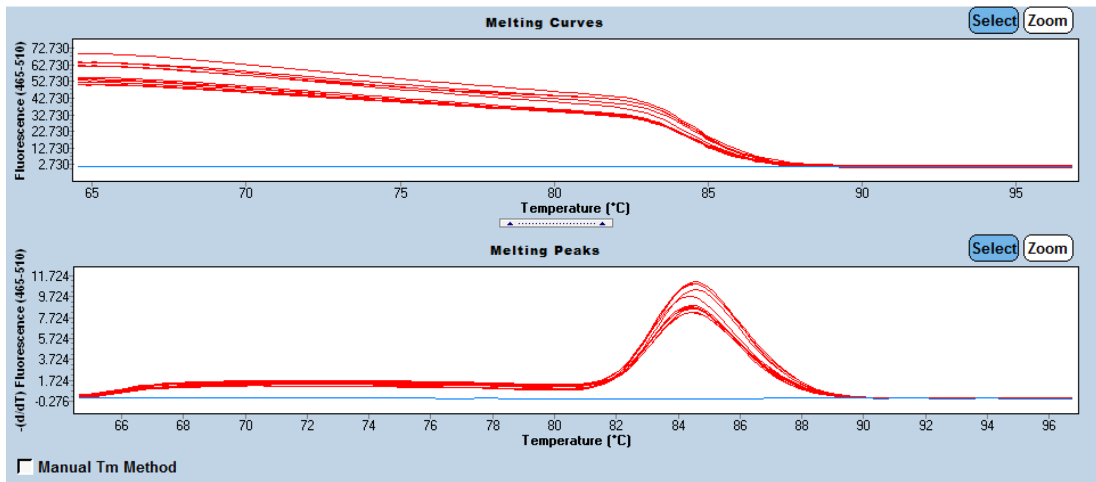


MiRNA17A – melting curve

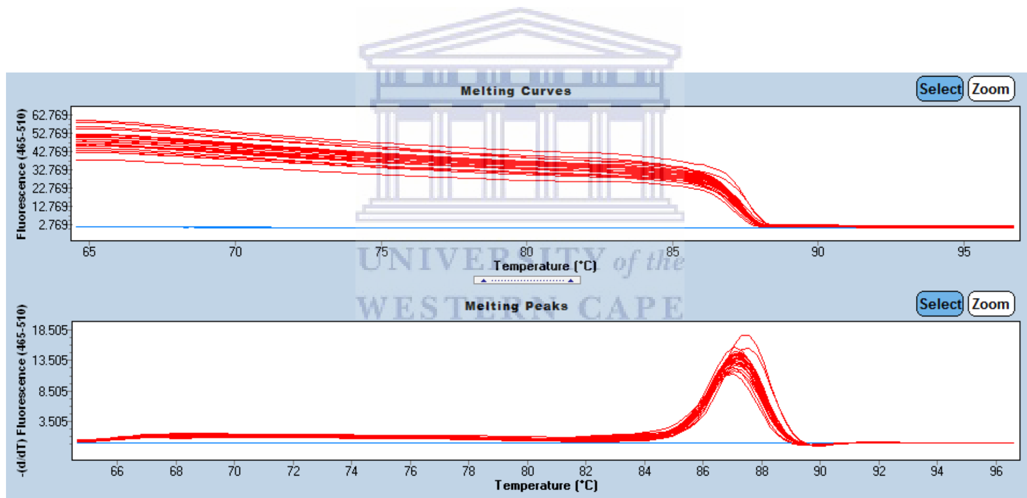

UNIVERSITY of the
WESTERN CAPE



MiRNA 191A- melting curve

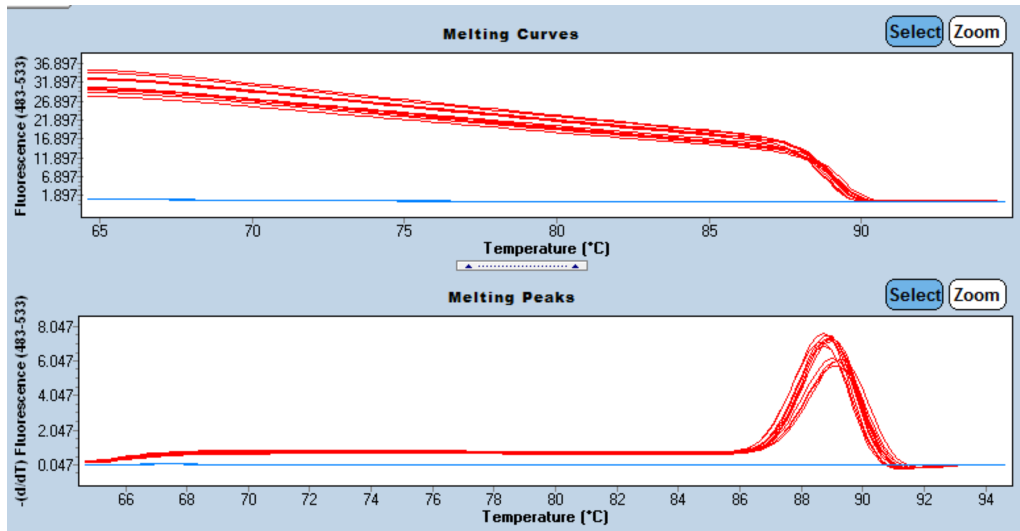


Mir-ur-1 melting curve



UNIVERSITY of the
WESTERN CAPE

Mir-ur-2 melting curve



Mir-ur-3 melting curve