Profiling circulating microRNAs in pregnancies complicated by type 1 diabetes, type 2 diabetes and gestational diabetes mellitus

By

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DECLARATION

By submitting this thesis, I Matladi Innocent Masete, hereby declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

ETHICS STATEMENT

The author, whose name appears on the title page of this dissertation/thesis, has obtained, for the research described in this work, the applicable research ethics approval. The author declares that he has observed the ethical standards required in terms of the University of Pretoria's Code of Ethics for Researchers and the Policy guidelines for responsible research.

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LIST OF ABBREVIATIONS

ADA	American Diabetes Association
AGO2	Argonaute RISC Catalytic Component 2
ADIPS	Australian Diabetes in Pregnancy Society
BCL2	B Cell Lymphoma 2
β	Beta
BRIP	Biomedical Research and Innovation Platform
BMI	Body Mass Index
ChREBP	Carbohydrate-Responsive Element-Binding Protein
cDNA	Complimentary DNA
COVID-19	Coronavirus Disease-19
CRP	C-Reactive Protein
Ct	Cycle Threshold
DNA	Deoxyribonucleic Acid
ELISAs	Enzyme-Linked Immunosorbent Assays
FPG	Fasting Plasma Glucose
FOXO1	Forkhead Box Protein O1
GDM	Gestational Diabetes Mellitus
HbA1c	Glycosylated Haemoglobin
HMW	High Molecular Weight
Human	*Homo Sapiens*
HIV	Human Immunodeficiency Virus
HUVECs	Human Umbilical Vein Endothelial Cells
HAPO	Hyperglycaemia and Adverse Pregnancy Outcomes
HIF1A	Hypoxia Inducible Factor-1A
Hsa-miR	Human microRNA
INSIG-1	Insulin Induced Gene 1
in utero	in the womb
IADPSG	International Association of Diabetes and Pregnancy Study Groups
IDF	International Diabetes Federation
KEGG	Kyoto Encyclopedia of Genes and Genomes
mRNA	Messenger RNA
MiRNAs	MicroRNAs

Mins	Minutes
Mouse	*Mus Musculus*
Mmu-miR	Mouse microRNA
NDDG	National Diabetes Data Group
NAFLD	Non-Alcoholic Fatty Acid Liver Disease
NCDs	Non-Communicable Diseases
ND	Not Determined
NR	Not Reported
N	Number
NPM1	Nucleophosmin 1
OGTT	Oral Glucose Tolerance Test
Pri	Primary
Pre	Precursor
PTEN	Phosphatase and Tensin Homolog
PI3K-Akt	Phosphoinositol-3-Kinase–Protein Kinase B
P	Statistical Significance
qRT-PCR	Quantitative Real Time PCR
RanGTP	Transporter protein
ROS	Reactive Oxygen Species
RNAs	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RISC	RNA-Induced Silencing Complex
RPM	Revolutions Per Minute
SARS-CoV-2	Severe Acute Respiratory Syndrome-Coronavirus-2
SAMRC	South African Medical Research Council
SEM	Standard Error of Mean
snRNAs	Small nuclear RNAs
snoRNAs	Small nucleolar RNAs
SREBP	Sterol Regulatory Element-Binding Proteins
TGs	Triglycerides
TRBP	Transactivation response element RNA-binding protein
tRNA	Transfer Ribonucleic Acid
T1DM	Type 1 Diabetes Mellitus

T2DM	Type 2 Diabetes Mellitus
USA	United States of America
UTR	Untranslated Region
VEGFA	Vascular Endothelial Growth Factor-A
WHO	World Health Organisation

ABSTRACT

Background

Maternal diabetes is associated with complications for both mother and child. Mothers have an increased risk of hypertension and caesarean section, while the risk of macrosomia, hypoglycaemia, respiratory distress syndrome, preterm delivery, malformations, and mortality is increased in children. Moreover, both mothers and children have a life-long risk of developing type 2 diabetes (T2DM) and cardiovascular disorders in future. Evidence suggests that women with pregestational type 1 diabetes (T1DM) and T2DM have a greater risk of complications compared to women with gestational diabetes mellitus (GDM), although the mechanisms that link maternal diabetes type with adverse outcomes are not elucidated. In recent years, epigenetic mechanisms, such as microRNAs (miRNAs), have emerged as key players in the pathophysiology of pregnancy complications. The aim of this study was to assess the effect of maternal diabetes type on circulating miRNA expression.

Methods

Demographic information and serum were obtained from pregnant women with T1DM (n=6), T2DM (n=26), GDM (n=17) and normoglycaemia (n=24) at 16-27 weeks of gestation. C-peptide, total and high molecular weight (HMW) adiponectin, triglycerides and C-reactive protein (CRP) concentrations were measured using enzyme-linked immunosorbent assays. Serum miRNAs were profiled using the Human Serum/Plasma miScript miRNA PCR array (n=4 per group).

Results

Women with T2DM (p=0.04) and GDM (p=0.015) were older than women with T1DM. Glucose concentrations were higher in women with maternal diabetes compared to normoglycaemia ($p<0.001$), and women with T1DM (P=0.039) and T2DM (p=0.019) had higher levels of glycated haemoglobin than women with GDM. Pulse rate was higher in women with T2DM (p=0.003) and GDM (p=0.003) compared to women with normoglycaemia. All metabolic parameters varied significantly across the four groups. Pairwise analysis showed higher levels of insulin in women with T1DM (p=0.008), T2DM (p=0.013) and GDM (p=0.014) compared to normoglycaemia. C-peptide levels were higher in women with GDM compared to normoglycaemia (p=0.010) and T1DM (p=0.051). Total

adiponectin concentrations were lower in women with T2DM (p=0.005) and GDM (p=0.030) compared to normoglycaemia, while HMW adiponectin concentrations were lower in women with T2DM (p=0.038) and GDM (p=0.052) compared to T1DM. Triglyceride levels were higher in women with GDM compared to controls (p=0.030), while CRP levels were higher in women with T2DM compared to normoglycaemia (p=0.008).The expression of miRNAs varied between groups, with significance observed for miR-19b-3p (↓9.8-fold; p=0.033) in women with GDM, miR-20a-5p (↓4.5-fold; p=0.047) in T1DM, and miR-29a-3p (↑1.8-fold; p=0.002) in T2DM compared to women with normoglycaemia. Bioinformatic analysis of miRNA gene targets showed that the forkhead box protein O1 (FOXO1) signalling and miRNAs in cancer pathways were common to all three miRNAs, while 15 KEGG pathways were common between miR-20a-5p and miR-29a-3p, and 27 pathways were unique to specific miRNAs.

Conclusion

We identified miRNA signatures associated with T1DM, T2DM and GDM, which may contribute towards advancing our knowledge and understanding of mechanisms underlying the different types of diabetes in pregnancy. Further exploration of these miRNA signatures and their association with pregnancy outcomes, may offer potential as biomarkers to predict adverse outcomes.

Word count: 486

Keywords: microRNAs; pregnancy; type 1 diabetes mellitus; type 2 diabetes mellitus; gestational diabetes mellitus.

RESEARCH OUTPUTS

Conference presentation:

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1. **Matladi Masete**, Nompumelelo Malaza, Stephanie Dias, Sumaiya Adam and Carmen Pheiffer (2021) MicroRNAs as diagnostic biomarkers of diabetes during pregnancy. *Journal of Diabetes Research.* ID 9045205 (Review Submitted)

2. **Matladi Masete**, Stephanie Dias, Nompumelelo Malaza, Sumaiya Adam and Carmen Pheiffer (2021) Investigating the significance of miR-19b, miR-20a and miR-29a expression in pregnancies complicated by different types of diabetes. *Diabetes, obesity and metabolism* (Original Article in progress)

THESIS OUTLINE

CHAPTER 1: Literature review

Chapter 1 summarises literature pertaining to the research topic and presents the research problem, hypothesis, study aim and objectives.

CHAPTER 2: Review

Chapter 2 presents a review article "*MicroRNAs as diagnostic biomarkers of diabetes during pregnancy*" that was submitted to the *Journal of Diabetes Research*. The review discusses the role that miRNAs play during pregnancy and in pregnancies complicated by diabetes.

CHAPTER 3: Materials and Methods

Chapter 3 presents the experimental design and the materials and methods that were used in this study.

CHAPTER 4: Results

Chapter 4 presents the results generated in this study. Results are displayed as tables, graphs or heatmaps where appropriate.

CHAPTER 5: Discussion and Conclusion

Chapter 5 summarises and discusses the thesis findings within the context of existing literature. It highlights the novelty and significance of the study findings. Moreover, the strengths and limitations of the study, and recommendations for future research are stated.

CHAPTER 1: LITERATURE REVIEW

1.1 Maternal diabetes

Maternal diabetes is an escalating health burden and a global public health crisis. The condition is associated with an increased risk of pregnancy complications for both mother and child and also increases their risk of developing non-communicable diseases (NCDs) such as type 2 diabetes (T2DM) and cardiovascular disease in later life (Dornhorst and Banerjee, 2010). Several lines of evidence suggest that the frequency and severity of adverse pregnancy outcomes are correlated with the severity of maternal diabetes, with pregestational diabetes associated with worse pregnancy outcomes than diabetes first detected in pregnancy (Fong et al., 2014). The specific mechanisms through which pregestational diabetes elicits more severe pregnancy complications are unknown. However, it has been speculated that higher levels and longer intrauterine exposure to hyperglycaemia may be potential mechanisms that link pregestational diabetes with worse outcomes (Fetita et al., 2006; Fong et al., 2014). These effects may be facilitated by metabolic and/or molecular mediators, which have not been elucidated to date. In recent years, epigenetic mechanisms such has miRNAs have garnered interest as tools to understand diabetes pathophysiology during pregnancy (Zhao et al., 2011; Tagoma et al., 2018). This literature review provides an overview of diabetes in pregnancy and the role of miRNAs, highlighting their role during disease and pregnancy.

1.2 The global burden

Maternal diabetes can be classified as either pregestational type 1 diabetes mellitus (T1DM) or T2DM, T1DM or T2DM first detected in pregnancy, or gestational diabetes mellitus (GDM), a milder form of hyperglycaemia that develops during pregnancy and usually resolves after delivery (Dornhorst and Banerjee, 2010; Msollo et al., 2019). It is estimated that 21.3 million pregnancies worldwide (16%) are complicated by diabetes, of which, ~6.2% are due to pregestational T1DM and T2DM, ~7.4% due to T1DM and T2DM first detected in pregnancy, and ~84% are due to GDM (International Diabetes Federation, 2019) (Figure 1). Alarmingly, these statistics may be an underestimation since many cases of maternal diabetes remain undetected, particularly in poorly resourced health care settings (International Diabetes Federation, 2019). In Africa, it has been estimated that up to 60% of diabetes cases are undiagnosed, while suboptimal diabetes screening and diagnostic strategies during

pregnancy may miss many cases of maternal diabetes (International Diabetes Federation, 2019). A recent study conducted in an urban setting in South Africa reported that a significant number of women with GDM were missed when using risk factor-base selective screening rather than universal screening which is widely adopted at various academic units in the country (Adam and Rheeder, 2017).

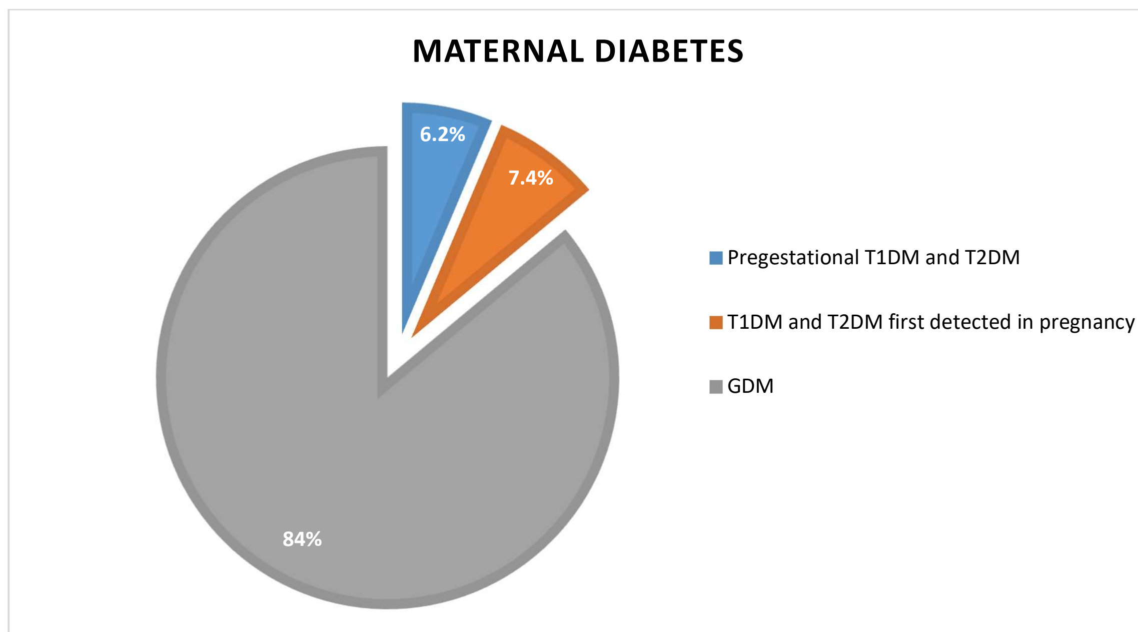

Figure 1. Global prevalence of maternal diabetes types (International Diabetes Federation, 2019)
Abbreviations: GDM, gestational diabetes; T1DM, Type 1 diabetes; T2DM, Type 2 diabetes
(Chart created with Microsoft Excel, 2010)

The health care costs of treating maternal diabetes have been estimated to be ~25.1% more than treating pregnant women without diabetes (Kolu et al., 2012), while in the long-term these women pose an additional burden to the health system due to their increased risk of developing or worsening NCDs (Kim et al., 2002; Bellamy et al., 2009). This dire situation is compounded by the rapidly rising obesity prevalence, arguably the single largest modifiable risk factor for diabetes (Al-Goblan et al., 2014). Epidemiological studies have consistently reported high rates of obesity, particularly amongst women of reproductive age (Hruby and Hu, 2015). Unfortunately, these high rates of obesity, spurred by urbanisation and the consumption of carbohydrate and fat dense diets, may also occur in poorly resourced health care settings that are least equipped to deal with maternal diabetes. Other risk factors for maternal diabetes include being older than 35 years of age, family history of diabetes or

history of stillbirth, macrosomia or previous GDM, or lifestyle factors such as consuming an unhealthy diet, being physically inactive, smoking and alcohol consumption (Chan et al., 2009; Imoh et al., 2016; Larrabure-Torrealva et al., 2018).

1.3 Pathophysiology

All types of maternal diabetes are characterised by hyperglycaemia, although the mechanisms that underlie them differ (Figure 2). Maternal T1DM is defined as a chronic autoimmune disease characterised by destruction of pancreatic beta- (β) cells (Vargas et al., 2010). These women become insulin deficient and rely on exogeneous insulin administration to achieve blood glucose control. In contrast, the pathogenesis of maternal T2DM is primarily associated with obesity and insulin resistance, and the inability of insulin sensitive tissues to respond appropriately to insulin. Subsequently, this may lead to defective insulin secretion by pancreatic β-cells, thereby leading to hyperglycaemia (Nolan et al., 2011). Unfortunately, many cases of pregestational T2DM are first diagnosed during pregnancy due to poor access to health care (Chivese et al., 2019). It is argued that the pathogenesis of T2DM and GDM are similar, with both conditions associated with insulin resistance and pancreatic (β)-cell dysfunction (Kim et al., 2002; Herath et al., 2017). However, GDM is a milder form of hyperglycaemia that develops due to maternal metabolic adaptation during pregnancy (Plows et al., 2018). It is believed that GDM develops in women who are unable to mount a compensatory β-cell response to counteract normal insulin resistance that develops during pregnancy. The development of insulin resistance during pregnancy, which is mainly due to placental hormone production (growth hormone, corticotropin, placental lactogen, oestrogen and progesterone) (Handwerger and Freemark, 2000), plays a crucial role in fetal nutrition and allows glucose to be shunted to the growing fetus (Coustan, 2020).

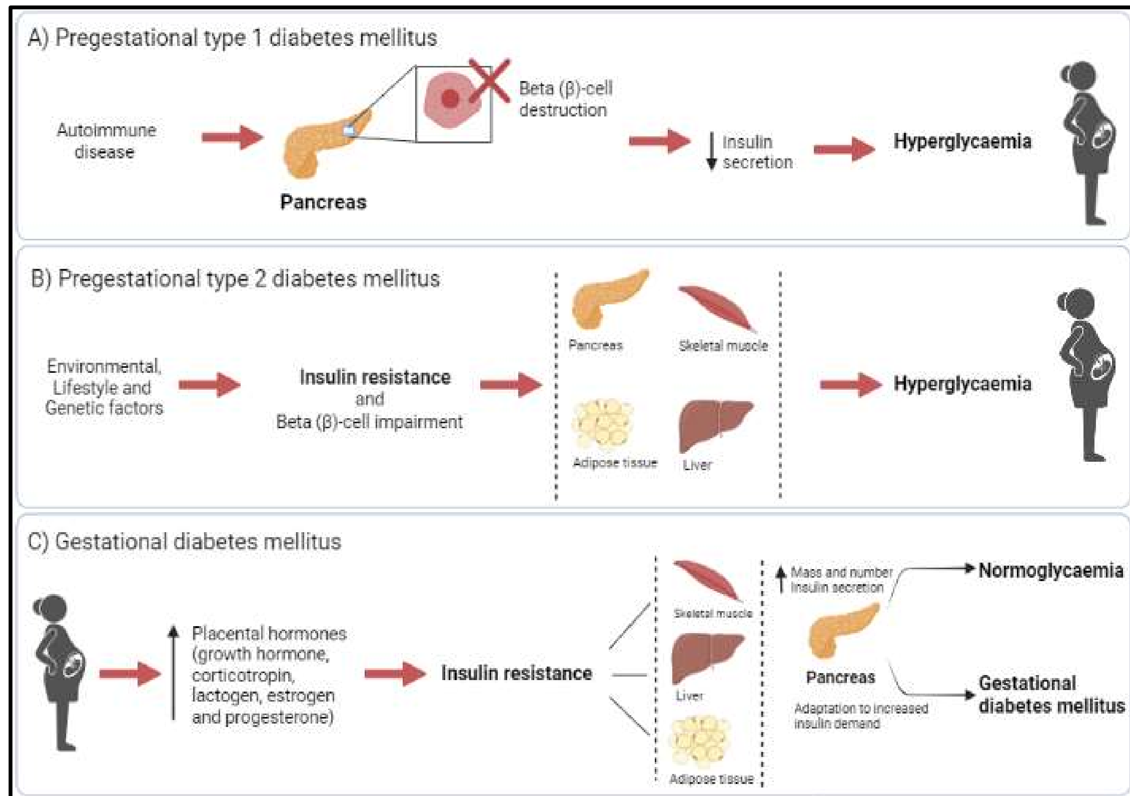

Figure 2. Schematic diagram illustrating progression to maternal diabetes

(Image created with BioRender.com)

A) Pregestational type 1 diabetes (T1DM) occurs when the immune system attacks pancreatic β-cells causing insulin deficiency and hyperglycaemia, B) Pregestational type 2 diabetes (T2DM) occurs due to environmental, lifestyle and genetic factors that causes insulin resistance in peripheral tissue such as skeletal muscle, liver and adipose tissue and β-cell impairment in the pancreas, leading to hyperglycaemia, C) Gestational diabetes mellitus (GDM) occurs due to an increase in maternal placental hormones, which causes insulin resistance in peripheral tissue such as skeletal muscle, liver and adipose tissue. As a result, pancreatic β-cells compensates by increasing insulin secretion and production, leading to normoglycaemia, while failure to adapt to the increased insulin demand leads to hyperglycaemia and subsequently GDM.

Metabolic pathways such as insulin signalling and secretion, lipid metabolism and inflammation are dysregulated during maternal diabetes (Mazaki-Tovi et al., 2007; Pendeloski et al., 2017). Insulin is a peptide hormone produced by β-cells in the pancreatic islets of Langerhans, and is important for glucose homeostasis (Wilcox, 2005). High levels of insulin in pregnancy are associated with the development of GDM (Georgiou et al., 2008; Liang et al., 2016), while an association between decreased insulin sensitivity and pregnancy-related hypertensive disorders have been reported (Kaaja et al., 1999). C-peptide is the

cleavage product generated during the conversion of proinsulin to mature insulin (Venugopal et al., 2021), and is used to assess the insulin secretory reserve and β-cell function (Leighton et al., 2017). Although T1DM is characterised by insulin deficiency, high levels of C-peptide and improvement in β-cell function and glycaemic control have been reported in women with T1DM during pregnancy (Nielsen et al., 2009). Adiponectin is an adipocyte-derived hormone with insulin-sensitizing and anti-inflammatory characteristics that plays an important role in maintaining energy balance (Pheiffer et al., 2021). This adipokine exists in three forms, low, medium and high molecular weight (HMW) molecules, with the latter being the predominant isoform in circulation and considered the most active biological isoform, particularly in relation to insulin sensitivity (Liu et al., 2007). Low levels of adiponectin are associated with increased insulin resistance during pregnancy (Catalano et al., 2006; Guelfi et al., 2017) and the development of GDM (Bao et al., 2015). Triglycerides (TG) are ester compounds made up of glycerol and three fatty acid molecules. During pregnancy, TG levels increase in response to oestrogen stimulation (Ghio et al., 2011; Ryckman et al., 2013), and have been associated with an increased risk of GDM (Ryckman et al., 2015; Eppel et al., 2021). C-reactive protein (CRP) is a plasma protein that serves as a common biomarker for systemic inflammation (Ridker et al., 2000) and is associated with GDM and T2DM (Nesto, 2004; Alamolhoda et al., 2020).

1.4 Complications

Maternal diabetes is associated with adverse short- and long-term pregnancy outcomes for both mother and child (Benhalima et al., 2019; Wu et al., 2020). In the short term, maternal complications include preeclampsia and caesarean section, while fetal complications include macrosomia, shoulder dystocia, hypoglycaemia, hyperbilirubinaemia and congenital malformations (O'Sullivan et al., 2011; Benhalima et al., 2019; Dias et al., 2019a). The mechanisms that mediate fetal complications are not fully elucidated, but it has been speculated that maternal hyperglycaemia induces reactive oxygen species (ROS), which causes deoxyribonucleic acid (DNA) damage in the fetus, leading to apoptosis and abnormalities in organs (Ornoy et al., 2015). Another proposed mechanism whereby maternal diabetes may elicit fetal complications is mediated by hyperinsulinaemia, leading to increased fetal growth and macrosomia (Barnes-Powell, 2007; Sugrue and Zera, 2018). Moreover, hyperinsulinaemia has been shown to deplete fetal oxygen storage capability, leading to fetal hypoxia and hypertension (Schwartz and Teramo, 2000; Barnes-Powell, 2007).

The long-term consequences of maternal diabetes have gained increased interest in recent years, particularly in women with GDM. Epidemiological studies have demonstrated that women with GDM have a ~7-fold risk of developing T2DM (Bellamy et al., 2009), with ~70% of women with GDM developing T2DM within 3 years in high risk populations (Kim et al., 2002). A recent study conducted in South Africa reported that ~ one third of women with GDM develop T2DM within 5-6 years (Chivese et al., 2019). Women with pregestational diabetes have an increased risk of developing diabetic complications, such as retinopathy, nephropathy, neuropathy, and ischemic heart disease in later life (Schaefer-Graf et al., 2018; Sugrue and Zera, 2018). Maternal diabetes is associated with abnormal intrauterine programming through the "Developmental Origins of Health and Disease" concept , which postulates that an unfavourable intrauterine environment increases the future metabolic risk of children (Mandy and Nyirenda, 2018). Indeed, children born to mothers with diabetes have an increased risk of developing obesity, insulin resistance, hypertension and cardiovascular disease in later life (Sugrue and Zera, 2018).

The risk of pregnancy complications is related to glucose concentrations maintained during pregnancy. The Hyperglycaemia and Adverse Pregnancy Outcomes (HAPO) study, which is one of the largest birth cohort studies conducted to date, followed 23,316 women during pregnancy, and showed that even mild levels of hyperglycaemia during pregnancy, is directly correlated with adverse outcomes including high birth weight, caesarean delivery, and neonatal hypoglycaemia (Metzger et al., 2008). Therefore, achieving optimal glucose control during pregnancy is of utmost importance, with the risk of pregnancy complications reduced in women who are able to achieve tight glucose control (McCance, 2015). Epidemiological studies have provided evidence that the severity of maternal diabetes is related to the frequency and severity of pregnancy complications (Benhalima et al., 2019; Wu et al., 2020). For example, complications are more frequent and severe in women with pregestational diabetes compared to GDM, most likely due to preconceptual and longer exposure to hyperglycaemia during pregnancy (Fetita et al., 2006; Fong et al., 2014).

1.5 Diagnosis

Maternal diabetes that develops during pregnancy may be diagnosed using various methods including random or fasting blood glucose concentrations, or the oral glucose tolerance test

(OGTT). Currently, the most widely accepted method of diagnosis is based on screening for risk factors such as age ≥ 35 years, obesity, family history of diabetes, history of stillbirth, macrosomia or previous GDM, followed by blood glucose testing with the OGTT (Dias et al., 2019b). There is currently no accepted diagnostic criteria that is universally used although many international bodies advocate for the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria (Metzger and Coustan, 1998; World Health Organisation, 2013; International Association of Diabetes and Pregnancy Study Groups Consensus Panel, 2010). The IADPSG criteria advocate for universal screening of all pregnant women at 24–28 weeks of gestation, based on findings of the HAPO study, which showed that mild levels of glucose intolerance, even at glucose concentrations previously considered normal, are associated with adverse pregnancy outcomes (Metzger et al., 2008). However, to date, there is lack of evidence that the use of the IADPSG criteria leads to significant clinical improvements in maternal and fetal outcomes (Shang and Lin, 2014; Adam et al., 2019), while limited resources hamper the use of these criteria in low-resource settings (Rheeder et al., 2017). Pregestational diabetes is usually diagnosed by self-report, medical records, or medication usage.

1.6 Treatment

Optimal glucose control in pregnancies complicated by diabetes may improve pregnancy outcomes and reduce the risk of maternal and fetal complications (American Diabetes Association, 2010). Methods to control blood glucose include regular self-monitoring of blood glucose levels, pregnancy education, lifestyle management and pharmacological therapy preconception and during pregnancy (Magon and Chauhan, 2012; Sugrue and Zera, 2018). Self-monitoring of blood glucose levels allows for dietary modifications to normalise glucose fluctuations timeously (American Diabetes Association, 2010). The pregnancy glucose targets recommended by the American Diabetes Association (ADA) are: fasting glucose < 5.3 mmol/L, one-hour postprandial glucose < 7.8 mmol/L and two-hour postprandial glucose < 6.7 mmol/L (American Diabetes Association, 2019). Pregnancy education includes preconception care and counselling for diabetic women of child bearing age, with emphasis placed on the management of hyperglycaemia and adverse outcomes in pregnancy (Bell, 2001; Hofmanova, 2006), while physical activity and a healthier diet is recommended during pregnancy as it improves insulin sensitivity and stimulates glucose uptake (King et al., 1988; American Diabetes Association, 2015). Plant-based diets and

physical activity regimens have shown ameliorative properties against maternal diabetes (Mijatovic-Vukas et al., 2018; Fedullo et al., 2021; Schiattarella et al., 2021). Pharmacological therapeutics, such as insulin (Karter et al., 2010), metformin (Holman, 2007), or both metformin and insulin with or without intensive lifestyle management (American Diabetes Association, 2015; Sikorskaya et al., 2021), have been shown to regulate glucose concentrations during pregnancy.

1.7 Epigenetics

Epigenetic mechanisms refer to changes in gene expression without changes in the underlying nucleotide sequence and play an important role in normal development, where they regulate the expression of genes involved in diverse biological processes such as cell proliferation, differentiation, apoptosis and metabolism (Chuang and Jones, 2007; Wu et al., 2018). Accordingly, the dysregulation of epigenetic processes is implicated in the development of various diseases. Studies have shown that epigenetic changes are gene-environment interactions, which can be induced by factors such as diet, physical activity, smoking and disease (Bovell et al., 2013; Florio et al., 2020; Karere et al., 2021). The most studied epigenetic mechanisms include DNA methylation, histone modifications and non-coding ribonucleic acids (RNAs) . DNA methylation, which is primarily associated with gene-silencing, is the best characterised epigenetic mark, while several studies have reported its potential role in the pathophysiology of diabetes (Pheiffer et al., 2016; Wu et al., 2018; Dias et al., 2019c). Histone modifications play an important role in the processes that regulate DNA accessibility and transcription, and have been shown to regulate the expression of genes associated with diabetes (Muka et al., 2016). Non-coding RNAs are RNA molecules that do not encode functional proteins and play a prominent role in the regulation of gene expression. MicroRNAs (miRNAs) and long non-coding RNAs are considered the most common regulatory RNAs in epigenetic control and disease (Ha and Kim, 2014). MiRNAs are arguably the most studied non-coding RNAs with regard to maternal diabetes and will be reviewed in detail below.

1.7.1 MiRNAs

MiRNAs are a class of short (~ 22 nucleotides), highly conserved, single stranded noncoding RNA molecules that regulate gene expression through post-transcriptional mechanisms (O'Brien et al., 2018). MiRNAs were first discovered in *Caenorhabditis elegans* in 1993 (Lee

et al., 1993) and since then over 2 500 miRNAs have been identified in humans (Alles et al., 2019; Satake et al., 2018), which regulate ~ 60% of genes in the genome (Zhang and Wang, 2017). MiRNAs are synthesised in the nucleus and via a complex series of steps are transported to the cytoplasm to perform their function (Ha and Kim, 2014). They are known as master regulators of gene expression and control various biological processes including cell proliferation and differentiation, apoptosis, metabolism and also facilitate cell–cell communication (Bayraktar et al., 2017). Accordingly, dysregulated miRNA expression is implicated in the pathophysiology of metabolic disease and is associated with conditions including cancer, obesity, T2DM and cardiovascular disease (Croce, 2009; Méndez-Mancilla et al., 2018). MiRNA patterns are altered during pregnancy, with dysregulated expression observed in pregnancies complicated by diabetes (Wang et al., 2019), and associated with maternal and perinatal complications (Satake et al., 2018). In 2014, Kappil and Chen demonstrated that *in utero* (in the womb) exposure to maternal diabetes influence offspring outcomes through epigenetic pathways such as miRNA expression (Kappil and Chen, 2014).

1.7.2 MicroRNA nomenclature

A nomenclature system has been adopted for miRNAs. The numbering of miRNAs is consecutive, with the prefix "mir" followed by a dash (-). The uncapitalised "mir" stands for premature miRNA, while the capitalised "miR" stands for mature miRNA. The prefix is followed by the unique identification number assigned to each miRNA in the order of their discovery (for example, miR-1, miR-2, miR-3, etc.) (Wright and Bruford, 2011). When two mature miRNAs originate from the 3' or 5' arms of the same primary miRNA , they are identified by a "-3p" or "-5p" suffix at the end (for example, miR-1-3p or miR-1-5p) (Issler and Chen, 2015). When one arm's mature miRNA is more abundant than the other, the miRNA with the lowest concentration gets an asterisk (*) next to its name (Bartel, 2004). MiRNAs with nearly identical mature sequences are labelled with a lower-case letter to indicate their structural similarity (for example, miR-1a and miR-1b). The inclusion of a number (for example, miR-1a-1 and miR-1a-2) distinguishes distinct precursor sequences and genomic loci from different sections of the genome that express similar mature sequences (Issler and Chen, 2015). MiRNAs are also denoted with a three-letter prefix and annotated according to the species in which they are found. Hsa-miR-1-3p, for example, is found in *Homo sapiens* (human), but mmu-miR-1-3p is found in *Mus musculus* (mouse) (Ambros et al., 2003).

1.7.3 Biogenesis and mechanism of action

MiRNA biogenesis is a complex process that begins in the nucleus of cell (Figure 3). RNA polymerase II (and possibly RNA polymerase III) transcribes miRNA genes located in intra- and intergenic regions of the genome, to produce primary miRNA transcripts of ~1000 nucleotides (Bartel, 2004). A microprocessor complex Drosha (RNase III endonuclease) cleaves primary miRNA transcripts to generate stem-loop precursor miRNAs that are ~70 nucleotides long. Precursor miRNAs are transported to the cytoplasm by Ran-GTP and the export receptor (Exportin-5), where after Dicer (RNase III endonuclease) cleaves them to produce mature miRNAs. The RNA-induced silencing complex (RISC) guides mature miRNA complexes to messenger RNA (mRNA) for degradation or silencing (Bartel, 2004; Guo et al., 2010). MiRNA-RISC complex binds to the 3′ untranslated region (UTR) of mRNA inducing mRNA degradation or translational repression (Guo et al., 2010). The interaction is dependent on the complementarity at the miRNA seed region, a conserved sequence that is usually located at positions 2-8 from the miRNA 5′-end. Although miRNAs are mainly implicated in gene silencing, studies have suggested that miRNAs may have additional functions such as promoting protein synthesis (Vasudevan et al., 2007; Ørom et al., 2008), regulating gene transcription possibly through interacting with other epigenetic mechanisms such as DNA methylation (Pheiffer et al., 2016) and influencing nuclear transcript stability and alternative splicing events when reimported into the nucleus (Roberts, 2014).

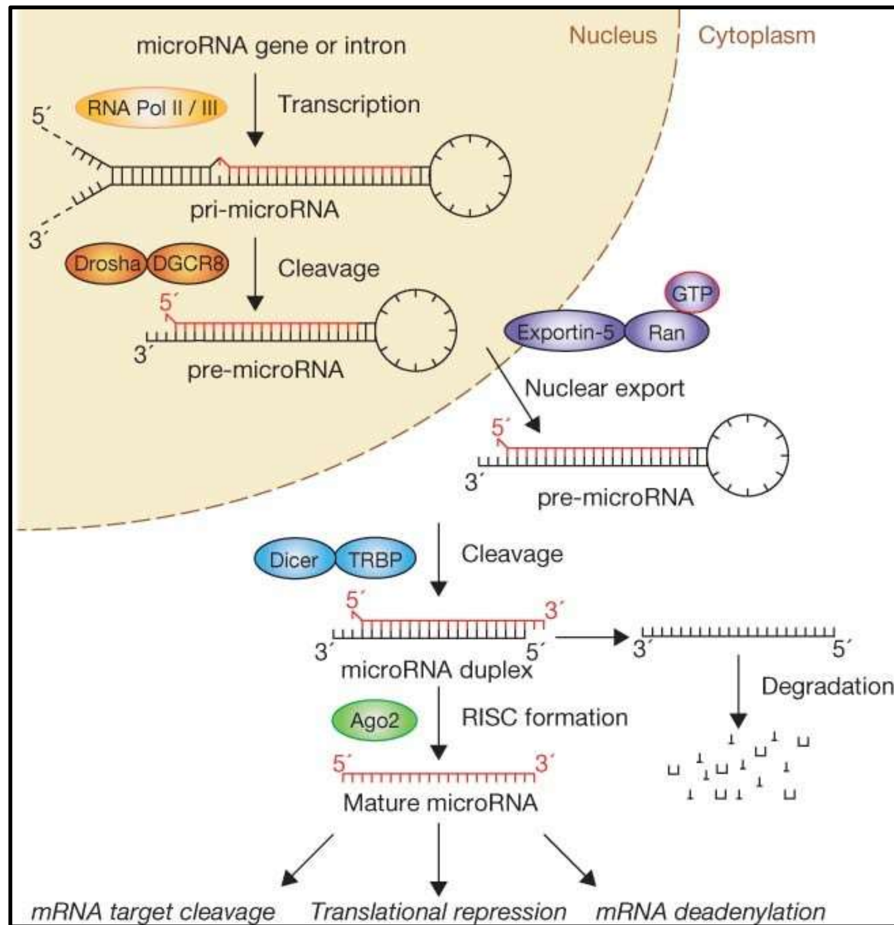

Figure 3. A diagram illustrating the biogenesis of miRNAs and its mode of action
(Image taken from Winter et al., 2009)

MiRNA transcripts (pri-miRNA) are transcribed by RNA Polymerase (pol II or III) and processed to pre-miRNAs by microprocessors (Drosha and DGCR8), and thereafter transported to the cytoplasm by Exportin-5 and RanGTP. Pre-miRNAs are processed into short miRNA duplexes by Dicer and its associated binding protein TRBP. The RNA-induced silencing complex (miRISC)-associated argonaut protein (Ago2) binds to the 5' mature miRNA strand and silences their messenger RNA target region (image adopted from Winter et al., 2009). Abbreviations: pri, primary ; pre, precursor; RanGTP, transporter protein; TRBP, transactivation response element RNA-binding protein; Ago, argonaut; RNA, ribonucleic acid; RISC, RNA-induced silencing complex; mRNA, messenger RNA.

1.7.4 Circulating miRNAs

In recent years, studies have provided evidence that miRNAs are located in circulation. These circulating miRNAs are released from cells as exosomes, microvesicles, apoptotic bodies, or are non-vesicle bound and are thought to play a central role in cell-to-cell communication (Bayraktar et al., 2017). Several studies have reported that miRNA expression in serum or

plasma may reflect tissue expression (Liang et al., 2007; Ma et al., 2019; Mompeón et al., 2020). Circulating miRNAs are stably expressed and can be quantified with relative ease using common laboratory techniques such as quantitative real time polymerase chain reaction (qRT-PCR) (Li and Kowdley, 2012). Accordingly, circulating miRNAs have received increasing interest as potential biomarkers of disease or to explain disease pathophysiology (Soifer et al., 2007). Circulating miRNAs are highly stable due to their association with various carriers such as lipoproteins, RNA-binding proteins (AGO2 and nucleophosmin 1 (NPM1)) and microvesicles such as exosomes, which has sparked interest in their potential role as biomarkers.

1.7.5 MiRNAs during pregnancy

Pregnancy enacts enormous stresses on the mother's physiology, causing several physiological, biochemical, and metabolic adaptations to accommodate fetal growth and development (Soma-Pillay et al., 2016). In recent years, an important role for miRNAs during pregnancy and pregnancy-related diseases has emerged (Cai et al., 2017; Poirier et al., 2017). MiRNAs are key metabolic and developmental regulators and facilitate maternal metabolic adaption by responding to physiological changes during pregnancy and may serve as cell-to-cell communicators between maternal cell types and tissues, and between maternal and fetal tissues (Mitchell et al., 2015). In 2013, genome-wide analysis found that more than 600 miRNAs are expressed in the placenta (Chen and Wang, 2013), which suggests an important role for miRNAs during pregnancy. The placenta plays an important role in maternal metabolic adaptation to pregnancy; thus, the differential expression of placental miRNAs may underlie pregnancy-related pathophysiological changes. Placental miRNAs are secreted into maternal circulation and can be quantified in plasma or serum (Chim et al., 2008), thus placental-derived circulating miRNAs may serve as biomarkers of placental dysfunction and pregnancy-related disorders. Many miRNAs have been identified in placental, uterine tissue and in maternal blood using techniques such as qRT-PCR, microarrays and sequencing. These miRNAs regulate processes that are critical for adequate placentation in early pregnancy and facilitate the flow of blood and nutrients between the mother and her fetus. Accordingly, many studies have reported that maternal circulating miRNAs are associated with pregnancy complications including placental weight (Miura et al., 2014), placental abruption (Miura et al., 2016), placental previa (Hasegawa et al., 2015), preeclampsia, gestational hypertension, and fetal growth restriction (Hromadnikova et al., 2013)

preeclampsia and intrauterine growth restriction (Hromadnikova et al., 2017), macrosomia (Jiang et al., 2015) and GDM (Pheiffer et al., 2018a; 2019). Although pregnancy adaption is mainly driven by placental factors, the importance of other maternal metabolic tissues during pregnancy is increasingly recognised (Poirier et al., 2017).

1.8 The COVID-19 pandemic and risk of diabetes during pregnancy

The novel Coronavirus disease-19 (COVID-19) is a highly contagious disease caused by severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) (Huang et al., 2020). The first outbreak of COVID-19 occurred in Wuhan, China, in December 2019. COVID-19 was declared a pandemic by the World Health Organisation (WHO) shortly after, and strict lockdown restrictions were imposed on most aspects of everyday life, including employment, social life, leisure time, and healthcare (World Health Organisation, 2020). The COVID-19 pandemic has exacerbated the incidence of maternal diabetes. Recently, Justman et al. (2020) reported that COVID-19 lockdown restriction is linked to a higher risk of developing diabetes in pregnancy (Justman et al., 2020), perhaps due to the consumption of unhealthy diets or maternal stress and cortisol production, factors that are associated with insulin resistance, a major risk factor for diabetes (Rosmond, 2003; Joseph and Golden, 2017). Telemedicine, remote diabetes education and technology implementation, including women's perspectives, is key to provide effective regulation of patients' glucose management, and in turn greatly improves pregnancy outcomes affected by COVID-19 (Murphy, 2020).

1.9 Research problem

Maternal diabetes is a formidable health burden and poses an increasing threat to the health of mother and child in the short- and long-term (Benhalima et al., 2019; Wu et al., 2020). All types of diabetes in pregnancy are associated with pregnancy complications, although the risk is increased in women with pregestational diabetes (Fong et al., 2014). The specific mechanisms through which pregestational diabetes elicit more severe pregnancy complications are largely unknown, although more severe and longer *in utero* exposure to hyperglycemia may be implicated in this process. Deciphering the mechanisms that underlie maternal T1DM, T2DM and GDM may aid the understanding of how these different types of diabetes are linked with pregnancy outcomes. The expression of miRNAs is altered during pregnancy and diabetes, thus profiling these epigenetic markers may offer potential as tools to elucidate the mechanisms that underlie the different types of maternal diabetes.

1.10 Hypothesis, Aim and Objectives

Evidence in the literature indicates that unique molecular mechanisms may underlie the different types of maternal diabetes. The expression of miRNAs, important epigenetic markers that regulate the expression of various pregnancy and diabetes associated genes, are altered during disease. Therefore, we hypothesised that miRNAs are altered during maternal diabetes, and that expression would differ between T1DM, T2DM and GDM.

The aim of the study was to assess the effect of maternal diabetes type on circulating miRNA expression.

The three objectives to achieve the study aim were:

i. To assess the metabolic state of pregnant women with diabetes by measuring serum insulin, C-peptide, total adiponectin, HMW adiponectin, TG and CRP concentrations using enzyme-linked immunosorbent assays (ELISAs).
ii. To profile miRNAs in the serum of pregnant women with T1DM, T2DM and GDM using the Human Serum/Plasma miScript miRNA PCR array.
iii. To explore the functional significance of differentially expressed miRNAs by conducting bioinformatics to identify miRNA gene targets and their pathways.

CHAPTER 2: REVIEW

MicroRNAs as diagnostic biomarkers of diabetes in pregnancy

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Contribution:

Conceptualisation and original review draft; literature search and study selection; data extraction; manuscript writing

2.1 Abstract

Diabetes in pregnancy poses a serious health risk to both mother and child. Several lines of evidence suggest that pregestational diabetes is associated with more severe pregnancy complications than gestational diabetes (GDM), most likely due to preconceptual exposure, higher level of hyperglycaemia and different pathophysiological mechanisms. In recent years, microRNAs (miRNAs), particularly placental-derived miRNAs, have emerged as key players in the pathophysiology of pregnancy-related disorders. MiRNA signatures in pregnant women with type 1 diabetes (T1DM), type 2 diabetes (T2DM) and GDM may offer insight into their different underlying mechanisms and health consequences. In this review we provide an overview of diabetes in pregnancy and describe miRNA biology focusing on the potential role of miRNAs during pregnancy and pregnancy-related complications. Studies that have profiled miRNAs in pregnancies complicated by diabetes are summarised. Our findings highlight the lack of miRNA profiling in pregnant women with T1DM and T2DM, and the challenges posed by miRNAs. We identified four miRNAs that were similarly differentially expressed in women with GDM compared to pregnant women with normoglycaemia in two or more studies. These miRNAs may serve as potential biomarkers for GDM. Further studies to investigate the importance of miRNAs in the development of the placenta and their contribution to cell-to-cell communication in pregnancies complicated by T1DM, T2DM and GDM are warranted. Such studies will provide insight into the mechanisms that underlie the different types of diabetes and could identify predictive biomarkers of adverse pregnancy outcomes, potentially allowing mitigation of these complications.

Keywords: microRNAs; pregnancy; type 1 diabetes mellitus; type 2 diabetes mellitus; gestational diabetes mellitus.

2.2 Introduction

Diabetes in pregnancy is a significant cause of morbidity for mothers and their children. The prevalence of diabetes in pregnancy is increasing globally and has been exacerbated by the recent COVID-19 pandemic. For example, the COVID-19 lockdown restrictions are associated with an increased risk of developing diabetes during pregnancy (Justman et al., 2020), possibly due to increased maternal stress, cortisol production and insulin resistance. (Rosmond, 2003; Joseph and Golden, 2017). Type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM) and gestational diabetes mellitus (GDM) are the three main types of diabetes that complicate pregnancies. Globally, ~15.8% of live births (20.4 million) are associated with diabetes in pregnancy, of which 83.6% are due to GDM, 7.9% due to pre-existing T1DM or T2DM, and 8.5% due to T1DM and T2DM first detected in pregnancy (International Diabetes Federation, 2019). Although all types of diabetes are associated with adverse health consequences for mother and child, pregestational diabetes has poorer pregnancy outcomes than GDM, possibly due to different pathophysiological mechanisms, level of hyperglycaemia and exposure to a hyperglycaemic environment earlier in placental development (Huynh et al., 2015; Xiang et al., 2018). Pregestational diabetes is associated with an increased risk of fetal and neonatal loss, congenital malformations, preterm delivery, macrosomia, pre-eclampsia, caesarean deliveries, and maternal mortality. Although GDM also carries an increased risk for these adverse outcomes, the complications are not as severe or common (Mathiesen and Vaz, 2008; Shand et al., 2008). Women with pregestational diabetes are treated with insulin and/or metformin (Kitzmiller et al., 2008; Simmons, 2010), whereas the first line of treatment for GDM is diet and exercise, followed by pharmacological therapy if women fail to reach their glucose targets using diet and exercise (Dias et al., 2019b). Studies have reported that plant-based diets and physical activity can reduce the risk of developing GDM (Mijatovic-Vukas et al., 2018; Fedullo et al., 2021; Schiattarella et al., 2021), possibly by improving immune function and decreasing the expression of pro-inflammatory cytokines such as interleukin 6, which is of particular importance during the COVID-19 pandemic (Fedullo et al., 2021), and ameliorating oxidative stress and insulin resistance (Schiattarella et al., 2021). An improved understanding of the pathophysiological mechanisms that underlie diabetes in pregnancy may facilitate strategies to mitigate adverse pregnancy outcomes.

MicroRNAs (miRNAs) are short, single stranded non-coding RNA molecules that have attracted considerable interest as biomarkers to improve diagnostic strategies and to further our understanding of the pathophysiological processes that underlie disease development (Ardekani and Naeini, 2010; Turchinovich et al., 2012; Guay and Regazzi, 2017). MiRNAs regulate various biological pathways and their dysregulation play a critical role in disease development. Furthermore, the important role that miRNAs play during pregnancy and its complications is increasingly recognised (Cai et al., 2017; Poirier et al., 2017).

This narrative review provides a comprehensive update of studies that have profiled miRNAs in diabetic pregnancies. Firstly, we provide an overview of diabetes in pregnancy, describing miRNA biology and focusing on its role during pregnancy and how miRNAs may impact maternal metabolism and fetal development. We also summarise studies that have profiled miRNAs in pregnancies complicated by diabetes. Four databases, Pubmed, Web of Science, EBSCOhost and Scopus were searched using the search terms “type 1 diabetes”, “type 2 diabetes”, “gestational diabetes mellitus”, “pregestational diabetes”, “maternal diabetes”, “microRNA” and “pregnancy”, including corresponding synonyms and associated terms for each word. We included all studies that profiled miRNAs in pregnancies complicated by T1DM, T2DM or GDM and excluded articles that investigated non-diabetic, pregnant women.

2.2.1 Overview of diabetes in pregnancy

The increasing prevalence of diabetes in pregnancy, both pregestational (T1DM and T2DM) and GDM is partly attributed to the increasing obesity and T2DM epidemics (Ali and Dornhorst, 2011; Dornhorst and Banerjee, 2010; McCance, 2015). T1DM occurs due to the failure of pancreatic beta (β) cells to produce insulin and accounts for approximately 5-10% of diabetes cases worldwide (American Diabetes Association, 2013). T2DM, which accounts for 90-95% of diabetes cases globally (American Diabetes Association, 2013), primarily develops due to obesity and insulin resistance (Kahn et al., 2006). GDM, a milder form of hyperglycaemia with onset in the second trimester, is thought to develop in pregnant women who are unable to mount a compensatory β-cell response to counteract insulin resistance, a characteristic of pregnancy (Kühl, 1991). The screening and diagnosis of GDM is contentious with some international bodies advocating for universal screening at 24-28 weeks of gestation (International Association of Diabetes and Pregnancy Study Groups Consensus Panel, 2010),

while others recommend risk-factor screening (Dias et al., 2019b). The recommendation for universal screening is based on findings from the Hyperglycaemia and Adverse Pregnancy Outcome (HAPO) study, which shows that mild levels of glucose intolerance, even at glucose concentrations previously considered normal, are associated with adverse pregnancy outcomes (Metzger et al., 2008). Despite this recommendation, many countries and institutions still use selective or risk-factor screening to diagnose GDM, which, unfortunately, has poor predictive value and fails to identify a large proportion of women with GDM (Dias et al., 2019b).

Diabetes in pregnancy is considered a high-risk condition for both mother and baby, with perinatal complications more common than in non-diabetic pregnant women (Ali and Dornhorst, 2011; Dornhorst and Banerjee, 2010; McCance, 2015). It has been suggested that pregestational diabetes is associated with more severe pregnancy complications than GDM, most likely due to preconceptual and longer exposure to hyperglycaemia during pregnancy (Fong et al., 2014). Optimal glucose control during the first 12 weeks of pregnancy is critical. Hyperglycaemia leads to excess reactive oxygen species production and oxidative stress, which damages embryonic DNA and modifies key apoptotic regulatory genes, thereby increasing the risk of congenital malformations (Dornhorst and Banerjee, 2010; Guerin et al., 2007). In the second trimester, maternal hyperglycaemia stimulates fetal insulin secretion leading to accelerated fetal growth. Other growth factors, including human placental growth hormone, fetal insulin-like growth factor and tumour necrosis factor α, are also influenced by maternal hyperglycaemia. Moreover, maternal hyperglycaemia may have detrimental long-term effects on fetal pancreatic islets, potentially increasing the risk of diabetes in later life. Persistent maternal hyperglycaemia and fetal hyperinsulinaemia creates a metabolic environment that predisposes to pregnancy complications such as miscarriage, stillbirth, fetal macrosomia, congenital malformations, transient neonatal morbidity including hypoglycaemia, neonatal death due to congenital malformation and prematurity, and an increased risk developing obesity and/or diabetes in later life (Dornhorst and Banerjee, 2010). The risk increases with worsening glycaemic control, maternal obesity and increasing age. As such, optimal antenatal management and strict maternal glycaemic control is essential to improve pregnancy outcomes (McCance, 2015). Key to such initiatives is the identification of sensitive biomarkers of glucose control that may serve as predictors of poor maternal and fetal outcomes in pregnancies complicated by diabetes.

2.2.2 MicroRNA biology

MiRNAs were first identified in *Caenorhabditis elegans* in 1993 (Lee et al., 1993) and have since emerged as powerful regulators of diverse biological processes including development, proliferation, differentiation, apoptosis and metabolism (Ardekani and Naeini, 2010; Cai et al., 2017; Regazzi, 2018). MiRNAs are short, non-coding, single-stranded RNA molecules that are ~22 nucleotides in length and are produced in a stepwise process involving multiple enzymes. MiRNA genes, which are located in intra- and intergenic regions of the genome, are transcribed in the nucleus by RNA polymerase II (and possibly by RNA polymerase III), to produce primary miRNA transcripts of ~1000 nucleotides in length (Bartel, 2004). Primary transcripts are cleaved by the microprocessor complex Drosha RNase III endonuclease to generate stem-loop precursor miRNAs that are ~70 nucleotides long. Ran-GTP and the export receptor (Exportin-5) transports miRNAs to the cytoplasm, where Dicer, a RNase III endonuclease, cleaves them to produce mature miRNAs. Mature miRNAs complex with the RNA-induced silencing complex (RISC), which guides it to messenger RNA (mRNA) for degradation or silencing (Bartel, 2004; Guo et al., 2010). MiRNAs, complexed to RISC, bind to the 3′ untranslated region (UTR) of mRNA inducing degradation or translational repression of the mRNA transcript (Guo et al., 2010). This interaction is dependent on the complementarity at the miRNA seed region, a conserved sequence that is usually located at positions 2-8 at the 5′end. Although miRNAs are mainly implicated in gene silencing, studies have suggested that miRNAs may have additional functions such as promoting protein synthesis (Ørom et al., 2008; Vasudevan et al., 2007), regulating gene transcription by interacting with other epigenetic mechanisms such as DNA methylation (Pheiffer et al., 2016) and influencing nuclear transcript stability and alternative splicing events when reimported into the nucleus (Roberts, 2014). Furthermore, recent studies have increasingly recognised the important role extracellular miRNAs play in cell-to-cell communication by either acting locally (paracrine or autocrine) or at distance (endocrine/exocrine) (Gupta et al., 2010; Turchinovich et al., 2012; Guay and Regazzi, 2017). Extracellular miRNAs are highly stable due to their association with various carriers such as lipoproteins, RNA-binding proteins (Argonaute RISC Catalytic Component 2 (AGO2) and nucleophosmin 1 (NPM1)) and microvesicles (e.g., exosomes), which has sparked interest in their potential role as biomarkers.

2.2.3 MicroRNAs and pregnancy

Pregnancy enacts enormous stress on the mother's physiology, causing several physiological, biochemical, and metabolic adaptations to accommodate fetal growth and development (Soma-Pillay et al., 2016). In recent years, an important role for miRNAs during pregnancy and pregnancy-related diseases has emerged (Cai et al., 2017; Poirier et al., 2017). MiRNAs are key metabolic and developmental regulators that facilitate maternal metabolic adaption by responding to physiological changes during pregnancy, serving as cell-to-cell communicators between maternal cell types and tissues, and between maternal and fetal tissues (Mitchell et al., 2015). A large number of miRNAs have been identified in placental and uterine tissue and in maternal blood using techniques such as quantitative real time PCR, microarrays and sequencing. These miRNAs regulate processes that are critical for adequate placentation in early pregnancy and facilitate the flow of blood and nutrients between the mother and her fetus. Although pregnancy adaption is mainly driven by placental factors, the importance of other maternal metabolic tissues is increasingly recognised (Poirier et al., 2017).

Placental-derived circulating miRNAs, which are primarily secreted by exosomes, may reflect the physiological state of the pregnancy and fetal development, thus may offer potential as biomarkers to predict pathologies and pregnancy outcome (Mitchell et al., 2015). This theory is supported by several studies. Exosomes, which are ~40-120 nanometres in size, are secreted into maternal circulation, primarily from syncytiotrophoblasts (Luo et al., 2009), as early as the 6th week of gestation and increase significantly during pregnancy (Salomon et al., 2014; Cai et al., 2017; Poirier et al., 2017). Furthermore, exosomes are released during normal physiological conditions, while the rate of release increases in response to pathological conditions such as hyperglycaemia (Mitchell et al., 2015), suggesting that circulating placental miRNAs may be useful to diagnosis placental dysfunction and may serve as a clinically useful early pregnancy screening test to identify women at risk of developing pregnancy complications. The early detection of women at risk of pregnancy complications may provide an opportunity for risk stratification and intervention to improve maternal and neonatal outcomes. Accordingly, studies have reported that maternal circulating miRNAs are associated with placental weight (Miura et al., 2014), placental abruption (Miura et al., 2016), placental previa (Hasegawa et al., 2015), preeclampsia, gestational hypertension, and fetal growth restriction (Hromadnikova et al.,

2013), preeclampsia and intrauterine growth restriction (Hromadnikova et al., 2017), macrosomia (Jiang et al., 2015) and gestational diabetes (Pheiffer et al., 2018a).

Although T1DM, T2DM and GDM represent distinct pathophysiological states, miRNAs with shared expression between these diabetes types were reported. Using Agilent miRNA arrays, Collares et al. (2013) reported a set of common miRNAs in peripheral blood mononuclear cells of seven female and male patients with T1DM, seven female and male patients with T2DM and six women with GDM (Collares et al., 2013). Nine miRNAs, miR-126, miR-1307, miR-142-3p, miR-142-5p, miR-144, miR-199a-5p, miR-27a, miR-29b and miR-342-3p were common to T1DM, T2DM and GDM, which may reflect metabolic and inflammatory pathways that are shared between T1DM, T2DM and GDM. Interestingly, these authors identified several miRNAs that were unique to each diabetes type. Eleven miRNAs, let-7f, let-7g, miR-103, miR-1260, miR-1274a, miR-1274b, miR-130a, miR-150, miR-20b, miR-21 and miR-720 were unique to T1DM. Five miRNAs, miR-140-3p, miR-199a-3p, miR-222, miR-30e and miR-451 were unique to T2DM. Ten miRNAs, miR-101, miR-1180, miR-1268, miR-181a, miR-181d, miR-26a, miR-29a, miR-29c, miR-30b and miR-595 were unique to GDM (Collares et al., 2013). Specific miRNAs may represent biological markers for each type of diabetes, warranting further investigation as potential mechanisms that underlie the different diabetes types. Unfortunately, patients with T1DM and T2DM were not pregnant and included both females and males. MiRNAs are dynamic and altered in response to physiological changes during pregnancy (Mitchell et al., 2015), while sex differences in miRNA expression have been reported (Karere et al., 2021). Thus, studies on miRNA profiling in pregnant women with T1DM, T2DM and GDM may be more suitable to identify potential biomarkers of pregnancy health and disease, fetal development and pregnancy outcomes.

2.2.4 MicroRNA profiling in pregnancies complicated by diabetes

Our comprehensive literature search identified a total of 1785 articles, of which 41 met the inclusion criteria and are included in the review (Figure 4). Studies that did not profile miRNAs in pregnant women with diabetes were excluded. The 41 included studies were case-control studies on GDM conducted between 2011 and 2021 (Table 1). GDM was defined using different diagnostic criteria (diagnostic criteria cut-off values Appendix 1, Table 10) including the 2010 International Association of Diabetes and Pregnancy Study

Groups (IADPSG) criteria (n=11); American Diabetes Association (ADA) criteria in 2004 (n=2), 2006 (n=1), 2010 (n=2), 2011 (n=1), 2012 (n=1) and 2016 (n=1); World Health Organisation (WHO) criteria, 2014 (n=1) and IADPSG/WHO, 2013 (n=1); 2011 Italian National Health System guidelines (n=2); 2015 Australasian Diabetes in Pregnancy Society (ADIPS)/WHO, 2014 criteria (n=1); Chinese society for Diabetes Mellitus criteria (n=1); 2013 Endocrine Society Clinical Practice Guideline (n=1); 2016 Guidelines of the Society of Obstetricians and Gynaecologists of Canada (n=1) and the 1979 National Diabetes Data Group (NDDG) criteria (n=1). Thirteen studies did not state what diagnostic criteria were used. None of the included studies profiled maternal miRNAs in pregnant women with T1DM and T2DM. The majority of studies were conducted in Chinese and European women, with only one study conducted in Africa. Sample size varied considerably between studies ranging from three to 204 women. MiRNAs were profiled in various biological samples including serum (n=13), plasma (n=8), placenta (n=11), whole blood (n=9), urine (n=1), human umbilical vein endothelial cells (HUVECs) (n=1), skeletal muscle tissue (n=1) and omental adipose tissue (n=1). Different techniques were employed. The majority of studies profiled miRNAs using quantitative real-time PCR (qRT-PCR) with SYBR Green (n=23), Taqman probes (n=15) and TB Green (n=1). Other techniques included Taqman Low Density arrays (n=1), miRNA sequencing (n=3), Illumina TrueSeq Small RNA kit (n=1), miRCURY LNA™ Array (n=2), TaqMan array microfluidics (n=1), miScript array T & B cell activation (n=1), Agilent miRNA Microarrays (n=1), NanoString nCounter human miRNA assay (n=1) and AFFX chip microarrays (n=1). Gestational age at the time of miRNA profiling ranged between 6 and 40 weeks. U6 and *C. elegans* miR-39 were the most common normalisation controls used.

A wide range of miRNAs were found to be differentially expressed, with only a few similarly expressed across studies. MiRNAs that were investigated in two or more studies included miR-16 (n=7), miR-222 (n=6), miR-29a (n=6), miR-19b (n=5), miR-19a (n=5), miR-330 (n=4), miR-132 (n=4), miR-20a (n=3), miR-517 (n=3), miR-223 (n=3), miR-210 (n=3), miR-17 (n=3), miR-155 (n=3), miR-29b (n=2), miR-657 (n=2), miR-92a (n=2), miR-137 (n=2), miR-483 (n=2), miR-342 (n=2), miR-30d (n=2), let-7g (n=2), miR-195 (n=2), miR-21 (n=2), miR-9 (n=2) and miR-494 (n=2). Seven studies reported on miR-16 expression. Of these, three studies reported higher miR-16 expression in the serum and plasma of women with GDM compared to pregnant women with normoglycaemia (Cao et al., 2017; Sørensen et al.,

2021; Zhu et al., 2015). In contrast, Herrera-Van Oostdam et al. (2020) reported lower miR-16 expression in urine samples of women with GDM in the third trimester, but higher expression in the first and second trimesters (Herrera-Van Oostdam et al., 2020). Three studies reported no difference in miR-16 expression between women with or without GDM (Hocaoglu et al., 2019, 2020; Pheiffer et al., 2018b). Of the six studies that profiled miR-222, two studies reported higher expression of miR-222 in omental adipose tissue and plasma of women with GDM compared to pregnant women with normoglycaemia (Shi et al., 2014; Tagoma et al., 2018), while two studies reported lower expression of miR-222 in serum of pregnant women with GDM compared to pregnant women with normoglycaemia (Pheiffer et al., 2018b; Zhao et al., 2011). Wander et al. (2017) demonstrated no difference in the expression of miR-222 in plasma of pregnant women with GDM compared to pregnant women with normoglycaemia (Wander et al., 2017). Herrera-Van Oostdam et al. (2020) reported differences in miR-222 expression during pregnancy. These authors demonstrated higher expression of miR-222 in urine samples of women with GDM compared to pregnant women with normoglycaemia during the first trimester, but observed no significant difference in expression in the second trimester, and lower expression in the third trimester (Herrera-Van Oostdam et al., 2020). Inconsistencies in miR-29a expression across studies were also observed. Three studies showed that miR-29a expression was higher in serum of women with GDM compared to pregnant women with normoglycaemia (Gillet et al., 2019; Martínez-Ibarra et al., 2019; Sørensen et al., 2021), one study reported lower expression of miR-29a in serum during GDM (Zhao et al., 2011), while two studies reported no difference in miR-29a expression in the serum and plasma of pregnant women with GDM compared to pregnant women with normoglycaemia (Wander et al., 2017; Pheiffer et al., 2018b). Five studies profiled both miR-19a and miR-19b. Two studies reported increased expression of miR-19a and miR-19b in serum and plasma samples of GDM patients (Zhu et al., 2015; Wang et al., 2019a). However, two studies reported no difference in the expression of miR-19a and miR-19b in plasma and serum samples between pregnant women with GDM and pregnant women with normoglycaemia (Cao et al., 2017; Pheiffer et al., 2018b). Stirm et al. (2018) reported conflicting results. These authors observed higher expression of both miR-19a and miR-19b in the whole blood of women with GDM compared to normoglycaemia in the screening group using miRNA sequencing, but reported no difference in miR-19a and miR-19b expression using SYBR green in the validation sample (Stirm et al., 2018). All four of the studies that profiled miR-330 found that its expression was higher in serum and plasma of pregnant women with GDM compared to women with normal pregnancies (Sebastiani et al.,

2017; Martínez-Ibarra et al., 2019; Pfeiffer et al., 2020; Xiao et al., 2020). These studies used serum and plasma samples to profile miR-330 expression using qRT-PCR in Latin and Chinese women with GDM compared to women with normoglycaemia. Four studies profiled miR-132 expression during GDM. Two studies showed that miR-132 expression was lower in the serum and placenta of Chinese women with GDM compared to pregnant women with normoglycaemia (Zhao et al., 2011; Zhou et al., 2019). Pheiffer et al. (2018b) saw a trend towards decreased miR-132 expression in South African women with GDM (Pheiffer et al., 2018b). However, in contrast, Gillet et al. (2019) reported that miR-132 expression was higher in the serum of Canadian women with GDM compared to pregnant women with normoglycaemia (Gillet et al., 2019).

MiRNAs investigated in three studies include miR-517, which exhibited higher expression during GDM in the first and second trimesters but lower expression in the third trimester (Herrera-Van Oostdam et al., 2020). The other two studies that profiled miR-517 showed no difference in expression in the serum of women with GDM compared to pregnant women with normoglycaemia (Wander et al., 2017; Gillet et al., 2019). Three studies profiled miR-20a, of which two studies reported higher expression of miR-20a in women with GDM when compared to pregnant women with normoglycaemia (Zhu et al., 2015; Cao et al., 2017). However, Pheiffer et al. (2018b) reported lower expression of miR-20a in serum samples of South African women with GDM when compared to controls (Pheiffer et al., 2018b). Two studies reported increased expression of miR-223 in serum and plasma of women with GDM (Yoffee et al., 2019; Abdeltawab et al., 2020), however, Wander et al. (2017) reported no difference in the expression of miR-223 in plasma of American women with GDM compared to pregnant women with normoglycaemia (Wander et al., 2017). Expression of miR-210 was shown to be higher in serum samples of women with GDM compared to women with normoglycaemia (Gillet et al., 2019), lower in placental samples of women with GDM (Ding et al., 2018), while no difference in miR-210 expression was observed in plasma (Wander et al., 2017). MiR-17 was differentially expressed in three studies. Two studies reported higher expression of miR-17 in plasma samples of Chinese women with GDM when compared to pregnant women with normoglycaemia (Zhu et al., 2015; Cao et al., 2017). Pheiffer et al. (2018) observed a trend towards decreased miR-17 expression in South African women with GDM compared to pregnant women with normoglycaemia (Pheiffer et al., 2018b). Three studies profiled miR-155 with conflicting results reported. Wander et al. (2017) reported higher expression in plasma samples of American women with GDM when compared to

pregnant women with normoglycaemia (Wander et al., 2017). Hocaoglu et al. (2019) reported no change in the expression of miR-155 in the whole blood of Turkish women with GDM (Hocaoglu et al., 2019), but in a more recent study, these authors showed lower expression of miR-155 in the whole blood of Turkish women with GDM compared to pregnant women with normoglycaemia (Hocaoglu et al., 2020).

Twelve miRNAs were investigated in two studies. Circulating levels of three miRNAs, miR-342 (Tagoma et al., 2018; Gillet et al., 2019), let-7g (Stirm et al., 2018; Tagoma et al., 2018) and miR-195 (Tagoma et al., 2018; Wang et al., 2020) were higher in plasma/serum/whole blood of women with GDM compared to pregnant women with normoglycaemia. In contrast, conflicting results were reported for eight circulating miRNAs, including miR-92a and miR-30d (Li et al., 2015; Tagoma et al., 2018), miR-9 (Li et al., 2015; Martínez-Ibarra et al., 2019), miR-137 (Li et al., 2015; Peng et al., 2018), miR-483 (Sebastiani et al., 2017; Gillet et al., 2019), miR-21(Wander et al., 2017; Hocaoglu et al., 2019), miR29b (Gillet et al., 2019; Sun et al., 2020) and miR-494 (He et al., 2017; Gillet et al., 2019). The expression of miR-657 was higher in placental and placental-derived mononuclear macrophages of women with GDM compared to pregnant women with normoglycaemia (Wang et al., 2018, 2019b). Twelve articles included in this review reported differential miRNA expression, yet these were identified in single studies only (Zhao et al., 2014; Floris et al., 2015; Cao et al., 2016; Xu et al., 2017; Lamadrid-Romero et al., 2018; Li et al., 2018; Monfared et al., 2018; Nair et al., 2018; Zhang and Chen, 2020; Balci et al., 2020; Feng et al., 2020; Hu et al., 2021).

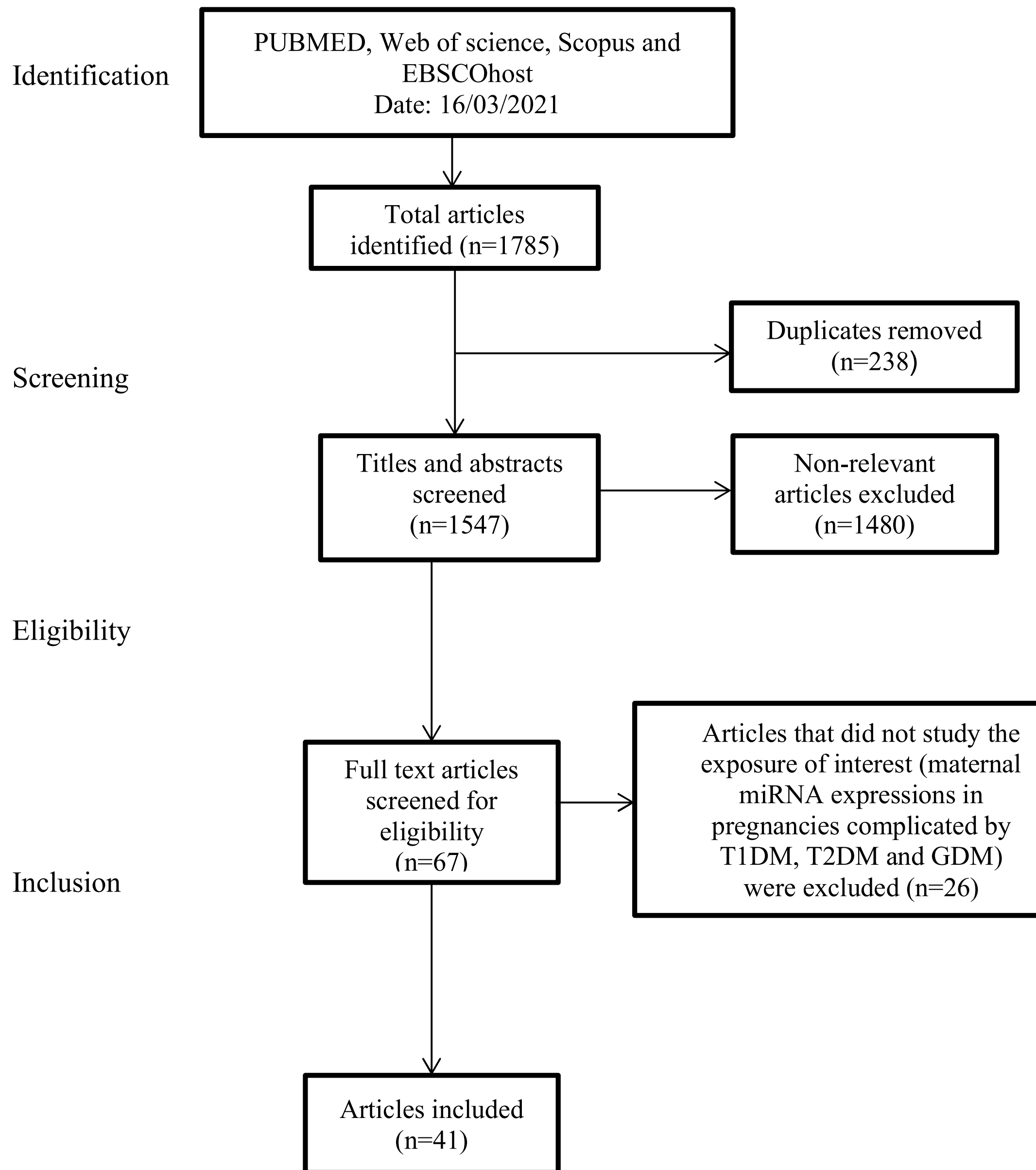


Figure 4. Flow diagram illustrating study selection

Table 1. Studies profiling maternal miRNAs in pregnancies complicated by GDM

Author	Country	Study design	Gestational age (weeks)	GDM diagnostic criteria	Sample size	Biological source	Method	Normalization	Outcomes (GDM vs control)
(Zhao et al., 2011)	China	Case-control	16-19	ADA, 2004	**Discovery sample** GDM = 24 Controls = 24 **Internal validation** GDM = 36 Controls = 36 **External validation 1** GDM = 16 Controls = 16 **External validation 2** GDM = 16 Controls = 16	serum	**Discovery** TaqMan Low Density Arrays **Validation** Individual SYBR Green qRT-PCR	**Discovery** RNU6B cel-miR-39 **Validation** cel-miR-39	**Discovery sample** ↓ miR-132 (p=0.042), miR-29a (p=0.032), miR-222 (p=0.041). **Internal validation** ↓ miR-132 (p=0.034), miR-29a (p=0.045), miR-222 (p=0.016). **External validation 1** ↓ miR-29a (p=0.001), miR-222 (p=0.017). **No significant change** miR-132 (p=0.235), **External validation 2** ↓ miR-132 (p=0.001), miR-29a (p=0.001), miR-222 (p=0.001).
(Monfared et al., 2018)	Iran	Case-control	16-19	NR	GDM = 30 Controls = 30	serum	SYBR Green qRT-PCR	RNU6B	↑ miR-135a (p=0.001)
(Lamadrid-Romero et al., 2018)	Mexico	Case-control	First trimester Second trimester Third trimester	ADA, 2016	**First trimester** GDM = 13 Controls = 12 **Second trimester** GDM = 24 Controls = 24 **Third trimester** GDM = 20 Controls = 16	serum	SYBR Green qRT-PCR	cel-miR-39	**First trimester** ↑ miR-183-5p (p<0.002), miR-200b-3p (p<0.009), miR-125b-5p (p<0.02), miR-1290 (p<0.03) **Second trimester** ↑ miR-183-5p (p=0.03), ↓ miR-200b-3p (p=0.04). **Third trimester** ↑ miR-183 (p<0.0001), ↓miR-200b-3p

									(P<0.001).
(Pheiffer et al., 2018b)	South Africa	Case-control	13-31	IADPSG, 2010	GDM = 28 Controls = 53	serum	SYBR Green qRT-PCR	cel-miR-39	↓ miR-20a-5p (2.7-fold; p=0.038), miR-222-3p (2.6-fold; p=0.027). **No significant change** miR-16-5p (1.9-fold; p=0.120), miR-17-5p (2.5-fold; p=0.121), miR-19a-3p (2.6-fold; p=0.056), miR-19b-3p (1.9-fold; p=0.625), miR-29a-3p (2.0-fold; p=0.768), miR-132-3p (2.4-fold; p=0.070).
(Martínez-Ibarra et al., 2019)	Mexico	Case-control	Second-third trimester	IADPSG, 2010	GDM = 18 Controls = 22	serum	TaqMan qRT-PCR	miR-454	↑ miR-9 (p=0.03), miR-29a (p=0.01), miR-330 (p=0.004)
(Gillet et al., 2019)	Canada	Case-control	6-15	Guidelines of the Society of Obstetricians and Gynaecologists of Canada, 2016	GDM = 23 Controls = 46	serum	SYBR Green qRT-PCR	cel-miR-39	↑ miR-520h (p=0.03), miR-1323 (p=0.03), miR-136-5p (p=0.03), miR-342-3p (p=0.008), miR-29a-3p (p=0.03), miR-29b-3p (p=0.04), miR-122-5p (p=0.01), miR-132-3p (p=0.03), miR-182-3p (p=0.01), miR-210-3p (p=0.02). **No significant change** miR-494-3p (p=0.10), miR-517-5p (p=0.12), miR-517a-3p, miR-376c-5p, miR-483-3p.
(Zhou et al., 2019)	China	Case-control	24-28	ADA, 2012	GDM = 108 Controls = 50	serum/ placenta	SYBR Green qRT-PCR	U6	↓ miR-132 (p<0.001)
(Wang et al., 2019a)	China	Case-control	24-28	NR	GDM = 100 Controls = 100	serum	TaqMan qRT-PCR	U6	↑ miR-19a (4.0-fold; p=0.001), miR-19b (4.77-fold; p=0.02).
(Xiao et al., 2020)	China	Case-control	16-28	Italian National Health System guidelines, 2011	GDM = 30 Controls = 10	serum	SYBR Green qRT-PCR	U6	↑ miR-330-3p (p<0.001)
(Wang et al.,	China	Case-control	24-28	IADPSG, 2010	GDM = 102	serum	SYBR Green qRT-	U6	↑ miR-195-5p (p<0.01).

2020)					Controls = 102		PCR		
(Pfeiffer et al., 2020)	Spain	Case-control	26-30	NDDG, 1979	GDM = 31 Controls = 29	serum	TaqMan qRT-PCR	cel-miR-39	↑ miR-330-3p (p = 0.003) **No significant change** miR-224-5p, miR-103-3p, miR-206.
(Abdeltawab et al., 2020)	NR	Case-control	Third trimester	NR	GDM = 109 Controls = 103	serum	TaqMan qRT-PCR	cel-miR-39	↑ miR-223 ($p<0.001$)
(Sørensen et al., 2021)	Europe	Case-control	<20	IADPSG, 2010 WHO, 2013	GDM = 82 Controls = 41	serum	SYBR Green qRT-PCR	cel-miR-39	↑ miR-29a-3p (p=0.004), miR-134-5p (p=0.046), miR-16-5p (p=0.008)
(Zhu et al., 2015)	China	Case-control	16-19	ADA, 2011	GDM = 10 Controls = 10	plasma	MiRNA sequencing, SYBR Green qRT-PCR	miR-221	↑ miR-16-5p ($p=5.36 \times 10^{-11}$), miR-17-5p ($p=1.10 \times 10^{-10}$), miR-19a-3p ($p=6.57 \times 10^{-43}$), miR-19b-3p ($p=1.73 \times 10^{-74}$), miR-20a-5p ($p=5.27 \times 10^{-37}$).
(Wander et al., 2017)	USA	Case-control	7-23	ADA, 2004	GDM = 36 Controls = 80	plasma	SYBR Green qRT-PCR	cel-miR-39 and miR-423-3p	↑ miR-155-5p (p=0.028), miR-21-3p (p=0.005), miR-146b-5p (p=0.068). **No significant change** miR-517-5p, miR-126-3p, miR-210-3p, miR-222-3p, miR-223-3p, miR-518a-3p, miR-29a-3p
(Cao et al., 2017)	China	Case-control	24-28	NR	GDM = 85 Controls = 72	plasma	TaqMan qRT-PCR	cel-miR-39, cel-miR-54, cel-miR-238	↑ miR-16-5p ($p<0.01$), miR-17-5p ($p<0.01$), miR-20a-5p ($p<0.01$). **No significant change** miR-19a-3p and miR-19b-3p
(Sebastiani et al., 2017)	Italy	Case-control	24-33	Italian National Health System guidelines, 2011	GDM = 21 Controls = 10	plasma	TaqMan array microfluidics **Validation** TaqMan qRT-PCR	miR-374, miR-320	**Taqman arrays** ↑ miR-330-3p (p=0.029), miR-483-5p (2.01-fold; p=0.028); ↓ miR-548c-3p (p=0.028), miR-532-3p (p=0.028). **TaqMan qRT-PCR** ↑ miR-330-3p (p=0.01)

									No significant change miR-548c-3p
(Tagoma et al., 2018)	Estonia	Case-control	23-31	IADPSG, 2010	GDM = 13 Controls = 9	plasma	MiScript miRNA PCR array Human T Cell & B Cell Activation, SYBR Green qRT-PCR	cel-miR-39	↑ let-7e-5p (p=0.03), let-7g-5p (p=0.01), miR-100-5p (p=0.04), miR-101-3p (p=0.03), miR-146a-5p (p=0.03), miR-18a-5p (p=0.05), miR-195-5p (p=0.03), miR-222-3p (p=0.03), miR-23b-3p (p=0.02), miR-30b-5p (p=0.04), miR-30c-5p (p=0.02), miR-30d-5p, (p=0.03), miR-342-3p (p=0.04), miR-423-5p (p=0.02), miR-92a-3p (p=0.05).
(Peng et al., 2018)	China	Case-control	24-28	ADA, 2010	GDM = 11 Controls = 12	plasma	SYBR Green qRT-PCR	U6	↑ miR-137 (p<0.01)
(Yoffe et al., 2019)	Italy/Spain	Case-control	9-12	IADPSG, 2010	GDM = 23 controls = 20	plasma	NanoString nCounter human miRNA assay **Validation** TaqMan qRT-PCR	cel-miR-39	↑ miR-23a ($p=1.92 \times 10^{-2}$), miR-223 ($p=1.42 \times 10^{-7}$). **Validation** ↑ miR-223 (p= 0.009), miR-23a (p=0.03).
(Balci et al., 2020)	Turkey	Case-control	NR	ADA, 2010	GDM = 30 Controls = 30	plasma	TaqMan qRT-PCR	NR	↑miR-7-5p (p<0.05)
(Zhao et al., 2014)	China	Case-control	37-40	NR	GDM = 40 Controls = 40	placenta	TaqMan qRT-PCR	U6	↑ miR-518d (p<0.01)
(Li et al., 2015)	China	Case-control	Delivery	Endocrine Society Clinical Practice Guideline, 2013	**Discovery** GDM = 5 Controls = 5 **Validation** GDM = 10 Controls = 10	placenta	**Discovery** Agilent Human miRNA Microarray **Validation** SYBR Green qRT-PCR	U6	↑ miR-508-3p (p<0.01); ↓ miR-27a (p<0.05), miR-9 (p<0.05), miR-137 (p<0.05), miR-92a (p<0.05), miR-33a (p<0.05), miR-30d (p<0.05), miR-362-5p (p<0.05), miR-502-5p (p<0.05). **No significant change** miR-148b, miR-10a, miR-370, miR-25, miR-15b.
(Cao et al.,	China	Case-control	37-40	NR	GDM = 193	placenta	SYBR Green qRT-	U6	↑ miR-98 (p<0.05)

2016)					Control = 202		PCR		
(Ding et al., 2018)	China	Case-control	Third trimester	IADPSG, 2010	**Discovery** GDM = 8 Controls = 8 **Validation** GDM = 20 Controls = 18	placenta	miRNA sequencing, SYBR Green qRT-PCR	U6	↑ miR-202-5p (p<0.01); ↓ miR-138-5p (p<0.01), miR-210-5p(p<0.05), miR-3158-5p (p<0.01), miR-4732-3p (p<0.05).
(Li et al., 2018)	China	Case-control	NR	NR	**Screening** GDM = 3 Controls = 3 **Validation** GDM = 15 Controls = 15	placenta/ whole blood	miRCURY LNA™ microRNA Array, qRT-PCR	NR	↓ miR-96 (p<0.01)
(Wang et al., 2019b)	China	Case-control	37-40	NR	GDM = 30 Controls = 29	placenta	TaqMan qRT-PCR	NR	↑ miR-657 (p<0.01)
(Sun et al., 2020)	China	Case-control	>37	IADPSG, 2010	GDM = 204 Controls = 202	placenta	TaqMan qRT-PCR	U6	↓ miR-29b (p<0.05)
(Wang et al., 2018)	China	Case-control	NR	NR	GDM = 48 Controls = 46	placental-derived mononuclear macrophages	SYBR Green qRT-PCR	NR	↑ miR-657 (p<0.001)
(Nair et al., 2018)	Australia	Case-control	≥37	ADIPS, 2015 WHO, 2014	GDM = 12 Controls = 12	placenta, plasma exosomes, skeletal muscle tissue	Illumina TrueSeq Small RNA kit, SYBR Green qRT-PCR	RNU6B	**Placenta, plasma exosomes, skeletal muscle tissue:** ↑ hsa-miR-125a-3p (p<0.05), hsa-miR-99b-5p (p<0.05), hsa-miR-197-3p (p<0.05), hsa-miR-22-3p (p<0.05),hsa-miR-224-5p (p<0.05), hsa-miR-27b-3p (p<0.05), hsa-miR-200a-3p (p<0.05), hsa-miR-141-3p (p<0.05). **Placenta:** ↓ hsa-miR-133a-3p (p=0.003)

									Plasma exosomes: ↓hsa-miR-133a-3p ($p=0.003$) **Skeletal muscle tissue:** ↑hsa-miR-133a-3p ($p<0.05$)
(Xu et al., 2017)	China	Case-control	NR	NR	**Placenta** GDM = 3 Controls = 3 **Whole blood** GDM = 25 Controls = 25	placenta/ whole blood	miRCURY LNA™Array, qRT-PCR	NR	↑ miR-503 ($p<0.01$).
(Hocaoglu et al., 2019)	Turkey	Case-control	33 ± 4.1	IADPSG, 2010	GDM = 19 Controls = 28	whole blood	SYBR Green qRT-PCR	U6	↓ miR-21-3p ($p = 0.001$) **No significant change** miR-16-5p, miR155-5p.
(He et al., 2017)	China	Case-control	NR	NR	GDM = 20 Controls = 20	whole blood	TaqMan qRT-PCR	U6	↓ miR-494 ($p<0.01$)
(Stirm et al., 2018)	Germany	Case-control	24-32	IADPSG, 2010	**Screening** GDM = 8 Controls = 8 **Validation** GDM = 30 Controls 30	whole blood	miRNA sequencing, SYBR Green qRT-PCR	U6	**Screening group** ↑ miR-19a ($p=1.48 \times 10^{-03}$), miR-19b ($p=5.28 \times 10^{-03}$), miR-142 ($p=3.36 \times 10^{-04}$), miR-143 ($p=8.72 \times 10^{-04}$), let-7g-5p ($p=4.32 \times 10^{-04}$), miR-340 ($p=7.06 \times 10^{-04}$) **Validation group** ↑ miR-340 ($p=0.03$) **No significant change** miR-19a, miR-19b, miR-142, miR-143, let-7g-5p.
(Zhang and Chen, 2020)	China	Case-control	NR	NR	GDM = 30 Controls = 30	whole blood	SYBR Green qRT-PCR	U6	↑ miR-770-5p ($p<0.01$)
(Hocaoglu et al., 2020)	Turkey	Case-control	33 ± 4.1	IADPSG, 2010	GDM = 14 Controls = 27	whole blood	SYBR Green qRT-PCR	U6	↓ miR-155-5p ($p=0.04$) **No significant change** miR-16-5p

(Feng et al., 2020)	China	Case-control	24-28	Chinese society for Diabetes Mellitus	GDM =12 Controls = 12	Whole blood	TaqMan qRT-PCR	U6	↑ miR-33a-5p ($p<0.01$)
(Hu et al., 2021)	China	Case-control	24-28	IADPSG, 2010	GDM =35 Controls =35	whole blood	TB Green qRT-PCR	U6	↓ miR-4646 ($p<0.001$), miR-5196 ($p<0.001$), miR-3679 ($p=0.009$) **No significant change** miR-8061
(Herrera-Van Oostdam et al., 2020)	Mexico	Case-control	**First trimester** (8-20) **Second trimester** (24-28) **Third trimester** (32-39)	WHO, 2014	GDM = 27 Controls = 34	urine	TaqMan qRT-PCR	U6	**First trimester** ↑ miR-16-5p ($p<0.05$), miR-222-3p ($p<0.05$), miR-516b-5p ($p<0.05$), miR-517-5p ($p<0.05$), miR-518-3p ($p<0.05$) **Second trimester** ↑ miR-16-5p ($p=0.009$), miR-516b-5p ($p=0.043$), miR-517-5p ($p=0.034$), miR-518-3p ($p=0.021$) **No significant change** miR-222-3p ($p=0.387$), **Third trimester** ↓ miR-16-5p ($p<0.01$), miR-222-3p ($p<0.01$), miR-516b-5p ($p<0.05$), miR-517-5p ($p<0.05$), miR-518-3p ($p<0.01$)
(Shi et al., 2014)	China	Case-control	38-39	ADA, 2006	GDM = 13 Controls = 13	omental adipose tissue	AFFX miRNA expression chips microarrays, TaqMan qRT-PCR	miR-16	↑ miR-222 ($p<0.01$)

(Floris et al., 2015)	Italy	Case-control	24-28	NR	GDM = 22 Controls = 24	Human umbilical vein endothelial cells	SYBR Green qRT-PCR	beta-actin	↑ miR-101 ($p<0.01$)

(↑) up- and (↓) down- regulation, p value and fold regulation reported if given in article, ADA: American Diabetes Association, ADIPS: Australasian Diabetes in Pregnancy Society, GDM: gestational diabetes mellitus, IADPSG: International Association of Diabetes in Pregnancy Study Group, NDD: National Diabetes Data Group, NR: not reported, qRT-PCR: quantitative reverse transcription PCR, WHO: World Health Organisation, Chinese society for Diabetes Mellitus (year not specified), TB Green: Premix Ex Taq II (Tli RNase H Plus)

2.3 Discussion

MiRNA signatures in pregnant women with T1DM, T2DM and GDM may offer insight into their different underlying mechanisms and health consequences for mother and child (Dornhorst and Banerjee, 2010; Mitchell et al., 2015; Poirier et al., 2017; Cai et al., 2017). This review provides an overview of diabetes in pregnancy, focusing on the potential role of miRNAs as biomarkers of pregnancy health and disease. We summarise all studies that have profiled miRNAs in pregnancies complicated by diabetes. Our main finding is the lack of studies that have profiled miRNAs in pregnant women with pregestational diabetes (T1DM and T2DM) or in women who developed overt or T2DM during pregnancy. We highlight a set of miRNAs that are differentially expressed in women with GDM in two or more studies. Lastly, findings of this review support the challenges of miRNA profiling and the concerns that must be addressed before miRNAs can become clinically applicable.

Despite our search identifying 41 research articles that profiled miRNAs in diabetic pregnancies, none of these quantified miRNAs in pregnancies complicated by T1DM and T2DM. All pregnancies complicated by diabetes are considered high-risk, with the risk positively correlated with the level of hyperglycaemia (Dornhorst and Banerjee, 2010; Ali and Dornhorst, 2011; McCance, 2015). Accordingly, pregestational diabetes is associated with more severe adverse pregnancy outcomes than GDM (Ali et al., 2020). Profiling miRNAs, potential markers of pregnancy health and glucose control, during T1DM, T2DM and GDM may lead to a better understanding of the mechanisms that link hyperglycaemia with adverse outcomes (Cai et al., 2017; Poirier et al., 2017). Although Collares et al. (2013) profiled miRNAs during T1DM, T2DM and GDM (Collares et al., 2013), this investigation included non-pregnant women with T1DM and T2DM and thus does not reflect placental-derived miRNAs and pregnancy pathophysiology. Our findings highlight the need for studies to profile miRNAs in pregnancies complicated by all types of diabetes to allow insight into the underlying mechanisms and to enable comparisons between T1DM, T2DM and GDM during pregnancy.

MiRNAs offer tremendous potential for GDM screening and diagnosis (Pheiffer et al., 2018a), however, population-specific factors such as ethnicity, age, gender and stage of disease influence miRNA expression (Bovell et al., 2013; Florio et al., 2020; Karere et al., 2021) and may hinder their widespread applicability (Dias et al., 2018). We identified four

circulating miRNAs that were consistently expressed at higher levels in women with GDM compared to women with normoglycaemia in different populations, using different methodologies and during different gestational ages. These include miR-330 (n=4), miR-342 (n=2), let-7g (n=2) and miR-195 (n=2). Of interest is miR-330 as circulating levels of this miRNA was consistently higher in serum and plasma isolated from women with GDM compared to women with normoglycaemia across four studies conducted in Italy, Mexico, Spain, and China using different methodologies (Martínez-Ibarra et al., 2019; Pfeiffer et al., 2020; Sebastiani et al., 2017; Xiao et al., 2020). Similarly, miR-342 was consistently expressed at higher levels in women with GDM compared to women with normoglycaemia in studies conducted in Canada and Estonia (Gillet et al., 2019; Tagoma et al., 2018), let-7g was expressed at higher levels during GDM in studies conducted in Germany and Estonia (Tagoma et al., 2018; Stirm et al., 2018), and miR-195 was expressed at higher levels during GDM in studies conducted in China and Estonia (Tagoma et al., 2018; Wang et al., 2020). MiRNAs that are commonly expressed across diverse populations, biological samples and using different measurement platforms present opportunities as biomarkers for GDM with widespread applicability.

Our findings support the challenges of miRNA profiling (Dias et al., 2018). Although circulating miRNAs are stable and easily quantified using techniques that are readily available in the laboratory, the ability to replicate findings across studies have proved difficult (McDonald et al., 2011). Factors that contribute to the variability in miRNA expression include populations differences such as ethnicity, socioeconomic status, environmental factors and viral infections (Huang et al., 2011; Bovell et al., 2013; Pheiffer et al., 2019; Florio et al., 2020; Karere et al., 2021), differences in the methodologies and measurement platform used, biological source and normalisation methods (Pritchard et al., 2012; Wang et al., 2012; Dias et al., 2017), varying sample sizes (Stirm et al., 2018) and gestational age (Herrera-Van Oostdam et al., 2020). Furthermore, normalisation controls for miRNA profiling is not standardised and may affect quantification. Diagnostic criteria for GDM and glucose cut-off values vary across studies, which may have contributed to the varied miRNA profiles observed. Furthermore, glycaemic control during pregnancy may be another factor influencing miRNA levels, which is an important factor to consider in future studies. Due to the heterogeneity of miRNA expression and its role in the regulation of

multiple genes, we recommend that a miRNA signature based on a pool of miRNAs may have more clinical applicability than individual miRNAs.

2.4 Conclusion and future perspectives

The prevalence of diabetes in pregnancy is increasing globally and is associated with adverse pregnancy outcomes for mother and child (Dornhorst and Banerjee, 2010; Ali and Dornhorst, 2011; McCance, 2015). Profiling miRNAs during pregnancy could offer promising avenues for the discovery of biomarkers of pregnancy health and disease, fetal development and pregnancy outcomes (Cai et al., 2017; Poirier et al., 2017; Pheiffer et al., 2019). Although miRNAs offer tremendous potential, much work still needs to be done before they can become clinically applicable (Dias et al., 2018). Further studies to investigate the importance of miRNAs in the development of the placenta and their contribution to cell-to-cell communication in pregnancies complicated by T1DM, T2DM and GDM are warranted. Such studies will provide important insight into the mechanisms that underlie the different types of diabetes and could identify predictive biomarkers of adverse pregnancy outcomes, potentially allowing mitigation of these complications.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study overview

The experimental outline of this cross-sectional study is shown in Figure 5. Briefly, 126 pregnant women were recruited, of whom 53 participants did not meet the inclusion criteria. The 73 women who participated in the study were categorised into four groups, i.e. pregestational T1DM (n=6) and T2DM (n=26), GDM (n=17) and normoglycaemia (control) (n=24). Serum insulin, C-peptide, total and HMW adiponectin, TG and CRP concentrations were measured by ELISA. Circulating miRNAs were extracted from serum and quantified using the Nanodrop spectrophotometer and Agilent Bioanalyser. Serum miRNAs were profiled using the Human Serum/Plasma miScript miRNA PCR array, followed by bioinformatic analysis to identify miRNA gene targets and their enriched pathways.

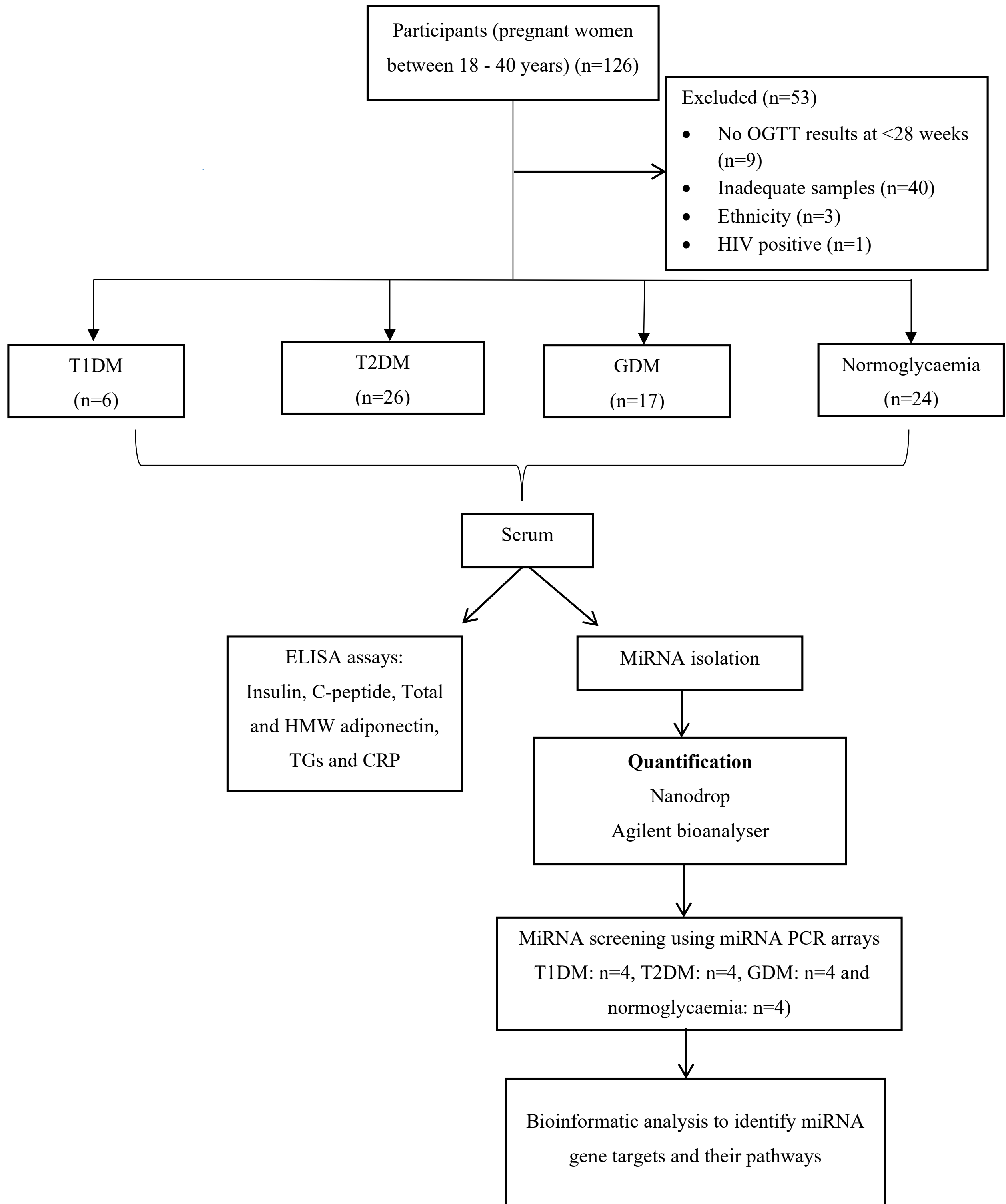


Figure 5. Experimental design

3.2 Participant recruitment

Women (n=126) attending the diabetic antenatal clinic at Steve Biko Academic Hospital, Pretoria, South Africa were recruited to the study. Of these, 53 were excluded because they did not meet the inclusion criteria, which were 1) age $\geq 18 \leq 40$ years of age, 2) Black African ethnicity, 3) $\leq$ 28 weeks pregnant, 4) singleton pregnancy, 5) human immunodeficiency virus (HIV) negative and 6) unknown congenital abnormalities. A total of 73 women were enrolled in the study. After participants were informed about the study aims and procedures, written informed consent was obtained. The study was approved by the Human Research Ethics Committee of the University of Pretoria (ethics no: Protocol 743/2020) and was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). All data collected was anonymised and participant information was kept confidential.

3.3 Clinical Data

Clinical data was collected in the form of a standardised questionnaire documenting demographic information and general medical and family history of diabetes. During this time, a physical examination was conducted to measure pulse, blood pressure, body weight and height according to standard operating procedures at the clinic. Body mass index (BMI) was calculated using the equation: weight (kg)/height (m^2).

3.4 Diabetes diagnosis

Pregnant women were categorised into groups according to the type of diabetes. Pregestational or pre-existing T1DM and T2DM were based on self-report and medication history. Women with no history of diagnosed diabetes underwent a 75g 2 hour OGTT as recommended by IADPSG (International Association of Diabetes and Pregnancy Study Groups Consensus Panel, 2010) and WHO (World Health Organisation, 2013). These women were classified as having GDM, overt diabetes (T2DM) or normoglycaemia according to these criteria (Table 2).

Table 2. IADPSG, 2010 and WHO, 2013 diagnostic criteria

Measurement	GDM*	T2DM±	Normoglycaemia¥
0-h Glucose (mmol/L)	≥ 5.1-7.0	≥ 7.0	< 5.1
1-h Glucose (mmol/L)	≥ 10	-	< 10
2-h Glucose (mmol/L)	≥ 8.5-11	≥ 11.1	< 8.5
Random Glucose (mmol/L)	-	≥ 11.1	-
HbA1c (%)	-	≥ 6.5	-

*One or more of these values must be met or exceeded for the diagnosis of GDM

±One of these values must be met or exceeded to identify the patient as having overt diabetes (T2DM)

¥Considered normal if all values are less than the threshold for GDM and T2DM

Abbreviations: GDM, gestational diabetes mellitus; T2DM, type 2 diabetes mellitus; HbA1c, glycated haemoglobin

3.5 Blood collection

Whole blood was collected from participants under non-fasting conditions by venipuncture. Blood was collected in BD Vacutainer® SST™ Tubes (BD Vacutainer, Woodlands, Cape Town, South Africa), which are coated with silicone and micronised silica particles to accelerate clotting of blood and a gel that separates blood cells from serum (Figure 6). For serum isolation, blood tubes were stored at room temperature for approximately 2 hours, where after tubes were centrifuged at 4 000 rpm for 15 mins at 4ºC (OHAUS Frontier™ Multi FC5706, Parsippany, New Jersey, United States) to separate blood cells from serum. After separation, serum was aliquoted into 2 ml microfuge tubes and stored at -40ºC. Samples were initially stored at Steve Biko Academic Hospital (Pretoria, South Africa), and thereafter transported to the laboratories at the Biomedical Research and Innovation Platform (BRIP), South African Medical Research Council (SAMRC), Cape Town, South Africa and stored at -80ºC.

Figure 6. An illustration of BD Vacutainer SST Tubes
(Image from bd.com)

3.6 Enzyme-linked immunosorbent assays

Serum levels of insulin, C-peptide, total and HMW adiponectin, CRP and TGs were measured using commercial ELISAs.

3.6.1 Insulin

Insulin was measured using the human insulin ELISA kit (Merck Millipore, Burlington, Massachusetts, USA), according to the manufacturer's instructions. Briefly, the plates were washed with 300 µl of wash buffer, thereafter, 20 µl of standards, quality controls (QC1, QC2) and samples were added in duplicate to the ELISA plates coated with mouse monoclonal anti-human insulin antibodies. Following this, 20 µl of pre-titered biotinylated mouse monoclonal anti-human insulin antibody was added, and the plates were sealed and incubated for 60 mins on an IKA®MS 3 digital universal small shaker (Merck KGaA, Darmstadt, Germany) (450 rpm). Thereafter, plates were washed with 300 µl of wash buffer thrice and 100 µl of enzyme solution (pre-titered streptavidin-horseradish peroxidase conjugate) was added. The plates were then sealed and incubated for 30 mins on a shaker (450 rpm). After shaking, the plates were washed five times with 300 µl of wash buffer and 100 µl of substrate solution (3, 3', 5, 5'-tetramethylbenzidine) was added. The plates were sealed, protected from light and incubated at room temperature for 20 mins. After incubation, 100 µl of stop solution was added and the absorbance was read at 450 nm on a SpectraMax i3 plate reader (Molecular Devices®, San Jose, California, USA). Data were analysed using my assays sigmoidal four parameter logistic regression accessible at https://www.myassays.com/four-parameter-fit.assay.

3.6.2 C-peptide

C-peptide was measured using the human C-peptide ELISA kit (Merck Millipore, Burlington, Massachusetts, USA), according to the manufacturer's instructions. Briefly, plates were washed with 300 µl of wash buffer. Thereafter, 10 µl of matrix solution and 40 µl of assay buffer were added to standards and control wells, and 50 µl of assay buffer was added to sample wells. Following this, 10 µl of assay buffer, standards, QC1, QC2 and samples were added in duplicates into appropriate wells. Thereafter, 20 µl of detection antibody was added into each well, the plate was sealed and incubated at room temperature for 2 hours on an IKA® MS 3 digital universal small shaker (Merck KGaA, Darmstadt, Germany) (450 rpm). After shaking, the plates were washed five times with 300 µl of wash buffer. After

incubation, 80 µl of enzyme solution was added into each well. Thereafter, the plates were incubated at room temperature for 30 mins while shaking on small shaker and thereafter washed five times with 300 µl of wash buffer. For development, 80 µl of substrate solution was added, the plates were sealed protected from light and incubated at room temperature for 20 mins. After incubation, 80 µl of stop solution was added and the absorbance was read at 450 nm on a SpectraMax i3 plate reader (Molecular Devices®, San Jose, California, USA). Data were analysed using my assays sigmoidal four parameter logistic regression accessible at https://www.myassays.com/four-parameter-fit.assay.

3.6.3 Total Adiponectin

Adiponectin was measured using the Human Adiponectin ELISA kit (Merck Millipore, Burlington, Massachusetts, USA), according to the manufacturer's instructions. Briefly, serum was diluted 1:5 in 1X assay buffer and the plates were prepared by incubating in 300 µl of wash buffer for 5 mins at room temperature, whereafter the wash buffer was discarded. Thereafter, 60 µl of assay buffer A was added to wells, followed by the addition of 20 µl of assay buffer A to blank wells, and standards, QC1, QC2 and samples in duplicate to the ELISA plate coated with mouse monoclonal anti-human adiponectin antibodies. Following this, 20 µl of pre-titered biotinylated mouse monoclonal anti-human adiponectin antibody was added and the plate was sealed and incubated for 60 mins on an IKA® MS 3 digital universal small shaker (Merck KGaA, Darmstadt, Germany) (450 rpm). Thereafter, plates were washed with 300 µl of wash buffer thrice, and 100 µl of enzyme solution was added. The plate was then sealed and incubated for 30 mins on a shaker (450 rpm). After shaking, the plates were washed five times with 300 µl of wash buffer and 100 µl of substrate solution was added. The plates were sealed protected from light and incubated at room temperature for 20 mins. After incubation, 100 µl of stop solution was added and the absorbance was read at 450 and 490 nm on a SpectraMax i3 plate reader (Molecular Devices®, San Jose, CA, USA). Data were analysed using my assays sigmoidal four parameter logistic regression accessible at https://www.myassays.com/four-parameter-fit.assay, and final results were multiplied by dilution factor 1:500.

3.6.4 High molecular weight Adiponectin

HMW Adiponectin was measured using the Human HMW Adiponectin ELISA kit (Merck Millipore, Burlington, Massachusetts, USA), according to the manufacturer's instructions.

Briefly, samples were first centrifuged for 5 min at 10,000 rpm (5415R, Eppendorf, Hamburg, Germany). Thereafter, 10 µl sample was added to 85 µl of sample digestion buffer and vortexed well. Sample digestion solution was diluted by adding 30 µl to 270 µl of sample digestion buffer in a polypropylene microfuge tube, vortexed well, and 5 µl of diluted sample digestion solution was added to each sample tube, vortex well and incubated at 37ºC for 2 hours. After incubation, 95 µl of 1X sample dilution buffer was added to a second set of microcentrifuge tubes for each digested sample, and 5 µl of digested samples were transferred to the second set of tubes, and vortexed well. ELISA plates were prepared by incubating in 300 µl of wash buffer for 5 mins at room temperature. Thereafter, 90 µl of running buffer was added to all the wells, and 10 µl of assay buffer to blank wells, and standards, QC1, QC2 and pre-treated samples were added in duplicate to the plate coated with mouse monoclonal anti-human adiponectin antibodies. The plates were sealed, agitated and incubated for 2 hours on a shaker (450 rpm). After incubation, plates were washed with 300 µl of wash buffer thrice. Thereafter, 100 µl of human HMW Adiponectin detection antibody was added to the plates, sealed, agitated and incubated for 1 hour on a shaker (450 rpm). After incubation, plates were washed with 300 µl of wash buffer thrice. Thereafter, 100 µl of enzyme solution was added to the plates, sealed, agitated and incubated for 30 mins on a shaker (450 rpm). After incubation, the plates were washed thrice with 300 µl of wash duffer. Following this, 100 µl of substrate solution was added to all wells, the plates were sealed with foil and incubated at room temperature for 20 mins away from light. Thereafter, 100 µl of stop solution was added and the absorbance read at 450 and 590 nm on a SpectraMax i3 plate reader (Molecular Devices®, San Jose, California, USA). Data were analysed using my assays sigmoidal four parameter logistic regression accessible at https://www.myassays.com/four-parameter-fit.assay, final results were multiplied by dilution factor 1:200.

3.6.5 C-reactive protein

CRP was measured using the Human CRP ELISA kit (Merck Millipore, Burlington, Massachusetts, USA), according to the manufacturer’s instructions. Briefly, samples were diluted 1:4000 with wash buffer, by first diluting each sample 1:1000, followed by a 1:4 dilution. The ELISA plate was prepared by incubating in 300 µl of wash buffer for 5 mins at room temperature and discarded. Thereafter, 100 µl of standards, QC1, QC2 and samples were added in duplicate to the ELISA plate coated with mouse monoclonal anti-human CRP

antibodies and the plate was sealed and incubated for 30 mins on a shaker (450 rpm) at room temperature. After shaking, the plates were washed five times with 300 µl of wash buffer. After incubation, 100 µl of enzyme solution (anti-human CRP horseradish peroxidase conjugate) was added and incubated for 30 mins on a shaker (450 rpm) at room temperature. After shaking, the plates were washed five times with 300 µl of wash buffer. After incubation, 100 µl of substrate solution was added. The plate was sealed protected from light and incubated at room temperature for 20 mins. After incubation, 100 µl of stop solution was added and the absorbance was read at 630 nm on a SpectraMax i3 plate reader (Molecular Devices®, San Jose, California, USA). Data were analysed using my assay sigmoidal four parameter logistic regression accessible at https://www.myassays.com/four-parameter-fit.assay,values were multiplied by 4000 to obtain the actual serum concentrations.

3.6.6 Triglycerides

Serum TGs were measured using the Triglyceride Quantification kit (Sigma-Aldrich, St. Louis, Missouri, USA). Briefly, 40 µl of 1 mM TG standard was diluted with 160 µl of assay buffer to prepare a 0.2 mM standard solution. Following this, 0, 10, 20, 30, 40, and 50 µl of the 0.2 mM standard, 0.3 µl sample, were added to wells, followed by the addition of assay buffer to bring the volume in each well to 50 µl. Thereafter, 2 µl of lipase was added to each sample and standard well, mixed well on a shaker (450 rpm) and incubated for 20 mins at room temperature. Thereafter, 50 µl of the master reaction mix (made up of assay buffer (46 µl), triglyceride probe (2 µl), and enzyme mix (2 µl) was added to each plate. Plates were sealed with aluminium foil, mixed well on a shaker (450 rpm) and incubated for 60 mins at room temperature (25ºC). The absorbance was measured at 570 nm on a SpectraMax i3 plate reader (Molecular Devices®, San Jose, California, USA). The amount of TGs present in the samples was determined using the below equation (Equation 1):

Equation 1. Calculation used to determine TG concentration

Concentration of TG: Sa/Sv = C

Sa = Amount of Triglyceride in the unknown sample (nmole) from standard curve

Sv = Sample volume (µL) added into the wells

C = Concentration of Triglyceride in sample

TG molecular weight: 885.4 g/mole

Sample Calculation Amount of Triglyceride (Sa) = 5.84 nmole (from standard curve)
Sample volume (Sv) = 50.0 µL
Final concentration of TG in sample
5.84 nmole/50.0 µL = 0.117 nmole/µL
0.117 nmole/µL × 885.4 ng/nmole = 104 ng/µL

3.7 RNA isolation

MiRNA-enriched total RNA was isolated using the miRNeasy Serum/Plasma kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, serum samples were removed from -80ºC and allowed to thaw on ice at room temperature for 30 mins. Thereafter, 1 ml of QIAzol lysis reagent was added to 200 µl of serum, mixed by vortexing for 15 sec on a Vortex-Mixer VM-10 orbital shaker (witeg Labortechnik GmbH, Wertheim, Germany), and incubated at room temperature (25ºC) for 5 mins. Thereafter, 3.5 µl of MiRNeasy serum/plasma spike-in-control (*Caenorhabiditis elegans* miR-39) (1.6 x 10^8 copies/µl) was added as an exogenous synthetic miRNA to control for technical variation during RNA extraction, complimentary DNA (cDNA) synthesis and qRT-PCR. Following this, 200 µl of chloroform was added to each sample and vortexed for 15 sec for phase separation. Samples were incubated at room temperature (25ºC) for 3 mins, and thereafter centrifuged at 4ºC for 15 mins at 12 000 rpm (5415R, Eppendorf, Hamburg, Germany). After centrifugation, the upper colourless aqueous phase containing approximately 600 µl of RNA was transferred to a new collection tube and mixed thoroughly with 900 µl of 100% ethanol. Thereafter, 700 µl of the sample was pipetted into a RNeasy MinElute spin column and centrifuged at 14 000 rpm for 15 sec at room temperature. This step was repeated, and the flow through was discarded both times. Samples were subsequently washed with 700 µl of buffer RWT and centrifuged at 14 000 rpm for 15 sec, followed by the addition of 500 µl of buffer RPE and centrifuged at 14 000 rpm for 15 sec. For a final wash, 500 µl of 80% ethanol was added into the RNeasy MinElute spin column and centrifuged for 2 mins at 14 000 rpm at room temperature. The spin column was placed in a new 2 ml tube and centrifuged at 14 000 rpm for 5 mins to completely dry the spin column membrane. After drying, the spin column was placed in a 1.5 ml tube, and 14 µl of RNase-free water was added directly onto the spin column membrane and centrifuged at 8 000 rpm for 1 min to elute the RNA. RNA samples were stored at -80ºC.

3.8 RNA quantification

Total RNA concentrations were determined by measuring the absorbance at a wavelength of 260 nm (A260) using a NanoDrop™ One/OneC Microvolume UV-Vis spectrophotometer (Nanodrop Technologies, Wilmington, Delaware, USA) according to the manufacturer's instructions (Figure 7). RNA purity was assessed by calculating the A260/280 (protein contamination) ratio, with a ratio of 1.8–2.0 indicating an acceptable level of purity for gene expression analysis, and a A260/230 ratio less than 1.8–2.0 indicates contamination with wash solutions, chaotropic salts and phenols (Wilfinger et al., 1997).

Figure 7. An illustration of the nanodrop spectrophotometer
(Image captured in the laboratory)

3.9 MicroRNA quantification

MiRNA concentrations were measured using the Agilent bioanalyser and the Small RNA assay kit at the Central Analytical Facilities, Stellenbosch University (Stellenbosch, South Africa). The Bioanalyser LabChip instrument (Figure 8) using the Small RNA assay provides a fast and sensitive analysis to resolve small nucleic acids in the size range of 6 to 150 nucleotides and enables the detection and quantification of miRNAs (15–40 nucleotides) within a small RNA fraction (Agilent Technologies Inc., 2016). MiRNA concentration and its percentage relative to total RNA were assessed using the Small RNA (Pico) kit according to

manufacturer's instructions (Agilent Technologies, Santa Clara, California, USA). The concentration of miRNA is calculated as an absolute amount (pg/µl) and as a percentage (%) of small RNA in the total RNA sample. Figure 9 represents an electrophoretogram of small RNAs (miRNAs, transfer RNAs, and small ribosomal RNAs) that form part of the total RNA sample.

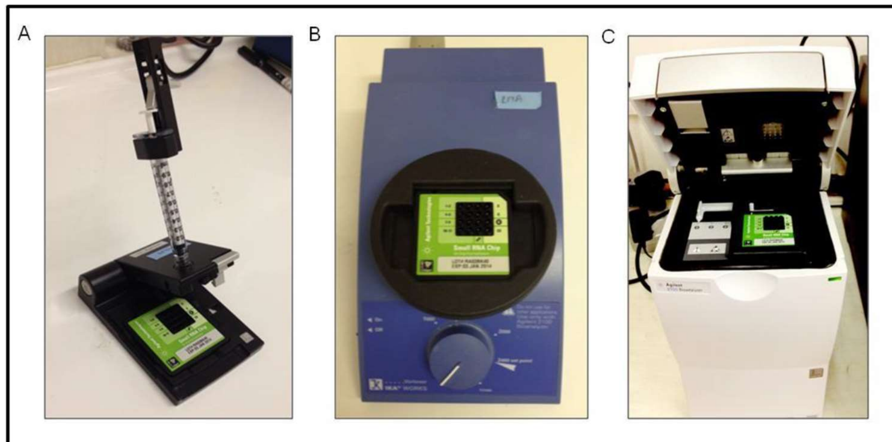


Figure 8. An illustration of the Agilent LabChip instrument
A) The priming station and plunger, B) the Agilent chip vortex, and C) the Agilent 2100 Bioanalyser
(Image captured in the laboratory)

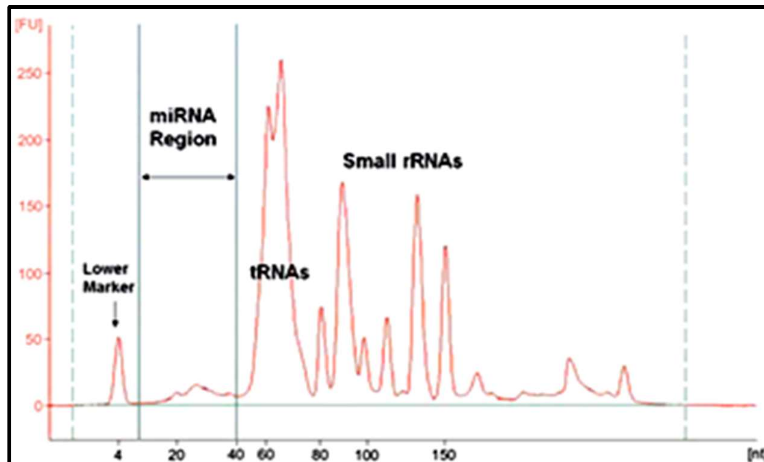


Figure 9. Representative image of an electropherogram for small RNA analysis
(Adapted from Mueller et al., 2004)

3.10 MiRNA expression profiling

Sixteen patient samples (T1DM (n=4), T2DM (n=4), GDM (n=4) and normoglycaemia (n=4) were selected for qRT-PCR using the Human Serum/Plasma miScript miRNA PCR array (Qiagen, Hilden, Germany) to identify differentially expressed miRNAs. Samples were matched for age, gestational age and BMI as far as possible.

3.10.1. Reverse transcription

MiRNAs were reverse transcribed using the miScript II RT Kit and the miScript HiSpec Buffer according to the manufacturer's instructions (Qiagen, Hilden, Germany). Briefly, the RNA samples and reagents were thawed on ice for 30 mins. Thereafter, reverse transcription components (Table 3) were added to samples, mixed using a Vortex-Mixer VM-10 orbital mixer (Witeg Labortechnik GmbH, Wertheim, Germany) and briefly centrifuged using the myFuge™ 8 Mini Centrifuge (Benchmark Scientific, Sayreville, New Jersey, USA). Following this, samples were incubated for 60 mins at 37ºC, followed by 5 mins at 95ºC on the Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The cDNA was diluted 1:10 by adding 200 µl of RNase-free water and used for qRT-PCR.

Table 3. Reverse transcription reaction components

Component	**Volume/reaction (µl)**
5x miScript HiSpec Buffer	4
10x miScript Nucleics mix	2
miScript reverse transcriptase mix	2
RNase free water	12
Template RNA (ng/ul)	1

3.10.2 Quantitative real time PCR

Quantitative real time PCR was conducted using the Human Serum/Plasma miScript miRNA PCR array (Qiagen, Hilden, Germany). The Human Serum/Plasma miScript miRNA PCR array profiles the expression of 84 miRNAs using SYBR Green. SYBR green is a cyanine dye that binds double-stranded DNA and is commonly used to quantify gene expression in qRT-PCR (Ponchel et al., 2003). Other miRNAs that are stably present in serum are included as positive controls on the array. These miRNAs include C. *elegans* miR-39 (endogenous

spike-in control), snoRNAs (small nucleolar RNAs) (SNORD61, SNORD68, SNORD72, SNORD95, and SNORD96A) (miScript PCR controls/references genes), snRNA (small nuclear RNA) RNU6 and reverse transcription and PCR positive controls (Figure 10).

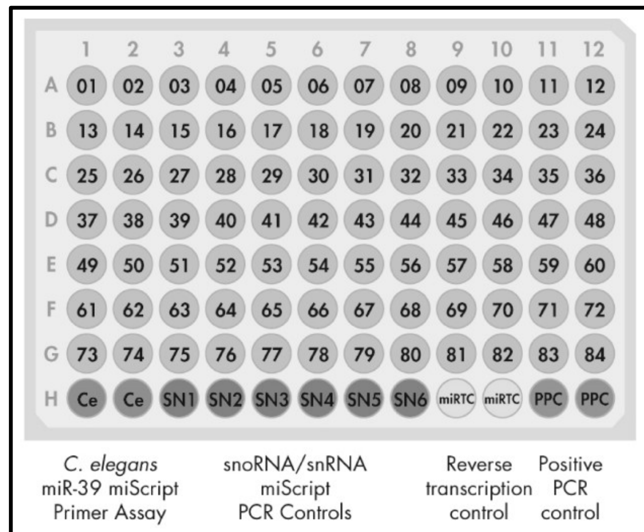


Figure 10. The Human Serum/Plasma miScript miRNA PCR array layout

A miScript Primer Assay for a mature miRNA is present in each of the wells A1 to G12 (1–84). Reference miRNAs include C. *elegans* miR-39 miScript Primer Assays (Ce), snoRNA/snRNA miScrpt PCR controls (SN1 (SNORD61), SN2 (SNORD68), SN3 (SNORD72), SN4 (SNORD95), SN5 (SNORD96A), SN6 (RNU6B/RNU6-2)), Reverse transcription controls (miRTC) (H9 and H10) and Positive PCR controls (PPC) (H11 and H12) (image taken from miScript PreAMP Handbook/www.qiagen.com/miRNA).

To conduct qRT-PCR, cDNA was mixed with 2x QuantiTect SYBR Green PCR Master Mix, 10x miScript Universal Primer, and RNase-free water in a total volume of 1100 µl as described in the miScript PreAMP Handbook (Table 4). After mixing, samples were dispensed into wells of the PCR array plate. The plate was sealed with a MicroAmp optical adhesive film (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and centrifuged for 1 min at 2 500 rpm at room temperature (Benchmark Scientific, Sayreville, New Jersey, USA). PCR was conducted on the Quantstudio 7™ Flex Real-Time PCR System (Applied Biosystems, Foster City, California, USA) (Table 5). Default baseline and cycle threshold (Ct) settings were used and the PCR array values were exported to an excel file to create a table of Ct values, which was uploaded onto the Qiagen GeneGlobe Design and Analysis Hub web portal (https://dataanalysis.qiagen.com/pcr/arrayanalysis.php). A cut-off Ct value of 35 cycles was applied and samples with multiple melt curves were removed. Gene expression data were normalised to cel-miR-39 and was quantified using the ΔΔCT method of relative

quantification (Rao et al., 2013) (Equation 2). PCR arrays were assessed for reproducibility, reverse transcription and PCR efficiency.

Equation 2. ΔΔCT method of relative quantification

$$\Delta\Delta CT \text{ method} = \Delta CT(\text{target sample}) - \Delta CT(\text{reference sample})$$

Table 4. Reaction mix for the Human Serum/Plasma miScript miRNA PCR

Array format: Component	**384-well (4 x 96) Formats E, G***
2x QuantiTect SYBR Green PCR Master Mix	550 µl
10x miScript Universal Primer	110 µl
RNase-free water	340 µl
Template cDNA	100 µl
Total volume	**1100 µl**

Table 5. Cycling conditions for real-time PCR

Step	**Time**	**Temperature**
Initial activation step	15 min	95ºC
3-step cycling for 40 cycles		
Denaturation	15 s	94ºC
Annealing	30 s	55ºC
Extension	30 s	70ºC

3.11 Bioinformatics

Bioinformatics was conducted to explore the functional significance of differentially expressed miRNAs. MiRNA gene targets and their pathways were identified using miRNA Pathway Dictionary Database (miRPathDB) 2.0 (accessible at https://mpd.bioinf.uni-sb.de/) (Kehl et al., 2020). MiRPathDB identifies miRNA gene targets using MiRanda 3.3a, miRTarBase 7 and TargetScan 7.1 and maps gene targets to pathways using various

databases (Gene Ontology, Kyoto Encyclopedia of Genes and Genomes (KEGG), miRbase, miRCarta, Reactome and WikiPathways). The software conducts statistical analysis using R, version 3 using the over-representation analysis (ORA) test. The Benjamini-Hochberg method was employed to control for the false discovery rate. Only KEGG pathways were selected for the analysis in the thesis. The KEGG database is a collection of manually drawn pathway maps that may be used for biological evaluation of genomic sequences and other high-throughput data consisting of an integrated database with three generic categories of systems information, genetic information, and chemical information, as well as a human-specific health information category (Kanehisa et al., 2019).

3.12 Statistics

Statistical analysis was conducted using Graphpad Prism® version 8 (GraphPad Software, La Jolla, USA). Data are presented as the mean and standard error of the mean for normally distributed data or as the median and interquartile range (25th-75th percentiles) for skewed data. Differences between groups were compared using one-way Anova and the Holm-Sidak's multiple comparisons test or the non-parametric Kruskal Wallis and Dunn's multiple comparisons test, followed by an unpaired student t-test or Mann-Whitney, as appropriate, to further explore differences. To investigate relationships between variables, Spearman's correlation was conducted. A p-value ≤ 0.05 was considered statistically significant.

CHAPTER 4: RESULTS

4.1 Clinical characteristics of participants

The clinical characteristics of participants are shown in Table 6. Participants included pregnant women with normoglycaemia (n=24), pregestational T1DM (n=6) and T2DM (n=26) and GDM (n=17). Age was significantly different across the four groups (p=0.008). Women with T1DM were significantly younger than women with T2DM (p=0.040) and GDM (p=0.015). Although the BMI of women with T2DM and GDM were higher than women with normoglycemia and T1DM, the difference was not statistically significant. As expected, women with pregestational T1DM (p=0.039) and T2DM (p=0.019) had higher HbA1c levels than women with GDM. Moreover, as expected, fasting plasma concentrations were higher in pregnant women with T2DM ($p<0.001$) and GDM ($p<0.001$) compared to women with normoglycaemia. Similarly, 2-hour glucose concentrations were higher in pregnant women with GDM ($p<0.001$) compared to women with normoglycaemia. Pulse rate was higher in women with T2DM (p=0.003) and GDM (p=0.003) compared to women with normoglycaemia. No differences in blood pressure were observed between the groups.

4.2 Metabolic parameters

Maternal diabetes is a metabolic disorder characterised by dysregulation in insulin sensitivity and secretion, lipid metabolism and inflammation (Mazaki-Tovi et al., 2007; Vrachnis et al., 2012; Ebert et al., 2020). Therefore, we measured the circulating levels of insulin, C-peptide, total and HMW adiponectin, TGs and CRP in the serum of pregnant women with pregestational T1DM and T2DM, GDM and normoglycaemia (Table 7). Insulin levels varied significantly between groups (p=0.012), with pairwise analysis showing higher levels in women with T1DM (p=0.008), T2DM (p=0.013) and GDM (p=0.014) compared to women with normoglycaemia (Figure 11A). High levels of insulin in women with T1DM may be attributed to the administration of exogenous insulin. Therefore, we measured expression of C-peptide, which is a marker of β-cell function and insulin secretion (Leighton et al., 2017; Venugopal et al., 2021). C-peptide levels varied between groups (p=0.043), with higher levels observed in women with GDM compared to normoglycaemia (p=0.010) and a trend towards significance observed between women with T1DM and GDM (p=0.051) (Figure 11B). Total adiponectin levels were significantly different between groups (p=0.017), with women with T2DM (p=0.005) and GDM (p=0.030) displaying lower levels of total

adiponectin compared to women with normoglycaemia (Figure 11C). HMW adiponectin levels, which is considered to be the biologically active form of adiponectin (Pereira et al., 2012), differed between groups (p=0.040), and pairwise analysis showed lower levels in women with T2DM (p=0.038) and GDM (p=0.052) than in women with T1DM (Figure 11D). Serum TGs were higher in women with GDM compared to women with normoglycemia (p= 0.030) (Figure 11E). CRP levels varied between maternal diabetes groups (p=0.036), and were higher in women with T2DM compared to women with normoglycaemia (p= 0.008) (Figure 11F).

Table 6. Participant characteristics

Variable	Normoglycaemia	Pregestational diabetes		GDM	P-value (difference between groups)
		T1DM	T2DM		
N	24	6	26	17	
Age (years)	30 (27-37)	29 (27-29)[a,b]	36 (32-37)[a]	36 (34-37)[b]	0.008
Body mass index (kg/m^2)	25.9 (19.3-38.5)	26.9 (24.4-35.6)	31.2 (28.8-38.5)	37.3 (29.8-43.9)	0.333
Glycated haemoglobin (%)	ND	9.3 (0.3)[a]	8.6 (0.5)[b]	5.7 (0.3)[a,b]	0.014
Fasting glucose (mmol/L)	3.9 (3.6-4.1)[c,d]	ND	8.0 (6.8-14.2)[c]	5.6 (5.3-6.3)[d]	< 0.001
2-hour glucose (mmol/L)	4.6 (4.3-5.6)[c]	ND	ND	7.8 (6.2-9.6)[c]	< 0.001
Pulse (bmp)	86.3 (2.3)[e,f]	94.7 (5.4)	99.0 (2.2)[e]	100.9 (3.7)[f]	0.001
Systolic blood pressure (mm Hg)	117.7 (2.5)	120.7 (2.4)	118.3 (2.1)	119.4 (2.4)	0.712
Diastolic blood pressure (mm Hg)	70.6 (2.3)	73.7 (2.5)	71.5 (1.8)	72.6 (3.3)	0.963

Data presented as the mean (SEM) for normally distributed variables or as the median and interquartile range (25th and 75th percentiles) for skewed variables.

Statistical differences between groups were assessed with the one-way anova for normally distributed data or the Kruskal Wallis test with Dunn's multiple comparisons test for skewed data. Similar superscripts indicate statistical significance, [a,b] $p < 0.05$, [e,f] $p<0.01$, [c,d] $p<0.001$

Abbreviations: GDM, gestational diabetes mellitus; ND, not determined; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus

Table 7. Participant metabolic parameters

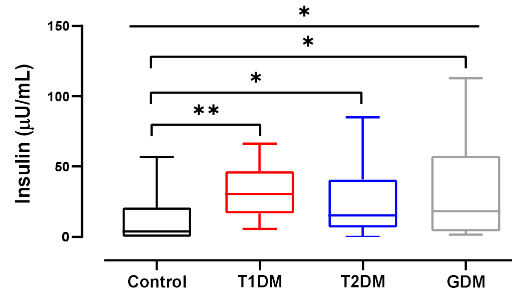
Variable	Normoglycaemia	Pregestational diabetes: T1DM	Pregestational diabetes: T2DM	GDM	P-value (difference between groups)
N	24	7	26	17	
Insulin (µU/mL)	3.8 (0-20.8)[a,b,c]	30.5 (16.6-46.6)[c]	15.4 (6.7-40.8)[a]	18.1 (4.0-57.5)[b]	0.012
C-peptide (ng/mL)	1.0 (0.7-2.0)[a]	1.1 (0-1.6)[#]	1.8 (0.9-2.3)	1.9 (1.1-4.4)[a,#]	0.043
Total Adiponectin (µg/mL)	8.1 (4.7-12.0)[c,a]	7.0 (4.6-14.6)	4.1 (3.1-6.1)[c]	4.1 (3.5-7.1)[a]	0.017
HMW Adiponectin (µg/mL)	4.3 (1.9-6.7)	7.4 (3.4-14.7)[a,#]	2.0 (1.3-3.0)[a]	2.3 (1.1-4.8)[#]	0.040
Triglycerides (mg/dL)	324.1 (27.9)[a]	388.8 (51.0)	408.2 (44.96)	416.4 (28.53)[a]	0.270
C-Reactive Protein (µg/mL)	4.5 (3.4-13.0)[c]	7.7 (7.3-9.4)	15.2 (7.6-27.2)[c]	8.1 (4.6-17.3)	0.036

Data presented as the median and interquartile range (25th and 75th percentiles) or as the mean and SEM for normally distributed data.

Statistical differences between groups were assessed with the Kruskal Wallis test and Dunn's multiple comparisons test or with the one-way ANOVA and Holm-Sidak's multiple comparison test for normally distributed data. Paired tests were conducted using the Mann-Whiney test or the unpaired t-test. Similar superscripts indicate statistical significance, [a,b]$p < 0.05$, [c]$p<0.01$, [#]$p=0.05$

Abbreviations: HMW, high molecular weight; GDM, gestational diabetes mellitus; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus.

A



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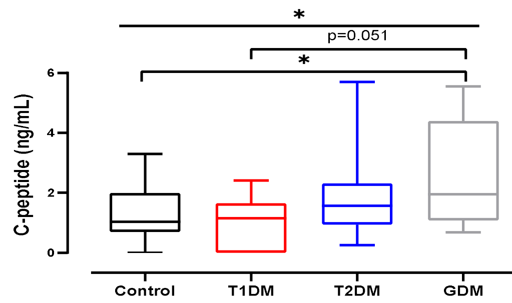

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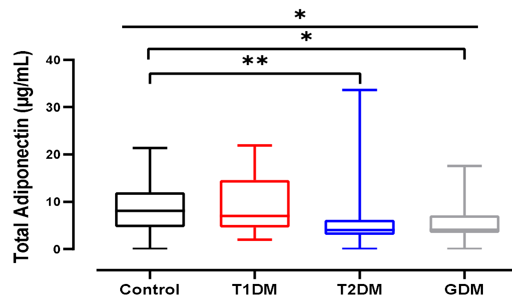

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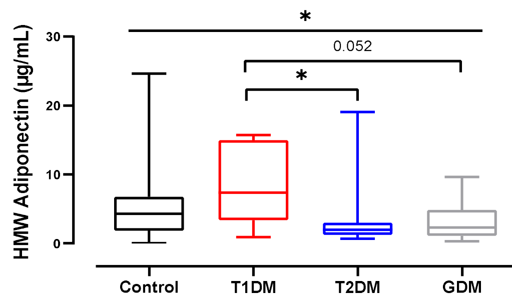

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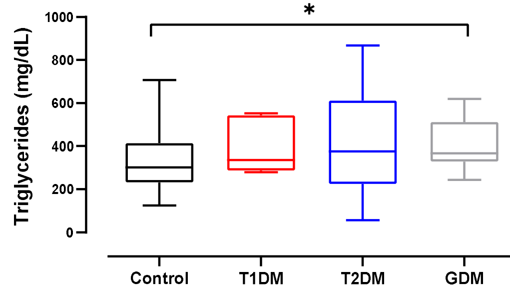

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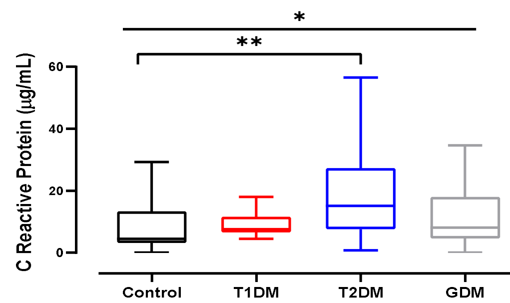


Figure 11. Metabolic parameters in pregnant women

The concentration of A) insulin, B) C-peptide, C) total adiponectin, D) high molecular weight (HMW) adiponectin, E) triglycerides and F) C reactive protein were measured in the serum of pregnant women with pregestational type 1 diabetes (T1DM) and type 2 diabetes (T2DM), gestational diabetes (GDM) and normoglycaemia (controls) using commercial enzyme-linked immunosorbent assays (ELISAs). Results are expressed as box whisker plots indicating the median and interquartile range in boxes and whiskers representing the minimum and maximum values (n=7-26 per group), *p<0.05, **p<0.01.

4.3 Association between clinical and metabolic parameters

Metabolic dysregulation during diabetes is complex, involving the aberrant expression of various parameters. Therefore, we next explored the association between clinical and metabolic parameters. As expected, insulin and C-peptide concentrations were correlated with each other, while both were correlated with higher TG, CRP, Fasting glucose (FPG), BMI and weight, and with lower total and HMW adiponectin concentrations. Total and HMW adiponectin were correlated with each other and with younger age and lower TG, FPG, 2-hour glucose concentrations and weight, while total adiponectin was correlated with lower pulse rate and HMW adiponectin was correlated with lower CRP and BMI. TG and CRP were correlated with each other and with higher pulse rate, BMI and weight. FPG and 2-hour glucose were correlated with each other and with higher HbA1c and pulse rate, while 2-hour glucose was also correlated with older age. Pulse rate was correlated with higher BMI and weight. SBP and DBP were correlated with each other and with weight, while SBP was also correlated with 2-hour glucose (Table 8).

Table 8. Metabolic parameter correlations

Variable	**Insulin**	**C-peptide**	**Total adiponectin**	**HMW adiponectin**	**TG**	**CRP**	**Age**	**HbA1c**	**FPG**	**2hr glucose**	**Pulse**	**SBP**	**DBP**	**BMI**	**Weight**
Insulin	1***	0.66***	-0.24*	-0.19#	0.29*	0.34**	-0.12	0.26	0.33*	0.13	0.19	0.07	0.18	0.39**	0.37**
C-peptide	0.66***	1***	-0.25*	-0.19	0.34**	0.29*	0.07	-0.07	0.28*	0.07	0.11	0.08	0.07	0.67***	0.59***
Total adiponectin	-0.24*	-0.25*	1***	0.64***	-0.20#	-0.14	-0.23*	0.02	-0.35*	-0.38**	-0.31**	0.06	-0.14	-0.17	-0.23#
HMW adiponectin	-0.19#	-0.19	0.64***	1***	-0.29*	-0.26*	-0.27*	-0.10	-0.31*	-0.32*	-0.10	-0.04	-0.15	-0.28#	-0.32**
TG	0.29*	0.34**	-0.20	-0.29*	1***	0.33**	0.13	0.06	0.26	0.22	0.35**	0.12	0.17	0.36*	0.33**
CRP	0.34**	0.29*	-0.14	-0.26*	0.33**	1***	-0.03	-0.01	0.24	0.21	0.26*	0.11	0.11	0.32*	0.36**
Age	-0.12	0.07	-0.23*	-0.27*	0.13	-0.03	1***	-0.04	0.24	0.36**	0.06	-0.02	0.08	0.09	0.12
HbA1c	0.26	-0.07	0.02	-0.10	0.06	-0.01	-0.04	1***	0.67*	0.97***	-0.21	-0.06	0.13	-0.79***	-0.67***
FPG	0.33*	0.28*	-0.35*	-0.31*	0.26	0.24	0.24	0.67*	1***	0.80***	0.37**	0.19	0.09	0.13	0.29*
2hr glucose	0.13	0.07	-0.38**	-0.32*	0.22	0.21	0.36**	0.42*	0.80***	1***	0.37**	0.32*	0.22	-0.23	0.15
Pulse	0.19	0.11	-0.31**	-0.10	0.35**	0.26*	0.06	-0.21	0.37**	0.37**	1***	0.20	0.15	0.23	0.32**
SBP	0.07	0.08	0.06	-0.04	0.12	0.11	-0.02	-0.06	0.19	0.32*	0.20	1***	0.53***	0.10	0.23
DBP	0.18	0.07	-0.14	-0.15	0.17	0.11	0.08	0.13	0.09	0.22	0.15	0.53***	1***	0.10	0.24*
BMI	0.39**	0.67***	-0.17	-0.28#	0.36*	0.32*	0.09	-0.79***	0.13	-0.23	0.23	0.10	0.10	1***	0.96***
Weight	0.37**	0.59***	-0.23	-0.32**	0.33**	0.36**	0.12	-0.67***	0.29*	0.15	0.32**	0.23	0.24*	0.96***	1***

The Spearman rank coefficient is indicated. *p<0.05, **p<0.001, ***p<0.001, #p<0.1

Abbreviations: GDM, gestational diabetes mellitus; ND, not determined; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; New T2DM, T2DM first diagnosed in pregnancy. Statistical differences between groups were assessed with the Kruskal Wallis test with Dunn's multiple comparisons test. #p=0.054

4.4 MicroRNA expression analysis

4.4.1 RNA concentration and yield

The concentration and yield of miRNA-enriched total RNA varied between 6.1 and 149 ng/µl and 92 and 2229 ng, respectively (Appendix 1, Table 9). RNA purity was assessed by calculating the A260/280 (protein contamination) (Appendix 1, Table 9).

4.4.2 MiRNA percentage and concentration

The concentration and percentage of miRNA in the total RNA sample varied between 4 and 91% and 49.2 and 960 pg/µl, respectively (Appendix 1, Table 9).

4.4.3 PCR arrays

MiRNAs isolated from the serum of pregnant women with T1DM, T2DM, GDM and normoglycaemia were profiled using the miScript miRNA serum/plasma PCR Array. The assay passed the PCR Array Reproducibility quality control check which is determined by an average PPC Ct of 19 ± 2 across PCR plates. No significant differences in the distribution of Ct values for miRNAs across the different sample groups were observed (Figure 12). The majority of miRNAs were undetected (Ct > 35). Using a 1.5-fold cut-off for differential expression, a number of miRNAs were shared between diabetes groups, although their fold regulation varied relative to the controls (Figure 13). A total of 36 miRNAs were upregulated in pregnant women with diabetes compared to women with normoglycaemia (Figure 14). Of these, one was unique to women with T1DM, 14 were unique to women with T2DM, two were unique to women with GDM, five shared between T1DM and T2DM, five shared between T2DM and GDM, one shared between T1DM and GDM, and eight shared across all three groups. The identities of these miRNAs are shown in Figure 14. A total of 35 miRNAs were decreased in women with diabetes compared to women with normoglycaemia (Figure 15). Of these, three miRNAs were unique to women with T1DM, one was unique to women with T2DM, fourteen were unique to women with GDM, three shared between T2DM and GDM, nine shared between T1DM and GDM, and five shared across all three groups. The identities of these miRNAs are shown in Figure 15. Of the differentially expressed miRNAs, the expression of only three was statistically significant (Figure 16). The expression of miR-19b-3p was lower in women with GDM (9.8-fold; p=0.033) (Figure 16A), miR-20a-5p was lower in women with T1DM (4.5-fold; p=0.047) (Figure 16B), and miR-29a-3p was higher in women with T2DM (1.8-fold; p=0.002) (Figure 16C) compared to controls.

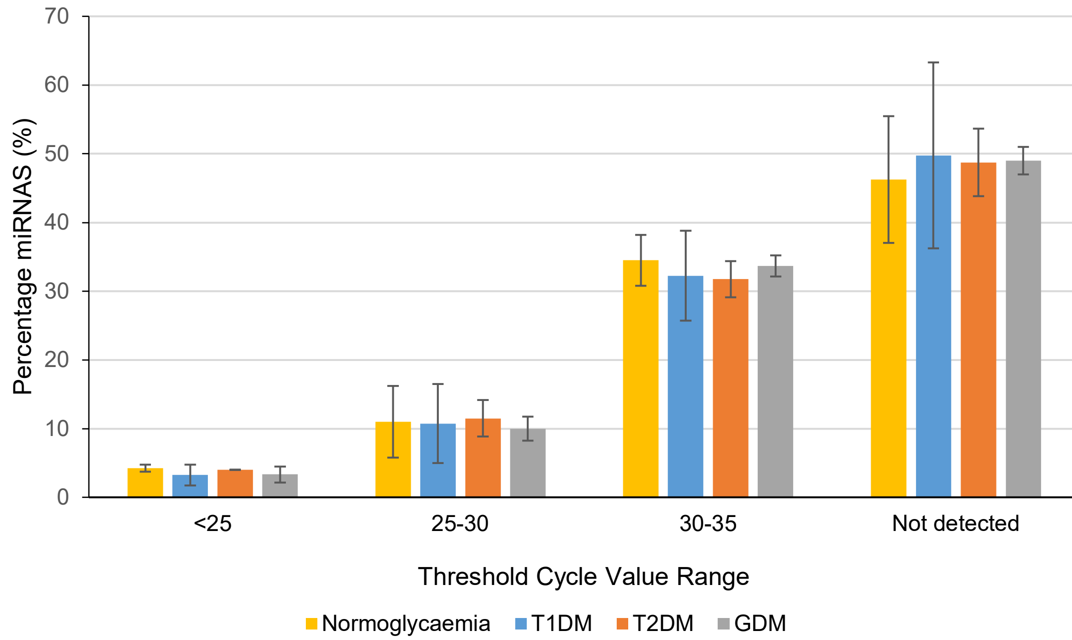


Figure 12. Distribution of Ct values for miRNAs

MiRNAs were profiled in the serum of pregnant women with type 1 diabetes (T1DM) (n=4), type 2 diabetes (T2DM) (n=4), gestational diabetes (GDM) (n=3) and normoglycaemia (controls) (n=4) using the miScript miRNA serum/plasma PCR Array. The range of Ct values for miRNAs are shown.

Abbreviations: Ct, cycle threshold; GDM, gestational diabetes; T1DM, type 1 diabetes; T2DM, type 2 diabetes.

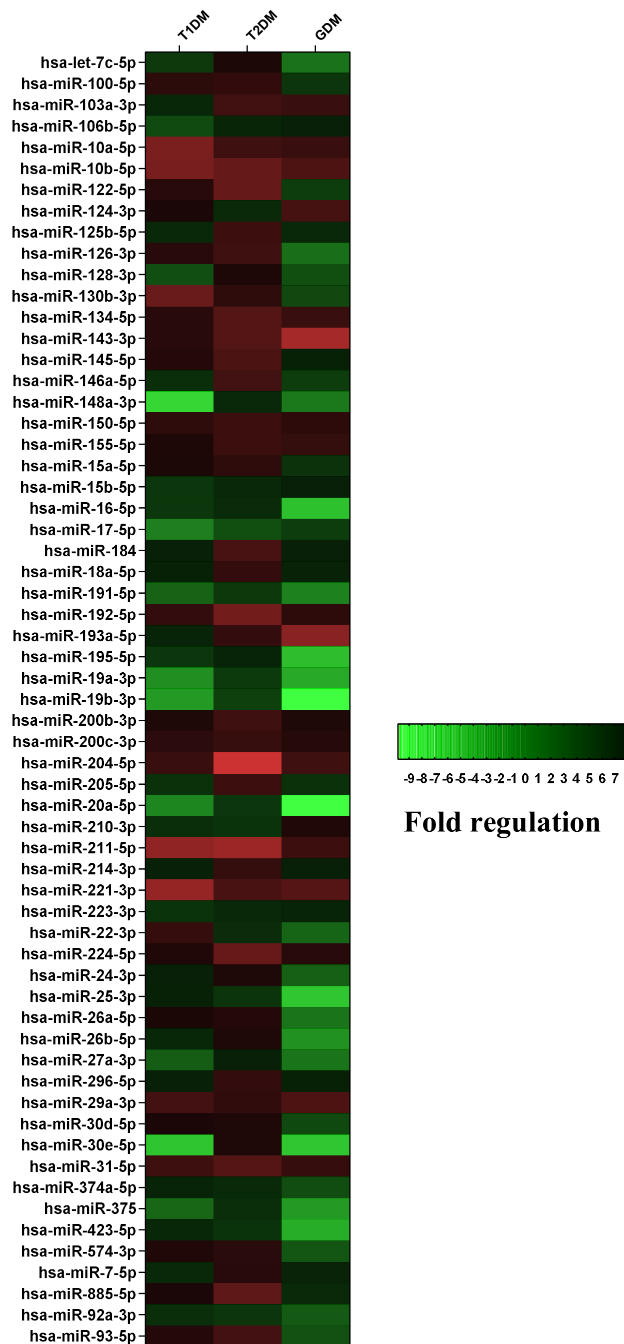


Figure 13. Heat map illustrating fold regulation of miRNAs in maternal diabetes

Fold regulation was calculated relative to miRNA expression in controls and the data is visualised in the heatmap where the fold regulation is relative to the change in colour intensity. High and low miRNA fold regulations are indicated by red and green, respectively.

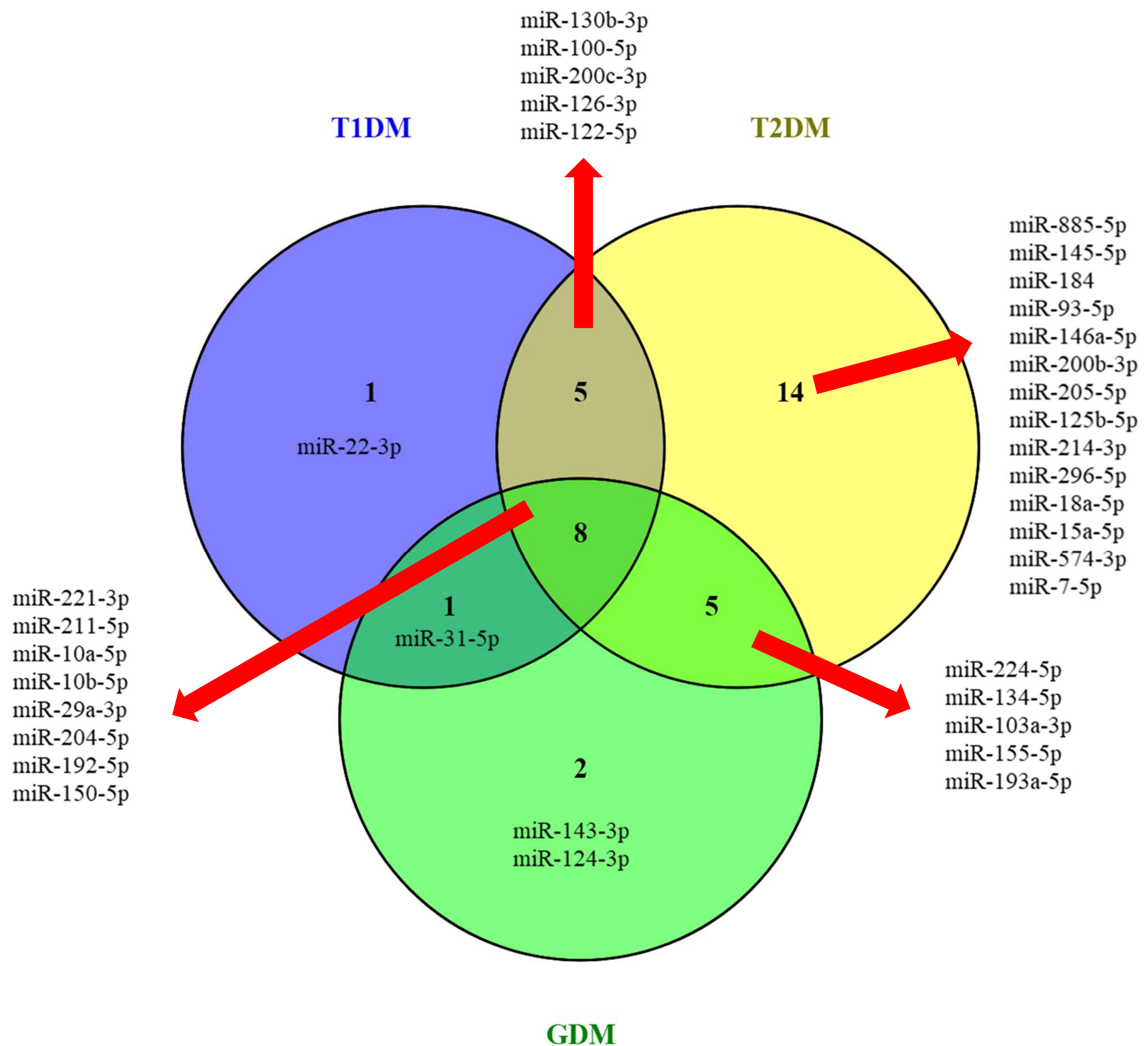


Figure 14. MiRNAs upregulated during maternal diabetes

MiRNAs were profiled in the serum of pregnant women with type 1 diabetes (T1DM) (n=4), type 2 diabetes (T2DM) (n=4), gestational diabetes (GDM) (n=3) and normoglycaemia (controls) (n=4) using the miScript miRNA serum/plasma PCR Array. The Venny diagram illustrates miRNAs with > 1.5-fold higher expression during maternal diabetes compared to controls (https://academo.org/demos/venn-diagram-generator/).

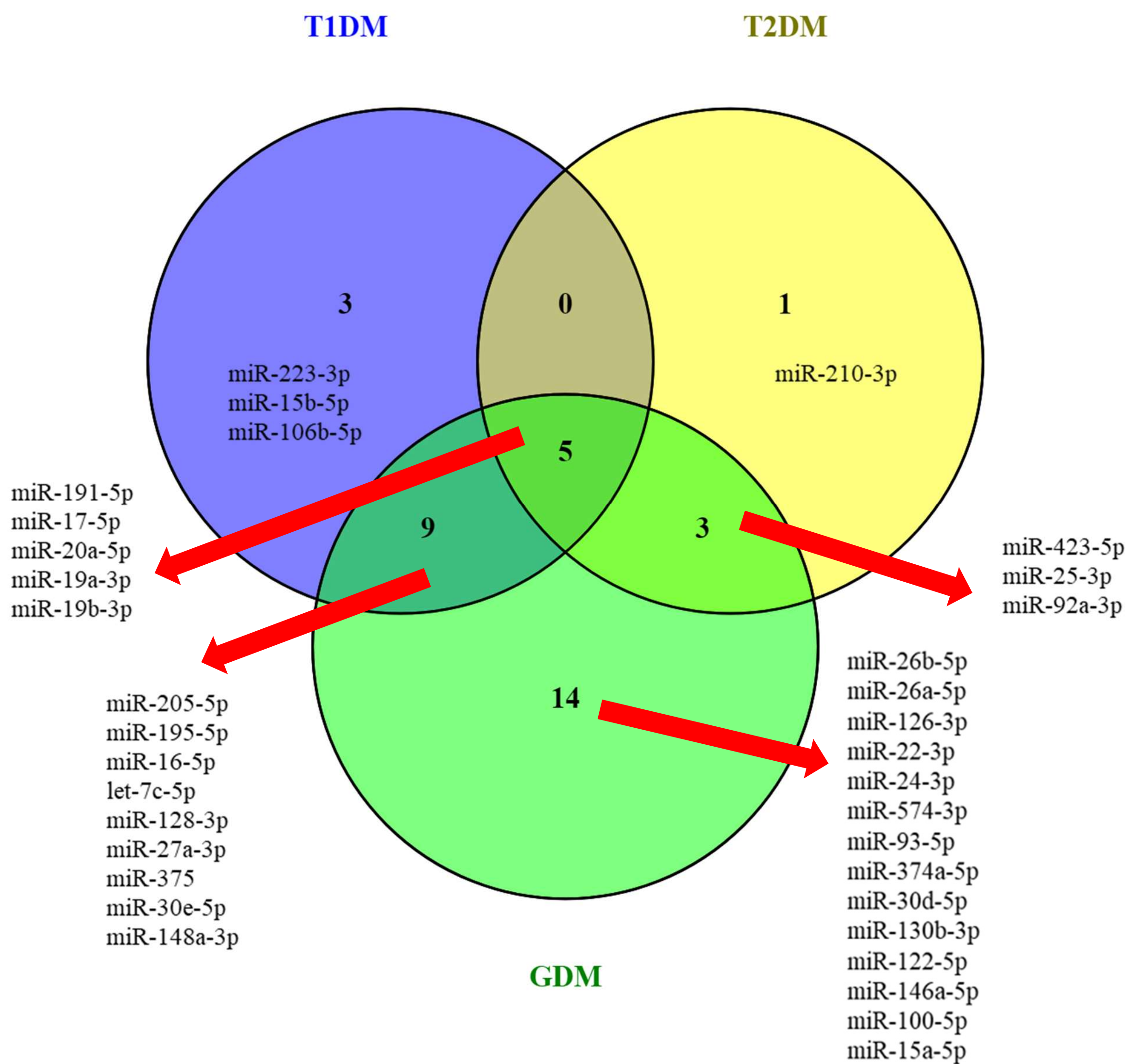
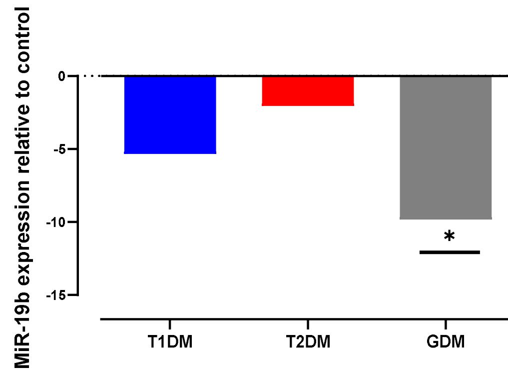


Figure 15. MiRNAs downregulated during maternal diabetes

MiRNAs were profiled in the serum of pregnant women with type 1 diabetes (T1DM) (n=4), type 2 diabetes (T2DM) (n=4), gestational diabetes (GDM) (n=3) and normoglycaemia (controls) (n=4) using the miScript miRNA serum/plasma PCR Array. The Venny diagram illustrates miRNAs with > 1.5-fold lower expression during maternal diabetes compared to controls (https://academo.org/demos/venn-diagram-generator/).

A



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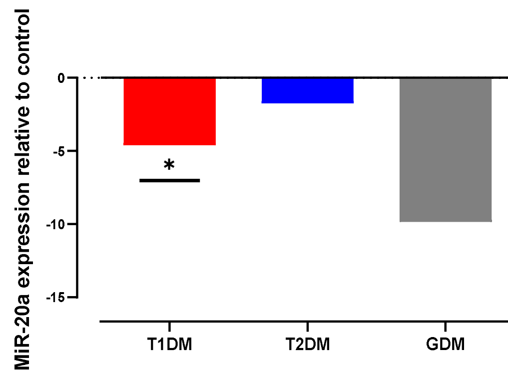

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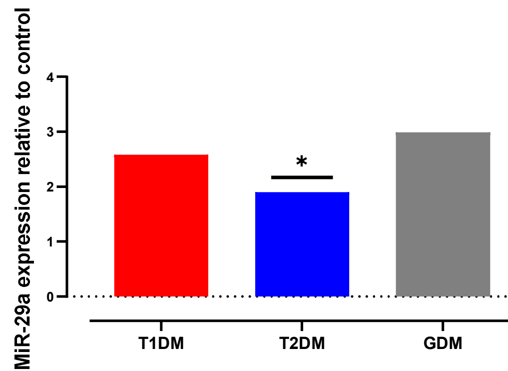

Figure 16. Fold regulation of statistically significant MiRNAs during maternal diabetes

MiRNAs were profiled in the serum of pregnant women with type 1 diabetes (T1DM) (n=4), type 2 diabetes (T2DM) (n=4), gestational diabetes (GDM) (n=3) and normoglycaemia (controls) (n=4) using the miScript miRNA serum/plasma PCR Array. The fold regulation of A) miR-19b-3p, B) miR-20a-5p and C) miR29a-3p relative to controls are represented in the graphs, *p<0.05.

4.5 Bioinformatics

To further explore the functional significance of the differentially expressed miRNAs, bioinformatic analysis was conducted to identify miRNA gene targets and their enriched biological pathways. Messenger RNA target prediction and pathway analysis for the three differentially expressed miRNAs (miR-19b-3p, miR-20a-5p and miR-29a-3p) were conducted using miRPathDB 2.0. Significant KEGG pathways with strong experimental evidence for each miRNA are illustrated in Figure 17. The forkhead box protein O1 (FOXO1) signalling and miRNAs in cancer pathways were common to all three miRNAs, while 15 pathways were common across miR-20a-5p and miR-29a-3p only. In addition, nine pathways were specific to miR-20a-5p and 18 pathways were specific to miR-29a-3p.

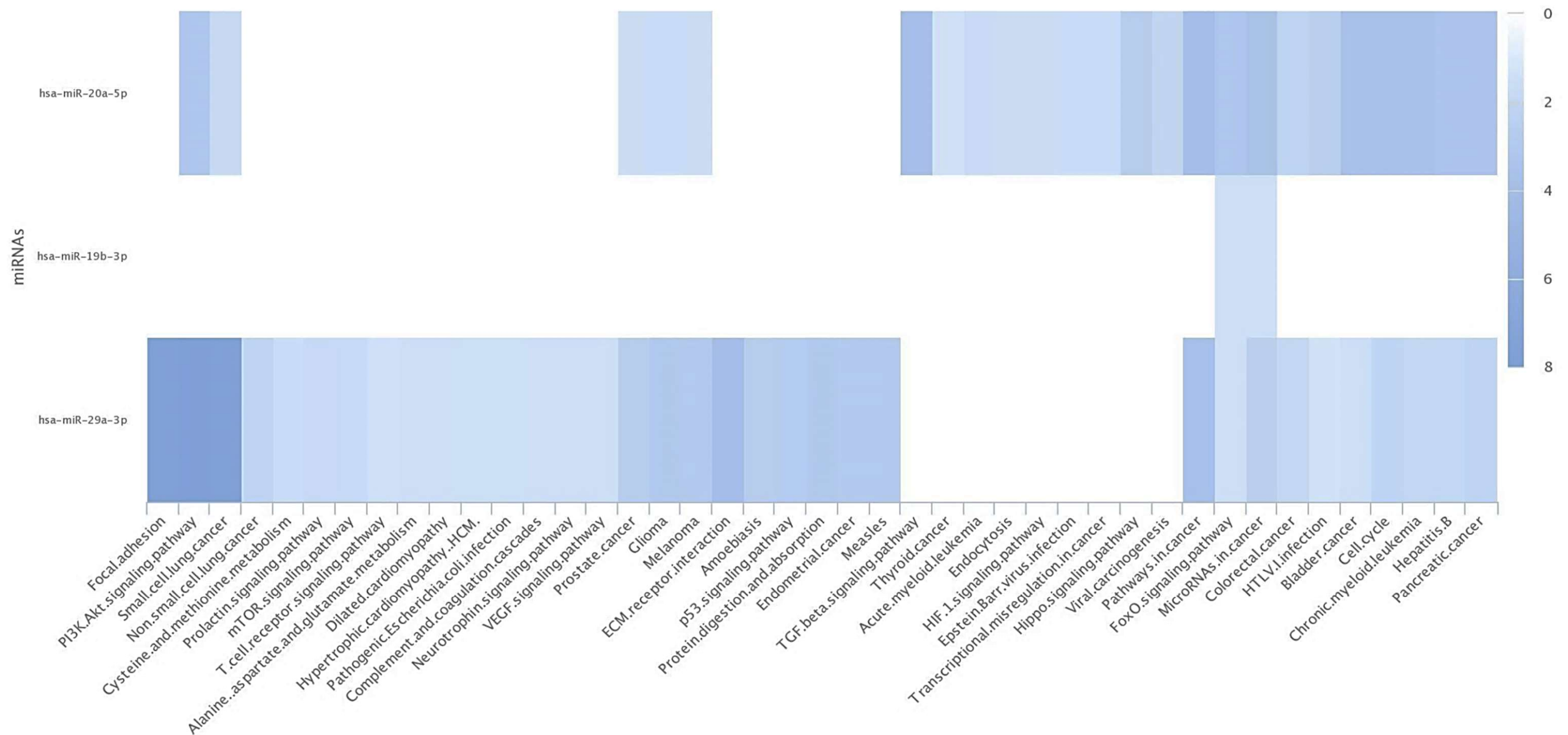

Figure 17. A heatmap illustrating KEGG pathways enriched for three significant miRNAs

A heatmap displaying KEGG pathways enriched for miR-19b-3p, miR-20a-5p, and miR-29a-3p with strong experimental evidence. Two pathways were common in miR-19b-3p, miR-20a-5p and miR-29a-3p, 15 pathways were common in miR-20a-5p and miR-29a-3p, nine pathways were specific to miR-20a-5p and 18 pathways were specific to miR-29a-3p. The p-values were used to calculate the indicated enrichment scores. The higher enrichment score, the greater the significance of the relevant miRNAs in the pathway**.** Higher enrichment scores are indicated by the darker blue colour intensity.

CHAPTER 5: DISCUSSION AND FUTURE PERSPECTIVES

5.1 Introduction

Maternal diabetes is associated with an increased risk of complications for both mother and child with several studies providing evidence that the frequency and severity of these adverse pregnancy outcomes are related with the severity of hyperglycaemia (Benhalima et al., 2019; Wu et al., 2020). For example, women with pregestational T1DM and T2DM have a higher risk of adverse outcomes (fetal and neonatal loss, congenital malformations, preterm delivery, macrosomia, preeclampsia, caesarean deliveries, and maternal mortality), compared to women with GDM (Mathiesen and Vaz, 2008; Shand et al., 2008). This may be attributed to GDM being a milder metabolic disorder than pregestational diabetes or due to the fact that pregestational diabetes is associated with longer intrauterine exposure to hyperglycaemia than GDM. Despite the factors linking maternal diabetes with adverse pregnancy outcomes being largely unknown, it is plausible that the molecular mechanisms that underlie the different types of maternal diabetes may provide insight. In recent years, epigenetic mechanisms such as miRNAs have garnered interest as tools to understand disease pathophysiology during pregnancy (Soifer et al., 2007). Several studies have provided evidence that the expression of miRNAs, including circulating miRNAs in serum, correlate with hyperglycaemia during pregnancy. Therefore, profiling miRNAs in pregnancies complicated by diabetes may aid in elucidating the pathophysiological mechanisms that underlie the different types of maternal diabetes. We hypothesised that circulating miRNAs will differ between the different types of maternal diabetes, thereby representing markers of diabetes type and offering insight into the mechanisms that orchestrate pregnancy complications.

The main findings of the study are 1) the identification of miRNAs with shared and unique expression across the different types of maternal diabetes, 2) the identification of three miRNAs with significant differential regulation, which was related to maternal diabetes type, 3) variation in peripheral metabolic markers between different types of maternal diabetes.

5.2 MiRNAs with shared and unique expression during maternal diabetes

MiScript miRNA PCR Arrays have gained popularity as tools for sensitive and specific mature miRNA expression profiling to identify miRNAs associated with biological processes and disease (Qiagen, Hilden, Germany). More recently, these techniques have also been applied to the assessment of relatively stable, extracellular miRNAs in serum and plasma samples. These circulating miRNAs are of great interest as potential noninvasive biomarkers for a variety of diseases, while recent studies have provided evidence that extracellular miRNAs may be involved in disease pathophysiology (Condrat et al., 2020). In this study, the Human Serum & Plasma miScript miRNA PCR Array, which enables rapid profiling of the 84 most relevant miRNAs associated with serum and plasma was used to profile miRNAs isolated from the serum of pregnant women with pregestational T1DM and T2DM, GDM and normoglycaemia.

The majority of the expressed miRNAs demonstrated shared expression across the different types of diabetes, although their expression levels differed. These findings highlight the ambiguous and non-specific nature of miRNAs, with a single miRNA speculated to regulate up to 200 genes involved in different biological pathways (Krek et al., 2005). Moreover, these results may also reflect the physiological similarities and shared biological pathways between the different types of diabetes. For example, T2DM and GDM are reported to have similar aetiologies, particularly the involvement of insulin resistance, inflammation and lipid dysregulation (Buchanan et al., 2007) and could share a common genetic background. It has been reported that T2DM and GDM share common genetic risk variants (Huerta-Chagoya et al., 2015) and that the determinants of these diseases are similar, with both being driven mainly by lifestyle factors such as obesity and physical inactivity (Sigal et al., 2006; Yen et al., 2019). Indeed, women with GDM have a ~7-fold increased risk of developing T2DM in later life (Bellamy et al., 2009), supporting the idea of a shared aetiology. Evidence, although limited, suggest that GDM and T2DM are more related to each other than with T1DM, which is an autoimmune disease. However, T1DM and GDM do share some risk variants and a low percentage of GDM patients have tested positive for T1DM autoimmune markers (Weng et al., 2002). Differences in the expression of lipid and glucose metabolism genes have been demonstrated in the placenta of mothers with T1DM and GDM (Radaelli et al., 2009), unfortunately our study did not assess placental gene expression in mothers with T2DM.

5.3 Three miRNAs with significant differential expression related to maternal diabetes type

The expression of three miRNAs was significantly different to controls and varied according to diabetes type. These miRNAs were similarly regulated in all diabetes types, yet their fold regulation compared to controls was significant in one type of diabetes only. For example, miR-19b-3p was downregulated in T1DM, T2DM and GDM compared to controls, although only the decreased expression in GDM was significant. Similarly, miR-20a-5p was downregulated in all diabetes types compared to controls, yet only the decreased expression during T1DM was statistically significant. MiR-29a-3p was increased in all types of diabetes, but only the regulation in T2DM was significant.

MiR-19b-3p has been reported to regulate the expression of FOXO1, sterol regulatory element-binding proteins (SREBP-1c and SREBP-2), insulin induced gene 1 (INSIG-1) and carbohydrate-responsive element-binding protein (ChREBP), key genes involved in biological pathways including insulin signalling, lipid and cholesterol biosynthesis and non-alcoholic fatty acid liver disease (NAFLD) (Sud et al., 2017). A previous study conducted in the same population as investigated in the current study similarly reported decreased expression of miR-19b-3p in serum of pregnant women with GDM when compared to normoglycaemia, however, the differences observed in this study were not statistically significant (Pheiffer et al., 2018b). In contrast, three studies conducted in Chinese populations reported conflicting results. Studies by Zhu et al. (2015) and Wang et al. (2019a) reported that miR-19b-3p was upregulated using SYBR Green and TaqMan qRT-PCR in plasma and serum, respectively, of pregnant women with GDM when compared to normoglycaemia (Zhu et al., 2015; Wang et al., 2019a), while one study by Cao et al. (2017) reported no difference in expression of miR-19b-3p using TaqMan qRT-PCR in plasma of pregnant women with GDM compared to normoglycaemia (Cao et al., 2017). Similarly Stirm et al. (2018) showed no difference in the expression of miR-19b-3p using SYBR Green qRT-PCR in whole blood of pregnant women with GDM compared to normoglycaemia in Germany population (Stirm et al., 2018).

MiR-20a-5p belongs to the miR-17-92 cluster, which is associated with angiogenesis, a key physiological process during pregnancy (Wang et al., 2012). The gene targets of miR-20a-5p, endothelial growth factor-a (VEGFA), hypoxia inducible factor-1a (HIF1A), phosphatase and

tensin homolog (PTEN), a protein implicated in the pathogenesis of insulin resistance, and the anti-apoptotic gene B cell lymphoma 2 (BCL2), are increasing during GDM, which suggests a corresponding decrease in miR-20a-5p expression (Pheiffer., et 2018b). Accordingly, in our study, the expression of miR-20a-5p was decreased during GDM, which is consistent with a previous study conducted in a similar population in South Africa. The expression of miRNA-20a-5p was similarly decreased during T1DM and T2DM, however only the former was significant (Pheiffer et al., 2018b). In contrast two studies reported upregulation of miR-20a-5p using SYBR green and Taqman qRT-PCR in plasma and placental tissue, respectively, of pregnant Chinese women with GDM when compared to normoglycaemia (Zhu et al., 2015; Cao et al., 2017). A study conducted in Spain, similarly, demonstrated differential expression of miR-20a-5p in placental tissue of women with T1DM and T2DM (Ibarra et al., 2018). These results suggest that miR-20a-5p regulates shared pathways in T1DM, T2DM and GDM, which may be related to the effect of maternal diabetes on angiogenetic processes.

MiR-29b-3p belongs to the miR-29 family and is among the most abundantly expressed miRNAs in metabolically active tissues such as the pancreas, liver and muscle (Dooley et al., 2016). Members of the miR-29 family act as an important regulators of β-cell function and insulin secretion, insulin-stimulated glucose metabolism and lipid oxidation, and have been shown to potentiate obesity and insulin resistance (Zhou et al., 2016). Studies have reported that miR-29b-3p was upregulated during T2DM (Kong et al., 2011; Massart et al., 2017), which supports our findings in pregnant women with T2DM and confirms its relevance to the pathophysiological processes during diabetes. Although, not significant, the expression of miR-29a-3p was increased in pregnant women with T1DM and GDM, consistent with its function in the pancreas and insulin resistance. Other studies have similarly reported increased expression of miR-29a-3p using Taqman and SYBR green qRT-PCR in serum of Canadian, Mexican and European women with GDM when compared to normoglycaemia (Gillet et al., 2019; Martínez-Ibarra et al., 2019; Sørensen et al., 2021), although conflicting results have also been reported (Pheiffer et al., 2018b; Zhao et al., 2011).

These conflicting results allude to the complexities of miRNA profiling, which are affected by various analytical factors including the method of normalisation and measurement platform used, and population factors including age, ethnicity, BMI and lifestyle factors (such

as smoking, diet and physical exercise) (Bovell et al., 2013; Florio et al., 2020; Karere et al., 2021). Furthermore, the concentration and yield of miRNAs are affected by the extraction procedure and technical variables that occur during experimental steps (Faraldi et al., 2019). The complexities of miRNA profiling are further confirmed in the study conducted by Sud et al. (2017) which showed that dietary factors such as fructose and fat may differentially regulate miRNA expression (Sud et al., 2017).

Bioinformatics confirmed the functional role of these miRNAs in biological pathways such as FoxO1 signalling pathways and the phosphoinositol-3-kinase–Protein Kinase B (PI3K-Akt) pathway, which have been implicated in the pathogenesis of diabetes (Colomiere et al., 2009; Wang et al., 2014; Maiese, 2015). The FoxO1 signalling pathway regulates various pathways including insulin signalling and gluconeogenesis (Gross et al., 2008). These findings strengthen the candidacy of these miRNAs as mechanisms that may underlie the different types of maternal diabetes, although future work in larger sample sizes and preferably longitudinal studies that profile miRNAs during pregnancy progression are required to validate our findings.

5.4 Variation in clinical presentation and peripheral metabolic markers in different types of maternal diabetes

Consistent with the widely held notion that age and BMI are risk factors for T2DM and GDM (Dias et al., 2019a), women with T2DM and GDM were older and heavier than women with T1DM. Also, as expected, HbA1c, which reflects long-term glucose control (Glinianaia et al., 2012), and glucose concentrations were higher in women with pregestational diabetes compared to GDM. C-peptide concentrations were higher in women with T2DM and GDM compared to women with T1DM, while total and HMW adiponectin concentrations were lower in women with T2DM and GDM. These findings support the notion that T2DM and GDM are more closely related to each other than with T1DM (Bellamy et al., 2009). As expected, metabolic markers of dysregulation were more closely linked with each other. For example, insulin and C-peptide concentrations were correlated with higher glucose concentrations, TGs, systemic inflammation, BMI and weight, while the opposite was observed for total and HMW adiponectin. Others have similarly reported negative correlations between adiponectin, measures of adiposity, inflammation and insulin resistance (Lihn et al., 2005). Similarly, lower adiponectin concentrations are associated with increased

adiposity and insulin resistance during pregnancy (Catalano et al., 2006) and with an increased risk of pregnancy disorders such as GDM (Pheiffer et al., 2021).

5.5 Strengths and limitations

To the best of our knowledge, this study is the first to compare miRNA expression in pregestational T1DM and T2DM, and GDM and provides novel findings on miRNA expression during maternal diabetes. Moreover, our study provided a comprehensive analysis of various metabolic markers during maternal diabetes, which has also been limited to date. However, it is also important to note that the study has several limitations. A major limitation of the study was the lack of validation of differentially expressed miRNAs identified by the PCR Array, in a bigger sample. While every effort was made to purchase assays and reagents for validation, delivery delays due to COVID-19 and lockdown restrictions significantly hampered such efforts and therefore these experiments were not conducted timeously, and the results thereof are not included in this thesis. Another limitation of this study is the use of serum rather than placental tissue, which may be more appropriate to study disease mechanisms during pregnancy. Although our protocol included the collection of placental tissue for analysis, challenges due to time of delivery resulted in failure to obtain these specimens. Importantly, studies have reported that placental miRNAs may be reflected in circulation, which supports our use of serum (Xu et al., 2017; Li et al., 2018). MiRNA expression is dynamic and changes with advancing gestation (Rosero et al., 2010), thus the wide gestational age range of our participants, may have contributed to the heterogeneous miRNA expression observed in our study and the failure to attain statistical significance. Furthermore, our sample size was small, and substantial heterogeneity between women may have led to the large variances observed in our data. Lastly, our study was limited by the absence of data for lifestyle and environmental factors, such as diet, physical activity, smoking and alcohol consumption, which are well known to contribute to changes in miRNA expression (Alegría-Torres et al., 2011).

5.6 Future perspectives

Although miRNA research during pregnancy, particularly GDM, has exploded in the last few years, there is a scarcity of studies profiling miRNAs during pregestational diabetes. In future, studies on miRNA profiling should be conducted in pregnancies complicated by the different types of diabetes, to provide insight into their underlying mechanisms, which may

be related to pregnancy outcome. In addition, our findings, in a small population of Black South African pregnant women attending the Steve Biko Academic Hospital in Pretoria, South Africa, should be confirmed in replication studies across different ethnicities and populations in South Africa and globally, to assess the generalisability of our findings. Longitudinal studies to profile miRNAs across pregnancy trimesters are required to assess causality, and to determine whether differences are involved in disease pathogenesis. Furthermore, studies to correlate miRNA expression with pregnancy outcomes are required to give insight into the potential role that miRNAs play in diabetes-related pregnancy complications.

Other research questions that could be addressed in future to further this research include: 1) Are the altered miRNA signatures in maternal diabetes the same across different ethnicities and population groups? 2) Could the unique miRNAs identified in this study be used as predictive markers for maternal diabetes? 3) Could miRNAs be used as predictive markers for short- and long- term pregnancy outcomes complicated by diabetes? Although numerous studies have demonstrated that miRNAs have potential as predictive biomarkers for diabetes in pregnancy, future studies should focus on reducing experimental and technical challenges with miRNA profiling. These could be achieved by tightly controlling miRNA processing during detection and normalisation, data processing and optimisation, and by adjusting for confounding factors that are known to affect miRNA patterns.

5.7 Conclusion

Our findings show that several circulating miRNAs have shared and unique differential expression across maternal diabetes types, although their expression levels differed. Of the differentially expression miRNAs, three miRNAs, miR-19b-3p, miR-20a-5p and miR-29a-3p, showed statistical significance when compared to controls, and varied according to maternal diabetes type. Moreover, variations in peripheral metabolic markers across different maternal diabetes types were observed. These findings suggest that the identification of miRNA signatures associated with diabetes type may contribute towards advancing our knowledge and understanding the mechanisms underlying the different types of diabetes in pregnancy.

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APPENDIX 1

RNA concentrations

Table 9. Serum RNA concentration, yield, purity and miRNA concentration

Sample ID	Nanodrop results				Agilent assay (CAF)	
	Nucleic Acid (ng/µl)	RNA yield (ng)	A260/A280	A260/A230	miRNA %	concentration pg/µl
1	10.95	164.25	0.96	0.16	57	209.3
2	16.11	241.65	1.37	0.18	48	234.9
3	15.28	229.2	1.32	0.73	56	43.3
4	18.08	271.2	1.36	0.28	38	199.6
5	22.915	343.725	1.38	0.06	53	118.9
6	32.22	483.3	1.44	0.06	25	178.7
7	43.61	654.15	1.14	0.22	52	64.5
8	32.55	488.25	1.28	0.42	72	252.8
9	20.72	310.8	1.30	0.13	65	127.7
10	28.27	424.05	1.401	0.074	53	284
11	66.35	995.25	1.45	0.56	72	100.9
12	17.2	258	1.06	0.2	69	239
13	7.18	107.7	1.30	0.31	58	255.1
14	36.59	548.85	1.4	0.56	55	97.9
15	13.12	196.8	1.35	0.58	51	237.60
16	18.55	278.25	1.37	0.08	37	191.1
17	22.5	337.5	1.49	0.03	55	70.9
18	24.19	362.85	1.3	0.46	62	274.3
19	11.19	167.85	1.37	0.26	59	276.4
20	7.0	105	1.35	0.2	49	87.3
21	19.36	290.4	1.35	0.11	89	125.7
22	15.62	234.3	1.22	0.12	62	165.3
23	13.28	199.2	1.13	0.21	57	119
24	11.59	173.85	1.24	0.37	63	160.1
25	25.7	385.5	1.41	0.05	65	82.3
26	21.47	322.05	1.06	0.28	4	141
27	33.46	501.9	1.33	0.75	44	129.9
28	22.74	341.1	1.33	0.51	63	97.6
29	12.51	187.65	0.96	0.09	49	55.2

30	15.26	228.9	1.15	0.12	53	95.2
31	6.12	91.8	1.32	0.27	51	128.6
32	19.75	296.25	1.19	0.08	50	135.3
33	18.16	272.4	1.37	0.04	72	66
34	8.0	120	1.06	0.09	58	152.8
35	108.99	1634.85	1.3	0.4	43	114.8
36	18.8	282	1.29	0.05	65	126.2
37	14.64	219.6	1.18	0.08	57	91.1
38	12.07	181.05	1.38	0.37	57	69.4
39	23.86	357.9	1.45	0.03	57	75.1
40	14.59	218.85	1.4	0.06	52	56.2
41	11.25	168.75	1.17	0.57	59	91.4
42	15.52	232.8	1.41	0.06	41	152.1
43	36.15	542.25	1.34	0.57	57	54.9
44	8.61	129.15	1.02	0.23	63	44.4
45	20.89	313.35	1.34	0.18	54	57.2
46	19.48	292.2	1.39	0.55	57	82.5
47	13.9	208.5	1.41	0.06	65	64.8
48	23.74	356.1	1.31	0.58	59	49.8
49	14.67	220.05	1.3	0.51	56	138.1
50	25.26	378.9	1.22	0.08	74	110
51	76.92	1153.8	1.46	0.09	60	134.9
52	30.38	455.7	1.01	0.12	57	98.2
53	31.69	475.35	1.35	0.19	49	119.7
54	24.7	370.5	1.29	0.14	57	109.9
55	118.19	1772.85	1.43	0.19	50	43.2
56	23.05	345.75	1.37	0.07	49	96
57	20.65	309.75	1.18	0.41	50	113.6
58	20.58	308.7	1.47	0.63	27	353.6
59	18.89	283.35	1.23	0.37	61	95.8
60	148.59	2228.85	1.36	0.47	51	160.7
61	18.76	281.4	1.37	0.11	42	146.7
62	17.16	257.4	1.26	0.17	49	81.6
63	20.07	301.05	1.34	0.29	51	83
64	24.68	370.2	1.18	0.17	66	71.5
65	27.36	410.4	1.19	0.24	55	156.3
66	11.31	169.65	1.31	0.37	54	134.5

67	42.4	636	1.39	0.05	56	77.5
68	24.98	374.7	1.34	0.34	55	167.1
69	14.98	224.7	1.25	0.1	50	58.1
70	22.57	338.55	1.22	0.34	62	37.8
71	18.77	281.55	1.27	0.33	71	15.8
72	13.08	196.2	1.11	0.25	66	18.1
73	13.11	196.65	1.21	0.51	91	960.3

ND-Not determined

Table 10. Common diagnostic criteria's for GDM

Diagnostic criteria	Approach step	Glucose challenge	Glucose threshold (mmol/L)			
			fasting	1 h	2 h	3 h
(O'sullivan and Mahan, 1964)	2	100 g OGTT	5.0	9.2	8.1	6.9
(National Diabetes Data Group, 1979)	2	100 g OGTT	5.8	10.6	9.2	8.1
(Carpenter and Coustan, 1982	2	100 g OGTT	5.3	10.0	8.6	7.8
(World Health Organisation, 1999)	1	75 g OGTT	7.0	-	7.8	-
(American Diabetes Association, 2013)	2	100 g OGTT	5.3	10.0	8.6	7.8
(International Association of Diabetes and Pregnancy Study Groups Consensus Panel, 2010)	1	75 g OGTT	5.1	10.0	8.5	-
(National Institute for Health and Care Excellence, 2015)	1	75 g OGTT	5.6	-	7.8	-

Abbreviation: OGTT, oral glucose tolerance test

Ethics Certificate

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG # IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

24 November 2020

Approval Certificate
New Application

Ethics Reference No.: 743/2020
Title: Profiling circulating microRNAs in pregnancies complicated by type 1 diabetes, type 2 diabetes and gestational diabetes mellitus

Dear Mr MI Masete

The **New Application** as supported by documents received between 2020-11-11 and 2020-11-18 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2020-11-18 as resolved by its quorate meeting.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-11-24.
- Please remember to use your protocol number (743/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

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