INVESTIGATING MOLECULAR BIOMARKERS DURING GESTATIONAL DIABETES MELLITUS

School of Medicine, Department of Obstetrics and Gynaecology,

Faculty of Health Sciences

In fulfilment of the requirements for the degree

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In the subject

Reproductive Biology

by

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DECLARATION

I, Stephanie Dias, declare that the dissertation/thesis, which I hereby submit for the degree Philosophiae Doctor at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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 she 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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DEDICATION

This thesis is dedicated to my parents, Charmaine and Stephen Dias, who have sacrificed so much to ensure that my dreams are fulfilled, and to my husband, Jason Sieas, for all his encouragement, understanding and patience during my study.

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LIST OF ABBREVIATIONS

ACOG	American College of Obstetricians and Gynaecologists
ADA	American Diabetes Association
ADIPOQ	Adiponectin
ANOVA	One-way analysis of variance
ART	Anti-retroviral therapy
BMI	Body mass index
C5orf34	Chromosome 5 open reading frame 34
CAMK1D	Calmodulin dependent protein kinase 1d
CAMTA1	Calmodulin binding transcription activator 1
CAPN10	Calpain 10
CCDC124	Coiled-coil domain containing 124
CD36	Cluster of differentiation 36
CDC123	Cell division cycle 123 homolog
CDH8	Cadherin-8
CDKAL1	Cdk5 regulatory subunit associated protein 1 like 1
CDKN2A/2B	Cyclin dependent kinase inhibitor 2a/2b
CDS	Coding domain sequences
CI	Confidence interval
COPS8	Constitutive photomorphogenic homolog subunit 8
CpG	Cytosine-phosphate-guanine
CRP	C-reactive protein
CYP26B1	Cytochrome P450 family 26 subfamily b member 1
DGCR8	Digeorge critical region 8
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase

DPSG	Diabetes in Pregnancy Study Group
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
Expo5	Exportin-5
FDR	False discovery rate
FIGO	International Federation of Gynaecology and Obstetrics
FOXP3	Forkhead box p3
FTO	Fat mass and obesity-associated
Fwd	Forward
GC	Group-specific
GCK	Glucokinase
GCKR	Glucokinase regulator
GCT	Glucose challenge test
GDM	Gestational diabetes mellitus
GO	Gene ontology
GRα	Glucocorticoid receptor alpha
GRβ	Glucocorticoid receptor beta
GTF21	General transcription factor 21
HAAO	3-hydroxyanthranilate 3,4-dioxygenase
HAPO	Hyperglycaemia and Adverse Pregnancy Outcome
HbA1c	Glycated haemoglobin
HIV	Human immunodeficiency virus
HOMA	Homeostatic model assessment
HOOK2	Hook microtubule-tethering protein 2
hPL	Human placental lactogen
HRM	High resolution melt-curve analysis

HWE	Hardy-Weinberg Equilibrium
IADPSG	International Association of Diabetes in Pregnancy Study Group
IDF	International Diabetes Federation
IGT	Impaired glucose tolerance
IGF2BP2	Insulin like growth factor 2 mRNA binding protein 2
IL-10	Interleukin-10
IL-6	Interleukin-6
IRS1	Insulin-receptor substrate 1
KASP	Kompetitive allele specific PCR
KCNJ11	Potassium voltage-gated channel subfamily j member 11
KCNQ	Potassium voltage-gated channel, KQT-like subfamily
KCNQ1	Potassium voltage-gated channel subfamily q member 1
KEGG	Kyoto Encyclopedia of Genes and Genomes
KHDRBS2	KHbRNA binding domain containing, signal transduction associated 2
KLHDC3	Kelch domain-containing protein 3
LADA	Latin American Diabetes Association
LC-ESI/MS/MS	Liquid chromatography ionisation/multi-stage mass spectrometry
LDR	Ligase detection reaction
MAPK	Mitogen activated protein kinase
MEA1	Male-enhanced antigen 1
MIF	Macrophage migration inhibitory factor
miRISC	MiRNA-induced silencing complex
miRNAs	MicroRNAs
mRNA	Messenger ribonucleic acid

MSH5	Muts protein homolog 5
MTHFR	Methylenetetrahydrofolate reductase
MTNR1B	Melatonin-receptor 1B gene
NCD	Non-communicable disease
NDDG	National Diabetes Data Group
NICE	National Institute for Health Care Excellence
NLRC5	NOD-like receptor family CARD domain containing 5
NOOB	Norma-exponential out-of-band
NPM1	Nucleophosphin 1
NRC1	Non-imprinted glucocorticoid receptor
NRG1	Neuregulin 1
NUDT6	Nucleoside diphosphate-linked moiety x motif 6
OGTT	Oral glucose tolerance test
OSR1	Odd-skipped related transcription factor 1
PCA	Principle component analysis
PCR	Polymerase chain reaction
PI3K	Phosphoinositide-3-kinase
PI3KR5	Phosphoinositide-3-kinase, regulatory subunit 5
PPARG2	Peroxisome proliferator-activated receptor-gamma 2
PPFIBP2	Protein-tyrosine phosphatase, receptor-type, f polypeptide-interacting protein-binding protein 2
Pre-miRNAs	Precursor miRNAs
Pri-miRNAs	Primary transcripts
qRT-PCR	Quantitative real time-polymerase chain reaction
R1	Region 1
RASA3	Ras p21 protein activator 3

RBP4	Retinol binding protein 4
RDH12	Retinol dehydrogenase 12
Rev	Reverse
RFLP	Restriction fragment length polymorphism
RFTN1	Raftlin, lipid raft linker 1
SA	South Africa
SD	Standard deviation
SEM	Standard error of the mean
SEMA6D	Semiphorin 6d
SEMDSA	Society for Endocrinology, Metabolism and Diabetes of South Africa
SHBG	Sex hormone-binding globulin
SLC30A8	Solute carrier family 30 member 8
SLC9A3	Solute carrier family 9 member 3
SNIP1	SMAD nuclear interacting protein 1
SNPs	Single nucleotide polymorphisms
SP-1	Specificity protein 1 transcription factor
STK11	Serine/threonine kinase 11
STOX2	Storkhead box
SWAP70	Switching b cell complex subunit swap 70
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TCF7L2	Transcription factor 7-like 2
TET	Ten-eleven translocation
TFAP2A	Transcription factor AP2-alpha
TRBP	Transactivation-responsive RNA binding protein

UNC5C	Unc-5 netrin receptor c
UTR	Untranslated region
VDR	Vitamin D receptor
WHO	World Health Organisation
WNT6	Wingless-type MMTV integration site family, member6
XBP1	X-Box binding protein 1

ABSTRACT

Introduction

Gestational diabetes mellitus (GDM) is a significant public health concern, due to its association with short- and long-term complications in both mothers and offspring. DNA methylation and single nucleotide polymorphisms (SNPs) offer potential to serve as molecular biomarkers, which may lead to improved detection of GDM with positive effects on health outcomes.

Aim

The aim of this study was to investigate whether DNA methylation and SNPs are associated with GDM and may offer potential as molecular biomarkers for GDM in South Africa (SA).

Methods

This study followed a two-pronged approach. Firstly, literature searches were conducted to collate and synthesise all published articles reporting on the prevalence of GDM in SA, the screening and diagnostic strategies used, and the current status of DNA methylation and SNPs as biomarkers for GDM. Secondly, we conducted experiments to investigate global (n=201), genome-wide (n=24) and gene-specific DNA methylation (n=286) of the adiponectin gene *(ADIPOQ)* in whole blood of women with and without GDM, using an Enzyme-Linked Immunosorbent Assay, a methylationEPIC BeadChip Array and pyrosequencing, respectively. In addition, genotype and allele frequencies of *ADIPOQ* rs266729 and rs17300539, and

methylenetetrahydrofolate reductase (*MTHFR)* rs1801133 were determined, using quantitative real-time PCR (n=449) and DNA sequencing for validation.

Results

The literature search showed that the prevalence of GDM in SA has increased over the years. Furthermore, it showed that the lack of uniformity in screening and diagnosis between and within countries hamper the accurate detection of GDM. Lastly, the literature search identified several studies that support the use of DNA methylation and SNPs as potential biomarkers for GDM. Experimentally, we showed no differences in global DNA methylation between GDM and non-GDM groups. Interestingly, global DNA methylation levels were 18% (p=0.012) higher in obese compared to non-obese pregnant women. Genome-wide methylation analysis identified 1046 differentially methylated CpG sites (associated with 939 genes) (Cut-off threshold: M>0.06 and p<0.01). Among the top five CpG sites identified, one CpG mapped to the calmodulin-binding transcription activator 1 *(CAMTA1)* gene, which has been shown to regulate insulin production and secretion. Two CpG sites (-3410: p=0.048 and -3400: p=0.004) in the *ADIPOQ* promoter were hypomethylated during GDM in HIV negative, but not in HIV positive women. Lastly, no association between the *ADIPOQ* and *MTHFR* polymorphisms and GDM was observed in our population.

Conclusion

To our knowledge, this is the first study to investigate the association between DNA methylation or *ADIPOQ (*rs266729 and rs17300539) and *MTHFR* (rs1801133) polymorphisms and GDM in SA. Findings suggest that gene-specific, but not global methylation nor SNPs rs266729, rs17300539 and rs1801133, may offer potential as molecular biomarkers of GDM in this population. Future longitudinal studies in larger

samples that include both HIV negative and positive pregnant women are warranted to explore the candidacy of DNA methylation as molecular biomarkers for GDM.

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Key words: Global DNA Methylation, Gene-specific Methylation, Single Nucleotide Polymorphism (SNP), Adiponectin *(ADIPOQ),* Methylenetetrahydrofolate Reductase *(MTHFR),* Molecular Biomarkers, Gestational Diabetes Mellitus (GDM), South Africa (SA), Human Immunodeficiency Virus (HIV).

SUMMARY OF THESIS

The structure of the thesis is as follows:

The INTRODUCTION provides a brief overview of GDM, succinctly summarising previous research on the study topic. Thereafter, the problem statement provides context to the study, and emphasises why it is important to address the problem. The rationale highlights the importance of conducting the study, thus justifying the significance. The rationale is followed by the study hypothesis, aims and objectives.

CHAPTER 1 is a detailed review of the literature allowing deeper insight into GDM. The chapter begins by outlining the history of GDM and describes the prevalence and pathophysiology of the disease. Thereafter, the short- and long-term complications in both mothers and offspring are briefly reviewed, and the challenges and caveats of GDM screening, diagnosis and treatment strategies are discussed. Lastly, DNA methylation and SNPs are discussed, together with their clinical relevance as potential molecular biomarkers for GDM.

CHAPTER 2 outlines the overall study design, describes participant recruitment and details the inclusion and exclusion criteria that were used. In addition, this chapter illustrates participant selection for each empirical chapter and provides a brief description of the research methodologies used in these chapters. More detailed methods are given in the respective chapters.

CHAPTER 3 is a review article that collates and synthesises all published articles on the prevalence of GDM in South Africa (SA). The review provides an update of the prevalence of GDM in SA and highlights treatment and management strategies

currently employed in the country. The review was published in the *South African Medical Journal*: **Dias S**, Adam S, Rheeder P, Pheiffer C. Prevalence of and risk factors for gestational diabetes mellitus in South Africa. 2019;109:463-467–467.

CHAPTER 4 is a review article that highlights the varied GDM screening and diagnostic strategies currently employed in SA. The review describes the evolution of the major GDM screening and diagnostic strategies and discusses the controversy surrounding GDM screening. In addition, the review highlights novel screening and diagnostic methods that are being explored and makes recommendations for GDM screening, diagnosis and management. The review was published in the *South African Medical Journal*: **Dias S**, Pheiffer C, Rheeder P, Adam S. Screening and diagnosis of gestational diabetes mellitus in South Africa: What we know so far. 2019;109:457-462.

CHAPTER 5 is a review article that summarises the current status of molecular biomarkers for GDM. This review summarises all studies that investigated SNPs and DNA methylation during GDM. Furthermore, the limitations of these molecular biomarkers, as well as future research recommendations, are discussed. The review was published in the *International Journal of Molecular Science*: **Dias S**, Pheiffer C, Abrahams Y, Rheeder P, Adam S. Molecular Biomarkers for Gestational Diabetes Mellitus. 2018;19(10): 2926.

CHAPTER 6 investigates the potential of global DNA methylation to serve as a biomarker for GDM. Global DNA methylation was quantified in the whole blood of women with or without GDM using an Enzyme-Linked Immunosorbent Assay. This work was published as a research article in *Biomarkers*: **Dias S**, Adam S, Wyk NV, Rheeder P, Louw J, Pheiffer C. Global DNA methylation profiling in peripheral blood cells of South African women with gestational diabetes mellitus. 2019;24:225–31.

CHAPTER 7 investigates genome-wide DNA methylation during GDM. DNA methylation was quantified in the whole blood of women with or without GDM using the Illumina MethylationEPIC Bead Chip Array. In addition, functional analysis of differentially methylated genes was conducted to identify pathways associated with GDM. This chapter was published as a research article in the *International Journal of Molecular Science:* **Dias S**, Adam S, Rheeder P, Louw J, Pheiffer C. Altered Genome-Wide DNA Methylation in Peripheral Blood of South African Women with Gestational Diabetes Mellitus. 2019;20(23):5828.

CHAPTER 8 explores the association between gene-specific methylation of the *ADIPOQ* gene and GDM in human immunodeficiency virus (HIV) negative and positive women. Methylation across eight CpGs within the promoter of *ADIPOQ* was quantified using pyrosequencing. *In silico* analyses was conducted to identify transcription factors that bind to CpGs with altered methylation. This chapter was submitted as a short report to *Clinical Epigenetics*: **Dias S**, Adam S, Abrahams Y, Rheeder P, Pheiffer C. Human immunodeficiency virus infection affects the association between adiponectin DNA methylation and gestational diabetes mellitus in South African women. 2019.

CHAPTER 9 investigates the association between genetic variants and GDM. SNPs in adiponectin (*ADIPOQ)* (rs266729 and rs17300539) and methylenetetrahydrofolate reductase (*MTHFR)* (rs1801133) genes were profiled using quantitative real-time PCR. In addition, we explored the association between SNPs and clinical characteristics. Furthermore, the association between *MTHFR (*rs1801133) and global methylation was investigated. This chapter will be submitted as a research article to the *Journal of Assisted Reproduction and Genetics*: **Dias S**, Adam S, Rheeder P, Pheiffer C. Genetic

variants of adiponectin and methylenetetrahydrofolate reductase genes in South African women with gestational diabetes mellitus.

CHAPTER 10 summarises and discusses the findings of the individual reviews and empirical chapters, followed by integration and synthesis of the overall thesis findings. We discuss the significance of the study findings, the novelty, and how the study findings contribute to existing knowledge, thus advancing research. Lastly, we discuss the potential impact of biomarkers on health systems, and highlight the strengths and limitations of the study, and recommendations for future research.

INTRODUCTION

1. STUDY OVERVIEW

Gestational diabetes mellitus (GDM) is a form of glucose intolerance that is diagnosed during the second or third trimester of pregnancy, that is not pre-existing or overt diabetes (American Diabetes Association, 2016). The prevalence of GDM is increasing rapidly, and in 2017 it was estimated to affect approximately 14% of pregnancies worldwide (International Diabetes Federation, 2017), representing about 18 million births. Without appropriate glucose management, GDM is associated with adverse maternal, foetal and perinatal complications (Alam et al., 2006; Mohammadbeigi et al., 2013; Young & Ecker, 2013), and an increased risk of future metabolic disease in both mothers and offspring (Mitanchez et al., 2015).

The oral glucose tolerance test (OGTT) conducted at 24-28 weeks of gestation is currently considered the gold standard for the diagnosis of GDM (IADPSG panel, 2010). However, the test is cumbersome to conduct, is time-consuming, requires fasting, multiple venesections, and is associated with nausea and vomiting, often leading to decreased patient compliance. Moreover, the test is expensive, resulting in many low- and middle-income countries using the risk factor-based selective screening approach, which often has poor sensitivity. The identification of simpler and more effective tests that can detect GDM early, may facilitate risk stratification and intervention strategies that could potentially lead to better management of GDM, thereby improving health outcomes.

In recent years, molecular mechanisms are increasingly explored as biomarkers for metabolic disease. Of these, DNA methylation, an epigenetic mechanism that reflects the interplay between gene-environment interactions (Christensen & Marsit, 2011; Ling & Rönn, 2019), has attracted considerable interest. Several studies have shown

that DNA methylation changes reflect metabolic adaptation during pregnancy (Bouchard et al., 2012; Houde et al., 2013; Cardenas et al., 2018) and may be implicated in the pathophysiology of GDM. Accordingly, altered DNA methylation has been demonstrated in maternal blood, placental tissue and cord blood of GDM-complicated pregnancies, supporting its potential as biomarkers for GDM (Finer et al., 2015; Reichetzeder et al., 2016; Haertle et al., 2017; Wu et al., 2018). In addition to DNA methylation, which reflects both genetics and the environment, screening for single nucleotide polymorphisms (SNPs) may detect genetic susceptibility to GDM and may be useful as biomarkers. Genetic risk variants for GDM have been identified in many genes (Huopio et al., 2013; Pagán et al., 2015; Popova et al., 2017; Ding et al., 2018), although limited studies have been conducted in SA, warranting further investigation.

Serum biomarkers for GDM are widely investigated. Of these, adiponectin, an insulin-sensitising adipokine (Brochu-Gaudreau et al., 2010; Ghadge, Khaire & Kuvalekar, 2018) that progressively declines with increasing insulin resistance during pregnancy (Catalano et al., 2006; Bao et al., 2015), has shown great potential. Several studies (Ranheim et al., 2004; Worda et al., 2004; Retnakaran et al., 2010), including one conducted in our population (Adam et al., 2018) reported that serum adiponectin concentrations are lower in pregnancies complicated by GDM. However, high inter-individual heterogeneity in adiponectin levels (Lacroix et al., 2013) hampers the identification of a clinically applicable threshold and its candidacy as a biomarker for GDM. The molecular mechanisms that regulate adiponectin expression may offer potential as biomarkers with improved sensitivity and specificity compared to serum adiponectin levels.

It has been reported that HIV infection alters DNA methylation (Zhang et al., 2016) which may have implications for biomarker discovery. This is particularly relevant in

SA, the country with the highest prevalence globally (HSRC Press, 2018). In SA it is estimated that 7.9 million people are living with HIV, of whom 26.3% are women of reproductive age, and that one-third of all pregnancies are complicated by GDM (HSRC Press, 2018).

2. PROBLEM STATEMENT

GDM is a growing public health problem worldwide. Recently, a GDM prevalence of 25.8% was reported in an urban setting in SA, a rate considerably higher than previously reported in this country (Adam & Rheeder, 2017), although, different diagnostic criteria could partly account for the increase observed. The short- and long-term consequences of GDM contribute to maternal and child morbidity and mortality (Sheiner et al., 2019) as well as to the rising burden of non-communicable diseases, posing a health threat to both mothers and their offspring. It is estimated that approximately 30% of offspring and 50% of women with previous cases of GDM are predisposed to develop type 2 diabetes (T2D) in later life (Garcia-Vargas et al., 2012). Thus, there is a need to identify strategies to better manage GDM and improve health outcomes.

3. RATIONALE

The identification of alternative screening and diagnostic methods to traditional risk factor screening, which has poor predictive value in a South African population (Adam & Rheeder, 2017), may lead to improved detection of GDM with positive effects on health outcomes and costs to the health system. Molecular mechanisms offer potential as biomarkers for GDM. Aberrant DNA methylation has been demonstrated during GDM (Kang et al., 2017, 2018; Wu et al., 2018), while genetic risk variants for

GDM are widely reported (Huopio et al., 2013; Pagán et al., 2015; Popova et al., 2017; Ding et al., 2018). Thus, identification of altered DNA methylation and SNPs offer potential as sensitive and specific molecular biomarkers for GDM. These DNA-based biomarkers can be isolated from small quantities of blood, which may be more acceptable to patients. Furthermore, they can be detected sub-clinically, before disease manifestation, thus additionally have the potential to detect disease earlier, facilitating GDM preventative and management strategies and minimizing costs to the health system.

4. HYPOTHESIS

We hypothesised that DNA methylation and SNPs are associated with GDM and may offer potential to serve as molecular biomarkers for GDM in a South African population.

5. AIMS AND OBJECTIVES

An overview of the aims, objectives and methodology of the study, together with the thesis's chapters, is illustrated as a flow diagram in Figure A.

1. Main Aim

To investigate whether DNA methylation and SNPs are associated with GDM and may offer potential to serve as molecular biomarkers for GDM in a South African population.

2. *Specific Aims:*

- To provide an update of GDM in SA, focusing on prevalence and the screening and diagnostic tools used
- To evaluate the current status of molecular biomarkers for GDM
- To explore the association between DNA methylation and GDM in SA
- To explore the association between SNPs and GDM in SA

3. *Objectives*

- Objective 1:
  - Collate and review all published articles reporting on the prevalence of GDM in SA
  - Consult with tertiary health care institutions to identify GDM screening and diagnostic strategies currently used in SA

- Objective 2:
  - Collate and review all published studies reporting on DNA methylation and SNPs during GDM
- Objective 3:
  - Assess the association between global DNA methylation and GDM using an Enzyme-Linked Immunosorbent Assay
  - Assess the association between gene-specific DNA methylation and GDM using:
    - MethylationEPIC Bead Chip Array to measure genome-wide DNA methylation
    - Pyrosequencing to quantify methylation in the promoter of the adiponectin *(ADIPOQ)* gene

- Objective 4:
    - Genotype SNPs in the adiponectin gene
    - Genotype SNPs in the methylenetetrahydrofolate reductase *(MTHFR)* gene

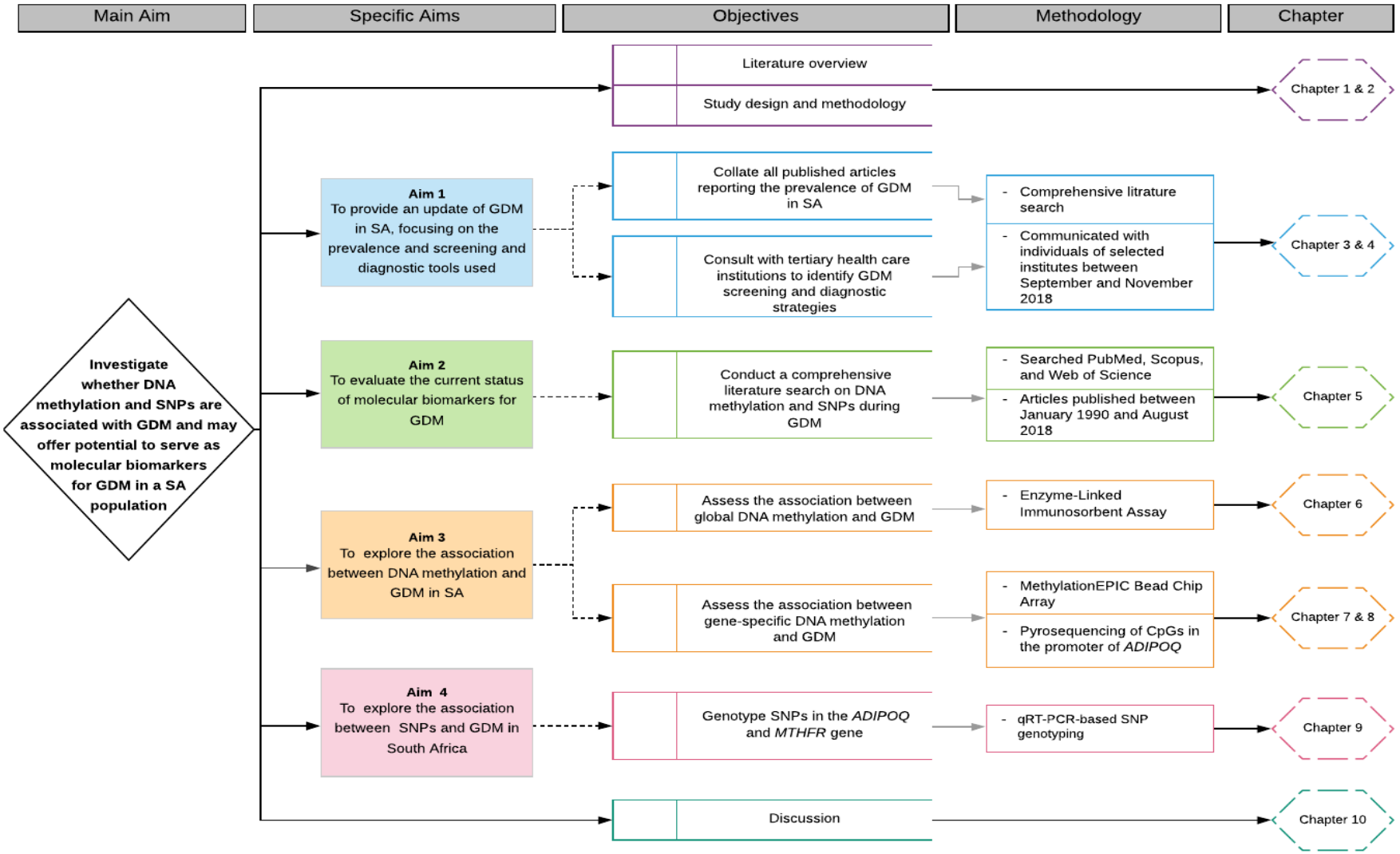

Figure A. Flow diagram illustrating aims, objectives and methodology in each chapter in the study.

CHAPTER 1

LITERATURE REVIEW

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1. HISTORY OF GESTATIONAL DIABETES MELLITUS

Diabetes in pregnancy was first described in 1824 by a German physician, Heinrich Bennewitz, who reported intense thirst and glycosuria in a pregnant woman, in whom the symptoms disappeared after delivery (Bennewitz, 1824). Until the discovery of insulin in 1923, there was no effective treatment for the condition, which often led to adverse pregnancy outcomes (Walker, 1928). By the 1940s, studies reported that a lesser degree of hyperglycaemia during pregnancy was also a risk for adverse outcomes and perinatal mortality (Miller, in press; Hurwitz & Jensen, 1946; Jackson, 1952). In 1964, John O' Sullivan performed a 100 g 3 hr oral glucose tolerance test (OGTT) using a two-step screening approach in 752 second and third trimester pregnant women (O'Sullivan JB & Mahan CM., 1964). Using this data, John O' Sullivan and statistician Claire Mahan, proposed the first statistically based criteria for assessing the upper limit for glycaemic normality during pregnancy (O'Sullivan JB & Mahan CM., 1964). This became the standard for detecting diabetes in pregnancy for the next decade. The modern term gestational diabetes mellitus (GDM) was first used by John O' Sullivan but was not recognised universally until popularised by Norbit Freinkel in 1980, when he published a major paper presenting several important insights into the pathophysiology of glucose metabolism in both mother and foetus. This led to an American sponsored workshop, where the definition of GDM as 'glucose intolerance first diagnosed during pregnancy' was established (Freinkel, 1980). For many years this traditional definition of GDM was accepted regardless of whether glucose intolerance predated pregnancy or persisted after birth. However, due to the rising obesity and diabetes epidemic, more women of childbearing age are afflicted with type 2 diabetes (T2D), resulting in an increase in the number of pregnant women with undiagnosed T2D (Lawrence et al., 2008). Thus, the World Health Organisation (WHO) classifies women with diabetes in the first trimester as having T2D, while GDM represents a milder form of hyperglycaemia that occurs in the latter

half of pregnancy, with return to normoglycemia after birth (WHO, 2013). According to the American Diabetes Association (ADA), GDM is defined as diabetes diagnosed in the second or third trimester of pregnancy that is not pre-existing diabetes (type 1 diabetes (T1D) or T2D) (American Diabetes Association, 2016).

2. PREVALENCE OF GESTATIONAL DIABETES MELLITUS

GDM is considered one of the leading causes of morbidity and mortality in mothers and infants worldwide (Sheiner et al., 2019). The World Health Organisation (WHO) classifies pre-existing diabetes or newly diagnosed T1D or T2D as severe hyperglycaemia during pregnancy, while GDM represents a milder form of hyperglycaemia. According to recent statistics, 21.3 million live births (16.2%) are affected by hyperglycaemia in pregnancy, of which 86.4% are due to GDM (International Diabetes Federation, 2017). The prevalence of GDM is rapidly increasing globally, ranging from 1% to 28%, depending on population characteristics and diagnostic criteria employed (Jiwani et al., 2012). The prevalence of GDM has been shown to be higher in low- and middle-income countries compared to high income countries where access to maternal health care is readily available (Seshiah et al., 2008; Jiwani et al., 2012; Kanguru et al., 2014; Goldenberg et al., 2016; Nguyen et al., 2018). Recently, a GDM prevalence of 25.8% was reported in an urban setting in SA, a rate much higher than previously reported in this country (Adam & Rheeder, 2017). Although different diagnostic criteria could partly account for the increased prevalence observed, other factors such as advanced maternal age and obesity may play a role. SA has an obesity crisis with ~69% of women regarded as overweight, of whom 42% are obese and 20% are morbidly obese (Ng et al., 2014; Statistics South Africa, 2017). The obesity epidemic is spurred by urbanisation, unhealthy diets and physical inactivity. Obesity is considered the major risk factor for metabolic syndrome and hyperglycaemia, particularly among females (Pons et al., 2015).

3. PATHOPHYSIOLOGY OF GESTATIONAL DIABETES MELLITUS

The exact mechanism underlying GDM pathophysiology is not completely elucidated, although progressive insulin resistance during pregnancy has been implicated (Metzger et al., 2007). During the first trimester of gestation, insulin sensitivity increases in order to promote the uptake of glucose into adipose stores to prepare for energy demands in later pregnancy (Di Cianni et al., 2003). However, as pregnancy progresses, an increase in placental hormones, including estrogen, progesterone, leptin, cortisol, placental lactogen and placental growth hormone are produced to facilitate nutrient transfer from mother to infant, leading to insulin resistance in peripheral tissues such as muscle, liver and adipose tissues (Catalano et al., 1991). Most women remain normoglycemic due to adequate pancreatic beta (β)-cell compensation with higher insulin secretion and production. However, GDM develops when β-cells become dysfunctional and are unable to meet the increasing insulin demands (Perkins, Dunn & Jagasia, 2007). A schematic diagram showing progression to GDM is illustrated in Figure 1.1.

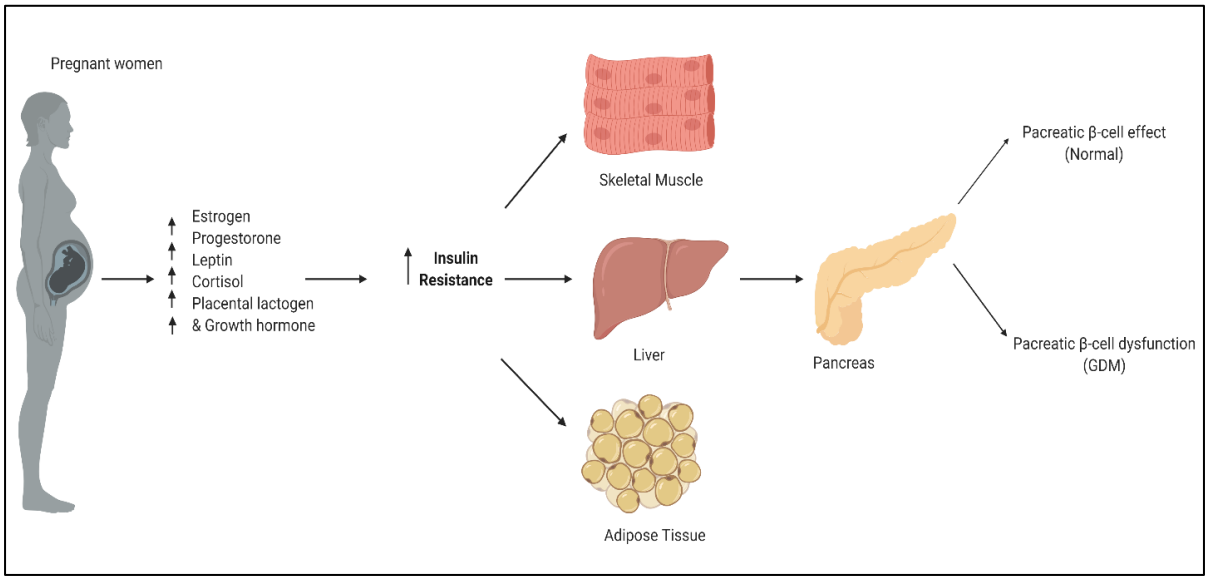

Figure 1.1. Schematic diagram illustrating progression to GDM. During pregnancy, maternal hormones increasse, leading to insulin resistance in peripheral tissues such as muscle, liver and adipose tissues. As a result, pancreatic beta (β)-cell compensated by increasing insulin secretion and production. However, when β-cells become dysfunctional, GDM occurs.

4. SHORT- AND LONG-TERM COMPLICATIONS OF GESTATIONAL DIABETES MELLITUS

Without appropriate diagnosis and management, GDM is associated with adverse maternal (pre-eclampsia, increase in caesarean deliveries, birth trauma), foetal (macrosomia, hypoglycaemia, shoulder dystocia) and perinatal (respiratory distress syndrome, jaundice, metabolic derangements) complications (Hod et al., 1991; Hadar & Hod, 2013; Moore, 2018), while both mothers and their offspring are at increased risk of developing T2D, obesity and other metabolic conditions in later life (Kim, Newton & Knopp, 2002; Clausen et al., 2008; Bellamy et al., 2009; Zhao et al., 2016). Generally, there is a continuous positive association between higher maternal hyperglycaemia and increased frequency of adverse outcomes. Recent estimates indicate that approximately 50% of women with previous cases of GDM (Kim, Newton & Knopp, 2002) and 30% of offspring (Garcia-Vargas et al., 2012) are predisposed to develop T2D within 10 years, thus posing significant health and economic burdens to health systems, particularly those in under-resourced countries such as SA.

5. SCREENING AND DIAGNOSIS OF GESTATIONAL DIABETES MELLITUS

Despite five decades of research, there is little consensus regarding the optimal GDM screening and diagnostic criteria (Long, 2011; Coustan, 2013). The lack of uniformity, both among countries and between obstetric and diabetes organisations within a single country, hamper the early detection and management of GDM, which negatively affects maternal and child health. The latest guidelines for GDM screening by the International Association of Diabetes in Pregnancy Study Groups (IADPSG) advocates for universal screening to promote appropriate treatment and improved

pregnancy outcomes (IADPSG panel, 2010). This implies that all pregnant women should be screened for GDM, regardless of risk factors (repeated glucosuria, previous GDM, family history of diabetes, history of stillbirth and congenital abnormalities, suspicion of polyhydramnios in pregnancy, obesity, previous macrosomic infant, history of polycystic ovarian syndrome and perinatal death, and women of South Asian descent) (SEMDSA, 2017). These guidelines were based on findings from the Hyperglycaemia and Adverse Pregnancy Outcome (HAPO) study, which assessed glucose concentrations and primary and secondary pregnancy complications in 23 316 pregnant women across 9 countries. The authors showed a strong linear correlation between maternal blood glucose concentrations and primary adverse pregnancy outcomes such as birth weight and cord blood C-peptide above the 90th percentile, and a weaker association for caesarean delivery and neonatal hypoglycaemia, at glucose concentrations previously considered normal (HAPO Study Cooperative Research Group et al., 2008). In addition, plasma glucose levels were associated with all five secondary adverse pregnancy outcomes including premature delivery, shoulder dystocia or birth injury, pre-eclampsia, hyperbilirubinemia and intensive neonatal care. In 2013, the World Health Organisation (WHO) revised their criteria, and endorsed those of the IADPSG (WHO, 2013).

The oral glucose tolerance test (OGTT) conducted at 24-28 weeks of gestation, when insulin resistance is likely to occur (Immanuel & Simmons, 2017), is currently considered the gold standard for the diagnosis of GDM (IADPSG panel, 2010). However, this test is time consuming, requires fasting, multiple blood draws, and is associated with nausea and vomiting, often leading to decreased patient compliance. In most cases fasting during pregnancy is not possible, since women from rural communities must travel far distances to receive clinical health care. In countries with a high prevalence of HIV, multiple blood draws are considered a health hazard, as it increases the risk of HIV transmission. In addition, in many resource-limited

countries, universal screening is considered expensive and unfeasible, resulting in many countries using risk-factor based selective screening as the predominant screening strategy. Unfortunately, these risk factors have poor predictive value, resulting in many women with GDM not receiving appropriate treatment (O'Sullivan et al., 1973; Cosson et al., 2014). In a previous study conducted in a South African population, Adam et al. showed that risk factors failed to identify approximately 10.6% of pregnant women with GDM (Adam & Rheeder, 2017). Thus, there is significant impetus to identify simpler, affordable and more effective tests that can detect women at high risk of developing GDM or diagnose GDM in pregnancy.

6. GESTATIONAL DIABETES MELLITUS TREATMENT

Treatment and management of hyperglycaemia in pregnancy is associated with reduced adverse obstetric and foetal outcomes (HAPO Study Cooperative Research Group et al., 2008). The first line of intervention for women with GDM is through exercise and nutritional counselling, which includes individualised physical activity guidelines and a dietary meal plan, usually provided by obstetricians, endocrinologists, dieticians or nurse educators. However, if lifestyle modification fails to achieve glucose control, pharmacological therapy is initiated. Several guidelines including the American Diabetes Association (ADA) and American College of Obstetrics and Gynaecology (ACOG) recommend insulin as the preferred treatment for GDM as it allows tight glucose control and does not cross the placenta to a measurable extent (American College of Obstetrics and Gynaecology, 2018; American Diabetes Association, 2019). Generally, oral agents are preferred to insulin in low- and middle-income settings due to its ease of administration and lower cost (Kelley, Carroll & Meyer, 2015). Recently, metformin and glyburide have been shown to adequately reduce hyperglycaemia during pregnancy and improve perinatal outcomes (Faraci et al., 2011). In guidelines of the National Institute for Health and

Care Excellence (NICE), the International Federation of Gynaecology and Obstetrics (FIGO) and the Society of Maternal-Fetal Medicine (SMFM), metformin is considered as a reasonable and safe first line pharmacological therapy alternative to insulin (Coustan et al., in press; Walker, 2008; Hod et al., 2015), and is seen as a treatment choice where maternal weight gain is an issue (Bettencourt-Silva et al., 2019). Patient-specific factors contributes to variation and should therefore be considered when selecting treatment and management strategies for GDM.

7. RECENT ADVANCES IN BIOMARKER DISCOVERY

Pregnancy is associated with metabolic adaptations which are reflected by changes in the expression of maternal proteins. These proteins are detectable in the serum and plasma during early pregnancy and have attracted increasing interest as potential screening and diagnostic proteins for GDM. Several of these proteins including adiponectin, sex hormone-binding globulin (SHBG), C-reactive protein (CRP) and glycosylated fibronectin have been widely investigated as biomarkers of GDM. Accordingly, Lian et al. showed that is it possible to detect decreased serum adiponectin concentrations from as early as 9 weeks of gestation in women who developed GDM (Lain et al., 2008). Similarly, Nanda et al. reported lower serum adiponectin and SHBG levels at 11 - 13 weeks gestation in women with GDM compared to controls. (Nanda et al., 2011). In a study investigating predictive tools to identify GDM risk in obese pregnant women, White et al. showed that using a combination of statistical modelling consisting of clinical variables and potential biomarkers such as adiponectin, they were able to identify a group of high-risk obese women of whom approximately 50% later developed GDM (White et al., 2016). In addition, Rasanen et al. reported that first-trimester serum concentrations of glycosylated fibronectin, adiponectin and high-sensitivity CRP were significantly associated with GDM. The authors showed that after adjusting for maternal factors

and other biomarkers, glycosylated fibronectin demonstrated an independent association with GDM with a positive predictive value of 63%. These studies suggest that maternal serum and plasma proteins together with clinical variables may have the potential as risk stratification tools to predict GDM in high risk women (Rasanen et al., 2013).

8. EPIGENETICS

In recent years, altered epigenetic mechanisms have been postulated to underlie the development of many diseases, including GDM. Epigenetics is the study of heritable changes in gene expression or phenotype that occurs without changes in the underlying DNA sequence (Bird, 2007). Each cell has a unique epigenetic signature which controls normal growth and development, eventually resulting in the phenotype of the cell and tissue. Genetics and environmental factors such as unhealthy diets and lack of physical activity induce epigenetic modifications that affect these biological systems (Mathers, Strathdee & Relton, 2010), making them important pathogenic mechanisms in complex multifactorial diseases. Due to their reversible nature, epigenetic changes may provide a window of opportunity for intervention strategies to prevent or reverse GDM and improve health outcomes. In addition, accumulating evidence suggest that intrauterine environmental exposure leads to persistent epigenetic modifications in developmentally important genes, that predisposes offspring to adverse health outcomes (Gabory, Attig & Junien, 2011). A number of studies focusing on the developmental origin of health and disease and metabolic programming have identified a link between environmental influences and epigenetic mechanisms such as DNA methylation, histone modifications and non-coding RNA regulation (miRNA) (Godfrey, Costello & Lillycrop, 2016), linking these epigenetic changes to the future health of offspring. For example, Godfrey et al. showed that epigenetic promoter methylation of retinoid X receptor-a (RXRA) at birth,

which correlated to maternal carbohydrate intake during early pregnancy, was associated with childhood adiposity at 9 years of age (Godfrey et al., 2011). Similarly, Ruchat et al. (2013) reported that GDM alters DNA methylation of genes involved in metabolic disease and growth in offspring, partly explaining the association between GDM and macrosomia (Ruchat et al., 2013). Moreover, other studies showed that altered miRNA expression was associated with pregnancy complications such as foetal macrosomia, preterm birth and small for gestational age (Li et al., 2015; Tsai et al., 2017), suggesting an important role of these epigenetic mechanisms as metabolic and developmental regulators during pregnancy.

9. DNA METHYLATION

DNA methylation is the most widely studied and best characterised epigenetic mechanism (Barres & Zierath, 2011) that predominantly, but not exclusively occur at the cytosine's within cytosine–phosphate–guanine (CpG) dinucleotides. DNA methylation is a biochemical process catalysed by the enzyme DNA methyltransferase (DNMT) that covalently adds a methyl group to the fifth carbon position of a cytosine residue within CpG dinucleotides, with S-adenosyl-methionine (SAM) serving as the methyl donor (Bird, 1980, 2002). SAM is converted to S-adenosyl-homocysteine (SAH) during this process. Methylation of CpG islands, which are regions with high levels of CpG dinucleotides primarily in the promoter regions of genes, is generally associated with transcriptional repression due to altered protein binding to target sites on DNA (Bird, 1980, 2002) (Figure 1.2). Approximately 55–90% of all CpG dinucleotides within CpG islands are methylated, constituting about 3% of the genome. DNA methylation is essential for normal developmental processes (Jin, Li & Robertson, 2011), and also plays an important role in regulating genes key in metabolic pathways such as glucose and lipid homeostasis, insulin signalling and β-cell function and, when dysregulated, contributes to metabolic disease (Fradin et al.,

2012; Gilbert & Liu, 2012; He, Zhang, et al., 2017). Aberrant global and gene-specific DNA methylation has been shown to be associated with metabolic conditions such as obesity (Van Dijk et al., 2015), T2D (Toperoff et al., 2012) and cardiovascular disease (Kim, Newton & Knopp, 2002). Evidence suggest that peripheral blood mirrors DNA methylation patterns in tissue, supporting its potential as biomarkers of various metabolic disease, including GDM (Li et al., 2012; Willmer et al., 2018). Several studies have demonstrated that DNA methylation is altered in the placenta and cord blood of women with GDM compared to women with normoglycemic pregnancies (Nomura et al., 2014; Finer et al., 2015; Haertle et al., 2017), thus increasing interest in screening maternal blood for biomarkers of GDM.

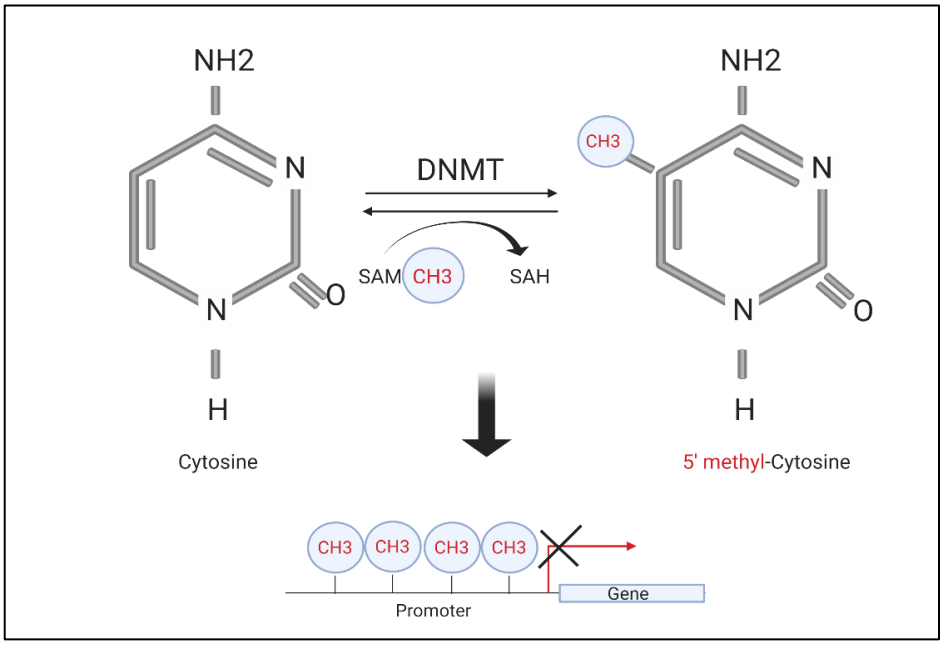

Figure 1.2. Characteristics of DNA methylation. The cytosine dinucleotide is converted to 5′ methyl-cytosine by the action of DNMT. Increased methylation in the promoter region is associated with gene silencing. SAM: S-adenosyl-methionine; SAH: S-adenosyl-homocysteine; DNMT: DNA methyltransferase; CH3: Methyl group

10. GENETIC VARIANTS

Single-nucleotide polymorphisms (SNPs) are the most common type of genetic variations among humans and refer to alterations of individual nucleotide bases (e.g. thymine to cytosine (T>C)) in a DNA sequence (Figure 1.3). SNPs occur roughly in every one site per 300 nucleotides, which translates to over 10 million SNPs in the human genome (Consortium, 2007). While most SNPs are silent and do not alter the function and expression of genes, others are biologically functional (Sachidanandam et al., 2001), and can lead to altered protein function and disease. In most common diseases, there are multiple genetic and environmental components which contribute to disease susceptibility (Hirschhorn et al., 2002). Thus, the identification of SNPs that are associated with the risk of disease within a population could partly explain the underlying biological causal mechanism. Several candidate gene and genome-wide association studies (GWAS) provide evidence that SNPs are associated with an increased risk of metabolic conditions including obesity, T2D, and cardiovascular disease (McCarthy, 2010; De Rosa et al., 2018). Variants in more than 50 and 80 loci were found to be associated with obesity (Rankinen et al., 2006) and T2D (Morris et al., 2012), respectively, and occur in genes that regulate glucose homeostasis and insulin signalling. Genetic alterations in genes responsible for metabolic changes during pregnancy predispose one to GDM. Several studies have highlighted genetic variants in common genes such as melatonin receptor 1B *(MTNR1B)* (Li et al., 2019), transcription factor 7 Like 2 *(TCF7L2)* (Franzago et al., 2018), receptor substrate 1 *(IRS1)* (Popova et al., 2017), adiponectin (*ADIPOQ*) (Takhshid, Haem & Aboualizadeh, 2015; Pawlik et al., 2017) and glucokinase (*GCK*) (Han et al., 2015) among others (Ao et al., 2015; Fatima et al., 2016; Jamalpour et al., 2018) to be associated with GDM risk, suggesting that SNPs may have potential to detect genetic susceptibility to GDM and may be useful as biomarkers.

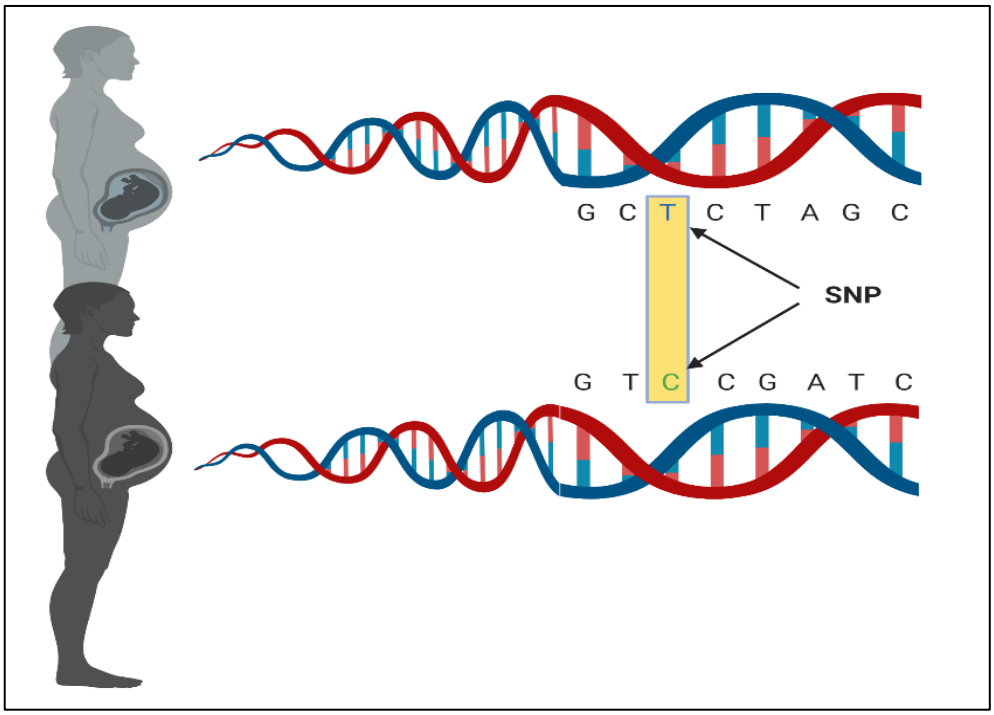

Figure 1.3. SNP characteristics. Example of a single nucleotide change from thymine to cytosine (T>C) in the DNA sequence. SNP: single nucleotide polymorphism

11. CURRENT STATUS OF BIOMARKERS

Proteins such as adiponectin, SHBG, CRP and glycosylated fibronectin, and DNA methylation and SNPs have attracted considerable interest as biomarkers of GDM. Despite showing potential, they are yet to become clinically applicable. Conducting more studies in different populations to identify optimal cut-offs could aid their predictive value, while rapid technological advances could overcome challenges associated with high costs and availability of resources. Vigorous research in this research field could lead to the development of a quick, cost effective and robust point-of-care test that could accurately identify women at high risk for GDM.

CHAPTER 2

STUDY DESIGN AND METHODOLOGY

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1. STUDY DESIGN

1.1.Participant Recruitment

This study forms part of a larger prospective cohort observational study on GDM screening, conducted at the Eyethu Yarona clinic (Lion Park Clinic) in Johannesburg, SA (Adam & Rheeder, 2017). The procedure for participant selection is illustrated in Figure 2.1. One thousand pregnant Black African women who were less than 26 weeks pregnant were enrolled at their first clinic visit and excluded if they had a multiple pregnancy, pre-existing or overt diabetes mellitus (type 1 (T1D) or type 2 (T2D) diabetes) or were acutely ill. Written informed consent was obtained from all participants. Gestational age was calculated using the last menstrual cycle reported. At recruitment, random glucose and glycated haemoglobin (HbA1c) levels were measured using a glucometer (Roche Diagnostics, Mannheim, Germany) and the point-of-care Afinion system (Alere Technologies, Oslo, Norway), respectively. Women with random glucose or HbA1c concentrations more than 11.1 mmol/L or 6.5%, respectively, were referred for glucose management and were excluded from further analysis. Of the 1000 pregnant women recruited, 82 (8.2%) had foetal losses, 163 (16.3%) moved away from the area, 194 (19.4%) were unreachable and 7 (0.7%) withdrew consent and did not continue with the study.

Five hundred and fifty-four women returned within two weeks in a fasted state for GDM testing and blood collection. GDM was diagnosed using the 75 g 2 hr OGTT at 24-28 weeks gestation if at least one glucose value was met (fasting plasma glucose ≥5.1 mmol/L, 1 hr OGTT ≥10 mmol/L or 2 hr OGTT ≥8.5 mmol/L), according to the International Diabetes and Pregnancy Study Group (IADPSG) criteria (IADPSG panel, 2010), and managed according to the International Federation of Gynaecology and Obstetrics (FIGO) recommendations with diet and lifestyle modifications, metformin

or insulin (Hod et al., 2015). Blood was collected for measuring fasting glucose concentrations (Vermaak and Partners, SA), and serum for insulin and C-reactive protein (CRP) concentrations (Pathcare laboratories, South Africa) and adiponectin concentrations using the human adiponectin enzyme-linked immunosorbent assay (ELISA) (Merck, Dermstadt, Germany). CRP is an acute-phase protein that is frequently used as marker of low-grade systemic inflammation, and elevated levels during pregnancy may be associated with an increased risk of GDM, while adiponectin, an adipose tissue-derived cytokine with insulin sensitizing properties have been shown to decrease in women with GDM compared to normoglycemic women, and may have potential as biomarkers of GDM. Moreover, human immunodeficiency virus (HIV) testing was offered to all pregnant women using rapid HIV kits, and results were confirmed with a different kit according to the guidelines of the SA Department of Health (National Department of Health South Africa, 2015). HIV positive women were treated with Atripla™; a fixed-dose coformulation of three anti-HIV drugs, efavirenz, emtricitabine and tenofovir given once-daily (National Department of Health South Africa, 2015). Anthropometric measurements were obtained according to standard procedures and demographic and socio-economic data were collected in the form of a standardised questionnaire. Whole blood was stored at -80 °C for DNA methylation profiling and SNP genotyping. For the studies presented in this thesis, 449 eligible women aged between 18 - 40 years, who had all data available and adequate blood samples for analysis were included.

1.2. Global DNA Methylation – Chapter 6

To assess the association between global DNA methylation and GDM, HIV negative women with (n=63) and without (n=138) GDM were selected and matched according to age, body mass index (BMI) and gestational age as far as possible.

1.3. Genome-Wide DNA Methylation – Chapter 7

To conduct genome-wide DNA methylation analysis, a subset of HIV negative women with (n=12) and without (n=12) GDM were selected and matched individually according to age, BMI and gestational age.

1.4. Gene-Specific DNA Methylation of The ADIPOQ Gene – Chapter 8

To explore the association between gene-specific methylation of the *ADIPOQ* gene and GDM 95 women with (n=95) and without (n=191) GDM were selected, of whom 181 were HIV negative (GDM: n=63 and non-GDM: n=118) and 105 were HIV positive (GDM: n=32 and non-GDM: n=73). One hundred and sixty-three samples were excluded due to insufficient serum samples for adiponectin measurement.

1.5. SNP Genotyping – Chapter 9

To investigate the association between genetic variants, *ADIPOQ* (rs266729 and rs17300539) and *MTHFR* (rs1801133), and GDM, a total of 118 women with (n=118) and without (n=331) GDM were selected.

*Detailed descriptions of the methodologies are outlined in the respective chapters.

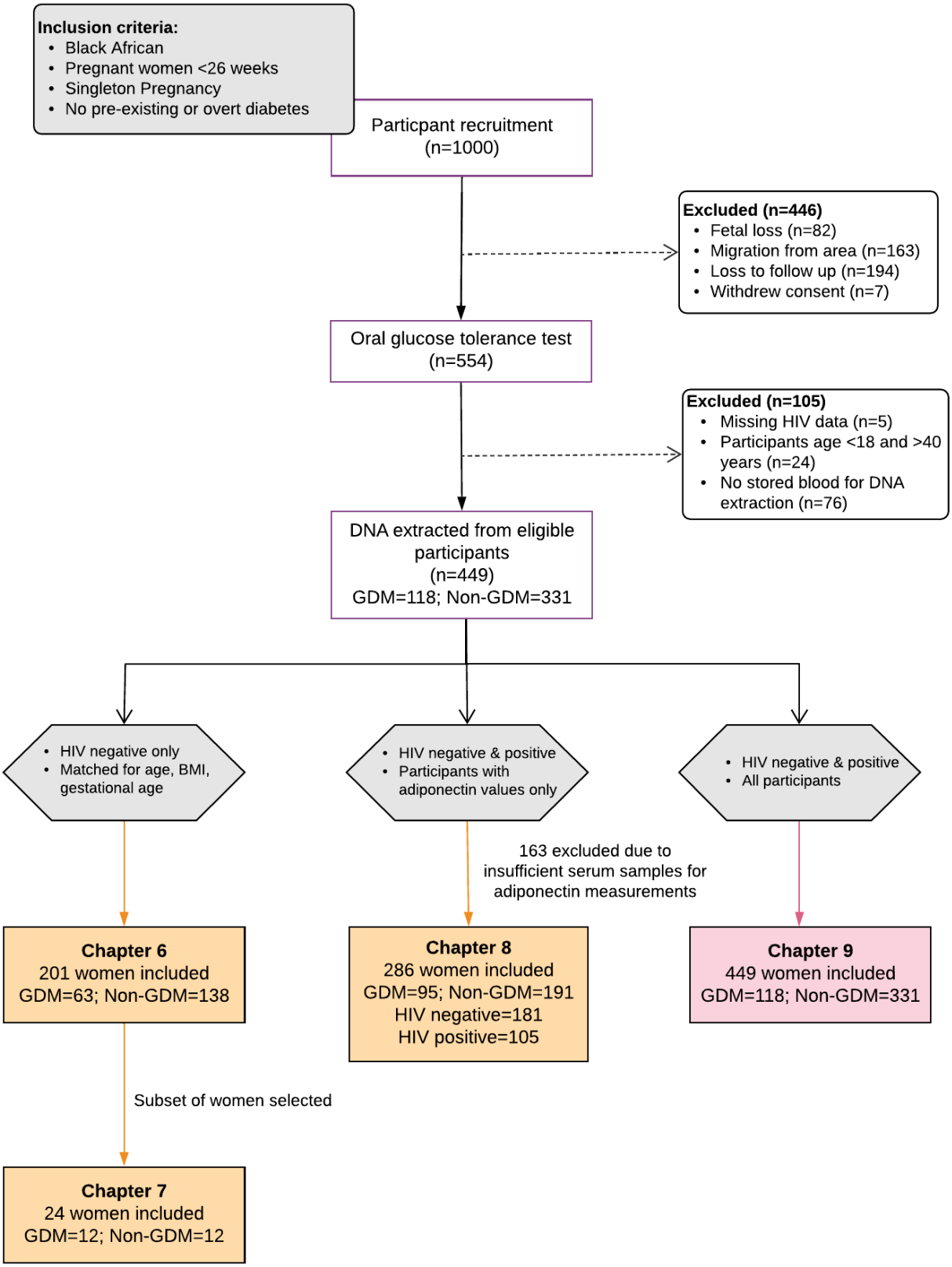

Figure 2.1. Flow diagram illustrating participant selection

CHAPTER 3

PREVALENCE OF AND RISK FACTORS FOR GESTATIONAL DIABETES MELLITUS IN SOUTH AFRICA

Adapted from:

Dias, S., Adam, S., Rheeder, P. & Pheiffer, C. 2019. Prevalence of and risk factors for gestational diabetes mellitus in South Africa. South African Medical Journal. 109(7):463-467–467. DOI: 10.7196/SAMJ.2019.v109i7.14127. *(Review article)*

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1. ABSTRACT

Gestational diabetes mellitus (GDM) is associated with adverse maternal, foetal and perinatal complications. Without appropriate glucose management, women with GDM and their offspring have an increased risk of developing type 2 diabetes (T2D) and other metabolic conditions later in life, thereby, adding to the growing burden of non-communicable diseases (NCDs). This review provides an update of GDM in South Africa (SA), showing that its prevalence is increasing, and highlights treatment and management strategies currently employed. Although the increase in GDM prevalence may partly be due to less stringent diagnostic criteria, the role of the increasing obesogenic environment in SA is an additional factor. Future research should focus on reducing the rising obesity epidemic and in so doing aim to prevent the development of GDM in SA. Such initiatives will have a positive impact on decreasing maternal and child morbidity and mortality and the future burden of NCDs.

2. INTRODUCTION

GDM, defined as glucose intolerance that is first diagnosed during the latter half of pregnancy, with return to normoglycemia after birth (WHO, 2013), is one of the leading causes of mortality and morbidity for both mothers and infants worldwide. Globally, approximately 16.2% (21.3 million) of live births are associated with hyperglycaemia in pregnancy, of which 86.4% are due to GDM, 6.2% due to pre-existing type 1 (T1D) or type 2 diabetes (T2D), and 7.4% due to T1D and T2D first detected in pregnancy (International Diabetes Federation, 2017). The prevalence of GDM is rapidly increasing worldwide, possibly due to advanced child-bearing age and obesity. This is particularly concerning, since South African women are considered amongst the most overweight women globally (Statistics South Africa, 2017). GDM is associated with maternal (preeclampsia, increase in caesarean deliveries, birth trauma), foetal (macrosomia, hypoglycaemia, shoulder dystocia), and perinatal (respiratory distress syndrome, jaundice, metabolic derangements) complications, while both mothers and their offspring are at risk for developing obesity, T2D and other metabolic conditions in later life. GDM thus poses a threat to maternal and child well-being, while its impact on the burden of non-communicable diseases (NCDs) is undeniable. This review provides an update of GDM in SA, focusing on risk factors and prevalence. Furthermore, we highlight recommendations for future research.

3. RISK FACTORS FOR GESTATIONAL DIABETES MELLITUS

The exact mechanism underlying GDM is not yet fully elucidated, although it is speculated that women who develop GDM may have underlying insulin resistance and genetic susceptibility (Perkins, Dunn & Jagasia, 2007). GDM is reported to affect about 14% of pregnancies globally, however, rates between 1-28% are reported in

different regions varying according to ethnicity (Hedderson, Darbinian & Ferrara, 2010), geographical location (urban vs. rural) (Mwanri et al., 2014), environmental factors and screening and diagnostic strategies employed (Pastakia et al., 2017). For example, in the USA, the prevalence of GDM was reported to be 11.1% in Asian Indians and 4.1% in non-Hispanic whites (Hedderson, Darbinian & Ferrara, 2010), while in Tanzania, the prevalence of GDM was 1% and 8.4% in a rural and urban setting, respectively (Mwanri et al., 2014). A systematic review conducted in six African countries showed that the prevalence of GDM ranged between 0% in Tanzania to 13.9% in Nigeria, although these studies varied with regard to their study design (Macaulay, Dunger & Norris, 2014). The prevalence of GDM in SA is estimated to vary between 1.6 – 25.8% based on the screening and diagnostic strategies employed (Ranchod, Vaughan & Jarvis, 1991; Adam & Rheeder, 2017).

Other definable risk factors that affect GDM prevalence include, repeated glucosuria, previous GDM, family history of diabetes, history of stillbirth and congenital abnormalities, suspicion of polyhydramnios in pregnancy, obesity, previous macrosomic infant, history of polycystic ovarian syndrome and perinatal death, and high risk ethnic groups (Table 3.1) (SEMDSA, 2017). Alarmingly, the rate of obesity is increasing dramatically, with SA now regarded as one of the world's most obese nations (Sartorius et al., 2015). According to recent statistics, approximately 69% of South African women are overweight or obese (Statistics South Africa, 2017). The increasing risk of obesity is largely due to changes in lifestyle, which have contributed to the increasing prevalence of T2D and metabolic syndrome, particularly among females (Sartorius et al., 2015). Although universal screening, whereby all pregnant women undergo the diagnostic 75 g oral glucose tolerance test (OGTT) at 24-28 weeks of gestation, has been widely advocated as the recommended screening strategy for GDM (IADPSG panel, 2010), at present, selective screening based on risk factors is predominantly utilised in SA, as it is considered less costly than subjecting all women

to laboratory testing. However, risk factor-based selective screening has been found to have poor sensitivity and specificity in many studies (O'Sullivan et al., 1973; Cosson et al., 2014), including our study (Adam & Rheeder, 2017), and performs poorly as a screening tool for GDM. Moreover, selective screening places a high demand on healthcare workers to identify patients who should be screened and is often poorly adhered to, leading to inadequate screening and testing of GDM.

Table 3.1. Risk factors for GDM

• Recurrent glycosuria
• Obesity (BMI ≥ 30 kg/m^2)*
• Family history of diabetes mellitus
• History of GDM
• History of polycystic ovarian syndrome
• High risk ethnic groups (eg. South Asian descent)
• Previous adverse pregnancy outcomes (stillbirth, congenital abnormalities, polyhydramnios, macrosomia etc.)

BMI: Body mass index, GDM: Gestational diabetes mellitus. *BMI cut-offs vary according to ethnicity (SEMDSA, 2017)

4. HIV AND GESTATIONAL DIABETES MELLITUS

SA has the highest burden of the Human Immunodeficiency virus (HIV) globally, with 7.1 million people living with the virus (UNAIDS, 2018) and ~19% of adults (15 – 49 years of age) affected by the disease (Shisana et al., 2014). HIV infection and antiretroviral therapy dysregulates glucose metabolism and is associated with an increased risk of glucose intolerance and metabolic disease (Dave et al., 2011, 2016; Noubissi, Katte & Sobngwi, 2018). There is evidence that antiretroviral therapy, particularly the first generation protease inhibitors induces insulin resistance in both

pregnant and non-pregnant women (Jao et al., 2013). The risk of developing GDM is shown to be more common in HIV-infected women receiving combined antiretroviral therapy, especially with the use of protease inhibitors (Martí et al., 2007; Soepnel et al., 2017). Although, protease inhibitors have been replaced by non-nucleoside reverse transcription inhibitors, which are considered safer, they too affect glucose homeostasis (Brown et al., 2005), thus require further investigation. Current data in SA show no association between HIV infection and antiretroviral therapy with GDM (Adam & Rheeder, 2017).

5. COMPLICATIONS OF GESTATIONAL DIABETES MELLITUS

GDM is associated with adverse short- and long-term pregnancy outcomes in both mothers and their offspring (Table 3.2). A study conducted on 57 629 GDM women reported that the prevalence of caesarean delivery, macrosomia, pre-term birth and pre-eclampsia was 27.8%, 15.7%, 8.4% and 2.6%, respectively (Billionnet et al., 2017). Foetal macrosomia is a common adverse obstetric outcome, affecting ~15-45% of neonates born to women with GDM, and is associated with an increased risk of caesarean delivery, shoulder dystocia, respiratory distress syndrome and neonatal hypoglycaemia (Kc, Shakya & Zhang, 2015; Ovesen et al., 2015). Furthermore, ~50% of women with GDM will develop T2D within 10 years (Kim, Newton & Knopp, 2002), while increasing evidence show that women with GDM have a 56% higher risk of developing cardiovascular disease (Kramer, Campbell & Retnakaran, 2019), and an increasing risk of metabolic syndrome in later life (De Souza, Ray & Retnakaran, 2011). GDM is associated with an abnormal intrauterine environment that negatively affects the long-term health of offspring, possibly through epigenetic changes. It is estimated that children born to mothers with GDM have an 8-fold increased risk of developing T2D and prediabetes compared to children born to mothers with normoglycemic pregnancies (Clausen et al., 2008). Treatment of GDM improves outcome, thus the

detection and appropriate management of GDM is critical to prevent pregnancy complications. However, a challenge which hampers the early detection and management of GDM is the current lack of global consensus on GDM screening and diagnosis, which varies between countries and institutions. Recently, a study conducted by Meek et al. found that women who were diagnosed with GDM according to the stringent International Association of Diabetes in Pregnancy Study Group (IADPSG) criteria, but were missed when using the National Institute for Health and Care Excellence (NICE) criteria, had a substantial risk of obstetric complications such as macrosomia, caesarean section and polyhydramnios (Meek et al., 2015).

Table 3.2. Adverse outcomes associated with GDM

	Mother	Offspring
Short-term	Spontaneous miscarriage Pre-eclampsia/ Pregnancy-induced hypertension Infections (e.g. urinary tract, puerperal sepsis) Caesarean delivery Preterm delivery Polyhydramnios Post-partum haemorrhage	Macrosomia Shoulder dystocia – birth trauma Premature birth – metabolic complications (hypoglycaemia, hypocalcaemia) Polycythaemia/Jaundice Respiratory distress syndrome Cardiomyopathy/Arrhythmias Stillbirth
Long-term	Weight gain/obesity GDM in subsequent pregnancies Type 2 diabetes Cardiovascular disease Metabolic syndrome	Obesity Type 1 diabetes Type 2 diabetes Metabolic syndrome

6. PREVALENCE OF GESTATIONAL DIABETES MELLITUS IN SOUTH AFRICA

SA is undergoing an epidemiological transition characterised by an increase in NCDs due to urbanisation, nutritional transition towards a diet consisting of high fat and refined sugars and sociocultural factors involving the perception of overweight as a measure of success and beauty. Approximately 69% of South African women are overweight, 40% are obese and 20% are morbidly obese (Ng et al., 2014; Statistics South Africa, 2017), and are considered amongst the most overweight women globally, particularly women of reproductive age. Accordingly, GDM has rapidly increased in SA, with prevalence ranging from 1.6% to 25.8% between 1969 to 2018 (Table 3.3). The first GDM prevalence study was conducted in 1969 amongst Indian women from Kwazulu-Natal using the 100 g OGTT and a prevalence of 23.8% and 8.3% was reported in women with and without risk factors, respectively (Notelovitz, 1969). In 1979, using risk factor screening and the 50 g OGTT, a GDM prevalence of 3% was reported in women of mixed ethnic ancestry in the Western Cape (Jackson & Coetzee, 1979). Using the World Health Organisation (WHO) 1985 diagnostic criteria, Ranchod et al. reported a GDM prevalence of 1.6% and 3.8% in women of Indian and mixed ethnic ancestry, respectively in Kwazulu-Natal (Ranchod, Vaughan & Jarvis, 1991). A population-based study conducted in rural Limpopo, reported a GDM prevalence of 8.8% in black women using the WHO 1999 diagnostic criteria (Mamabolo et al., 2007), while a study of overweight and obese pregnant women of mixed ethnic ancestry in Johannesburg, Gauteng, reported a GDM prevalence of 1.8% using random and fasting glucose concentrations (Basu, Jeketera & Basu, 2010). More recently, using the IADPSG criteria, a high prevalence of GDM (25.8%) was reported in black women from Johannesburg (Adam & Rheeder, 2017). The largest GDM prevalence study conducted in SA to date, found a GDM prevalence of 9.1% in black

women from Johannesburg using the revised WHO 2013 diagnostic criteria, which uses the same diagnostic cut-off criteria as the IADPSG criteria (Macaulay et al., 2018). The significantly higher prevalence observed by Adam et al. compared could be due to an over estimation of GDM prevalence as some women in the GDM group may have had pre-existing or overt diabetes.

Disparities in the prevalence of GDM was observed between these studies, which may be due to the different screening and diagnostic criteria used. More recent studies using the less stringent IADPSG or WHO 2013 criteria (Adam & Rheeder, 2017; Macaulay et al., 2018) show an increased prevalence of GDM compared to older criteria, which used higher glucose thresholds (Notelovitz, 1969; Jackson & Coetzee, 1979; Ranchod, Vaughan & Jarvis, 1991; Mamabolo et al., 2007; Basu, Jeketera & Basu, 2010). Thus, although the increased prevalence of GDM may partly be due to diagnostic criteria, the role of the increasing obesogenic environment in SA should not be underestimated (Statistics South Africa, 2017). Other factors, such as geographical setting, maternal age, study size, population and environmental differences could also account for the disparities in prevalence across studies (Adam & Rheeder, 2017; Macaulay et al., 2018).

Table 3.3. GDM prevalence studies conducted in South Africa

Author	Ethnicity	Sample size	Setting	Gestational age	Screening criteria	Diagnostic criteria	Threshold cut-off	Prevalence (95%, CI)
(Notelovitz, 1969)	Indian	568	King Edward VIII hospital, Durban	All trimesters	Risk factors	Institutional protocol 100 g OGTT	‡Venous blood ϕNormal = mean ± 2 SD GDM > mean ± 2 SD	Without risk factors 8.3% (5.4-12.0) With risk factors 23.8% (18.8-29.4)
(Jackson & Coetzee, 1979)	Mixed	558	Groote Schuur Hospital, Cape Town	All trimesters	Risk factors	Institutional protocol 50 g OGTT	‡Capillary blood Fasting ≥5.5 mmol 1 h glucose ≥10 mmol/L 2 h ≥6.7 mmol/L	3.0% (1.8-4.7)
(Ranchod, Vaughan & Jarvis, 1991)	Indian & Coloured	1717	Northdale hospital, Pietermaritzburg	All trimesters	75 g GCT	WHO 1985 DPSG EASD 75 g OGTT	Venous blood Fasting ≥7.8 mmol/L & 2 hr glucose ≥11.1 mmol/L Venous blood Fasting ≥5.2 mmol/L & 2 hr glucose ≥9 mmol/L	WHO: 3.8% (2.9-4.8) DPSG EASD 1.6% (1.0-2.2)

(Mamabolo et al., 2007)	Black	262	Rural Limpopo	28-36 weeks	No screening	WHO 1999 75 g OGTT	Venous blood IGT: Fasting <7 mmol/L & 2 hr glucose ≥7.8 mmol/L Diabetes: Fasting ≥7 mmol/L & 2 hr glucose 11.1 mmol/L	8.8% (6.0-12.9)
(Basu, Jeketera & Basu, 2010)	Mixed	767	Charlotte Maxeke Johannesburg Academic hospital, Johannesburg	23-32 weeks	Risk factors	Institutional protocol	Fasting ≥8 mmol/L or Random glucose ≥11 mmol/L	1.8% (1.0-2.9)
(Adam & Rheeder, 2017)	Black	554	Level 1 clinic, Johannesburg	24-28 weeks	Risk factor or no screening	IADPSG 75 g OGTT NICE 75 OGTT	Fasting ≥5.1 mmol/L 1 hr ≥10 mmol/L 2 hr ≥8.5 mmol/L Fasting ≥5.6 mmol/L 2 hr ≥7.8 mmol/L	Universal testing 25.8% (22.2-29.7) Selective testing 15.2% (12.3-18.4) Universal testing 16.9% (13.9-20.4) Selective testing 9.9% (7.6-12.7)

						WHO 1999	Venous blood IGT: Fasting <7 mmol/L & 2 hr glucose ≥7.8 mmol/L Diabetes: Fasting ≥7 mmol/L & 2 hr glucose 11.1 mmol/L	Universal testing 7.2% (5.2-9.7) Selective testing 3.6% (2.2-5.5)
(Macaulay et al., 2018)	Black	1906	Chris Hani Baragwanath Hospital, Soweto, Johannesburg	24-28 weeks	No screening	WHO 2013 75 g OGTT	Fasting ≥5.1 mmol/L 1 hr ≥10 mmol/L 2 hr ≥8.5 mmol/L	9.1% (7.9-10.5)

DPSG, Diabetes in Pregnancy Study Group; EASD, European Association for the Study of Diabetes; IADPSG, International Association of Diabetes in Pregnancy Study Group; NICE, National Institute for Health and Care Excellence; OGTT, Oral glucose tolerance test; WHO, World Health Organisation. Selective testing refers to risk factor screening: advanced maternal age, obesity, family history of diabetes mellitus, glycosuria, certain ethnicities and prior adverse pregnancy outcomes. ϕNormal calculated as the mean ± 2 SD, and all values outside that range were considered GDM (technique described by Herbert and Bournes and King (Wootton, 1974). ‡Institutional protocol

7. TREATMENT

Management and treatment of GDM are important to reduce adverse pregnancy outcomes and improve maternal and neonatal health (HAPO Study Cooperative Research Group et al., 2008). In SA, pregnant women with GDM are referred to the nearest secondary or tertiary hospital for management and treatment (Muhwava et al., 2018). At the tertiary level, management of GDM is through counselling and health education provided by obstetricians, endocrinologists, dieticians or nurse educators. The primary form of intervention for women with GDM is lifestyle modification, which involves counselling regarding diet and physical activity. A dietary meal plan is provided, which entails reduced sugar and starch intake, increased protein intake and reduced total calorie intake (Muhwava et al., 2018). However, in disadvantaged settings, these lifestyle changes are difficult to adhere to owing to the high cost of healthy food and lack of understanding and social support. A more sustainable approach is to counsel patients on how to reduce the glycaemic index of staple foods. The glycaemic index of carbohydrate-rich foods (e.g. potatoes, pap, pasta, rice) can be reduced by cooking, cooling and reheating, or by adding fats or acids such as lemon juice or vinegar (Kinnear et al., 2011).

Pharmacological therapy is initiated for women who fail to reach their glucose targets using diet and exercise. In SA, metformin is the drug of choice to treat GDM, while glyburide is used when metformin and insulin are not available. Although insulin is invasive and used only if metformin fails, it is effective, allows tight glucose control and is considered safe, as it does not cross the placenta. Generally, oral agents are preferred to insulin because of ease of administration, although studies conducted in other settings showed no substantial differences in major outcomes when comparing their benefits and risks (Nicholson et al., 2009; Rowan et al., 2009).

8. CURRENT PERSPECTIVES AND FUTURE RECOMMENDATIONS

- The rate of obesity is rising dramatically, consequently increasing the prevalence of GDM globally and in SA
- Untreated GDM negatively affects maternal and child health
- GDM increases the risk of developing T2D and other non-communicable diseases later in life
- Future research should focus on reducing obesity and preventing the development of GDM

9. CONCLUSION

The prevalence of GDM is rapidly increasing in SA and is becoming a major public health concern. Without appropriate glucose management, GDM is associated with adverse pregnancy outcomes and an increased risk of future metabolic conditions in mothers and their offspring, further contributing to the growing burden of NCDs. Although the significant increase in the prevalence of GDM observed in recent SA studies may be attributed to the lower diagnostic thresholds used, the role of the increasing obesogenic environment should not be underestimated. Future research should focus on reducing the rising obesity epidemic and in so doing aim to prevent the development of GDM. Such initiatives will have a positive impact on decreasing maternal and child morbidity and mortality and the future burden of NCDs.

CHAPTER 4

SCREENING AND DIAGNOSIS OF GESTATIONAL DIABETES MELLITUS IN SOUTH AFRICA: WHAT WE KNOW SO FAR

Adapted from:

Dias, S., Pheiffer, C., Rheeder, P. & Adam, S. 2019. Screening and diagnosis of gestational diabetes mellitus in South Africa: What we know so far. South African Medical Journal. 109(7):457-462–462. DOI: 10.7196/SAMJ.2019.v109i7.14064. *(Review article)*

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1. ABSTRACT

The early detection and management of gestational diabetes mellitus (GDM) present an ideal opportunity to decrease perinatal complications and adverse long-term health outcomes in mothers and their offspring. This review describes the major GDM screening and diagnostic strategies used worldwide, including novel screening and diagnostic methods that are being explored. It highlights the varied screening and diagnostic strategies currently employed in South Africa (SA). The lack of uniform GDM diagnostic criteria and variation in clinical practice hamper early detection and management of GDM, which negatively affects maternal and child health. We recommend that a SA diabetes-in-pregnancy study group, comprising interested obstetricians, physicians, endocrinologists, public health specialists, dieticians and scientists, be established to make evidence-based recommendations on affordable, accessible and applicable GDM screening and diagnostic and management strategies.

2. INTRODUCTION

GDM is defined as glucose intolerance that is first diagnosed during the second or third trimester of pregnancy, that is not pre-existing or overt diabetes (American Diabetes Association, 2016). The prevalence of GDM has significantly increased over the last 20 years (Ferrara, 2007), and in 2017 the International Diabetes Federation (IDF) estimated that approximately 14% of pregnancies are affected by GDM depending on diagnostic criteria used and the population studied (International Diabetes Federation, 2017). The World Health Organisation (WHO) classifies pre-existing diabetes or newly diagnosed type 1 (T1D) or type 2 (T2D) diabetes as severe hyperglycaemia during pregnancy, while GDM represents a milder form of hyperglycaemia that occurs in the latter half of pregnancy and usually resolves after delivery (WHO, 2013). Without appropriate glucose management, GDM is associated with perinatal complications and an increased risk for future metabolic disease in both mothers and their offspring.

The early detection and treatment of GDM are effective in preventing these adverse outcomes; therefore, universal screening and diagnosis of GDM are widely advocated as a strategy to promote appropriate treatment and improve pregnancy outcomes. The oral glucose tolerance test (OGTT) conducted at 24 - 28 weeks of gestation is currently considered the gold standard for the diagnosis of GDM (IADPSG panel, 2010; WHO, 2013). However, the test is cumbersome to conduct, as well as time-consuming, expensive and unfeasible in many low- and middle-income countries, resulting in many countries using risk factor-based selective screening. The lack of uniformity in GDM diagnosis and variations in clinical practice hamper its early detection and management, which negatively affects maternal and child health. Therefore, the identification of simple, cost-effective, sensitive and specific biomarkers, which do not

require fasting and multiple sampling, may offer potential as screening and diagnostic tools and have become a major focus in GDM research. This review describes the major GDM screening and diagnostic strategies used worldwide, including novel screening and diagnostic methods that are being explored. It highlights the varied screening and diagnostic strategies currently employed in SA. We also discuss challenges associated with these strategies and offer recommendations for future research.

3. SCREENING TESTS

The terms screening and diagnosis are often confusingly used interchangeably (Sacks, 2014). Screening tests identify asymptomatic GDM in apparently healthy pregnant women, facilitating diagnosis and management (Hartling et al., 2012). A negative screening test will obviate the need for the cumbersome OGTT, the gold standard for GDM diagnosis, which is costly and is associated with multiple sampling, nausea and vomiting. Currently screening for GDM is done by using traditional risk factors (Adam & Rheeder, 2017), the 50 g glucose challenge test (GCT) or an OGTT (Sacks, 2014). A number of other novel screening tests are being explored including fasting plasma glucose (Agarwal, 2016), glycated haemoglobin (HbA1c) (Khalafallah et al., 2016), cytokines (Abell et al., 2015) and molecular biomarkers (Dias et al., 2018), which will be discussed in more detail below. Screening for traditional risk factors remain the cornerstone of screening strategies in low-and middle-income countries due to costs and ease. However, several studies (Miailhe et al., 2015; Agbozo et al., 2018; Matta-Coelho et al., 2018) including ours (Adam & Rheeder, 2017) have reported that risk factors have poor predictive value and fail to identify a large percentage of women with GDM, limiting their use. Adam et al. reported that risk factors failed to identify ~10.6% of pregnant women with GDM in SA (Adam & Rheeder, 2017). The GCT is

commonly used to screen for GDM in the USA and involves administering a 50 g glucose load to pregnant women at 24 - 28 weeks of gestation, irrespective of fasting. If their 1 h plasma glucose concentrations exceed predetermined cut-off values, usually 7.2 mmol/L or 7.8 mmol/L, they are referred for GDM diagnosis. In 2010, the International Association of Diabetes in Pregnancy Study Groups (IADPSG) advocated for 'no screening' or 'universal testing', where all pregnant women undergo the diagnostic 75 g OGTT at 24 - 28 weeks of gestation (IADPSG panel, 2010). In addition, the IADPSG decreased the threshold for diagnosing GDM (Table 1). These recommendations were based on findings from the Hyperglycaemia and Adverse Pregnancy Outcome (HAPO) study that showed a linear correlation between maternal blood glucose concentrations and adverse pregnancy outcomes, even at glucose concentrations previously considered normal (HAPO Study Cooperative Research Group et al., 2008). The HAPO study assessed glucose concentrations and pregnancy complications in 23 316 pregnant women across 9 countries; therefore, the IADPSG considered this evidence sufficient to alter the diagnostic criteria for GDM. A few years later, the WHO endorsed the IADPSG universal testing strategy, but remains sceptical owing to poor quality of evidence, increased costs and the possibility of overdiagnosis (Sacks et al., 2018). Globally, there is no accepted screening criteria for GDM, and universal testing for diagnosing GDM remains the recommended strategy, although its implementation varies across countries and institutions.

4. DIAGNOSIS

The OGTT is the gold standard for GDM diagnosis. However, its use is not standardised worldwide and varies according to availability and access of standardised laboratories, resources, cost and GDM risk. The main issues of contention are whether a one-step or two-step procedure, which includes prior

screening is used, glucose load (75 g or 100 g), duration of test (2 h or 3 h), glucose cut-off values, and whether diagnosis is based on one or two high glucose values (Sacks et al., 2018). GDM diagnosis has evolved considerably over the years with older criteria based mainly on managing long-term health outcomes, while the more recent criteria focus on adverse perinatal outcomes. The landmark screening and diagnostic criteria for GDM are shown in Figure 4.1. In 1964, O'Sullivan and Mahan (O'Sullivan JB & Mahan CM., 1964) proposed a two-step approach, which involved screening with the GCT, followed by a confirmatory 100 g 3 h OGTT in women who tested positive for screening. The National Diabetes Data Group (NDDG) (National Diabetes Data, 1979) and Carpenter and Coustan (Carpenter & Coustan, 1982) revised these criteria in 1979 and 1984, respectively, correcting for the higher glucose concentrations in plasma compared with venous blood that was originally used by O'Sullivan and Mahan (O'Sullivan JB & Mahan CM., 1964). In 1985, the WHO recommended that a 75 g 2 h OGTT be performed to diagnose GDM, using the same thresholds as those for diagnosing diabetes in non-pregnant women (WHO, 1985). In 1999, the WHO revised their diagnostic criteria for GDM to include impaired glucose tolerance (IGT) and diabetes (WHO, 1999). The American Diabetes Association (ADA) adopted the Carpenter and Coustan (Carpenter & Coustan, 1982) criteria and recommended testing for GDM at 24 - 28 weeks of gestation using either a one-step approach with the 100 g OGTT or a two-step procedure with the GCT, followed by a diagnostic 100 g OGTT (American Diabetes Association, 2004). In the final report of the Pan American Conference on Diabetes and Pregnancy, the Latin American Diabetes Association (LADA) criteria were proposed for the diagnosis of GDM in selected countries of South America, using a two-step approach with a 75 g 2 h OGTT (Final report of the Pan American Conference, 2015). In 2010, as previously described, the IADPSG proposed universal testing, where a one-step 75 g 2 h OGTT is conducted for all pregnant women at 24 - 28 weeks of gestation (IADPSG panel, 2010). In 2013, the

WHO revised their criteria and endorsed those of the IADPGS (WHO, 2013). The National Institute for Health and Care Excellence (NICE) criteria are based on the WHO 1999 criteria, where GDM is diagnosed as IGT using the 75 g 2 h OGTT (NICE guidelines, 2015). They have not adopted the new recommended IADPSG/WHO 2013 diagnostic criteria, as evidence suggests relatively small differences in clinical outcomes and increased cost implications (Jacklin et al., 2017). Currently, the International Federation of Gynaecology and Obstetrics (FIGO) guidelines (Hod et al., 2015) recommend the use of a glucometer for point-of-care diagnosis of GDM in limited-resource settings due to its low cost, ease of use and ability to diagnose and treat GDM at the earliest possible opportunity. However, a study investigating the performance of the glucometer for diagnosis of GDM compared to the gold standard laboratory test showed poor correlation and reproducibility when GDM was diagnosed using the FIGO criteria (Adam & Rheeder, 2018).

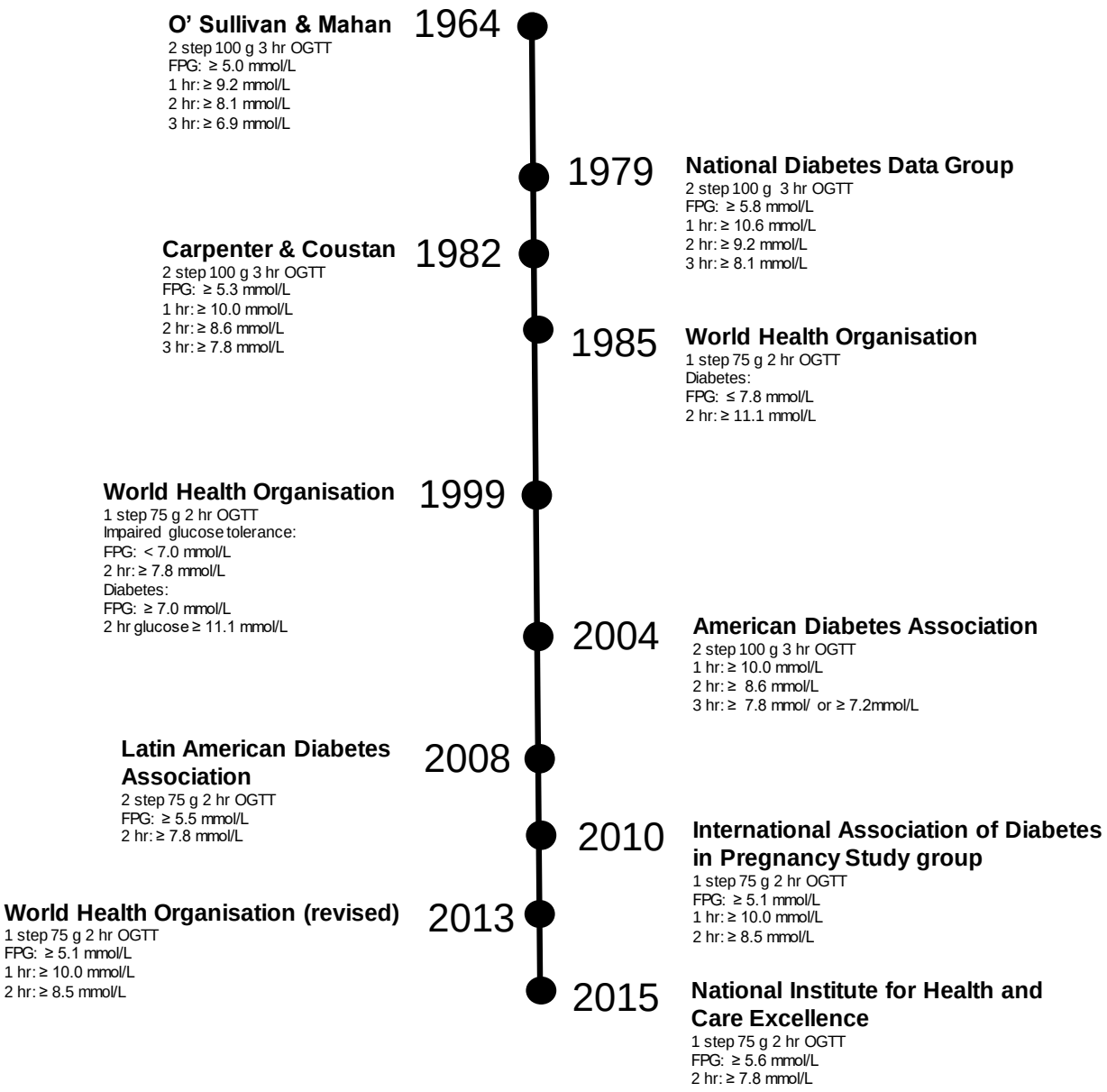

Figure 4.1. The evolution of GDM screening and diagnosis between 1964 and 2015. FPG: Fasting plasma glucose, OGTT: Oral glucose tolerance test

5. NOVEL SCREENING AND DIAGNOSTIC STRATEGIES

5.1. Glucose

The measurement of fasting glucose concentrations has shown promise as a screening (Agarwal, Punnose & Dhatt, 2004) and diagnostic test (Adam & Rheeder, 2017; Macaulay et al., 2018); however, the test still requires pregnant women to be in a fasted state and return to the clinic to obtain their laboratory results (Agarwal, 2016). Measurement of random glucose and HbA1c levels obviates the need for fasting and have been explored as alternative screening and diagnostic tests (Amreen et al., 2018). HbA1c, a measurement of the amount of glucose bound to haemoglobin, is currently the gold standard for long-term blood glucose monitoring. However, it is affected by factors such as ethnicity, anaemia, haemodilation or other blood disorders that hamper its accuracy as a diagnostic tool in both non-pregnancy and pregnancy (Hughes, Rowan & Florkowski, 2016; Amreen et al., 2018; Nguyen et al., 2019). While studies have reported various HbA1c cut-off values in different populations, the ideal cut-off values for the diagnosis of GDM have not been accurately defined and validated (Agarwal et al., 2001; Hird et al., 2016). Thus, although these tests do not require fasting and are convenient, fast, simple, inexpensive and can be done at point-of-care, the results are inconsistent, with low sensitivity and specificity, and has not been successful to date.

Other novel strategies investigated in the South African population include the 'breakfast test'– a non-standardised glucose load administered to pregnant women – instead of the OGTT (Marais et al., 2018). Because of the variability in carbohydrate content with a non-standardised glucose load (Marais et al., 2016), the breakfast test was revised to include a standardised carbohydrate content that is equivalent to the

75 g OGTT. Marais et al. (Marais et al., 2018) reported a correlation between blood glucose values obtained using the designed breakfast test and values obtained using the OGTT. These and other results suggest that a standardised breakfast test that is more palatable than the OGTT may offer an alternative method for assessing hyperglycaemia during pregnancy (Sutherland et al., 1989; Rey, 1997; Ramezani, 2008).

5.2. Serum Proteins

Adaptation to metabolic stress during pregnancy is reflected by changes in the expression of maternal proteins. These proteins are readily detected in plasma or serum and have recently attracted considerable interest as potential screening and diagnostic proteins for GDM. Several studies have reported on the potential of maternal plasma or serum biomarkers, such as adiponectin, sex hormone-binding globulin (SHBG), C-reactive protein (CRP) and glycosylated fibronectin, to serve as biomarkers for GDM (Smirnakis et al., in press; Nanda et al., 2011; Rasanen et al., 2013). Nanda et al. (Nanda et al., 2011) reported that maternal serum adiponectin and SHBG levels at 11 - 13 weeks of gestation were lower in women with GDM than in controls. Similarly, Smirnakis et al. (Smirnakis et al., in press) reported lower levels of serum SHBG and higher levels of CRP during the first and second trimesters in pregnant women who subsequently developed GDM. Furthermore, glycosylated fibronectin, adiponectin, CRP and human placental lactogen (hPL) concentrations at 5 - 13 weeks of gestation were shown to be associated with GDM (Rasanen et al., 2013). Together, these studies demonstrate that maternal proteins represent a promising first- and second-trimester screening test to identify women at risk of developing GDM. Further prospective studies are required to investigate the clinical applicability of these biomarkers.

5.3. Genetics

Variants in genes regulating glucose homeostasis are increasingly being implicated in the pathogenesis of GDM and thus present candidates for biomarkers of disease pathophysiology (Wu et al., 2016). To date, genetic studies have identified 8 genes commonly associated with the development of GDM in more than 2 independent populations. While genetic variants have been identified in other genes associated with GDM, these were demonstrated in single populations only (Dias et al., 2018). The genes identified in more than 2 independent populations include transcription factor 7-like 2 *(TCF7L2)*, adiponectin *(ADIPOQ)*, melatonin-receptor 1B gene *(MTNR1B)*, glucokinase *(GCK)*, glucokinase regulator *(GCKR)*, fat mass and obesity-associated *(FTO)*, insulin-receptor substrate 1 *(IRS1)* and potassium voltage-gated channel subfamily Q member 1 *(KCNQ)*. Due to variation across different populations, further studies are needed to confirm the association between risk alleles and GDM. Further analysis in diverse ethnic groups is required to examine whether these risk variants can be used as biomarkers to predict the development of GDM. Despite the association between genetics and GDM, the important role of the environment in GDM susceptibility is increasingly being recognised.

5.4. Epigenetics

Epigenetics is defined as changes in gene expression that occur without changes in the underlying DNA sequence (Christensen & Marsit, 2011). These changes reflect gene-environment interactions and are increasingly being implicated in the pathophysiology of metabolic diseases (Gu et al., 2013; Martín-Núñez et al., 2014). Epigenetic mechanisms include DNA methylation, chromatin and histone modifications, and non-coding RNAs such as microRNAs (miRNAs). DNA methylation is the most widely studied and best characterised epigenetic mechanism,

and refers to the addition of a methyl group to the fifth carbon position of a cytosine nucleotide, often leading to transcriptional repression (Lim & Maher, 2010). DNA methylation plays a key role in regulating genes involved in metabolic adaptation during pregnancy (Houde et al., 2013), and aberrant DNA methylation is implicated in the pathophysiology of GDM. Altered DNA methylation patterns have been demonstrated in maternal blood, placental tissue and cord blood of GDM complicated pregnancies, thus supporting its potential as biomarkers (Lesseur et al., in press; Ruchat et al., 2013; Nomura et al., 2014; Finer et al., 2015; Reichetzeder et al., 2016; Haertle et al., 2017).

Wu et al. (Wu et al., 2018) demonstrated that two genes, Hook microtubule-tethering protein 2 (*HOOK2*) and retinol dehydrogenase 12 (*RDH12*), are differentially methylated in placenta and whole blood of women with GDM. Interestingly, the changes in methylation status of these genes in whole blood occurred prior to the development of GDM, supporting their potential as screening biomarkers of GDM. In a study investigating maternal and cord blood in pregnant women and their offspring, Kang et al. (Kang et al., 2017) identified 200 genes that were differentially methylated in women with GDM compared to controls. Conversely, our recent study showed no differences in global DNA methylation between pregnant women with GDM and those with normoglycemia in SA (Dias, Adam, Wyk, et al., 2019). Global DNA methylation is a robust marker for overall genomic methylation; therefore, our failure could be due to subtle methylation differences between GDM and control groups. Perhaps, a more targeted approach using genome-wide gene-specific DNA methylation should be considered. Together, these studies show that altered DNA methylation in different biological material plays an important role the pathophysiology of GDM and offer opportunities as biomarkers.

MiRNAs, another epigenetic mechanism that is widely explored as biomarkers for GDM (Ge et al., 2015), have been shown to post transcriptionally regulate genes involved in diverse biological processes including glucose homeostasis (Bartel, 2004). Placental miRNA expression reflects metabolic adaptation, with aberrant expression observed during GDM. Interestingly, the expression of many of these altered miRNAs is mirrored in serum or plasma, thus offering potential as biomarkers for GDM. Zhao et al. (Zhao et al., 2011) reported that the expression of miR-29a and miR-222, miRNAs that are involved in insulin sensitivity, glucose homeostasis and beta-cell function, are decreased in serum of Chinese women with GDM compared with pregnant women without GDM. Pheiffer et al. (Pheiffer et al., 2018) similarly reported that the expression of miR-222 is decreased in the serum of South African women with GDM. They also reported the decreased expression of miR-20a, which was a significant predictor of GDM. Conversely, Tagoma et al. (Tagoma et al., 2018) reported increased expression of plasma-derived miR-222 in Finnish women with GDM compared with controls, while miR-20a was increased in Chinese women with GDM compared with controls (Zhu et al., 2015). Furthermore, many studies have demonstrated that placenta-specific miRNAs are altered in pregnancy complications, such as pre-eclampsia, macrosomia, preterm delivery, pregnancy loss and small-for-gestational-age babies, which further supports the use of miRNAs as predictive biomarkers for adverse pregnancy outcomes (Barchitta et al., 2017; Guarino et al., 2018).

GDM creates an abnormal intrauterine environment that negatively affects the long-term health of offspring, possibly through *in utero* programming of epigenetic mechanisms such as DNA methylation and miRNAs (El Hajj et al., 2013; Tryggestad et al., 2016; Haertle et al., 2017; Tsai et al., 2017). Using genome-wide methylation analysis, Heartle et al. (Haertle et al., 2017) identified 65 CpG sites associated with 52 genes that were differentially methylated in foetal cord blood from GDM and control

pregnancies. Of these, five candidate genes that play a role in metabolic pathways associated with oxidative damage, cardiovascular complications, glucose and amino acid metabolisms and adipocyte differentiation were validated. El Hajj et al. showed gene-specific methylation changes in the maternally imprinted *MESH* gene and non-imprinted glucocorticoid receptor (*NR3C1*) gene in both cord blood and placental tissue of GDM groups compared with controls (El Hajj et al., 2013). Recently, altered miRNA expression in the cord blood of offspring was shown to be associated with foetal complications (Tsai et al., 2017). Tryggestad et al. indicated that seven miRNAs were upregulated in human umbilical vein endothelial cells from infants born to mothers with GDM (Tryggestad et al., 2016). Despite their stability, relative ease of quantification and affordability, DNA methylation and miRNAs present several challenges that hinder their reproducibility across studies. Future research should explore risk scoring systems that can be used to combine molecular markers with maternal risk indicators to develop a clinical prediction tool for GDM.

6. SCREENING AND DIAGNOSIS IN SOUTH AFRICA

The four most common diagnostic criteria used in SA are the IADPSG/WHO 2013/FIGO, NICE, American College of Obstetricians and Gynaecologists (ACOG) and WHO 1999 criteria (Table 4.1). In 2017, the Society for Endocrinology, Metabolism and Diabetes of South Africa (SEMDSA) endorsed the IADPSG/WHO 2013 criteria and universal testing of all pregnant women (SEMDSA, 2017). However, the use of the IADPSG/WHO 2013 criteria is still being debated, as many clinicians consider these unfeasible in low- and middle-income countries such as SA. Their view is that it leads to overdiagnosis, and places a high demand on costs, workload and resources. Therefore, many local and regional health facilities continue to use risk-factor,

selective IADPSG/WHO 2013, NICE, ACOG or WHO 1999 criteria (Table 4.2) (McIntyre et al., 2015).

Table 4.1. Diagnostic criteria for GDM commonly used in South Africa.

Organisation	Glucose load (g)	0 hr glucose (mmol/L)	1 hr glucose (mmol/L)	2 hr glucose (mmol/L)	3 hr glucose (mmol/L)	Values for diagnosis
IADPSG/WHO/FIGO	75	5.1	10	8.5	-	≥1
NICE	75	5.6	-	7.8	-	≥1
ACOG	100	5.3	10	8.6	7.8	≥2
WHO 1999	75	7.0	-	7.8	-	≥1

ACOG, The American Congress of Obstetricians and Gynaecologists; FIGO, Federation of Gynaecology and Obstetrics; IADPSG, International Association of Diabetes in Pregnancy Study Groups; NICE, The National Institute for Health and Care Excellence; WHO, World Health Organisation; OGTT, oral glucose tolerance test.

Table 4.2. Current approach to GDM screening at selected Academic Centres in South Africa (Personal Communication).

Institution	Testing	Diagnostic Criteria	Level of Screening	Glucometer vs. Laboratory	[#]GDM Management
UP	Selective	IADPSG/WHO 2013	Clinic & Hospital	Glucometer & Laboratory	Tertiary hospital
WITS	Selective	NICE	Hospital	Laboratory	Tertiary hospital
UKZN	Selective & universal for: RK Khan hospital - Indian	IADPSG/WHO 2013	Hospital	Laboratory	Tertiary hospital
UCT	Selective	IADPSG/WHO 2013	Hospital	Laboratory	Tertiary hospital
US	Selective	NICE	Clinic & hospital	Glucometer	Tertiary hospital
UFS	Selective	IADPSG/ WHO 2013	Clinic & hospital	Glucometer & Laboratory	Tertiary hospital
SMU	Selective	[¥]Modified WHO	Hospital	Laboratory	Tertiary Hospital
Walter Sisulu	Selective	IADPSG/WHO 2013	Hospital	Laboratory	Tertiary Hospital

SMU, Sefako Makgatho Health Sciences University; UCT, University of Cape Town; UFS, University of Free State; UKZN, University of KwaZulu-Natal; UP, University of Pretoria; US, University of Stellenbosch; Wits, University of the Witwatersrand; WHO, World Health Organisation; IADPSG, International Association of Diabetes in Pregnancy Study Group; NICE, National Institute for Health Care Excellence; Selective: based on risk factors; Universal, all women get an OGTT. [#]All women are referred to a tertiary hospital for management of GDM with a positive OGTT. [¥]Modified WHO refers to the institute's own version of the WHO criteria, which has not yet been published.

7. CURRENT PERSPECTIVES AND FUTURE RECOMMENDATIONS

- Early screening and diagnosis of GDM improves health outcomes
- Although the OGTT is the gold standard for diagnosis, there is no consensus and GDM diagnosis is not standardised
- Novel screening and diagnostic strategies offer potential as biomarkers of GDM, but are yet to achieve clinical applicability
- Future longitudinal studies across SA are required to assess the risks and benefits of diagnostic criteria and pregnancy outcomes
- Experts are needed to establish and co-ordinate such initiatives and to make evidence-based recommendations on GDM screening and diagnosis

8. CONCLUSIONS

We have highlighted the varied screening and diagnostic strategies currently employed in SA. Although universal screening and diagnosis of GDM are widely advocated as a strategy to promote appropriate treatment and improve pregnancy outcomes, it is not feasible in many low-and middle-income countries, resulting in many countries using risk factor-based selective screening. The lack of uniform GDM screening and diagnostic criteria and variation in clinical practice negatively affect maternal and child health. There is limited evidence to support one approach over the other. There is a need for longitudinal studies across SA to investigate the association between diagnostic criteria and pregnancy outcomes, as well as long-term outcomes in mothers and their offspring. We recommend that a SA diabetes-in-pregnancy study group, comprising interested obstetricians, physicians, endocrinologists, public health specialists, dieticians and scientists, be established to co-ordinate such initiatives and

to make evidence-based recommendations on GDM screening, diagnosis and management.

CHAPTER 5

MOLECULAR BIOMARKERS FOR GESTATIONAL DIABETES MELLITUS

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1. ABSTRACT

Gestational diabetes mellitus (GDM) is a growing public health problem worldwide. The condition is associated with perinatal complications and an increased risk for future metabolic disease in both mothers and their offspring. In recent years, molecular biomarkers have received considerable interest as screening tools for GDM. The purpose of this review is to provide an overview of the current status of single nucleotide polymorphisms (SNPs), DNA methylation and microRNAs as biomarkers for GDM. PubMed, Scopus and Web of Science were searched for articles published between January 1990 and August 2018. The search terms included 'gestational diabetes mellitus', 'blood', 'single nucleotide polymorphism (SNP)', 'DNA methylation', and 'microRNAs' including corresponding synonyms and associated terms for each word. This review updates current knowledge of the candidacy of these molecular biomarkers for GDM with recommendations for future research avenues.

2. INTRODUCTION

GDM is defined as glucose intolerance that is first diagnosed during the latter half of pregnancy, with return to normoglycemia postpartum (WHO, 2013). The prevalence of GDM is increasing worldwide, with approximately 14% of pregnancies affected by GDM (International Diabetes Federation, 2017). The condition is associated with perinatal complications and an increased risk for future metabolic disease in both mothers and their offspring. The oral glucose tolerance test (OGTT) is considered the gold standard for the diagnosis of GDM. However, the test is cumbersome to conduct, requires fasting, multiple blood draws, and its association with nausea and vomiting leads to decreased patient compliance. Furthermore, the OGTT is conducted between 24-28 weeks of gestation (IADPSG panel, 2010; WHO, 2013), presenting a small window of opportunity to implement interventions to improve pregnancy outcomes. Earlier detection of GDM may lead to improved management, possibly preventing pregnancy complications. The identification of sensitive and specific biomarkers, which may offer potential for risk prediction and intervention strategies, has thus become a major focus in GDM research. Several studies have provided evidence for genetic predisposition to GDM (Wu et al., 2016), while gene-environment interactions could explain the population-specific variation in GDM prevalence (Pheiffer et al., 2018). Consequently, single nucleotide polymorphisms (SNPs) and epigenetic mechanisms are widely explored as molecular biomarkers for GDM.

The purpose of this review is to provide an overview of the current status of SNPs and the two most commonly investigated epigenetic mechanisms, DNA methylation and microRNAs (miRNAs), as molecular biomarkers for GDM. Three major databases, PubMed, Scopus and Web of Science were searched for studies published between January 1990 and August 2018 that investigated SNPs, DNA methylation and

miRNAs in the blood of women with GDM. Blood was selected as it is easily accessible as part of routine antenatal care. The search terms included 'gestational diabetes mellitus', 'blood', 'single nucleotide polymorphism (SNP)', 'DNA methylation' and 'microRNAs', including corresponding synonyms and associated terms for each word (Appendix, Table A1). Articles were selected if they reported case-control studies that investigated GDM in association with SNPs, DNA methylation or miRNAs in maternal blood, plasma or serum, and were conducted in humans. This review will begin with an overview of GDM, followed by a brief description of the characteristics of ideal biomarkers. Thereafter, studies profiling SNPs and DNA methylation in whole blood, and miRNAs in whole blood, plasma or serum of women with GDM are summarised, and the limitations of these molecular biomarkers are discussed. Finally, the current status of GDM biomarkers will be discussed, along with recommendations for future research.

3. OVERVIEW OF GESTATIONAL DIABETES MELLITUS

The exact mechanism underlying the development of GDM is not completely understood, however, it is speculated that women who develop GDM are unable to meet the increasing demand for insulin production during pregnancy (Barbour et al., 2007). GDM is associated with an increased risk of short-and long-term pregnancy complications. Women with GDM have a higher risk for pre-eclampsia, caesarean section and birth injury, while postpartum complications to offspring include macrosomia, shoulder dystocia, hyperinsulinemia, hypoglycaemia and hyperbilirubinemia (Alam et al., 2006; Mohammadbeigi et al., 2013; Young & Ecker, 2013). In the long-term both mothers and their offspring are predisposed to metabolic conditions such as obesity, type 2 diabetes (T2D) and cardiovascular disease (Mitanchez et al., 2015). Estimates are that approximately 30% of offspring (Garcia-

Vargas et al., 2012) and more than 70% of women with previous GDM (Kim, Newton & Knopp, 2002) are predisposed to develop type T2D in later life, thus posing a significant health and economic burden to health systems.

Recently, several studies have provided evidence that early detection and treatment of GDM improve health outcomes (Duran et al., 2014). Consequently, universal screening for GDM is advocated by most international organisations (McIntyre et al., 2015). However, only women who have traditional risk factors for GDM (obesity, ethnicity, advanced maternal age, glycosuria and previous adverse pregnancy outcomes) (Zhang, Rawal & Chong, 2016; Adam & Rheeder, 2017), are recommended for the OGTT in resource limited settings. Unfortunately, these risk factors have poor predictive value, resulting in many women with GDM not receiving appropriate treatment (Miailhe et al., 2015). Thus, there is significant impetus to identify biomarkers of GDM. Serum proteins such as adiponectin, insulin, sex hormone globulin, C-reactive protein and glycosylated fibronectin have been widely studied (Smirnakis et al., in press; Nanda et al., 2011; Rasanen et al., 2013; Adam & Rheeder, 2017), while the diagnostic utility of glycated haemoglobin (HbA1c) has also been explored (Renz et al., 2015). However, none of these potential biomarkers have yet achieved clinical applicability. Evidence for genetic susceptibility (Zhang et al., 2013) and dysregulated epigenetic regulation, in particular DNA methylation (Haertle et al., 2017) and miRNAs (Guarino et al., 2018), is increasingly being reported during GDM, sparking interest in their use as molecular biomarkers.

4. CHARACTERISTICS OF IDEAL BIOMARKERS

Biomarkers are indicators of normal biological processes that can be used to detect disease or other biological states of organisms. They are considered to be clinically

useful because they can potentially predict or diagnose disease, give insight into the pathophysiology of disease and can be used to monitor pharmacological responses to therapeutic intervention or predict clinical outcome (Strimbu & Tavel, 2010). Recent advancements in molecular biology have led to the development of molecular biomarkers that are easily measured in biological fluids such as whole blood, plasma and serum. The ideal biomarker should be cost effective and reproducible, easily accessible through non-invasive methods, stably expressed in biological fluids, sensitive to relevant changes in disease state, provide early detection of disease before clinical symptoms arise, and have the ability to differentiate between disease pathologies (Etheridge et al., 2011; Sahu et al., 2011). Commercial kits for SNPs (Huijsmans et al., 2007), DNA methylation (Mikeska & Craig, 2014) and miRNAs (Hydbring & Badalian-Very, 2013) are already clinically available for a number of other disorders.

5. SINGLE NUCLEOTIDE POLYMORPHISMS

Single nucleotide polymorphisms (SNPs) refer to alterations in the DNA sequence at individual nucleotide bases. They are the most common genetic variation, with over 10 million SNPs present in the human genome (Consortium, 2007). In most cases SNPs are silent, not altering the function or expression of genes (Sachidanandam et al., 2001), while others are biologically functional, and can lead to altered protein function and disease. The search for SNPs that influence disease susceptibility and outcome is a field of active research. Several studies have provided evidence that SNPs are associated with metabolic conditions including obesity, T2D and cardiovascular disease (McCarthy, 2010). Variants in more than 50 and 80 loci have been found to be associated with obesity (Rankinen et al., 2006) and T2D (Morris et al., 2012),

respectively, and occur in genes that regulate glucose homeostasis and insulin signalling.

5.1.Single Nucleotide Polymorphisms and Gestational Diabetes Mellitus

Genetic variants are increasingly being implicated in the pathogenesis of GDM (Wu et al., 2016). Evidence suggest that genetic alterations in genes responsible for metabolic changes during pregnancy predispose to GDM. In this review, a total of 76 studies were identified that investigated SNPs during GDM, using the search terms previously stated. However, to increase the likelihood of reporting a true association, only SNPs investigated in two or more populations were reported. Thirty-six SNPs investigated in 52 studies met the inclusion criteria and are summarised in Table 5.1.

Genetic studies of the Transcription factor 7 like 2 (*TCF7L2*) gene, which is arguably one of the most important T2D susceptibility genes (Hattersley, 2007), have produced varying results in GDM (Papadopoulou et al., 2011; Huopio et al., 2013; Stuebe et al., 2013; Reyes-López, Pérez-Luque & Malacara, 2014; Pagán et al., 2015; Michalak-Wojnowska et al., 2016; Anghebem-Oliveira, Martins, et al., 2017; Popova et al., 2017; Ding et al., 2018; Franzago et al., 2018). *TCF7L2* encodes a transcription factor, which is involved in the Wnt signalling, an important pathway that regulates glucose homeostasis. Twenty studies conducted in diverse populations have screened four SNPs (rs7903146, rs4506565, rs7901695 and rs12255372) in the *TCF7L2* gene. Four of the eight studies that investigated rs7903146 showed an association between the T allele and GDM (Papadopoulou et al., 2011; Huopio et al., 2013; Ding et al., 2018; Franzago et al., 2018). The other studies failed to observed an association between rs7903146 and GDM, possibly due to small samples size and lack of statistical power. (Reyes-López, Pérez-Luque & Malacara, 2014; Pagán et al., 2015; Michalak-Wojnowska et al., 2016). Both studies investigating rs4506565 reported an association

between the T allele and GDM (Pagán et al., 2015; Ding et al., 2018). One of the five studies investigating rs7901695 found an association between GDM and the T allele in American Caucasians (Stuebe et al., 2013), while one study found that the C allele, rather than the T allele was associated with GDM in a large Swedish population (Papadopoulou et al., 2011). The three studies that did not show an association had relatively small sample sizes (Pagán et al., 2015; Michalak-Wojnowska et al., 2016; Anghebem-Oliveira, Martins, et al., 2017). Of the five studies investigating rs12255372, two showed an association between the T allele and GDM, one was conducted in a large Swedish population and the other in a small Mexican population (Papadopoulou et al., 2011; Reyes-López, Pérez-Luque & Malacara, 2014). However, these results were not replicated in studies conducted in Russian, Spanish nor Brazilian populations (de Melo et al., 2015; Pagán et al., 2015; Popova et al., 2017) of moderate size, suggesting that ethnic or other confounding factors underlie these differences. The T-allele is associated with decreased insulin production and altered hepatic gluconeogenesis (Pilgaard et al., 2009), and therefore is a good candidate for further research in larger cohorts, despite these conflicting results obtained in these studies.

Adiponectin, an adipokine that regulates glucose and lipid metabolism (Bouchard et al., 2012; Bao et al., 2015), has been associated with GDM in many studies. Three SNPs within the adiponectin (*ADIPOQ*) gene, rs1501299, rs266729 and rs2241766, were investigated in ten studies. Markedly, seven studies that investigated rs266729 (Liang et al., 2010; Pawlik et al., 2017) and rs2241766 (Low et al., 2011; Beltcheva et al., 2014; Han et al., 2014; Takhshid, Haem & Aboualizadeh, 2015) found that the G allele was associated with GDM in various populations, while one study found no association between either rs266729 or rs2241766 and GDM in a Brazilian population (Gueuvoghlanian-Silva et al., 2012). Both of the studies investigating rs1501299

showed no association between this SNP and GDM (Beltcheva et al., 2014; Pawlik et al., 2017).

The melatonin receptor 1B gene (*MTNR1B)* encodes one of the receptors for melatonin, a hormone that is involved in regulating circadian rhythms, insulin signalling and glucose metabolism, amongst others (Sun et al., 2018). Two SNPs rs10830963 and rs1387153 within the *MTNR1B* gene were investigated. Eight of the nine studies that screened rs10830963 showed that the G-allele was associated with an increased risk for GDM in several Caucasian (Huopio et al., 2013; Stuebe et al., 2013; Popova et al., 2017; Rosta et al., 2017; Tarnowski, Malinowski, Safranow, et al., 2017a; Ding et al., 2018), as well as in Chinese and South Korean populations (Kim et al., 2011; Li et al., 2019). However, Wang et al. found that this SNP was not associated with GDM in a different Chinese population (Wang et al., 2011). The three studies that investigated rs1387153 reported an association between the T allele and GDM (Kim et al., 2011; Popova et al., 2017; Ding et al., 2018). Variants in *MTNR1B*, particularly the G allele of rs10830963 was previously shown to be associated with increased fasting glucose concentrations and reduced beta-cell function in Caucasians (Prokopenko et al., 2009).

Glucokinase *(GCK)* and the Glucokinase receptor *(GCKR)* play critical roles in glucose processing in the liver (Iynedjian, 2009). Two variants, rs1799884 and rs4607517, within the *GCK* gene were studied for GDM. For rs1799884, the minor allele, reported as either T (Popova et al., 2017) or A (Han et al., 2015) was associated with an increased risk of GDM. Tarnowski et al. also showed a trend towards a significant association between the T allele and risk of GDM in a Polish population (Tarnowski, Malinowski, Pawlak, et al., 2017). However, a large study in a Finnish population showed no association between rs1799884 and GDM (Huopio et al., 2013). No association between rs4607517 and GDM was observed (Wang et al., 2011; Huopio et al., 2013). Within the

GCKR gene, the C allele of rs780094 was associated with an increased risk of GDM in Malaysian, American Caucasian and Brazilian populations (Stuebe et al., 2013; Anghebem-Oliveira, Webber, et al., 2017; Jamalpour et al., 2018), but not in studies conducted in Polish or Finnish populations (Huopio et al., 2013; Tarnowski, Malinowski, Pawlak, et al., 2017). The C allele was increased in women with GDM from the Polish population, but this did not reach significance due to lack of statistical power.

The association between genetic variants within the fat mass and obesity-associated (*FTO*) gene and metabolic syndrome is widely reported (Wang, Dong, et al., 2012). *FTO* encodes an alpha-ketoglutarate dependent dioxygenase, that plays a role in adipocyte development and function (Merkestein & Sellayah, 2015). Three SNPs within the *FTO* gene have been studied for GDM. Of the six studies investigating rs9939609, one study in a Finnish population found an association between the A allele and an increased risk for GDM (Huopio et al., 2013), another study in a small Spanish population found an association between the T allele and GDM (Pagán et al., 2015), while four studies reported no association (de Melo et al., 2015; Popova et al., 2017; Saucedo et al., 2017; Franzago et al., 2018). Discrepancies between the studies are possibly due to ethnic and genotyping method differences. None of the studies investigating rs8050136 and rs1421085 found an association between these SNPs and GDM (de Melo et al., 2015; Anghebem-Oliveira, Martins, et al., 2017; Saucedo et al., 2017).

Insulin receptor substrate 1 *(IRS1)* is a protein that plays a key role in transmitting signals from the insulin and insulin-like growth factor-1 receptors to intracellular pathways that are associated with insulin response and risk of T2D (Gual, Le Marchand-Brustel & Tanti, 2005). Two genetic variants, rs1801278 and rs7578326,

within *IRS1* have been investigated during GDM. For rs1801278 the T allele was associated with an increased risk of GDM (Alharbi et al., 2014) in a Saudi Arabian, but not in a Russian population (Popova et al., 2017), while for rs7578326 the G allele was associated with a decreased risk of GDM in a Austro-Hungarian population (Rosta et al., 2017), but not in a Finnish population (Huopio et al., 2013). As previously stated, these conflicting results may be due to population and genotyping method differences.

Potassium Voltage-Gated Channel Subfamily Q Member 1 (*KCNQ1*) plays a role in insulin secretion, and variants of *KCNQ1* are associated with decreased insulin secretion and increased susceptibility to T2D (Yasuda et al., 2008). Two variants, rs2237895 and rs2237892, were investigated in different populations in four studies. In both variants, the C allele was associated with an increased risk of GDM (Kwak et al., 2010; Ao et al., 2015; Fatima et al., 2016). The Solute Carrier Family 30 Member 8 (*SLC30A8*) genes encodes a zinc transporter protein that plays a role in insulin secretion and variants of the gene are associated with T2D risk (Flannick et al., 2014). Rs13266634 was investigated in four studies with varying results. One study showed that the T allele was associated with a decreased risk of GDM in an Austro-Hungarian population, while the C allele was found to be associated with an increased risk of GDM in a Chinese and Swedish population (Liang et al., 2010; Dereke et al., 2016; Rosta et al., 2017). A large Finnish population showed no association between rs13266634 and GDM (Huopio et al., 2013).

As illustrated in Table 5.1, SNPs in 15 other genes were investigated in two studies, however, these showed either a positive association in one study only, or no association with GDM. Of these, SNPs within nine genes, CDK5 Regulatory Subunit Associated Protein 1 Like *(CDKAL1)*, Calpain 10 *(CAPN10)*, Potassium Voltage-Gated

Channel Subfamily J Member 11 *(KCNJ11)*, Retinol Binding Protein 4 *(RBP4)*, Group-Specific Component *(GC)*, Serine/Threonine Kinase 11 *(STK11)*, Macrophage Migration Inhibitory Factor *(MIF)*, Cyclin Dependent Kinase Inhibitor 2A/2B *(CDKN2A/2B)* and Insulin Like Growth Factor 2 mRNA Binding Protein 2 *(IGF2BP2)*, were associated with GDM in one population only, while SNPs within six genes, CD36 Molecule *(CD36)*, Peroxisome Proliferator Activated, Receptor Gamma 2 *(PPARG2)*, Vitamin D Receptor *(VDR)*, Cell Division Cycle 123 Homolog/Calmodulin Dependent Protein Kinase ID *(CDC123/CAMK1D)*, Interleukin 10 (IL-10) and Tumour Necrosis Factor-Alpha (TNF-α) were not associated with GDM in any of the populations investigated.

5.2. Limitations of Single Nucleotide Polymorphisms

There are inherent limitations in genetic association studies, particularly in studies of polygenic and multifactorial diseases such as GDM. As stated above, these limitations include inadequate sample size to detect statistically significant associations, and differences in allele frequencies and disease aetiology between ethnicities, which may explain why many genetic associations are not reproducible across populations. Furthermore, GDM diagnosis is not standardised internationally, thus different diagnostic criteria could have contributed to the discordant results observed between studies. Importantly, genetic variants do not solely contribute to the development of complex diseases and it is widely believed that disease arise due to the interaction of genetic predisposition and environmental factors (Welter et al., 2014). Thus, to accurately assess risk of GDM, biological and environmental factors, such as maternal age and diet (Popova et al., 2017), should be considered together with genetic variants.

Despite the variable results obtained across studies, many of the variants found to be associated with GDM, are also associated with T2D, supporting their biological

plausibility. Therefore, while the aetiology of GDM may differ from T2D, the genetic pathways through which the symptoms manifest are likely to overlap. In this review, only studies that profiled SNPs in DNA extracted from whole blood were reported on. However, the use of less invasive sources of genetic material such as buccal swabs is acknowledged (Andraweera et al., 2017). Furthermore, this review only included SNPs reported in two or more studies, and may have overlooked other important SNPs possibly associated with GDM. Thus, illustrating the importance of using the correct keywords and search terms for literature screening.

Table 5.1. Studies reporting on single nucleotide polymorphisms profiled in two or more populations with Gestational diabetes mellitus.

Author	Gene	SNP identification	Country	Detection method	Case/control	Associated allele or genotype	Risk for GDM
(Ding et al., 2018)	*TCF7L2*	rs7903146	Denmark & USA	qRT-PCR	2636/6086	T allele	Increased
(Franzago et al., 2018)			Italy	HRM	104/124	T allele	Increased
(Popova et al., 2017)			Russia	qRT-PCR	278/179	No association	-
(Michalak-Wojnowska et al., 2016)			Poland	qRT-PCR	50/26	No association	-
(Pagán et al., 2015)			Spain	Sequencing	45/25	No association	-
(Reyes-López, Pérez-Luque & Malacara, 2014)			Mexico	RFLP	90/108	No association	-
(Papadopoulou et al., 2011)			Sweden	qRT-PCR	826/1185	T allele	Increased
(Huopio et al., 2013)			Finland	MassARRAY	533/407	T allele	Increased
(Ding et al., 2018)		rs4506565	Denmark & USA	qRT-PCR	2636/6086	T allele	Increased
(Pagán et al., 2015)			Spain	Sequencing	45/25	T allele	Increased
(Anghebem-Oliveira, Martins, et al., 2017)		rs7901695	Brazil	qRT-PCR	127/125	No association	-

(Michalak-Wojnowska et al., 2016)			Poland	qRT-PCR	50/26	No association	-
(Pagán et al., 2015)			Spain	Sequencing	45/25	No association	-
(Stuebe et al., 2013)			USA / African American (AA) & Caucasian (C)	MassARRAY	26/362 (AA) & 56/843 (C)	No association (AA) T allele (C)	- Increased
(Papadopoulou et al., 2011)			Swedish	qRT-PCR	805/1116	C allele	Increased
(Popova et al., 2017)		rs12255372	Russia	qRT-PCR	278/179	No association	-
(de Melo et al., 2015)			Brazil	qRT-PCR	200/200	No association	-
(Pagán et al., 2015)			Spain	Sequencing	45/25	No association	-
(Reyes-López, Pérez-Luque & Malacara, 2014)			Mexico	RFLP	90/108	T allele	Increased
(Papadopoulou et al., 2011)			Swedish	qRT-PCR	826/1185	T allele	Increased
(Pawlik et al., 2017)	*ADIPOQ*	rs1501299	Poland	qRT-PCR	204/207	No association	-
(Beltcheva et al., 2014)			Bulgaria	qRT-PCR	130/130	No association	-
(Pawlik et al., 2017)		rs266729	Poland	qRT-PCR	204/207	G allele	Increased

(Beltcheva et al., 2014)			Bulgaria	qRT-PCR	130/130	G allele	Increased
(Liang et al., 2010)			China	MassArray & Gene chip	50/80 24/24	G allele	Increased
(Gueuvoghlanian-Silva et al., 2012)			Brazil	RFLP	79/169	No association	-
(Takhshid, Haem & Aboualizadeh, 2015)		rs2241766	Iran	RFLP	65/70	G allele	Increased
(Han et al., 2014)			China	RFLP	128/140	G allele	Increased
(Beltcheva et al., 2014)			Bulgaria	qRT-PCR	130/130	G allele	Increased
(Low et al., 2011)			Malaysia	RFLP	26/53	G allele	Increased
(Gueuvoghlanian-Silva et al., 2012)			Brazil	RFLP	79/169	No association	-
(Ding et al., 2018)	*MTNR1B*	rs10830963	Denmark and USA	qRT-PCR	2636/6086	G allele	Increased
(Li et al., 2019)			China	Sequencing	215/243	G allele	Increased
(Tarnowski, Malinowski, Safranow, et al., 2017a)			Poland	qRT-PCR	204/207	G allele	Increased
(Rosta et al., 2017)			Hungary & Austria	KASP	287/533	G allele	Increased

(Popova et al., 2017)			Russia	qRT-PCR	278/179	G allele	Increased
(Stuebe et al., 2013)			USA African American (AA) & Caucasian (C)	MassARRAY	26/362 (AA) & 56/843 (C)	No association (AA) G allele (C)	- Increased
(Wang et al., 2011)			China	qRT-PCR	725/1039	No association	-
(Kim et al., 2011)			South Korea	qRT-PCR	928/990	G allele	Increased
(Huopio et al., 2013)			Finland	MassARRAY	533/407	G allele	Increased
(Ding et al., 2018)		rs1387153	Denmark & USA	qRT-PCR	2636/6086	T allele	Increased
(Popova et al., 2017)			Russia	qRT-PCR	278/179	T allele	Increased
(Kim et al., 2011)			South Korea	qRT-PCR	928/990	T allele	Increased
(Tarnowski, Malinowski, Pawlak, et al., 2017)	*GCK*	rs1799884	Poland	qRT-PCR	204/207	No association	-
(Popova et al., 2017)			Russia	qRT-PCR	278/179	T allele	Increased
(Han et al., 2015)			China	PCR Invader assay	948/975	A* allele	Increased
(Huopio et al., 2013)			Finland	MassARRAY	533/407	No association	-
(Wang et al., 2011)		rs4607517	China	qRT-PCR	725/1039	No association	-

(Huopio et al., 2013)			Finland	MassARRAY	533/407	No association	-
(Jamalpour et al., 2018)	*GCKR*	rs780094	Malaysia	MassARRAY	267/855	C allele	Increased
(Tarnowski, Malinowski, Pawlak, et al., 2017)			Poland	qRT-PCR	204/207	No association	-
(Anghebem-Oliveira, Webber, et al., 2017)			Brazil	qRT-PCR	127/125	C allele	Increased
(Stuebe et al., 2013)			USA / African American (AA) & Caucasian (C)	MassARRAY	26/362 (AA) & 56/843 (C)	No association C allele	- Increased
(Huopio et al., 2013)			Finland	MassARRAY	533/407	No association	-
(Franzago et al., 2018)	*FTO*	rs9939609	Italy	HRM	104/124	No association	-
(Saucedo et al., 2017)			Mexico	qRT-PCR	80/80	No association	-
(Popova et al., 2017)			Russia	qRT-PCR	278/179	No association	-
(de Melo et al., 2015)			Brazil	qRT-PCR	200/200	No association	-
(Pagán et al., 2015)			Spain	Sequencing	45/25	T allele	Increased
(Huopio et al., 2013)			Finland	MassARRAY	533/407	A allele	Increased
(Saucedo et al., 2017)		rs8050136	Mexico	qRT-PCR	80/80	No association	-

(de Melo et al., 2015)			Brazil	qRT-PCR	200/200	No association	-
(Saucedo et al., 2017)		rs1421085	Mexico	qRT-PCR	80/80	No association	-
(Anghebem-Oliveira, Martins, et al., 2017)			Brazil	qRT-PCR	127/125	No association	-
(Popova et al., 2017)	*IRS1*	rs1801278	Russia	qRT-PCR	278/179	No association	-
(Alharbi et al., 2014)			Saudi Arabia	RFLP	200/300	T allele	Increased
(Huopio et al., 2013)		rs7578326	Finland	MassARRAY	533/407	No association	-
(Rosta et al., 2017)			Hungary & Austria	KASP	287/533	G allele	Decreased
(Fatima et al., 2016)	*KCNQ1*	rs2237895	Pakistan	RFLP / sequencing	208/429	C allele	Increased
(Kwak et al., 2010)			South Korea	qRT-PCR	869/632	No association	-
(Ao et al., 2015)		rs2237892	China	MassARRAY	562/453	C allele	Increased
(Kwak et al., 2010)			South Korea	qRT-PCR	869/632	C allele	Increased
(Rosta et al., 2017)	*SLC30A8*	rs13266634	Hungary & Austria	KASP	287/533	T allele	Decreased
(Dereke et al., 2016)			Sweden	RFLP	776/511	C allele	Increased
(Huopio et al., 2013)			Finland	MassARRAY	533/407	No association	-

(Liang et al., 2010)			China	MassArray & Gene chip	50/80 24/24	C allele	Increased
(Noury et al., 2018)	*CDKAL1*	rs7754840	Egypt	qRT-PCR	47/51	No association	-
(Rosta et al., 2017)			Hungary & Austria	KASP	287/533	C allele	Increased
(Popova et al., 2017)			Russia	qRT-PCR	278/179	No association	-
(Wang et al., 2011)			China	qRT-PCR	725/1039	No association	-
(Huopio et al., 2013)			Finland	MassARRAY	533/407	No association	-
(Castro-Martínez et al., 2018)	*CAPN10*	SNP43	Mexico	qRT-PCR & RFLP	116/83	No association	-
(Leipold et al., 2004)			Austria	RFLP	100/100	No association	-
(Castro-Martínez et al., 2018)		SNP63	Mexico	qRT-PCR & RFLP	116/83	No association	-
(Leipold et al., 2004)			Austria	RFLP	40/40	C allele	Increased
(Huopio et al., 2013)			Finland	MassARRAY	533/407	No association	-
(M et al., 2018)	*KCNJ11*	rs5219	India	RFLP	230/240	T allele	Increased
(Popova et al., 2017)			Russia	qRT-PCR	278/179	No association	-

(Huopio et al., 2013)			Finland	MassARRAY	533/407	No association	-
(Saucedo et al., 2014)	*RBP4*	rs3758539	Mexico	qRT-PCR	100/100	No association	-
(Ping et al., 2012)			China	LDR	505/687	G allele	Increased
(Hiraoka et al., 2011)			USA / Caucasian (C) & Filipino (F) & Pacific Islander (PI)	qRT-PCR	88 C, 82 F, and 19 PI / 315 C, 286 F, and 32 PI	No association	-
(Shi et al., 2016)	*GC*	rs16847024	China	MassARRAY	964/1021	T allele	Increased
(Wang et al., 2015)			China	qRT-PCR	692/802	No association	-
(Alharbi et al., 2015)	*STK11*	rs8111699	Saudi Arabia	RFLP	200/300	No association	-
(Bassols et al., 2013)			Spain	qRT-PCR	243/318	G allele	Decreased
(Aslani et al., 2011)	*MIF*	rs1007888	Iran	PCR-SSP	147/169	G allele	Increased
(Huopio et al., 2013)			Finland	MassARRAY	533/407	No association	-
(Noury et al., 2018)	*CDKN2A/2B*	rs10811661	Egypt /Egyptian	qRT-PCR	47/51	No association	-
(Ye et al., 2016)			Poland / Caucasian	qRT-PCR	204/207	C allele	Decreased
(Huopio et al., 2013)			Finland / Caucasian	MassARRAY	533/407	No association	-
(Popova et al., 2017)	*IGF2BP2*	rs4402960	Russia	qRT-PCR	278/179	No association	-

(Wang et al., 2015)			China	qRT-PCR	725/1039	T allele	Increased
(Huopio et al., 2013)			Finland	MassARRAY	533/407	No association	-
(Bartáková et al., 2018)	*CD36*	rs1527479	Czech Republic	qRT-PCR	293/70	No association	-
(Yang et al., 2018)			China	qRT-PCR	209/215	No association	-
(Franzago et al., 2018)	*PPARG2*	rs1801282	Italy	HRM	104/124	No association	-
(Anghebem-Oliveira, Martins, et al., 2017)			Brazil	qRT-PCR	127/125	No association	-
(Shi et al., 2016)	*VDR*	rs739837	China	MassARRAY	964/1021	No association	-
(Wang et al., 2015)			China	qRT-PCR	692/802	No association	-
(Tarnowski, Malinowski, Safranow, et al., 2017b)	*CDC123/CAMK1D*	rs1277970	Poland	qRT-PCR	204/207	No association	-
(Huopio et al., 2013)			Finland	MassARRAY	533/407	No association	-
(Gueuvoghlanian-Silva et al., 2012)	*IL-10*	rs1800896	Brazil	RFLP	79/169	No association	-
(Montazeri, Nalliah & Radhakrishnan, 2010)			Malaysia	RFLP	110/102	No association	-

(Gueuvoghlanian-Silva et al., 2012)	*TNF-α*	rs1800629	Brazil	RFLP	79/169	No association	-
(Montazeri, Nalliah & Radhakrishnan, 2010)			Malaysia	RFLP	110/102	No association	-

RFLP - Restriction fragment length polymorphism of PCR amplified fragments; KASP - Kompetitive Allele Specific PCR; qRT- PCR - Quantitative real-time PCR (TaqMan allelic discrimination assay); LDR - Ligase detection reaction; HRM - High resolution melt-curve analysis; MassARRAY - Sequenom MassARRAY iPLEX platform; PCR invader assay - Invasive cleavage reaction which uses a structure-specific flap endonuclease. *A is the minor allele also reported as T . *TCF7L2* - Transcription Factor 7 Like 2; *ADIPOQ* - Adiponectin; *MTNR1B* - Melatonin Receptor 1B; *CAPN10* - Calpain 10; *CDKAL1*- CDK5 Regulatory Subunit Associated Protein 1 Like; *CDKN2A/2B* - Cyclin Dependent Kinase Inhibitor 2A / 2B; *FTO* - Fat Mass And Obesity Associated; *GC* - Group-Specific Component (Vitamin D Binding Protein); *GCK* - Glucokinase; *GCKR* - Glucokinase Regulator; *IGF2BP2* - Insulin Like Growth Factor 2 mRNA Binding Protein 2; *IRS1* - Insulin Receptor Substrate 1; *KCNJ11* - Potassium Voltage-Gated Channel Subfamily J Member 11; *KCNQ1*- Potassium Voltage-Gated Channel Subfamily Q Member 1; *RBP4* - Retinol Binding Protein 4; *SLC30A8* - Solute Carrier Family 30 Member 8; *STK11* - Serine/Threonine Kinase 11; *MIF* - Macrophage Migration Inhibitory Factor; *CD36* - CD36 Molecule; *PPARG2* - Peroxisome Proliferator Activated Receptor Gamma 2; *VDR* - Vitamin D Receptor; *CDC123/CAMK1D* - Cell Division Cycle 123 Homolog / Calmodulin Dependent Protein Kinase I; IL-10 - Interleukin 10; TNF-α – Tumour Necrosis Factor Alpha.

6. DNA METHYLATION

DNA methylation, the most widely studied and best characterised epigenetic mechanism, occurs by the addition of a methyl group to the fifth carbon position of a cytosine residue within CpG dinucleotides (Lim & Maher, 2010). The process is catalyzed by the enzyme DNA methyltransferase (DNMT), with S-adenosyl-methionine serving as the methyl donor. Methylation of CpG islands, which are regions with high levels of CpG dinucleotides primarily in the promoter regions of genes, is generally associated with transcriptional repression due to altered protein binding to target sites on DNA (Bird, 1980, 2002). DNA methylation is a reversible process (Wu & Zhang, 2017). Ten-eleven translocation (TET) methylcytosine dioxygenases are able to cause the oxidation and demethylation of methylated cytosine to 5-hydroxymethylcytosine (Wu & Zhang, 2017), which is associated with gene activation. Recently, DNA methylation of CpG poor islands have been identified downstream of active promoters, either within (intragenic) or between (intergenic) genes, although the role of methylation in these regions are not fully elucidated (Pheiffer et al., 2016). Approximately 55-90% of all CpG dinucleotides within CpG islands are methylated, constituting about 3% of the genome. Global DNA hypomethylation is associated with genomic and chromosomal instability, while DNA methylation within the promoters of genes is generally associated with gene silencing. Both aberrant global and gene-specific DNA methylation has been shown to be associated with metabolic conditions such as obesity (Van Dijk et al., 2015), T2D (Toperoff et al., 2012) and cardiovascular disease (Kim et al., 2010). Thus, characterization of altered DNA methylation during disease processes could give insight into the pathophysiology of disease, and reveal novel diagnostic, prognostic, and therapeutic targets.

6.1.DNA Methylation and Gestational Diabetes Mellitus

DNA methylation during pregnancy plays a key role in modulating the transcriptional potential of the genome, and is known to affect gene expression pathways associated with a range of pathophysiological processes such as GDM (Bouchard et al., 2012; Houde et al., 2013). Several studies have demonstrated that DNA methylation is altered in the placenta and cord blood of women with GDM compared to women with normoglycemic pregnancies (Ruchat et al., 2013; Nomura et al., 2014; Finer et al., 2015; Reichetzeder et al., 2016). Intrauterine exposure to GDM leads to long lasting effects in the offspring and increases risk of disease in later life, possibly mediated by DNA methylation (El Hajj et al., 2013; Pinney, 2015). Importantly, it has been demonstrated that physiological and DNA methylation changes that occur during pregnancy are reflected in whole blood (Chim et al., 2008), thus increasing interest in screening maternal blood for biomarkers of GDM. DNA methylation profiling in pregnancies complicated by GDM is a relatively new research field, with limited studies conducted in maternal whole blood. Studies that have investigated DNA methylation in whole blood of women with GDM are summarised in Table 5.2.

Global DNA methylation provides an estimate of overall genomic methylation and is relatively easy and cost-effective to measure (Kurdyukov & Bullock, 2016). Currently, the only study that has investigated global DNA methylation during GDM was conducted in our laboratory (Dias, Adam, Wyk, et al., 2019). The study showed that global DNA methylation was not associated with GDM in a SA population, suggesting that the method may be too crude to detect subtle glucose intolerance and that gene-specific methylation is warranted in this population. Genome-wide DNA methylation profiling in maternal blood during GDM has been conducted using

methylation bead chip arrays (Enquobahrie et al., 2015; Kang et al., 2017; Wu et al., 2018). Methylation bead chip arrays can interrogate between 27,000 - 850,000 CpG sites across the genome at a single nucleotide resolution. In one of the earliest studies using bead chip arrays, Enquobahrie et al. reported that DNA methylation changes occurred early during pregnancy in six women with repeat pregnancies, one of which were complicated by GDM (Enquobahrie et al., 2015). They reported that 17 CpG sites were hypomethylated and 10 CpG sites were hypermethylated between GDM and normal pregnancies within the same women. Novel genes related to these CpG sites were found to be associated with cell cycle, cell morphology, cell assembly, cell organisation and cell compromise. Subsequently, using a newer bead chip array containing more CpG sites, Kang et al. showed that 200 CpGs corresponding to 151 genes, were differentially methylated in women with GDM (n=8) compared to controls (n=8). Amongst the differentially methylated genes were interleukin-6 (*IL-6*) and interleukin-10 (*IL-10*), which are key pro-inflammatory and anti-inflammatory cytokines, respectively (Kang et al., 2017). These cytokines function in a wide variety of inflammatory-associated diseases, including obesity and T2D. Moreover, a different study by Kang et al. showed that decreased methylation of *IL-10* during GDM was associated with increased serum IL-10 concentrations at the end of pregnancy (Kang et al., 2018). IL-10 serum concentrations have been shown to vary during pregnancy, suggesting that this cytokine plays an important role in the development of GDM. In another study using bead chip arrays, 100 differentially methylated CpG sites corresponding to 66 genes were identified in women with GDM (n=11) compared to controls (n=11) (Wu et al., 2018). Using more stringent statistical criteria to prioritize methylation sites, a total of five CpG sites within the Constitutive photomorphogenic homolog subunit 8 (*COPS8*), phosphoinositide-3-kinase, regulatory subunit 5 (*PIK3R5*), 3-hydroxyanthranilate 3,4-dioxygenase (*HAAO*), coiled-coil domain

containing 124 (*CCDC124*) and chromosome 5 open reading frame 34 (*C5orf34*) genes, were identified and validated using pyrosequencing. Since blood for DNA methylation profiling was collected prior to GDM diagnosis, these CpG sites may prove useful as predictive biomarkers for GDM. However, their candidacy as biomarkers require validation in larger studies.

6.2.Limitations of DNA Methylation

Although studies show that DNA methylation has potential as a diagnostic and prognostic biomarker, they are not without limitations (Levenson & Melnikov, 2012). Several factors including small sample size, lack of validation, differences in ethnicity, method of quantification and timing of methylation analysis during pregnancy, hinder reproducibility of findings across studies. Another limitation of the studies included in this review is the use of whole blood, which consist of a mixture of cell types such as lymphocytes, erythrocytes and platelets, and may confound methylation analysis (Reinius et al., 2012). Thus, future studies should consider purification of blood cell populations to separate specific cell types. Currently, there is no consensus on the best method to use for DNA methylation analysis. While global DNA methylation can easily be measured using crude DNA preparations, it is a measure of overall genomic methylation and does not offer the resolution required to detect subtle DNA methylation differences within genes (Dahl & Guldberg, 2003). In contrast, locus-specific DNA methylation such as bead chip arrays and pyrosequencing are expensive, requiring sophisticated equipment and bioinformatics expertise.

Table 5.2. Studies investigating DNA methylation in whole blood during Gestational diabetes mellitus.

Author	Study design	Country	Detection method	Main finding
(Dias, Adam, Wyk, et al., 2019)	63 GDM and 138 controls (~ 26 weeks gestation)	South Africa	Global DNA methylation using MDQ1 Imprint DNA Quantification Kit*	No difference in global DNA methylation between women with or without GDM. Global DNA methylation was associated with obesity and serum adiponectin concentrations.
(Enquobahrie et al., 2015)	6 women with 2 consecutive pregnancies with & without GDM (<20 weeks gestation)	United States	Illumina HumanMethylation27 BeadChip	17 CpG sites were hypomethylated and 10 CpG sites were hypermethylated in relation to GDM status
(Kang et al., 2017)	8 GDM and 8 controls (end of pregnancy)	Taiwan	Illumina Infinium HumanMethylationEPIC BeadChip	200 differentially methylated CpGs corresponding to 151 genes identified in women with GDM compared to controls
(Kang et al., 2018)	8 GDM and 24 controls (end of pregnancy)	Taiwan	MethyLight qRT-PCR assay	Decreased methylation of IL-10 during GDM, which was associated with increased serum IL-10 concentrations
(Wu et al., 2018)	11 GDM and 11 controls (12-16 weeks gestation)	United Kingdom	Illumina HumanMethylation450 BeadChip (450K) array and bisulfite pyrosequencing	100 differentially methylated CpGs corresponding to 66 genes were identified. Differential DNA methylation at 5 CpGs were validated in 8 of the 11 GDM women

qRT-PCR: Quantitative real-time PCR; GDM: Gestational diabetes mellitus. *Sigma-Aldrich

7. MICRORNAS

MiRNAs are short, highly conserved non-coding RNA molecules approximately 22 nucleotides in length that are powerful mediators of biological function. They regulate gene expression through post-transcriptional mechanisms by binding to the 3' untranslated region (UTR) of messenger RNA (mRNA) inducing gene silencing through translational repression or mRNA degradation (Brennecke et al., 2005). This interaction is dependent on the complementarity of the miRNA to the 'miRNA seed region', a region of 7 or 8 nucleotides contained within 3' UTR of mRNA. MiRNA binding requires a number of nucleotides to match the sequence flanking the seed region to direct the specificity of miRNA:mRNA interactions (Lewis, Burge & Bartel, 2005; Peterson et al., 2014). Since their initial discovery in *Caenorhabditis elegans* in 1993 (Lee, Feinbaum & Ambros, 1993), over 2000 miRNAs have been identified in humans and is believed to regulate about one third of the genome (Hammond, 2015).

MiRNAs are master regulators that control many biological processes including cell proliferation, differentiation, apoptosis and development (Du, 2005). Moreover, they regulate genes involved in metabolic processes such as glucose homeostasis, insulin signaling, pancreatic beta cell function, lipid metabolism and inflammation (Sliwinska, Kasinska & Drzewoski, 2017). Their dysregulation have been reported during many metabolic conditions, including obesity, T2D and cardiovascular disease (Papageorgiou et al., 2012; He, Ding, et al., 2017; Iacomino & Siani, 2017). Although they exert their function intracellularly, several studies have identified extracellular circulating miRNAs, which has sparked interest in their use as biomarkers of disease (Creemers, Tijsen & Pinto, 2012). Circulating miRNAs are associated with various complexes such as lipoproteins, exosomes, apoptotic bodies, microvesicles and ribonucleoproteins such as Ago1-4 or nucleophosphin 1 (NPM1), which serve to

protect these miRNAs from nuclease degradation, and act as carriers to transport them to their target mRNAs. This suggests that miRNA function in cell-to-cell communication, regulating gene expression in neighbouring cells by either acting locally (paracrine or autocrine signalling) or at a distance (endocrine/exocrine) (Creemers, Tijsen & Pinto, 2012; Turchinovich, Weiz & Burwinkel, 2012).

7.1.MicroRNAs and Gestational Diabetes Mellitus

MiRNAs are important metabolic and developmental regulators during pregnancy and have been shown to play a role in the development of GDM. In 2013, genome-wide analysis demonstrated that more than 600 miRNAs are expressed in the placenta (Chen & Wang, 2013). Recently, Poirier et al. reviewed placental miRNAs that are dysregulated during pregnancy and GDM (Poirier et al., 2017). The placenta plays an important role in maternal metabolic adaptation to pregnancy, and differential expression of placental miRNAs are believed to partly underlie these physiological changes. Placental miRNAs are released into maternal circulation (Chim et al., 2008), thus these miRNAs hold potential as biomarkers of placental dysfunction and GDM. Studies reporting circulating miRNA expression during GDM are summarised in Table 5.3.

In 2011, Zhao et al. were the first to profile the expression of serum miRNAs during GDM (Zhao et al., 2011). Using Taqman low density arrays, followed by confirmation with individual qRT-PCR, they identified three miRNAs, miR-132, miR-29a and miR-222, that were significantly downregulated in Chinese women with GDM (n=24) compared to controls (n=24) (Zhao et al., 2011). The differential expression of miR-29a and miR-222 were validated in an internal and two external validation cohorts. These miRNAs are thought to play a role in glucose homeostasis, insulin sensitivity and beta-cell function (Zhao et al., 2011). Several studies in other populations have

replicated these experiments with conflicting results. Recently, Pheiffer et al. reported decreased expression of miR-132, miR-29a and miR-222 in the serum of South African women with GDM (n=28) compared to controls (n=53), however, only the latter was statistically significant (Pheiffer et al., 2018). These findings demonstrate that the expression of these serum miRNAs is shared across South African and Chinese populations. In contrast to Zhao et al., Tagoma et al. showed that miR-222 was increased in plasma of women with GDM (n=13) compared to controls (n=9) (Tagoma et al., 2018). Wander et al., observed no differences in the expression of miR-222 or miR-29a in the plasma of American Caucasian women with GDM (n=36) compared to controls (n=80) (Wander et al., 2017). These discrepancies may be due to differences in biological samples used (serum or plasma), gestational age, or other unknown factors not accounted for.

Zhu et al. used high-throughput sequencing and qRT-PCR to investigate miRNAs in pooled plasma samples of Chinese women with (n=10) or without (n=10) GDM between 16-19 weeks of gestation. Five miRNAs (miR-16, miR-17, miR-19a, miR-19b, miR-20a) were significantly upregulated in GDM compared to controls (Zhu et al., 2015). Bioinformatic analysis revealed that the targets of these miRNAs are associated with the MAPK, insulin, TGF-β and mTOR signalling pathways, providing insight into the role of these miRNAs in GDM. Cao et al. investigated miR-16, miR-17 and miR-20a in a larger cohort of Chinese women at 16-19 weeks, 20-24 weeks and 24-28 week of gestation and found sustained increased expression in the plasma of women with GDM (n=85) compared to controls (n=72) at all the measured time points. However, they did not observe differences in the expression of miR-19a and miR-19b (Cao et al., 2017), as previously reported by Zhu et al. More recently, Pheiffer et al. reported conflicting results. The expression of all five miRNAs were decreased in

South African women with GDM, however, only the decreased expression of miR-20a was statistically significant (Pheiffer et al., 2018).

Functional analyses of these miRNAs have provided support for their role in the development of GDM (He, Bai, et al., 2017; Sebastiani et al., 2017; Lamadrid-Romero et al., 2018; Stirm et al., 2018). Many other miRNAs have been reported to exhibit altered expression during GDM, although these were identified in single studies only (Table 5.3).

7.2. Limitations of Circulating microRNA Profiling

The studies reviewed above highlight several miRNA candidates as biomarkers for GDM. However, the results are often discordant, possibly due to the different sample types and size, gestational age and the methods of analysis used.

Differences in miRNA expression have been reported in serum and plasma, suggesting that factors during the coagulation process could influence expression (Wang, Yuan, et al., 2012). Currently, there is no consensus on the best quantification method to use when profiling circulating miRNAs. Different methods of quantification are known to vary in sensitivity and specificity (Dias et al., 2017), which may impact the accuracy and interpretation of the data. Moreover, data normalization presents a significant challenge for the analysis of circulating miRNA profiling. Although strategies using exogenous miRNAs such as *C. elegans* miR-39 have proven to be less variable than endogenous reference genes, no ideal normalization strategy exists (Schwarzenbach et al., 2015). Thus, standardised guidelines for miRNA profiling would aid in the biological interpretation of miRNA data.

Table 5.3. Studies investigating circulating microRNAs during gestational diabetes mellitus.

Author	Study design	Country	Biological source	Detection method	Up-regulated	Down-regulated	No significant change	Normalization control
(Zhao et al., 2011)	24 GDM and 24 controls (16-19 weeks gestation); 36 GDM and 36 controls (Internal validation) 16 GDM and 16 controls (External validation)	China	Serum	Taqman low density array, qRT-PCR	-	miR-29a miR-132 miR-222	-	Cel-miR-39 (exogenous control)
(Pheiffer et al., 2018)	28 GDM and 53 controls (13-31 weeks gestation)	South Africa	Serum	qRT-PCR	-	miR-20a miR-222	miR-16 miR-17 miR-19a miR-19b miR-29a miR-132	Cel-miR-39 (exogenous control)
(Tagoma et al., 2018)	13 GDM and 9 controls (23-31 weeks gestation)	Estonia	Plasma	qRT-PCR	let-7e let-7g miR-100 miR-101	-	-	Cel-miR-39 (exogenous control)

					miR-146a miR-8a miR-195 miR-222 miR-23b miR-30b miR-30c miR-30d miR-342 miR-423 miR-92a		
(Wander et al., 2017)	36 GDM and 80 controls (7-23 weeks gestation)	United states	Plasma	qRT-PCR	miR-155 miR-21	miR-146b miR-517 miR-222 miR-210 miR-518a miR-29a miR-223 miR-126	Cel-miR-39 (exogenous control) and miR-423 (endogenous control)

(Zhu et al., 2015)	10 GDM and 10 controls (16-19 weeks gestation)	China	Plasma	Ion Torrent sequencing, qRT-PCR	miR-16 miR-17 miR-19a miR-19b miR-20a	-	-	miR-221 (endogenous control)
(Cao et al., 2017)	85 GDM and 72 controls (16-20, 20-24 and 24-28 weeks gestation)	China	Plasma	qRT-PCR	miR-16 miR-17 miR-20a	-	miR-19a miR-19b	RNU6 (endogenous control)
(Sebastiani et al., 2017)	21 GDM and 10 controls (24-33 weeks gestation)	Italy	Plasma	qRT-PCR	miR-330	-	miR-548c	miR-374 miR-320 (endogenous control)
(Stirm et al., 2018)	30 GDM and 30 controls (24-32 weeks gestation)	Germany	Whole blood	qRT-PCR	miR-340	-	-	RNU6B (endogenous control)
(He, Bai, et al., 2017)	20 GDM and 20 controls	China	Whole blood	qRT-PCR	-	miR-494	-	RNU6 (endogenous control)

(Lamadrid-Romero et al., 2018)	67 GDM and 74 controls (16-20, 20-24 and 24-28)	Not reported	Serum	qRT-PCR	miR-183 miR-200b miR-125b miR-1290	-	-	Cel-miR-39 (exogenous control)

8. CURRENT PERSPECTIVES AND FUTURE RECOMMENDATIONS

Advances in molecular biology have resulted in the identification of several molecular biomarkers for disease. Of these, genetic variants, DNA methylation and miRNAs are widely studied during GDM (Shaat & Groop, 2007; Georgiou et al., 2008; Moen et al., 2017). These molecular markers are stably expressed in biological fluids and hold potential as diagnostic or prognostic biomarkers of GDM. As reviewed above, many studies have provided evidence to support the use of these markers as biomarkers of GDM. However, despite these favourable results, molecular biomarkers face many challenges, which hinder their candidacy as biomarkers, and that must be addressed before they can be used clinically. As outlined above, SNPs, DNA methylation and miRNAs are all impacted by ethnicity and environmental factors. Furthermore, technical challenges during analysis contribute to inaccurate data and lack of reproducibility. Thus, standardization of analytical methods is critical when profiling molecular biomarkers. Moreover, large prospective cohort studies, conducted in populations with different ethnicities and environmental factors, are warranted to identify robust markers that are not influenced by these factors. The ideal biomarker for GDM would most likely be a combination of several molecular biomarkers to overcome the lack of sensitivity and specificity of individual factors. For example, a single miRNA regulates up to 200 different genes (Krek et al., 2005), thus miRNAs found to be associated with GDM, are non-specific and may possibly be involved in other conditions as well. To increase the predictive power of molecular biomarkers, future studies should consider using a combination of these markers in risk stratification models for predicting GDM risk.

9. CONCLUSION

GDM is a growing public health problem worldwide. The short and long-term consequences of GDM are likely to have an immediate negative impact on health systems and in addition, present a major reservoir of future disease. Screening and treatment of GDM leads to improved pregnancy outcomes (Duran et al., 2014), thus universal screening is widely advocated as a strategy to prevent adverse consequences. A growing body of evidence support the use of SNPs, DNA methylation and miRNAs as biomarkers that could aid in the early detection of GDM, thus facilitating intervention strategies to better manage GDM and improve health outcomes. Despite their potential, these molecular biomarkers face several challenges that need to be addressed before they can become clinically applicable. However, rapid technological advances could overcome these challenges and lead to the development of a quick, cost effective point-of-care test that could accurately identify women at high risk for GDM during early pregnancy. The establishment of an international body to standardize analytical conditions for molecular biomarkers, and large prospective cohort studies in different populations are required.

CHAPTER 6

GLOBAL DNA METHYLATION PROFILING IN PERIPHERAL BLOOD CELLS OF SOUTH AFRICAN WOMEN WITH GESTATIONAL DIABETES MELLITUS

Adapted from:

Dias, S., Adam, S., Van Wyk, N., Rheeder, P., Louw, J. & Pheiffer, C. 2019. Global DNA methylation profiling in peripheral blood cells of South African women with gestational diabetes mellitus. Biomarkers. 24(3):225–231. DOI: 10.1080/1354750X.2018.1539770. *(Original Article)*

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1. ABSTRACT

Background/Objective: Recently, several studies have reported that DNA methylation changes in tissue are reflected in blood, sparking interest in the potential use of global DNA methylation as a biomarker for gestational diabetes mellitus (GDM). This study investigated whether global DNA methylation is associated with GDM in South African women.

Methods: Global DNA methylation was quantified in peripheral blood cells of women with (n=63) or without (n=138) GDM using the MDQ1 Imprint® DNA Quantification Kit.

Results: Global DNA methylation levels were not different between women with or without GDM and were not associated with fasting glucose nor insulin concentrations. However, levels were 18% (p=0.012) higher in obese compared to non-obese pregnant women, and inversely correlated with serum adiponectin concentrations (p=0.005).

Discussion: Contrary to our hypothesis, global DNA methylation was not associated with GDM in our population. These preliminary findings suggest that despite being a robust marker of overall genomic methylation that offers opportunities as a biomarker, global DNA methylation profiling may not offer the resolution required to detect methylation differences in the peripheral blood cells of women with GDM. Moreover, global DNA methylation in peripheral blood cells may not reflect changes in placental tissue. Further studies in a larger sample are required to explore the candidacy of a more targeted approach using gene-specific methylation as a biomarker for GDM in our population.

2. CLINICAL SIGNIFICANCE

- The prevalence of GDM is rapidly increasing globally
- The 75 g 2 hr oral glucose tolerance test remains the gold standard for GDM diagnosis, however, several challenges hamper its use
- Risk factor-based screening has poor sensitivity for detecting GDM
- Thus, a more robust, non-invasive, simple and cost-effective screening tool is needed
- Altered DNA methylation patterns in peripheral blood cells holds potential as a biomarker for GDM screening

3. INTRODUCTION

GDM, defined as glucose intolerance that is first diagnosed during the latter half of pregnancy, with return to normoglycemia after birth (WHO, 2013), is a significant source of morbidity and mortality. In SA, a middle-income country, the prevalence of GDM has rapidly increased over the last few years (Mamabolo et al., 2007; Adam & Rheeder, 2017; Macaulay et al., 2018), paralleling the rising obesity epidemic. Recently, it was reported that 69% of South African women over the age of 15 years were either overweight or obese (Statistics South Africa 2017), a serious cause for concern since obesity is a major risk factor for the development of GDM. GDM is associated with adverse perinatal outcomes (Jensen et al., 2000) and increases susceptibility to future metabolic disorders in both mothers and their offspring (Damm, 2009), thus posing a significant burden to the already struggling and over-burdened South African health system.

The oral glucose tolerance test (OGTT), conducted between 24-28 weeks of gestation is the gold standard for the diagnosis of GDM (WHO, 1999). However, the test is

cumbersome to conduct and has several challenges including high costs, requirement for fasting, multiple blood draws, and is associated with nausea and vomiting. Currently, universal screening for GDM is recommended for all pregnant women (Hod et al., 2015). However, due to limited resources, selective screening based on traditional GDM risk factors such as obesity (body mass index (BMI) ≥30 kg/m^2), advanced maternal age (>35 years), family history of diabetes, history of GDM, previous macrosomia (baby weighing ≥4000 g), glycosuria, or previous adverse pregnancy outcomes (congenital abnormalities, unexplained still birth or recurrent pregnancy loss) is often performed in low and middle income countries. Unfortunately, these risk factors have poor sensitivity for detecting GDM in our population (Adam & Rheeder, 2017), resulting in a large number of GDM cases being missed. The identification of simple and cost-effective biomarkers to detect women with GDM could offer an alternative to the OGTT. Although a number of circulating biomarkers such as adiponectin, sex hormone binding globulin, C-reactive protein (CRP) and glycosylated fibronectin have been explored as biomarkers for GDM, none have yet achieved clinical applicability (Smirnakis et al., in press; Nanda et al., 2011; Rasanen et al., 2013; Adam et al., 2018).

Epigenetics reflect gene-environment interactions and is increasingly being implicated in the pathophysiology of metabolic diseases (Gu et al., 2013; Martín-Núñez et al., 2014). DNA methylation, the most widely studied and best characterised epigenetic mechanism, refers to the addition of a methyl group to the fifth carbon position of a cytosine residue within CpG dinucleotides, often leading to transcriptional repression (Lim & Maher, 2010). The process is reversible thus offering opportunities for risk stratification and intervention and has accordingly received considerable interest as biomarkers of disease. Although both gene-specific and global DNA methylation profiling have been explored, global DNA methylation, which

gives an estimate of overall genomic methylation can be quantified using non-invasive, inexpensive and simple methods, thus making it an attractive target for biomarker discovery. Several studies have reported that global DNA methylation is altered during hyperglycaemia (Matsha et al., 2016; Pinzón-Cortés et al., 2017) and in placental tissue of women with GDM (Nomura et al., 2014; Reichetzeder et al., 2016). Interestingly, hyperglycaemia-related DNA methylation changes in blood have been reported to correlate with changes observed in pancreatic β-cells and insulin responsive tissue such as skeletal muscle, liver and adipose tissue, thus, offering opportunities to use peripheral blood for DNA methylation analysis (Heyn & Esteller, 2012; Dayeh et al., 2016; Geach, 2016). We hypothesised that global DNA methylation in peripheral blood cells of black South African women is altered during GDM, and accordingly has potential as a biomarker for GDM in our population.

4. MATERIALS AND METHODS

4.1.Participants

The study was approved by the Health Sciences Ethics Committee of the University of Pretoria (180/2012). This research forms part of larger study investigating screening strategies for GDM in a South African population (Adam & Rheeder, 2017). One thousand pregnant women of black ethnicity, who were less than 26 weeks pregnant and who had a singleton pregnancy were recruited at a primary care clinic in Johannesburg, SA. Written informed consent was obtained from all participants. At recruitment, random glucose and glycated haemoglobin (HbA1c) levels were measured using a glucometer (Roche Diagnostics, Mannheim, Germany) and the point-of-care Afinion system (Alere Technologies, Oslo, Norway), respectively. Women with random glucose or HbA1c concentrations more than 11.1 mmol/L or 6.5%, respectively, were excluded. All women included in the study were requested to return within two weeks for fasting blood glucose measurements, and whole blood and serum collection. Of the 1000 women who were recruited, only 554 were scheduled for the 75 g 2 hr OGTT at 24-28 weeks of gestation due to foetal loss, migration, loss to follow up and withdrawal of consent (Figure 6.1). For this case control study, women with (n=63) and without (n=138) GDM, who were Human Immunodeficiency Virus (HIV) negative were selected and matched according to age, BMI and gestational age as far as possible.

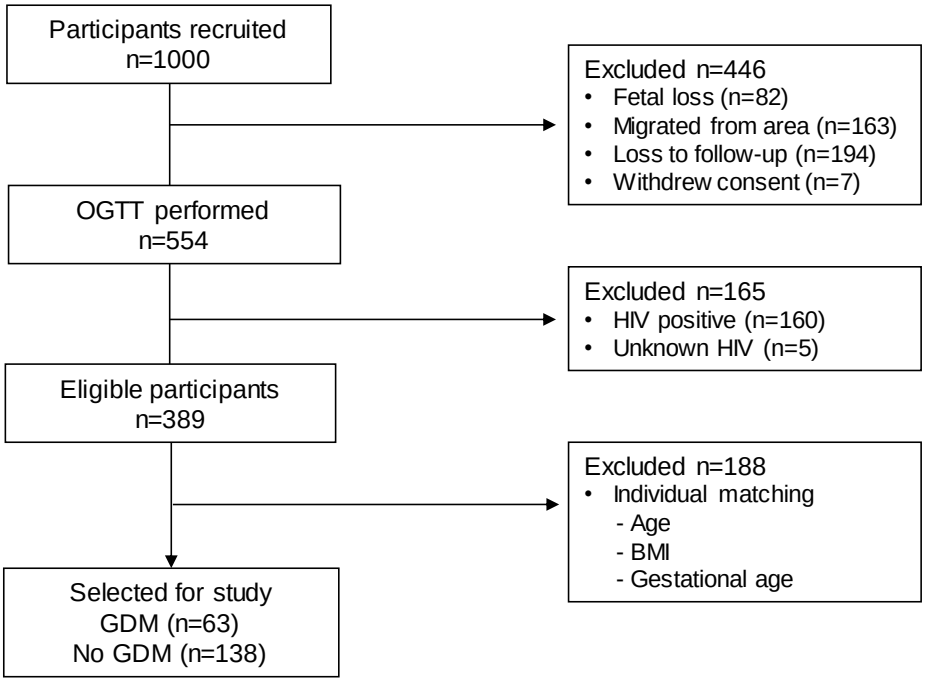

Figure 6.1. Flow diagram for study participants. For the current study, women with (n=63) or without (n=138) GDM were selected from a prospective cohort study in which 1000 pregnant women were recruited

4.2. Clinical and Biochemical Characteristics

Demographic information was obtained from a standardised questionnaire and anthropometric measurements were assessed according to standard procedures (Adam et al., 2018). The OGTT was conducted according to the International Association of Diabetes in Pregnancy Study Group (IADPSG) criteria (IADPSG panel, 2010). Briefly, women were given a 75 g glucose drink to ingest, and blood was collected for glucose measurements at 0 hr, 1 hr and 2 hr. At the time of OGTT, HbA1c concentrations were measured again by an accredited laboratory (Vermaak and Partners, Pretoria, South Africa). For comparative analysis, GDM was classified using the National Institute for Health and Care Excellence (NICE) and the World Health Organisation (WHO) 1999 criteria (Table 6.1) (WHO, 1999; NICE guidelines, 2015).

Fasting insulin and C-reactive protein (CRP) concentrations were measured in stored serum samples (Pathcare laboratories, Cape Town, South Africa). Serum adiponectin concentrations were quantified using the human adiponectin enzyme-linked immunosorbent assay (ELISA) (Merck, Darmstadt, Germany). The homeostatic model assessment (HOMA), a measure of insulin resistance was calculated using the equation: (fasting glucose in mmol/L x fasting insulin in mIU/mL)/22.5, using fasting plasma glucose and fasting serum insulin concentrations. Whole Blood for DNA methylation analysis was stored at -80^{o} C.

Table 6.1. GDM diagnostic criteria commonly used in South Africa

	Glucose concentration (mmol/L)		
Time[a]	IADPSG[b]	NICE[b]	WHO 1999[c]
0 hr OGTT	≥ 5.1 ≤ 7.0	≥ 5.6	≤ 7.0
1 hr OGTT	≥ 10	-	-
2 hr OGTT	≥ 8.5 ≤ 11.1	≥ 7.8	≥ 7.8

[a]Time after ingesting 75 g glucose drink; OGTT: oral glucose tolerance test; hr: hour; GDM: gestational diabetes mellitus; Impaired glucose tolerance: elevated glucose levels during pregnancy, that is not high enough to be classified as pre-existing or overt diabetes; IADPSG: International Association of Diabetes in Pregnancy Study Group; NICE: National Institute for Health and Care Excellence; WHO: World Health Organisation (WHO, 1999; IADPSG panel, 2010; NICE guidelines, 2015).
[b]One or more of these values are used to diagnose GDM.
[c]GDM diagnosed using impaired glucose tolerance.

4.3. Global DNA Methylation

DNA was extracted from 2 ml of stored whole blood in Ethylenediaminetetraacetic acid (EDTA) tubes using the QIAmp DNA Blood Midi Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany), and concentrations were measured using the Qubit Fluorometer dsDNA Broad Range Assay Kit (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Global DNA methylation

was quantified using the MDQ1 Imprint® Methylated DNA Quantification Kit according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, USA) as previously described (Pheiffer et al., 2014). Briefly, 100 ng of DNA was allowed to bind to the ELISA plate, where after the methylated fraction of DNA was detected using a 5-methylcytosine monoclonal antibody, and the absorbance was measured at 450 nm on a BioTek® ELX 800 plate reader (BioTek Instruments Inc., Winooski, USA). Global DNA methylation levels were calculated relative to the methylated positive control which was included in the kit. All samples were analysed in duplicate.

4.4. Statistical Analysis

Statistical analysis was conducted using STATA 14 (StataCorp, Texas, USA). Data were expressed as the mean ± standard error of the mean (SEM), or as the median and interquartile range (25^{th} and 75^{th} percentiles) for data that were not normally distributed. Categorical data were expressed as count (n) and percentage (%). The Shapiro-Wilk test was used to test for normality. The unpaired student t test or the Mann-Whitney test was used to compare variables across GDM groups, and the Chi-square test was used to analyse categorical variables. Spearman's rank correlation (r_s) was used to evaluate the relationship between global DNA methylation and serum adiponectin concentrations. A $p \leq 0.05$ was considered statistically significant.

5. RESULTS

The clinical characteristics of participants are presented in Table 6.2. As expected, fasting blood glucose ($p<0.001$), 1 hr OGTT ($p<0.001$), 2 hr OGTT ($p<0.001$) and HbA1c ($p=0.008$) concentrations were significantly higher in women with GDM compared to women without GDM. Similarly, fasting insulin ($p=0.067$) and HOMA ($p<0.001$) levels were increased in women with GDM. In contrast, women with GDM had lower concentrations of serum adiponectin than women without GDM ($p=0.018$).

Since the extent of hyperglycaemia may influence the association between global DNA methylation and GDM, GDM was classified using IADPSG, WHO and NICE criteria. Glucose concentrations differed significantly between women with GDM compared to women without GDM, using all three diagnostic criteria (Table 6.3). Fasting plasma glucose values were significantly lower in women with GDM using the WHO criteria compared to the IADPSG ($p=0.014$) and NICE ($p=0.005$) criteria, while the 2 hr OGTT values were significantly higher in women with GDM using the WHO criteria compared to the IADPSG criteria and NICE criteria ($p<0.001$) (Table 6.3).

However, no difference in global DNA methylation levels between women with or without GDM were observed when the different diagnostic criteria were used ($p>0.05$) (Figure 6.2 a-c). Global DNA methylation levels were 18% ($p=0.012$) higher in obese compared to non-obese pregnant women (Figure 6.3) and were inversely correlated with serum adiponectin concentrations ($r_s=-0.243$, $p=0.005$) (Figure 6.4).

Table 6.2. Clinical characteristics of the study population stratified according to GDM using the IADPSG criteria

Variable	GDM (n=63)	No GDM (n=138)	*p*-value
Age (years)[a]	28.0 (24.0-32.0)	28.0 (24.0-32.0)	0.810
BMI (kg/m^2)[a]	27.4 (23.4-31.2)	25.8 (23.5-29.8)	0.180
Fasting glucose (mmol/L)[a]	5.5 (5.2-5.9)	4.3 (4.0-4.6)	<0.001
OGTT 1 hr (mmol/L)[a]	6.5 (5.5-8.3)	5.5 (4.7-6.5)	<0.001
OGTT 2 hr (mmol/L)[a]	6.1 (5.2-7.2)	5.2 (4.6-5.9)	<0.001
HbA1c (%)[b]	5.2 (0.4)	5.1 (0.3)	0.008
Fasting insulin (mIU/L)[a]	6.5 (4.8-9.4)	5.7 (3.8-8.0)	0.067
HOMA[a]	1.6 (1.2-2.4)	1.1 (0.8-1.7)	<0.001
C-reactive protein (mg/L)[a]	6.9 (3.7-9.9)	5.4 (3.1-8.5)	0.209
Adiponectin (μg/ml)[a]	7.6 (4.9-11.8)	9.8 (6.6-14.7)	0.018
Education: n (%)[c]			
<grade 12	29.0 (46.7)	66.0 (49.6)	0.769
≥grade 12	33.0 (53.3)	67.0 (50.4)	
Risk factors: n (%)[c]			
None	27.0 (42.8)	79.0 (57.3)	0.143
≥1 Risk factors n (%)	36.0 (57.2)	59.0 (42.7)	

BMI: body mass index; OGTT: oral glucose tolerance test; HbA1c: glycated haemoglobin; HOMA: homeostatic model assessment. Data are expressed as the [a]median (25th–75th percentiles); [b]mean ± standard error of the mean or as [c]count (percentage). P-values for continuous data were calculated using the Mann-Whitney or the unpaired student t test. P-values for categorical data were calculated using the Chi-square test.

Table 6.3. GDM defined using the IADPSG, NICE and WHO 1999 diagnostic criteria

	Glucose concentration (mmol/L)								
	IADPSG			NICE			WHO 1999		
Time*	GDM	No GDM	*p*-value‡	GDM	No GDM	*p*-value‡	GDM	No GDM	*p*-value‡
n	63	138		41	160		13	188	
0 hr OGTT	5.5 (5.2-5.9)[a]	4.3 (4.0-4.6)	<0.001	5.8 (5.5-6.0)[b]	4.4 (4.0-4.8)	<0.001	5.2 (4.7-5.4)[a,b]	4.5 (4.1-5.1)	<0.05
1 hr OGTT	6.5 (5.5-8.3)	5.5 (4.7-6.5)	<0.001	-	-		-	-	
2 hr OGTT	6.1 (5.2-7.2)[c]	5.2 (4.6-5.9)	<0.001	6.6 (5.6-8.0)[d]	5.3 (4.7-5.9)	<0.001	8.6 (8.0-9.7)[c,d]	5.3 (4.7-6.0)	<0.001

*Time after ingesting 75 g glucose drink; ‡significant difference between women with or without GDM in each diagnostic criteria; similar superscripts indicate significant difference between groups; OGTT: oral glucose tolerance test; hr: hour; GDM: gestational diabetes mellitus; IADPSG: International Association of Diabetes in Pregnancy Study Group; NICE: National Institute for Health and Care Excellence; WHO: World Health Organisation (WHO, 1999; IADPSG panel, 2010; NICE guidelines, 2015). Data are expressed as median (25th–75th percentiles).

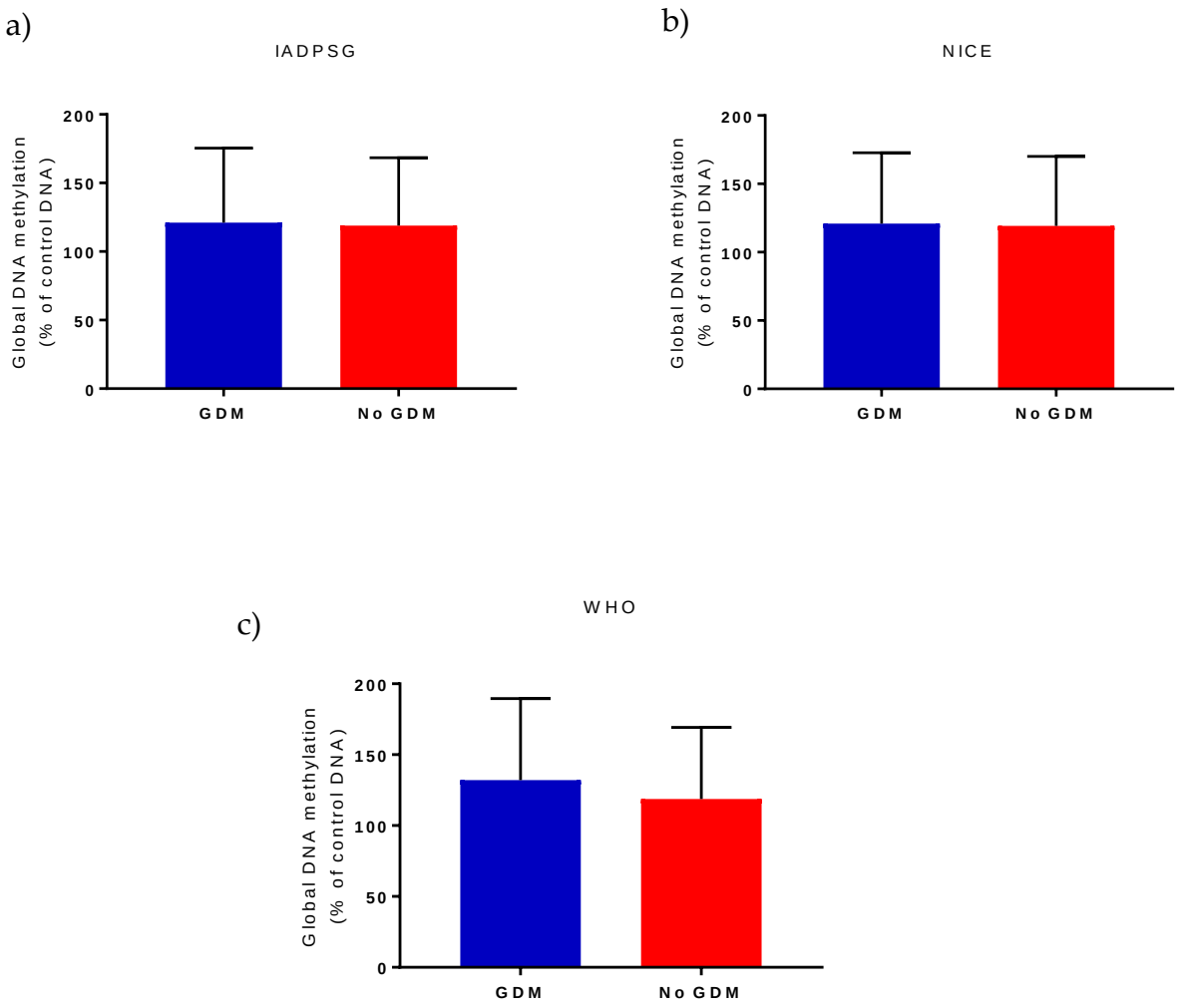

Figure 6.2. Global DNA methylation levels according to GDM status. Global DNA methylation was measured in the peripheral blood of women with or without GDM according to the IADPSG (GDM: n=63; non-GDM: n=138) (a), the NICE (GDM: n=41; non-GDM: n=160) (b) and the WHO (GDM: n=13; non-GDM: n=188) (c) criteria, using the MDQ1 Imprint® Methylated DNA Quantification Kit. Global DNA methylation levels were calculated relative to the methylated positive control which was included in the kit. Data are represented as the mean ± standard error of mean (SEM)

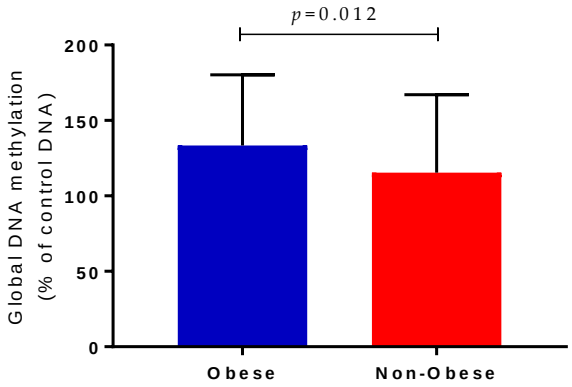

Figure 6.3. Global DNA methylation levels varies according to obesity status. Global DNA methylation was measured in the peripheral blood of obese (n=51) and non-obese (n=138) pregnant women using the MDQ1 Imprint® Methylated DNA Quantification Kit. Global DNA methylation levels were calculated relative to the methylated positive control which was included in the kit. Data are represented as the mean ± standard error of mean (SEM)

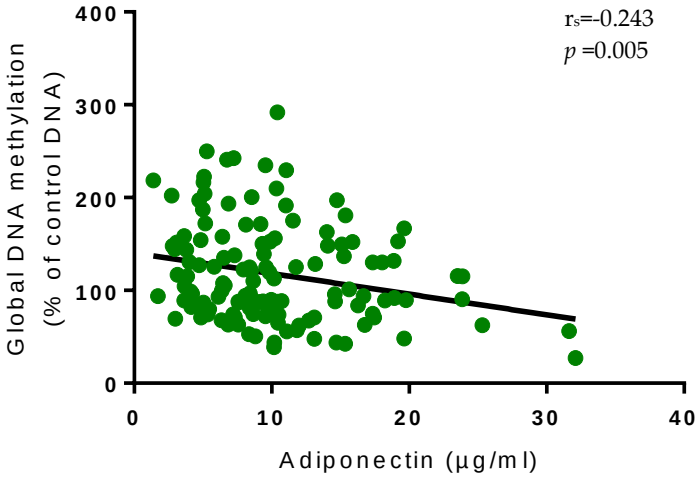

Figure 6.4. Global DNA methylation is inversely correlated with serum adiponectin concentrations. Each data point represents an individual (n=139) and indicates global DNA methylation levels relative to a methylated positive control and adiponectin concentration.

6. DISCUSSION

This study investigated whether global DNA methylation profiling has potential as a screening tool for GDM in black South African women. Contrary to our hypothesis, global DNA methylation levels in peripheral blood cells of black South African women were not associated with GDM. The IADPSG criteria is stringent, and the failure to observe differences in methylation between women with or without GDM may be due to small glucose concentration differences between groups. However, no difference in methylation was observed when GDM was classified according to NICE and WHO criteria, where glucose concentration differences between women with or without GDM were more pronounced. Previous studies have reported that global DNA methylation is associated with GDM (Nomura et al., 2014; Reichetzeder et al., 2016), however, these were conducted on placental tissue and used different methods to quantify global DNA methylation, possibly accounting for the discrepancies observed. Biological source affects global DNA methylation (Reinius et al., 2012), thus our failure to observe an association between GDM and global DNA methylation could be due to the use of peripheral blood cells rather than placenta. Furthermore, using liquid chromatography–mass spectrometry Reichetzeder et al. demonstrated that placental DNA methylation was increased during GDM (Reichetzeder et al., 2016), while using a luminometric methylation assay Nomura et al. reported that placental DNA methylation is decreased during GDM (Nomura et al., 2014), illustrating that method of quantification influences results. The ELISA, as used in this study, offers several advantages over other methods of quantifying global DNA methylation. It is cost-effective and does not require specialized equipment and expertise, making it more amenable for screening in low-and middle income countries (Kurdyukov & Bullock, 2016). Several studies have reported that the ELISA is able to detect aberrant global DNA methylation patterns during disease (Nakano, Boyle &

Firestein, 2012; Keller et al., 2014; Kagohara et al., 2015; Ramos et al., 2016), and in response to environmental factors (Guerrero-Preston et al., 2010; Tellez-Plaza et al., 2014; Ivorra et al., 2015; Sánchez, Reynoso-Camacho & Salgado, 2015).

Global DNA methylation levels were higher in obese compared to non-obese pregnant women. It has been widely reported that global DNA methylation is associated with obesity (Cash et al., 2011; Jintaridth et al., 2013; Piyathilake et al., 2013; Na et al., 2014), however studies in pregnant women are limited (Herbstman et al., 2013; Nomura et al., 2014). Consistent with our results, Nomura et al. reported that global DNA methylation was higher in obese compared to non-obese pregnant women (Nomura et al., 2014). However, in contrast to our findings, Herbstman et al. reported that global DNA methylation is decreased during pre-pregnancy obesity, while Michels et al. failed to see an association between global DNA methylation and obesity (Michels, Harris & Barault, 2011; Herbstman et al., 2013). These findings confirm the variability in assessing global DNA methylation levels according to biological source and methods of quantification.

Intriguingly, global DNA methylation was inversely correlated with serum adiponectin concentrations. Adiponectin is an adipokine with insulin-sensitising properties, which is dysregulated during obesity and metabolic disease (Cao, 2014). Similar to our findings, several studies have reported that adiponectin concentrations are decreased during GDM (Lacroix et al., 2013; Pala et al., 2015; Adam et al., 2018). Recently, it was reported that altered methylation at the adiponectin gene *(ADIPOQ*) locus is inversely correlated with circulating adiponectin concentrations during pregnancy (Bouchard et al., 2012), and is associated with decreased *ADIPOQ* gene expression levels in adult offspring of women with GDM (Houshmand-Oeregaard et al., 2017). To further explore the significance of the association between global DNA

methylation and adiponectin, pyrosequencing of *ADIPOQ* is currently being conducted in our laboratory.

Although quantification of global DNA methylation is a robust method to assess overall genomic DNA methylation, and has potential as a biomarker to facilitate risk stratification and intervention (Ramos et al., 2016), it may not offer the resolution required to detect subtle methylation differences in women with or without GDM. Furthermore, although the ELISA used in this study may offer several advantages for biomarker screening, validation using techniques such as liquid chromatography-electrospray ionization/multi-stage mass spectrometry (LC-ESI/MS/MS), which was demonstrated to be a stable and reliable method to detect global placental DNA methylation (Dwi Putra et al., 2014), would support the clinical applicability of ELISA screening. Another limitation of the study is the use of peripheral blood cells, which consists of a mixture of different cell types such as erythrocytes, lymphocytes and platelets which may confound methylation analysis (Reinius et al., 2012). Future studies should consider purification of blood cell populations to separate specific cell types (Reinius et al., 2012). Although DNA methylation in dysglycaemia-related tissues may be reflected in blood (Heyn & Esteller, 2012; Bacos et al., 2016; Dayeh et al., 2016), it should be noted that DNA methylation is considered to be highly tissue specific, and may not reflect changes in placental tissue.

It has been widely reported that DNA methylation is affected by environmental factors such as diet, smoking, alcohol consumption and physical activity (Joubert et al., 2012; Lim & Song, 2012; Ling & Rönn, 2014; Pauwels et al., 2017; Miyake et al., 2018). Thus, the lack to account for these environmental factors, pose a significant limitation to our study. However, women were recruited from the same community with similar life experiences, suggesting that they were likely to have similar

environmental exposures. In addition, women with and without GDM were not matched for age, BMI and gestational age when re-classifying them according the NICE and WHO 1999 criteria, which may have potential impact on DNA methylation patterns, and therefore presents another limitation.

7. CONCLUSION

Contrary to our hypothesis, global DNA methylation was not associated with GDM in our population. These preliminary findings suggest that despite being a robust marker of overall genomic methylation that offers opportunities as a biomarker, global DNA methylation profiling may not offer the resolution required to detect subtle methylation differences in the peripheral blood cells of women with GDM. Further studies in a larger sample are required to explore the candidacy of a more targeted approach using gene-specific methylation as a biomarker for GDM in our population. To our knowledge, this is the first study to investigate the association between global DNA methylation and GDM in SA.

CHAPTER 7

ALTERED GENOME-WIDE DNA METHYLATION IN PERIPHERAL BLOOD OF SOUTH AFRICAN WOMEN WITH GESTATIONAL DIABETES MELLITUS

Adapted from:

Dias, S., Adam, S., Rheeder, P., Louw, J. & Pheiffer, C. 2019. Altered Genome-Wide DNA Methylation in Peripheral Blood of South African Women with Gestational Diabetes Mellitus. International Journal of Molecular Sciences. 20(23):5828. DOI: 10.3390/ijms20235828. *(Original Article)*

TABLE OF CONTENTS

1. Abstract

Increasing evidence implicate altered DNA methylation in the pathophysiology of gestational diabetes mellitus (GDM). This exploratory study probed the association between GDM and peripheral blood DNA methylation patterns in South African women. Genome-wide DNA methylation profiling was conducted in women with (n=12) or without (n=12) GDM using the Illumina Infinium HumanMethylationEPIC BeadChip array. Functional analysis of differentially methylated genes was conducted using Gene Ontology and Kyoto encyclopaedia of genes and genomes pathway analyses. A total of 1046 CpG sites (associated with 939 genes) was differentially methylated between GDM and non-GDM groups. Enriched pathways included GDM-related pathways such as insulin resistance, glucose metabolism and inflammation. DNA methylation of the top five CpG loci showed distinct methylation patterns in GDM and non-GDM groups and was correlated with glucose concentrations. Of these, one CpG site mapped to the calmodulin binding transcription activator 1 (*CAMTA1*) gene, which have been shown to regulate insulin production and secretion and may offer potential as an epigenetic biomarker in our population. Further validation using pyrosequencing and conducting longitudinal studies in large sample sizes and in different populations are required to investigate their candidacy as biomarkers of GDM.

2. INTRODUCTION

GDM is defined as diabetes diagnosed in the second or third trimester of pregnancy that is not pre-existing or overt diabetes (American Diabetes Association, 2016). The prevalence of GDM is increasing, affecting approximately 14% of pregnancies globally (International Diabetes Federation, 2017), although rates vary between <1% and 28% according to the diagnostic criteria employed and population studied (Jiwani et al., 2012). GDM is associated with maternal (preeclampsia, caesarean section and birth injuries), foetal (macrosomia, shoulder dystocia, hyperinsulinemia, hypoglycaemia, hyperbilirubinemia) and perinatal (respiratory distress syndrome, metabolic derangements and jaundice) complications (Hod et al., 1991; Hadar & Hod, 2013; Moore, 2018), while both mothers and their offspring are at an increased risk of developing metabolic disease in later life (Kim, Newton & Knopp, 2002; Clausen et al., 2008; Zhao et al., 2016). Current estimates indicate that more than 50% of women with GDM develop type 2 diabetes (T2D) within 10 years, making GDM a strong predictor of T2D (Kim, Newton & Knopp, 2002; Damm et al., 2016). The identification of women with GDM who are at risk of developing T2D allows the introduction of timely measures to prevent or better manage disease progression.

Epigenetic mechanisms are increasingly being implicated in the pathophysiology of metabolic diseases, including GDM (Smith & Ryckman, 2015). DNA methylation, the most widely studied and best characterised epigenetic marker is a reversible process that refers to the addition of a methyl group to the fifth carbon position of a cytosine residue within a cytosine-phosphate-guanine (CpG) dinucleotide, and regulates gene expression through transcriptional mechanisms (Lim & Maher, 2010). Altered global and gene-specific DNA methylation are observed in the placenta of women with GDM (El Hajj et al., 2013; Reichetzeder et al., 2016). DNA methylation is a tissue specific

process, although recent evidence suggests that peripheral blood reflects DNA methylation in tissue (Willmer et al., 2018), while several studies report that maternal blood reflects pregnancy-associated DNA methylation changes (Enquobahrie et al., 2015; Kang et al., 2017; Wu et al., 2018), supporting its potential as epigenetic biomarkers for GDM.

DNA methylation during GDM has been studied using various techniques such as Enzyme Linked Immunosorbent Assays, Whole-genome bisulfite sequencing, Methylated DNA immunoprecipitation sequencing, Liquid chromatography coupled with mass spectrometry, Pyrosequencing, Bead Chip arrays and Methyl Light PCR (Reichetzeder et al., 2016; Haertle et al., 2017; Kang et al., 2017; Moen et al., 2017; Wu et al., 2018; Dias, Adam, Wyk, et al., 2019). Due to its comparatively low cost compared to sequencing, reproducibility and high sample throughput, Bead Chip arrays are currently the most widely used technique for genome-wide DNA methylation profiling (Pidsley et al., 2016; Nakabayashi, 2017). The current Bead Chip array version, the HumanMethylationEPIC, allows the interrogation of >850,000 CpG sites across the genome, enriched for promoters and enhancer sequences, covering 99% of RefSeq genes (McCartney et al., 2016). Previous versions, the HumanMethylation450 and HumanMethylation27, measured >480,000 and >27,000 CpG sites, respectively across the genome (Nakabayashi, 2017).

In South Africa (SA), the prevalence of GDM has increased from about 1.6% – 25.8% in recent years (Ranchod, Vaughan & Jarvis, 1991; Adam & Rheeder, 2017). The possible increase in future T2D cases will place a major burden on the already overburdened health system and creates an urgent need to identify preventative strategies. DNA methylation has attracted considerable interest as biomarkers that could facilitate risk stratification and offer opportunities for intervention strategies to

prevent or delay the development of T2D after pregnancy (Gillberg & Ling, 2015). The aim of this study is to explore the potential of DNA methylation to serve as biomarkers of GDM in black South African women. Genome-wide DNA methylation profiling was conducted in the peripheral blood of women with (n=12) or without (n=12) GDM using the Illumina methylationEPIC Bead Chip array. Functional analysis of differentially methylated genes was conducted to identify pathways associated with GDM in the South African population

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3. MATERIALS AND METHODS

3.1. Study Participants

Ethical approval for this study was granted by the University of Pretoria Health Sciences Ethics Committee (180/2012). The study was conducted according to the Declaration of Helsinki and all women gave written informed voluntary consent after the procedures had been fully explained in the language of their choice. One thousand pregnant women attending a primary care clinic in Johannesburg, SA were enrolled in the study. At recruitment, demographic and socio-economic data were obtained in the form of a standardised questionnaire and risk factors for GDM, i.e. advanced maternal age (age ≥35 years), obesity (BMI ≥30 kg/m^2), family history of diabetes mellitus, delivery of a previous baby more than four kilograms, glucosuria, previous recurrent pregnancy loss, stillbirth, or birth of a baby with congenital abnormalities) were assessed (Adam & Rheeder, 2017). Patients with pre-existing diabetes mellitus (Type 1 diabetes (T1D) and T2D) and those who were more than 26 weeks pregnant were excluded. At their first visit, random glucose and glycated haemoglobin (HbA1c) concentrations were measured. Women with random glucose and HbA1c concentrations less than 11.1 mmol/L and 6.5%, respectively, were requested to fast overnight and return to the clinic within two weeks. At this time, a 75 g oral glucose tolerance test (OGTT) was conducted, and GDM was diagnosed if at least one glucose value was met (fasting plasma glucose ≥5.1 mmol/L, 1 hr OGTT ≥10 mmol/L or 2 hr OGTT ≥8.5 mmol/L), according to the International Association of Diabetes in Pregnancy Study Group (IADPSG) criteria (IADPSG panel, 2010). Blood for measurement of adiponectin, C-reactive protein (CRP) and DNA methylation was collected at the first visit (<26 weeks of gestation) and stored at -80 ^{o}C. For this sub-study, a subset of women with (n=12) and without (n=12) GDM were selected for genome-wide DNA methylation analysis (Figure 7.1). The inclusion criteria were

pregnant women ≥18≤40 years of age, black ethnicity, human immunodeficiency virus (HIV) negative and women with a singleton pregnancy. All women were matched according to age, BMI and gestational age as far as possible.

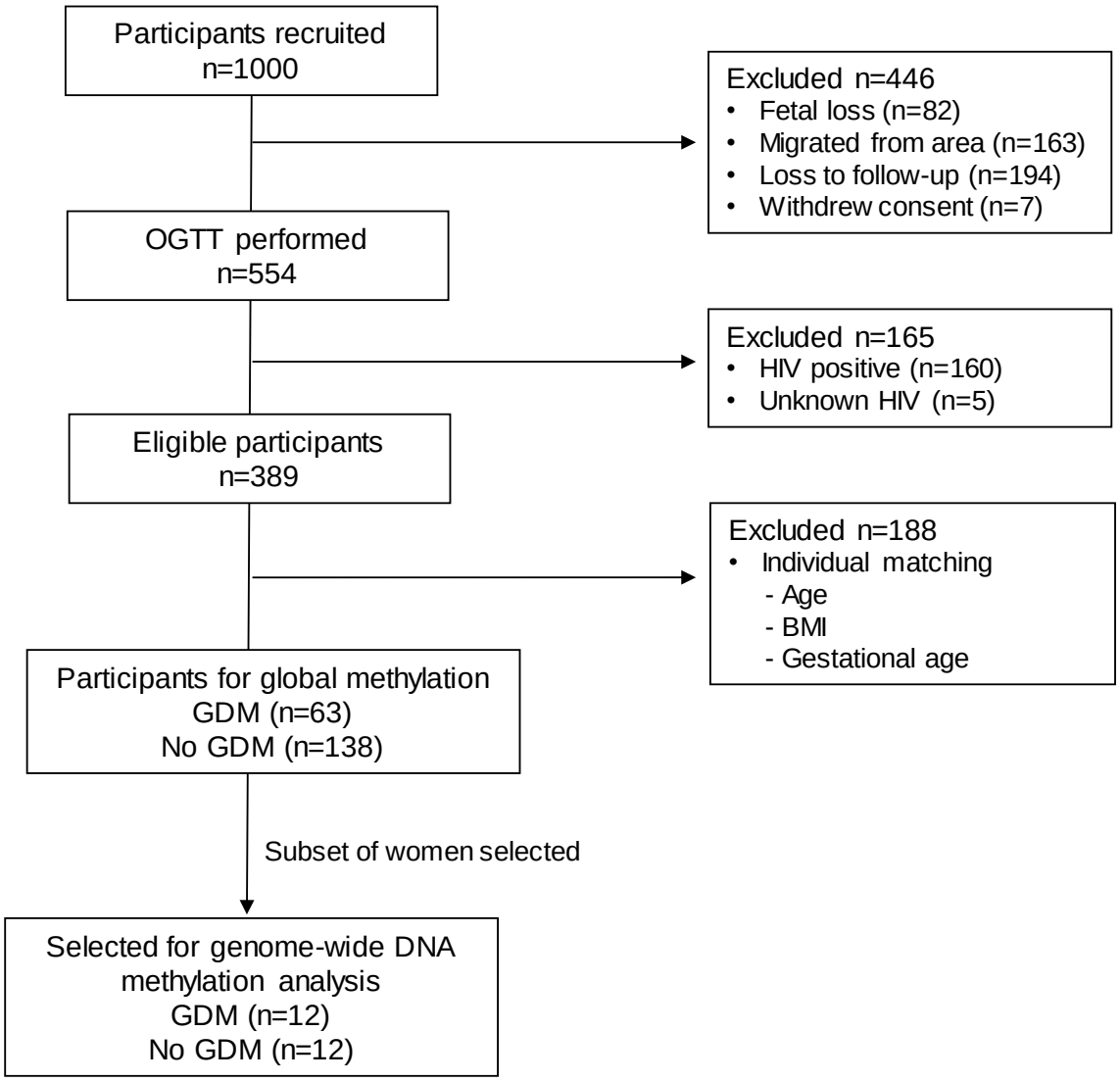


Figure 7.1. Flow diagram for study participants. For the current study, a subset of women with (n=12) and without GDM (n=12) were selected from a larger prospective cohort study.

3.2. DNA Extraction

Genomic DNA was extracted from 2 ml of peripheral blood collected in Ethylenediaminetetraacetic acid (EDTA) tubes using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany), as previously described (Dias, Adam, Wyk, et al., 2019). Briefly, white blood cells were lysed and loaded onto the QIAamp Midi column, bound DNA was washed and then eluted from the column membrane using 300 µl of elution buffer and centrifuged at 4500 x g for 2 mins. DNA concentration was measured using the Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and the Quanti-iT dsDNA Broad Range assay kit (ThermoFisher, Massachusetts, USA). One microgram of DNA in a volume of 45 µl was frozen and shipped on dry ice, as instructed by the University of Southern California Molecular Genomics Core (https://uscnorriscancer.usc.edu/core/molgen/) for genome-wide DNA methylation analysis using the Illumina Infinium HumanMethylationEPIC BeadChip (USC Molecular Genomics Core, Los Angeles, USA).

3.3. Genome-Wide DNA Methylation Profiling

Genome-wide DNA methylation profiling was conducted using the Illumina's Infinium HumanMethylationEPIC Bead Chip (HumanMethylationEPIC, Illumina Inc., San Diego, USA) according to manufacturer's instructions. Bisulfite conversion of 500 ng genomic DNA was performed using the Illumina-specific EZ DNA methylation kit (D5001, Zymo Research, Orange, USA), and quality control was conducted by quantitative real time PCR and melt curve analysis. Bisulfite converted DNA was amplified up to 1000-fold with DNA polymerase during the incubation step in the Illumina hybridization oven at 37 °C. Amplicons were then fragmented to 300-600 bp products, precipitated with isopropanol and loaded onto Illumina Infinium HumanMethylationEPIC Bead Chips prepared for hybridization in the capillary flow-

through chamber (Human MethylationEPIC, Illumina Inc.), according to the Infinium protocol (Illumina, Inc, n.d.). After annealing to locus-specific 50-mer probes, a single base extension occurs at the base immediately adjacent to the interrogated CpG site. Products were fluorescently labelled with either dinitrophenol-labelled ddATP/ddTTP or biotin-labelled ddCTP/ddGTP, depending on the methylation state of the interrogated CpG site. Fluorescence intensity was measured with the Illumina iScan system (iScan Control Software v.3.3.28) and was based on the ratio of methylated probe intensities and the overall intensity (sum of methylated and unmethylated probe intensities). The methylation scores were represented as raw beta- (β) values and were exported as 48 IDAT files.

3.4. Processing and Analysis of the Human MethylationEPIC Bead Chip Array

Data analysis was conducted by Partek (Partek, St. Louis, USA). IDAT files were imported to Partek (R) Genomics Suite (R) v.7.18.0803 software. Functional normalization with normal-exponential out-of-band (NOOB) background correction and dye correction was used (Fortin et al., 2014). Quality control was performed across all imported probes (865,859) for each sample. All samples passed the quality control, and those with detection $p<0.01$ were included in the analysis. Thereafter, β-values for imported probes were plotted and no outliers were detected, indicating that the data were technically sound. In addition, a histogram was used to illustrate distribution of methylation β-values across all CpG sites in each sample. Data filtering was conducted to remove polymorphic probes (n=22,139), cross-hybridising probes (n=40,762), non-CpG probes (n=1) and probes overlapping both the polymorphic and cross-hybridising probe lists (n=1,721) (Figure 3), according to McCartney et al. (McCartney et al., 2016). The clean data set consisted of 801,236 probes (referred to as CpG sites). Exploratory analysis was performed using principal component analysis (PCA). Cell

count estimation was performed empirically using methylation data from sorted blood cells using the 'Estimate Cell Count' function in the minfi package in R (Aryee et al., 2014). The function is based on a modification of the original method by Houseman et al. (Houseman et al., 2012) and the R package FlowSorted.Blood.450k (Jaffe, 2019). No differences in cell composition were identified, and cell composition was deemed unlikely to be a confounder (Figure S4). Therefore, cell composition was not corrected for in further analysis.

Following data processing, β-values were converted to M-values ($\log_2$ ratio [methylated signal intensity/unmethylated signal intensity]) to account for heteroscedasticity and allow for analyses assuming a Gaussian distribution (Du et al., 2010). M-values have a range of -∞ to +∞, with a value close to 0 indicating similar intensities between methylated and unmethylated probes. Positive M-values represent hyper-, while negative M-values represent hypo-methylation. M-values were then standardised (converted to Z-scores) to perform hierarchical clustering, using Euclidean distance and average linkage criteria for visualization of methylation signatures.

3.5.Functional Enrichment Analysis

All differentially methylated CpG sites were annotated to genes using the reference sequence database (RefSeq) build 87 and were subjected to functional analysis using Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis and Gene Ontology (GO) grouping categories (biological process, cellular component, and molecular function). The results of enriched pathways were ranked by enrichment scores to identify overrepresented pathways and then sorted by factor score to consider those pathways with the most significant p-value. A high enrichment score indicates that a significant number of the differentially methylated genes within a

pathway are present, while factor score enables comparison of pathways with similar enrichment scores between GDM and non-GDM groups.

3.6. Statistical Analysis

Participant characteristics were tested for normality using the Shapiro-Wilk test in STATA 14 (StataCorp, College Station, USA). Normally distributed data are expressed as the mean ± standard error of the mean (SEM), or as the median and interquartile range (25th and 75th percentiles) for data that were not normally distributed. An unpaired t-test or the Mann-Whitney test was used to compare variables across GDM groups. Categorical variables were analysed using the Chi-square test or the Fisher's exact test if the frequency was <5. A $p \leq 0.05$ was considered statistically significant. Due to the matched case control study design, a two-way analysis of variance ANOVA (one factor was the GDM status and the other was the pairing ID), was used to identify differentially methylated sites. To investigate the association between GDM and differentially methylated CpGs, univariate and multivariate generalised linear regression models were tested and adjust for confounding factors. Pearson's rank correlation (r) was used to evaluate the relationship between specific CpG DNA methylation (β-values; 0-1, as a percentage of methylated to unmethylated) states and clinical parameters. Pathway enrichment was based on the current publicly available human database, GRCh38, and statistical significance was calculated using Fisher's exact test. An enrichment score ≥3 was considered significant ($p<0.05$).

4. RESULTS

4.1. Study Participants

Participant characteristics are presented in Table 7.1. As expected, no difference in age, gestational age and BMI was observed between women with or without GDM. Women with GDM had significantly higher fasting ($p<0.001$) and 1 hr OGTT ($p<0.01$) glucose concentrations compared to women without GDM, while 2 hr OGTT ($p=0.07$) glucose concentrations showed a trend towards significance. In addition, fasting insulin concentrations, homeostatic model of assessment (HOMA), CRP levels were higher in women with GDM compared to women without GDM, although these were not statistically significant. No difference between groups were observed for HbA1c and adiponectin concentrations, nor for common risk factors (advanced maternal age (age ≥35 years), obesity (BMI ≥30 kg/m2), family history of diabetes mellitus, delivery of a previous baby more than four kg, glucosuria, previous recurrent pregnancy loss, stillbirth, or birth of a baby with congenital abnormalities), as well as education and employment status.

Table 7.1. Participant characteristics

Variables	Non-GDM (n=12)	GDM (n=12)	p-value
Age (years)[a]	27.3 (0.3)	27.3 (0.3)	1.00
Gestational age (weeks)[a]	19.3 (1.5)	19.3 (2.0)	1.00
BMI (kg/m²)[a]	27.1 (1.3)	27.6 (1.1)	0.77
Fasting glucose (mmol/L)[a]	4.3 (0.1)	5.5 (0.1)	<0.001
1 hr OGTT (mmol/L)[a]	5.2 (0.3)	6.6 (0.4)	0.01
2 hr OGTT (mmol/L)[a]	5.2 (0.3)	5.8 (0.3)	0.07
HbA1c (%)[a]	5.1 (0.1)	5.1 (0.1)	0.85
Fasting insulin (mIU/L)[b]	8 (7.5-9.0)	10.2 (6.3-12.7)	0.65
HOMA[b]	1.6 (1.6-1.8)	2.6 (1.5-2.9)	0.31
Adiponectin (μg/ml)[b]	10.4 (7.3-23.8)	9.7 (4.7-12.0)	0.28
C-reactive protein (mg/L)[a]	7.1 (1.2)	7.7 (1.1)	0.75
Risk factors: n (%)[c]			
None	10 (83.3)	7 (58.3)	0.37
≥1 risk factor	2 (16.7)	5 (41.8)	
*Education: n (%)[c]			
<grade 12	7 (63.6)	5 (41.7)	0.29
≥grade 12	4 (36.4)	7 (58.3)	
Employment: n (%)[c]			
None	8 (66.7)	7 (58.3)	1.00
Formal/informal employment	4 (33.3)	5 (41.7)	

BMI: body mass index; OGTT: oral glucose tolerance test; HbA1c: glycated haemoglobin; HOMA: homeostatic model assessment calculated according to the formula: fasting insulin (mIUL) x fasting glucose (mmol/L)/22.5; Risk factors: advanced maternal age (age >35 years), obesity (BMI >30 kg/m2), family history of diabetes mellitus, delivery of a previous baby more than four kilograms, glucosuria, previous recurrent pregnancy loss, stillbirth, or birth of a baby with congenital abnormalities. *One participant had missing data for education. Data are expressed as the [a]mean ± standard error of the mean, as [b]median (25th–75th percentiles) or as [c]count (percentage). p-values for continuous data were calculated using the Mann-Whitney or the unpaired student t test. p-values for categorical data were calculated using Chi-square test or Fisher's exact test if frequency was <5.

4.2. Genome-Wide DNA Methylation Profiling

The average detection p-values for all probes were calculated for each sample and are presented in supplementary Figure S1. Each sample showed p-values below the usual cut-off of 0.01, indicating that all samples passed the quality control. In addition, box and whisker plots showed concordance across samples without any outliers, suggesting good quality and consistency of samples (Figure 7.2). Median β-values ranged between 0.79 and 0.83 across the 24 samples. A histogram of β-values showing the frequency distribution of CpG methylation across all samples is illustrated in Figure S2. A clear separation between GDM and non-GDM groups is evident in the PCA score plot, with characteristic DNA methylation profiles aggregating together within the same group (Figure 7.3). The first three PCAs explain 27.6% of the variance observed. The β-values were then converted to M-values for statistical analysis. To identify differentially methylated CpG sites between GDM and non-GDM pregnancies, data were filtered using the criteria shown in Figure 7.4. An M-value cut-off threshold between >0.4 and >0.6 was explored in this study, which is within the threshold range suggested by Du et al (Du et al., 2010). In the first filtering step a M-value difference of >0.4 or <-0.4 and unadjusted $p<0.01$ was used, to permit comparison between differentially methylated probes. Further filtering steps including M-values which ranged between >0.5 or <-0.5 and >0.6 or <-0.6 with unadjusted $p<0.01$ were assessed. We identified 1046 differentially methylated CpG loci with M-value differences of >0.6 or <-0.6 and unadjusted $p<0.01$ (Table S1). To facilitate a more stringent analysis, a false discovery rate (FDR) <0.1 was added, which did not identify any significant probes. Hierarchical clustering was performed to determine whether these methylation patterns could distinguish between women with or without GDM. The heatmap in Figure 7.5 illustrates that there are distinct methylation patterns between the GDM and non-GDM groups.

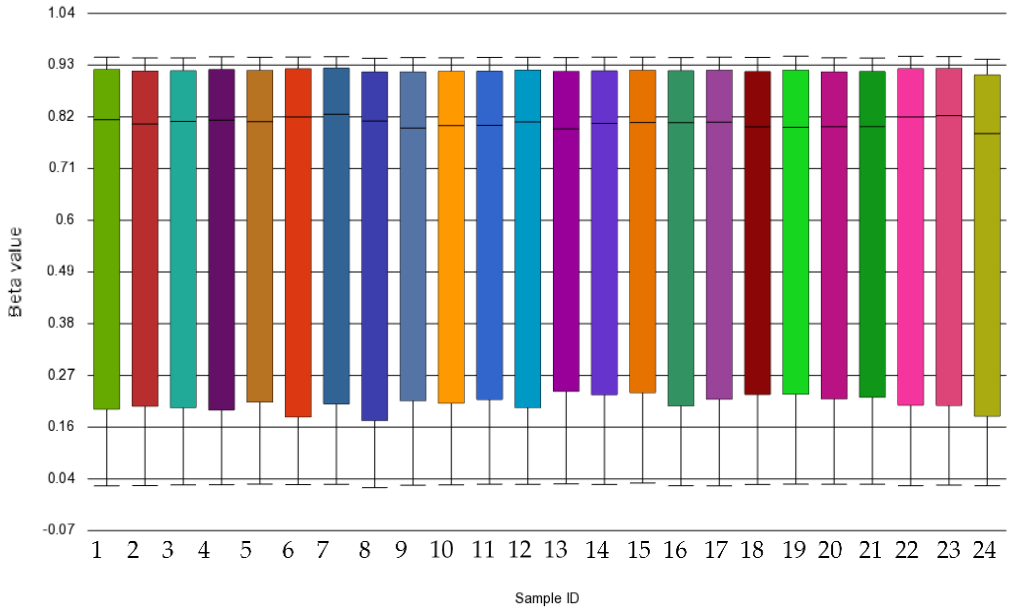


Figure 7.2. Box and whisker plots of β-values. Each box represents a sample (n=24). The median β-value is 0.042 with a minimum and maximum range of 0.785 and 0.827

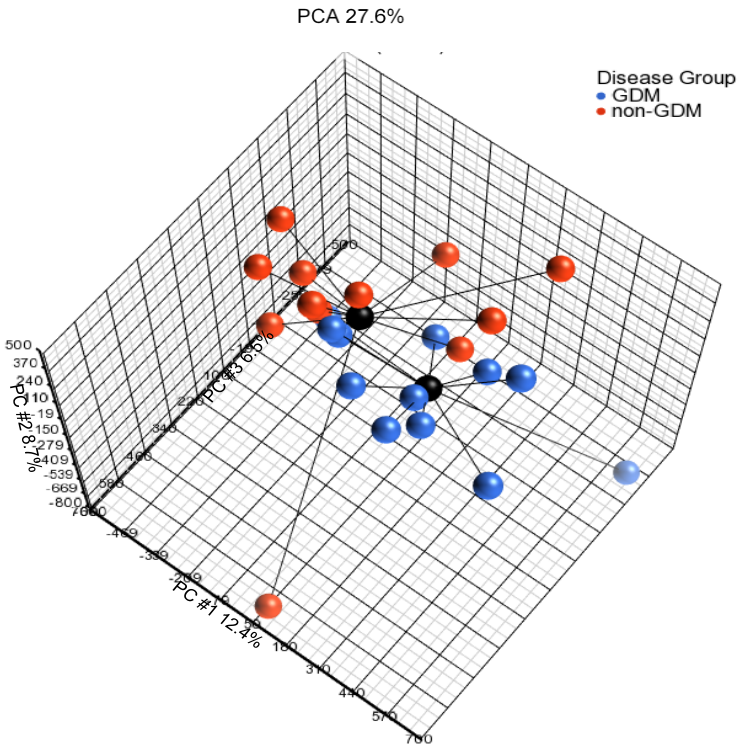


Figure 7.3. Principal component analysis (PCA) between GDM and non-GDM groups. Each dot represents a sample. Centroids (black) connect samples from the respective GDM (blue) or non-GDM (red) group and indicate the center of distribution. The first three PCAs explain 27.6% of the variance

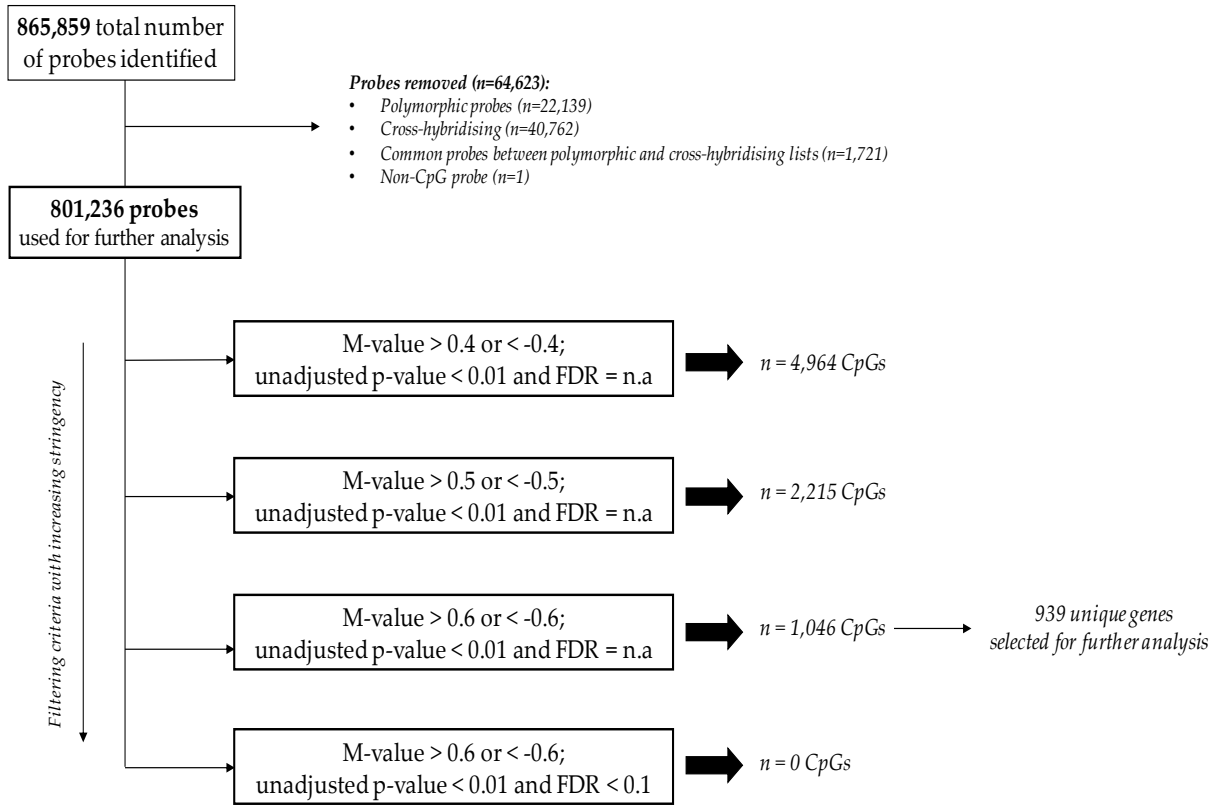


Figure 7.4. Filtering criteria for the identification of CpGs differentially methylated between GDM and non-GDM groups. A total number of 801,236 probes, derived through the removal of polymorphic, cross-hybridizing and non-CpG probes were used for analysis. FDR - false discovery rate; M-values closest to 0 indicate similar methylation intensities between probes

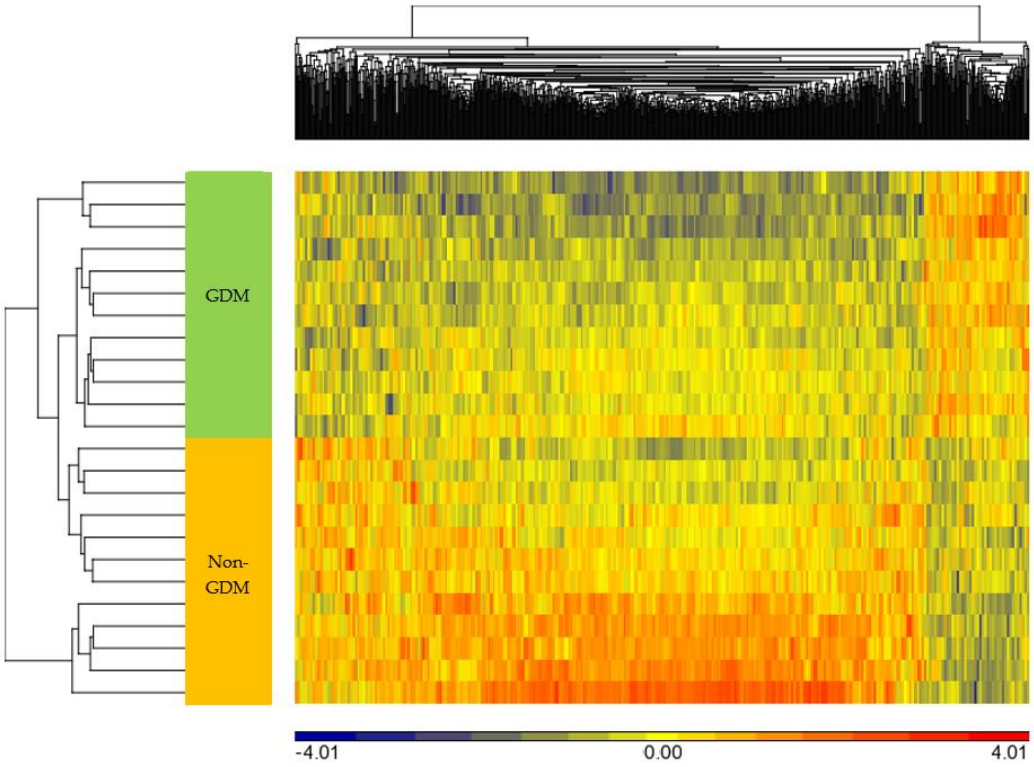


Figure 7.5. Heatmap showing methylation signatures of 1046 CpG sites in women with/without GDM. DNA methylation across 1046 CpG sites in each sample was analysed using Euclidean distance for both rows (observations) and columns (features) and average linkage criteria. Samples are shown in rows and are clustered in GDM (green) and non-GDM (orange) groups. Standardised M-values are depicted using a blue (hypomethylation in GDM) to red (hypermethylation in GDM) methylation gradient

Of the 1046 differentially methylated CpG loci, 148 CpG sites (14.2%) were hypermethylated and 898 CpG sites (85.8%) were hypomethylated in women with GDM compared to women without GDM. To increase the likelihood of identifying differentially methylated promoters, probes located 5 kbp upstream or up to 3 kbp downstream of the transcription start site were also included as promoter regions. The frequency of all CpG sites analysed and differentially methylated CpG sites in relation to their genomic location is shown in Figure 7.6. Of the differentially methylated CpGs, 16.3% were associated with 5'-untranslated regions (UTR), 49.7% with promoters, 6.2% with coding domain sequences (CDS), 19.1% with introns, 4.0% with

non-coding regions, 2.1% with 3'-UTRs and 4.6% with intergenic regions. Differentially methylated CpG sites were annotated to 939 unique genes using RefSeq build 87 (Table S2). The top five significantly differentially methylated CpG sites selected for further analysis, were associated with four unique genes, including Solute Carrier Family 9 Member A3 *(SLC9A3)*, Male-Enhanced Antigen 1; Kelch domain-containing protein 3 *(MEA1;KLHDC3)*, Calmodulin Binding Transcription Activator 1 *(CAMTA1)* and RAS P21 Protein Activator 3 *(RASA3)*, and one unknown gene. The probe ID, location, gene region and direction of methylation (GDM vs. non-GDM), as well as the nearest gene/regulatory region for the unknown gene is shown in Table 7.2. Of the differentially methylated CpG sites, cg22985016 and cg16306629 was shown to be significantly hypermethylated, while cg21910650, cg23643951 and cg07966372 was significantly hypomethylated in GDM compared to non-GDM groups. The association between GDM and the top five CpG sites remained significant for each CpG after linear regression adjusting for age BMI and gestational age (Table 7.3). To examine the degree to which DNA methylation levels at these CpGs are associated with clinical characteristics of GDM, Pearson's correlation analysis was performed (Table 7.4). For cg22985016 and cg16306629, a positive correlation between DNA methylation and fasting glucose concentrations was observed, while methylation at cg21910650, g23643951 and cg07966372 was inversely correlated with glucose concentrations. Furthermore, DNA methylation at cg22985016 and cg16306629 was correlated with 1 hr glucose, while methylation at cg07966372 was negatively correlated with fasting insulin concentrations. When adjusting for GDM, the association between the five CpGs and fasting glucose concentrations and between cg22985016 and cg16306629 and 1 hr OGTT was no longer significant, while he association between cg07966372 and fasting insulin remained significant (Table S3).

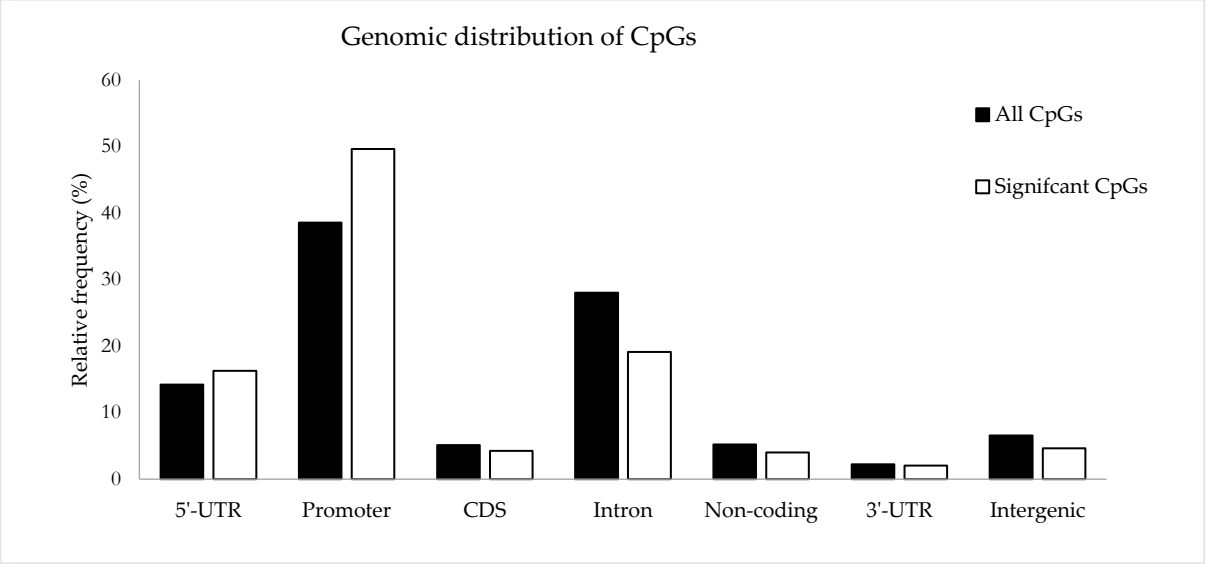


Figure 7.6. Relative frequency of all CpGs analysed (black bars) and differentially methylated CpGs identified in our study (white bars) in relation to genomic location across the genome. UTR- untranslated region; CDS- coding domain sequence

Table 7.2. The top five significantly differentially methylated CpG sites between GDM and non-GDM groups

Probe ID	Location	Gene symbol	Gene name	Region	p-value	Methylation
cg22985016	Chr5:492187-524227	SLC9A3	Solute Carrier Family 9 Member A3	Intron	1.84E-07	↑
cg21910650	Chr6:42976841-42986722	MEA1;KLHDC3	Male-Enhanced Antigen 1;Kelch domain-containing protein 3	Promoter/5′UTR	3.23E-06	↓
g23643951	Chr1:7151432-7309551	CAMTA1	Calmodulin Binding Transcription Activator 1	Intron	4.46E-06	↓
cg16306629	Chr8:119121060-119129059	COLECT10*	Collectin Subfamily member 10*	Enhancer*	9.22E-06	↑
07966372	Chr13:114782770-114898099	RASA3	RAS P21 Protein Activator 3	5′UTR/Intron	9.75E-06	↓

*nearest gene/regulatory region of cg16306629. ↑ - hypermethylation and ↓ - hypomethylation between GDM vs. non-GDM group

Table 7.3. Linear regression analysis of gestational diabetes mellitus and the top five significantly differentially methylated CpG sites, adjusting for age, body mass index and gestational age

	[a]Univariate			[b]Multivariate		
CpG site	Coefficient	95% CI	p-value	Coefficient	95% CI	p-value
cg22985016 *(SLC93A)*	0.028	0.019; 0.037	**<0.001**	0.028	0.019; 0.037	<0.001
cg21910650 *(MEA1;KLHDC3)*	-0.088	-0.117; -0.058	**<0.001**	-0.087	-0.118; -0.056	<0.001
cg23643951 *(CAMTA1)*	-0.056	-0.070; -0.042	**<0.001**	-0.056	-0.071; -0.042	<0.001
cg16306629 *(Unknown)*	0.274	0.183; 0.366	**<0.001**	0.275	0.192; 0.359	<0.001
cg07966372 *(RASA3)*	-0.015	-0.025; -0.004	**0.006**	-0.015	-0.026; -0.004	0.008

[a]Univariate linear regression: Association between CpG-specific methylation and GDM. [b]Multivariate linear regression: Adjusting for age (years), body mass index (kg/m2) and gestational age (weeks); CI – Confidence interval

Table 7.4. Correlation analysis showing the association between DNA methylation and fasting plasma, 1 hr OGTT, 2 hr OGTT and fasting insulin for the top five differentially methylated CpG sites

Variable	cg22985016 *(SLC93A)*		cg21910650 *(MEA1;KLHDC3)*		cg23643951 *(CAMTA1)*		cg16306629 *(Unknown)*		cg07966372 *(RASA3)*	
	rho	*p*-value	rho	*p*-value	rho	*p*-value	rho	*p*-value	rho	*p*-value
Fasting glucose (mmol/L)	0.728	**<0.001**	-0.694	**<0.001**	-0.735	**<0.001**	0.724	**<0.001**	-0.452	**0.026**
1 hr OGTT (mmol/L)	0.502	**0.012**	-0.377	0.069	-0.399	0.053	0.559	**0.004**	0.016	0.939
2 hr OGTT mmol/L)	0.297	0.168	-0.249	0.250	-0.338	0.115	0.266	0.219	0.098	0.658
Fasting insulin (mIU/L)	-0.037	0.888	-0.103	0.691	-0.204	0.433	0.109	0.674	-0.495	**0.043**

OGTT: oral glucose tolerance test; SLC93A: Solute Carrier Family 9 Member A3; MEA1;KLHDC3: Male-Enhanced Antigen 1;Kelch domain-containing protein 3; CAMTA1: Calmodulin Binding Transcription Activator 1; Unknown: gene nearest to this region is called Collectin Subfamily member 10; RASA3: RAS P21 Protein Activator 3. Pearson's correlation coefficient (rho) is shown with significance at p<0.05.

4.3.Functional Enrichment Analysis

Differentially methylated CpG sites (1046), annotated to 939 unique genes using M-values >0.6 and <-06 with unadjusted p<0.01 threshold criteria, were selected for functional enrichment analysis. Functional enrichment analysis identified 261 KEGG pathways, including pathways for T2D and insulin signalling (Table S4). Only 50 KEGG pathways were statistically significantly different between GDM and non-GDM groups (Table S5). Statistically significant pathways included cancer, brain signalling, cell growth, proliferation, viability and inflammation pathways. The most significant KEGG pathway was 'Signalling pathways regulating pluripotency of stem cells' with an enrichment score of 10.496, a p-value =2.76E-05 and 19 differentially methylated associated genes. In addition, GO terms were enriched by differentially methylated genes, categorized into 1181 biological processes, 167 molecular functions and 85 cellular components with a p-value <0.05 (Table S6). The top ten GO terms categorized into biological processes, molecular functions and cellular components are illustrated in Figure 7.7. Of these, homophilic cell adhesion via plasma membrane adhesion molecules (biological process), calcium ion binding (molecular function) and integral component of plasma membrane (cellular component) have the highest ranked enrichment score and p-value <0.001.

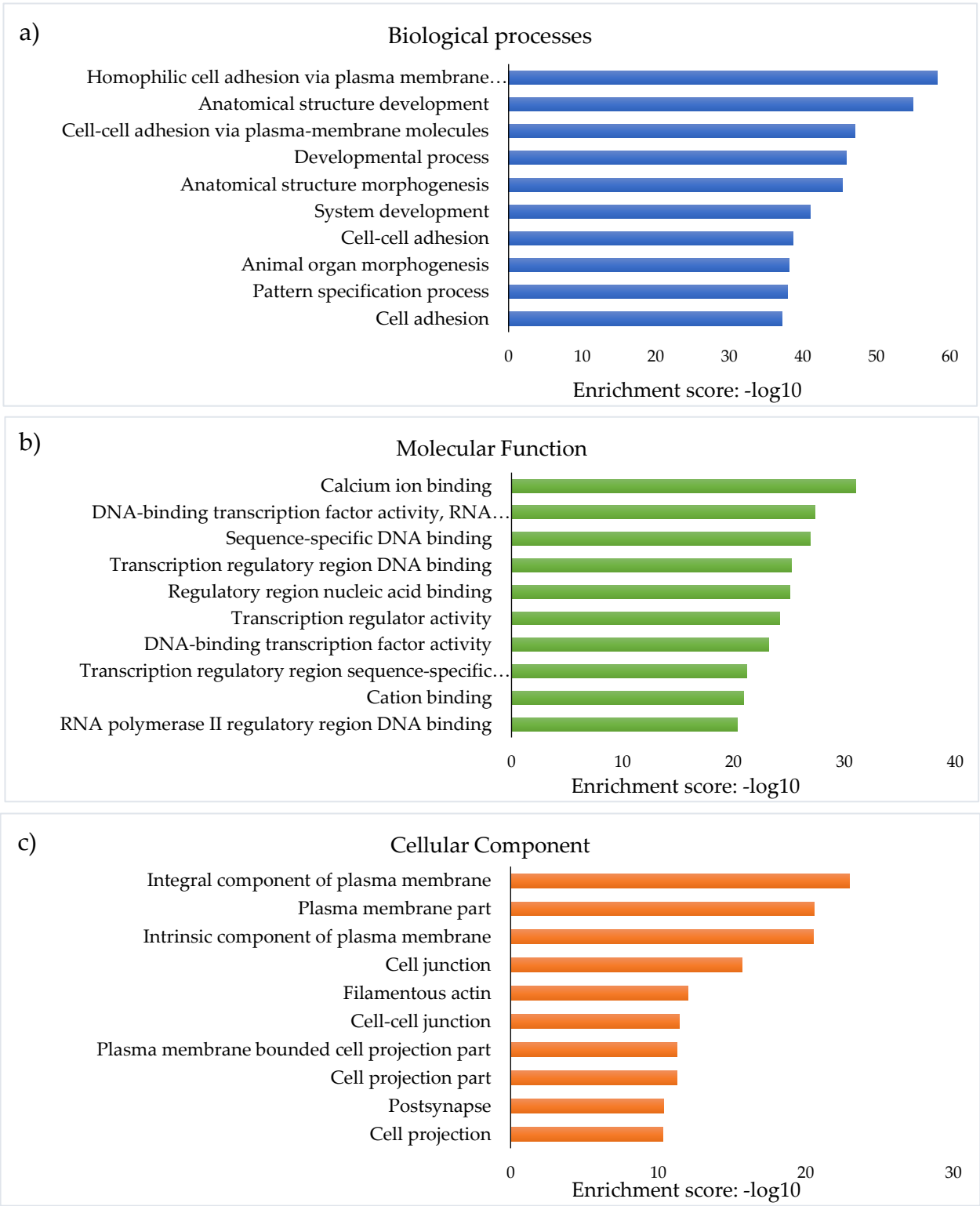


Figure 7.7. Top ten GO terms enriched by differentially methylated genes in GDM and non-GDM groups. Enriched GO terms were categorized into (a) biological processes, (b) molecular function and (c) cellular components. Data are presented as enriched scores express as –log10 (p value). Fisher p≤0.001

5. DISCUSSION

We report the differential methylation of 1046 CpG sites in the peripheral blood of black South African women with GDM compared to women with normoglycemic pregnancies. Functional analysis mapped these CpGs to genes in pathways key to metabolic regulation. Furthermore, differential methylation of the five CpG loci , within *SLC93A* was positively correlated with fasting and 1 hr glucose, while CpGs within *CAMTA, MEA1;KLHDC3* and *RASA3* was inversely correlated to fasting glucose, with distinct methylation profiles in GDM and non-GDM groups. *CAMTA1* is a transcriptional activator that was previously shown to regulate insulin production and secretion (Mollet et al., 2016). These results support the plausibility of the observed DNA methylation differences in GDM pathophysiology and potential as diagnostic biomarkers of GDM.

Genome-wide DNA methylation differences during GDM have been demonstrated in other populations. Kang et al. used the Illumina Infinium Human MethylationEPIC Bead Chip array to investigate DNA methylation in Chinese women with GDM, and showed that the top 200 differentially methylated loci mapped to 151 genes (Kang et al., 2017). Of these, 15 genes, *CAMTA1,* Smad Nuclear Interacting Protein 1 *(SNIP1)*, Protein-Tyrosine Phosphatase, Receptor-Type, F Polypeptide-Interacting Protein-Binding Protein 2 *(PPFIBP2),* Switching B Cell Complex Subunit *SWAP70 (SWAP70)*, Semiphorin 6D *(SEMA6D)*, Cadherin 8 *(CDH8)*, Cytochrome P450 Family 26 Subfamily B Member 1 *(CYP26B1),* Wnt Family Member 6 *(WNT6)*, Raftlin, Lipid Raft Linker 1 *(RFTN1),* Unc-5 Netrin Receptor C *(UNC5C),* Nucleoside Diphosphate-Linked Moiety X Motif 6 *(NUDT6),* Storkhead Box *(STOX2)*, MutS Protein Homolog 5 *(MSH5),* KH RNA Binding Domain Containing, Signal Transduction Associated 2 *(KHDRBS2),* and Neuregulin 1 *(NRG1)* were similarly shown to be differentially

methylated in our study, and has been illustrated in a venn diagram (Figure S3). Disparities in the number of differentially methylated CpG sites identified between studies could be due to population differences such as ethnicity, age and stage of pregnancy, and the data filtering criteria used. Although M-values were used to measure methylation differences in both studies, Kang et al. used a more stringent FDR adjusted p-value <0.05 for their analysis whereas we used an unadjusted p-value <0.01, since an FDR of <0.05 did not identify any significantly differentially methylated loci in our analysis. Despite using a higher FDR than Kang et al., the differential methylation of 15 genes were similar between studies (Kang et al., 2017). Other technical differences between studies which may affect methylation levels include sample preparation, loading during hybridization and batch effect bias (Soriano-Tárraga et al., 2013; Nakabayashi, 2017). Soriano-Tárraga et al. reported that the method of DNA extraction affects global DNA methylation levels (Soriano-Tárraga et al., 2013). Thus, standardization of analytical methods across laboratories is essential to enable comparison of DNA methylation patterns between studies. Other studies that used previous versions of the Bead Chip array similarly reported DNA methylation differences during GDM in Non-Hispanic Caucasian American and Caucasian English populations (Enquobahrie et al., 2015; Wu et al., 2018). As reported in these studies (Enquobahrie et al., 2015; Kang et al., 2017; Hjort et al., 2018; Wu et al., 2018), the majority of CpG differences in our study were hypomethylated in women with GDM compared to women without GDM. However, in contradiction, in our study most of the 1046 differentially methylated CpG sites occurred in promoter regions, whereas previous studies identified most of the differentially methylated CpGs in gene body regions (Huang et al., 2015; Hjort et al., 2018). Differences could be due to the method of analysis used. Our analysis included additional CpGs located 5 kbp upstream and 3 kbp downstream of the transcription start site to increase the probability of detecting differentially methylated promoter regions. Altered DNA

methylation in promoter regions influence the expression of specific genes (El Hajj et al., 2013; Kang et al., 2018; Wang, Yang, et al., 2018), which may enable the identification of genes/pathways involved in metabolic processes during GDM.

Recently, we demonstrated that global DNA methylation is not associated with GDM in South African women (Dias, Adam, Wyk, et al., 2019). We hypothesised that the failure to detect DNA methylation differences was due to technical limitations and that gene-specific methylation analysis would be able to identify GDM-associated methylation differences. Global DNA methylation quantification is a crude marker of overall genomic methylation and does not have the resolution to detect gene-specific differences, as observed in the current study. Similar findings were reported by Matsha et al., who showed no difference in global DNA methylation between 61 diabetic individuals on treatment and 287 normoglycemic subjects in a mixed ethnic ancestry South African population (Matsha et al., 2016). In addition, no difference in global DNA methylation was observed in peripheral blood mononuclear cells of a Danish population with obesity or T2D compared to controls (Simar et al., 2014).

The diagnosis of GDM is contentious and varies across countries and health institutions. Currently the IADPSG criteria is advocated by several international bodies and endorsed by the World Health Organisation (WHO) (WHO, 2013). However, concerns that the high costs and increased workload of IADPSG criteria outweigh the clinical effects of small glucose differences has hampered its universal use. We were able to see altered DNA methylation patterns despite small glucose differences between women with or without GDM, suggesting that epigenetic programming is evident even during mild hyperglycaemia. Kang et al. also demonstrated altered DNA methylation in women diagnosed with GDM according to IADPSG diagnostic criteria (Kang et al., 2017). These findings support The

Hyperglycaemia and Adverse Pregnancy Outcomes (HAPO) study, which showed that even mild hyperglycaemia is associated with adverse pregnancy outcomes and requires treatment (HAPO Study Cooperative Research Group et al., 2008). Furthermore, several clinical trials have confirmed that treatment of mild hyperglycaemia decreases maternal morbidity and adverse perinatal outcomes (Alwan, Tuffnell & West, 2009).

Functional analysis of differentially methylated CpG sites identified canonical pathways related to signal transduction, cell growth, proliferation, differentiation and apoptosis, insulin resistance, glucose metabolism, inflammation, neurological signalling, and oncogenesis. Altered DNA methylation of two signalling pathways, mitogen activated protein kinase *(MAPK)* and phosphoinositide 3-kinase *(PI3K)*, which play a role in cell growth and differentiation, and the metabolic action of insulin (Świderska et al., 2018), have previously been reported during GDM in other populations (Kang et al., 2017), identifying these CpG sites as likely biomarkers for the development of GDM. Our results demonstrated that pathways associated with cancer are differentially methylated in women with GDM compared to controls. Several studies have reported a link between GDM and cancer, particularly breast cancer (Sella et al., 2011; Park et al., 2017; Peng et al., 2019), identifying GDM as a potential risk factor for the development of cancer in later life , Nine of the top 10 GO terms enriched for biological processes were associated with structural organisation and developmental processes, supporting the influence of GDM on *in utero* programming of foetal growth and development (Monteiro et al., 2016). As expected, all 10 GO terms enriched for molecular functions were associated with regulatory or binding activities and offer insight into functions influenced by altered methylation at a molecular level during GDM.

A strength of our study is that women were matched for age, gestational age and BMI, to ensure that results were comparable between groups. In addition, DNA methylation analysis was conducted using the most comprehensive MethylationEPIC Bead Chip array currently available, which is considered a high-throughput method, that has a lower cost compared to sequencing, and is reproducible and time-efficient (Pidsley et al., 2016; Nakabayashi, 2017). . Our study has a number of limitations. The sample size (n=24) is small, although, it is larger than previously reported (Enquobahrie et al., 2015; Kang et al., 2017; Wu et al., 2018). No CpG sites reached FDR cut-off, suggesting that the study might have been underpowered. Albeit, 15 of the differentially methylated genes identified in our study were amongst the top 151 identified by Kang et al. Peripheral blood cells consist of a mixture of different cell types (Reinius et al., 2012), which may confound methylation analysis. In our study, cell type composition did not differ significantly between GDM and non-GDM groups and therefore was not adjusted for in further analysis due to the small sample size. Thus, methylation differences between cell types could have confounded our analysis. Furthermore, physical activity, diet, smoking and alcohol consumption, which are known to influence DNA methylation patterns, are not known, and could confound our analysis. However, women in our study were recruited from the same community and had similar lifestyle behaviours, education and employment status, suggesting that they had roughly similar environmental influences.

To our knowledge, this exploratory study is the first to profile genome-wide DNA methylation levels in the peripheral blood of South African women with GDM. We have identified five CpGs which are associated with GDM and offer potential as epigenetic biomarkers in our population. Further validation using pyrosequencing and conducting longitudinal studies in large sample sizes and in different populations are required to investigate their candidacy as biomarkers of GDM

CHAPTER 8

HUMAN IMMUNODEFICIENCY VIRUS INFECTION AFFECTS THE ASSOCIATION BETWEEN ADIPONECTIN DNA METHYLATION AND GESTATIONAL DIABETES MELLITUS IN SOUTH AFRICAN WOMEN

This Chapter has been submitted as a short report to *Clinical Epigenetics*. Dias S, Adam S, Abrahams Y, Rheeder P, Pheiffer C.

TABLE OF CONTENTS

1. ABSTRACT

Background: DNA methylation is increasingly being recognized as a potential biomarker for gestational diabetes mellitus (GDM), however the effect of human immunodeficiency virus (HIV) infection on its candidacy as a biomarker has not yet been investigated. This study explored the effect of HIV infection on methylation of the adiponectin *(ADIPOQ)* gene in South African women with GDM.

Results: DNA methylation levels at eight CpG sites within the *ADIPOQ* promoter was quantified in peripheral blood of women with (n=95) or without (n=191) GDM using pyrosequencing, where after women were stratified according to their HIV status. Of these, two CpG sites (-3410: p=0.048 and -3400: p=0.004) were hypomethylated during GDM in HIV negative, but not in HIV positive women. Methylation levels at these CpGs were inversely associated with serum adiponectin (-3410: p=0.023 and -3400: p=0.013) and fasting glucose (-3410: p=0.015 and -3400: p=0.001) concentrations. *In silico* analysis showed that transcription factors involved in adipocyte differentiation, stress response, and glucose and lipid metabolism may bind to the regions of altered DNA methylation.

Conclusion: Our results show that HIV infection affects the association between *ADIPOQ* DNA methylation and GDM in South African women. These findings have implications for biomarker discovery in high HIV prevalence settings such as SA, where approximately one in three pregnant women are HIV positive. We recommend that DNA methylation studies be conducted in both HIV negative and positive individuals.

2. INTRODUCTION

GDM is defined as glucose intolerance that is first diagnosed during the latter half of pregnancy, with return to normoglycemia after birth (WHO, 2013). Globally, the prevalence of GDM ranges from <1 to 28%, depending on the population studied and diagnostic criteria employed (Jiwani et al., 2012). Recently, a GDM prevalence of 25.8% was reported in an urban setting in SA, a rate considerably higher than previously reported in this country (Adam & Rheeder, 2017). Although, different diagnostic criteria could partly account for the increased prevalence, the study nevertheless highlights the growing challenge GDM poses on the health systems of under-resourced countries such as SA. Without appropriate diagnosis and management, GDM is associated with adverse maternal, foetal and perinatal outcomes (Hod et al., 1991; Hadar & Hod, 2013; Moore, 2018), while both mothers and their offspring are at increased risk of developing type 2 diabetes mellitus (T2D), obesity and other metabolic conditions in later life (Kim, Newton & Knopp, 2002; Clausen et al., 2008; Bellamy et al., 2009; Zhao et al., 2016). The early detection of GDM could facilitate risk stratification and intervention strategies that could potentially better manage GDM, thereby improving health outcomes.

Adiponectin is an insulin-sensitising hormone secreted by adipose tissue that regulates glucose and lipid homeostasis (Brochu-Gaudreau et al., 2010; Ghadge, Khaire & Kuvalekar, 2018). During pregnancy adiponectin concentrations progressively decline with increasing insulin resistance (Catalano et al., 2006; Bao et al., 2015). Furthermore, several studies (Ranheim et al., 2004; Worda et al., 2004; Retnakaran et al., 2010; Mohammadi & Paknahad, 2017), including ours (Adam et al., 2018) have reported that adiponectin concentrations are lower in women with GDM compared to women with normoglycemic pregnancies. Accordingly, adiponectin has

been widely investigated as a potential biomarker of GDM (Ranheim et al., 2004; Worda et al., 2004; Retnakaran et al., 2010; Guelfi et al., 2017; Mohammadi & Paknahad, 2017; Adam et al., 2018; Lobo et al., 2019). These studies show that it is possible to detect adiponectin dysregulation early during pregnancy (from 14 weeks) (Guelfi et al., 2017), that adiponectin concentrations correlate with glucose concentrations (Worda et al., 2004; Mohammadi & Paknahad, 2017) and the development of GDM (Ranheim et al., 2004; Retnakaran et al., 2010; Adam et al., 2018), and is able to predict GDM before clinical diagnosis at 24-28 weeks (Lobo et al., 2019). Thus, measurement of serum adiponectin concentrations offers tremendous potential to serve as a clinical biomarker for GDM.

DNA methylation is an important epigenetic mechanism that reflects the interplay between gene-environment interactions (Christensen & Marsit, 2011; Ling & Rönn, 2019). It is the most widely studied and best characterised epigenetic mechanism and refers to the addition of a methyl group to the fifth carbon position of a cytosine residue within a cytosine-phosphate-guanine (CpG) dinucleotide. This modification can alter chromatin structure and regulates gene expression primarily by transcriptional repression. Aberrant DNA methylation has been demonstrated in many diseases (Barres & Zierath, 2011; He et al., 2019; Krause et al., 2019). Evidence suggest that peripheral blood mirrors DNA methylation patterns in tissue, supporting its potential as biomarkers of various metabolic disease, including GDM (Li et al., 2012; Dias et al., 2018; Willmer et al., 2018). Recently we reported altered genome-wide DNA methylation patterns in South African women with GDM (Dias, Adam, Rheeder, et al., 2019). This study showed that 1046 CpG sites related to 939 genes displayed significant methylation differences between GDM and non-GDM groups, supporting the potential of DNA methylation as biomarkers for GDM in our population.

South Africa has the highest prevalence of human immunodeficiency virus (HIV) worldwide, with an estimated 7.9 million people infected (HSRC Press, 2018). HIV infection alters epigenetic mechanisms such as DNA methylation and microRNA expression (Zhang et al., 2016; Pheiffer et al., 2019), which may affect their potential as biomarkers of GDM. Given the high prevalence of HIV in South Africa, particularly in women of reproductive age (HSRC Press, 2018), it is important to explore the effect of infections such as HIV on potential biomarkers of GDM. Previous studies were conducted in HIV negative (Dias, Adam, Rheeder, et al., 2019) or without consideration of HIV status (Bouchard et al., 2012; Ott et al., 2018). This study investigated the effect of HIV infection on *ADIPOQ* DNA methylation in pregnant women with and without GDM. DNA methylation at eight CpG sites within the *ADIPOQ* promoter was quantified in the peripheral blood of women with (n=95) or without (n=191) GDM using pyrosequencing, where after women were stratified according to their HIV status. Furthermore, the association between CpG-specific methylation and clinical characteristics was assessed, while *in silico* analyses was conducted to identify transcription factors that bind to CpGs with altered methylation.

3. MATERIALS AND METHODS

3.1. Study Population

Ethical approval for this study was granted by the University of Pretoria Health Sciences Ethics Committee (180/2012). The study was conducted according to the Declaration of Helsinki and all women gave written informed voluntary consent after the procedures had been fully explained in the language of their choice (Adam & Rheeder, 2017). One thousand pregnant black African women attending a primary care clinic in Johannesburg, South Africa were recruited to the study. Women were excluded if they had twin pregnancies, pre-existing diabetes (Type 1 diabetes mellitus (T1D) and T2D) and if they had any other infections such as tuberculosis. At their first visit, random glucose and glycated haemoglobin (HbA1c) concentrations were measured using a glucometer (Roche Diagnostics, Mannheim, Germany) and the point-of-care Afinion system (Alere Technologies, Oslo, Norway), respectively. Women with random glucose concentrations <11.1 mmol/L and HbA1c concentrations <6.5%, were requested to return to the clinic within two weeks in a fasted state for GDM testing and blood collection. GDM was diagnosed using the 75 g 2 hr oral glucose tolerance test (OGTT) at 24-28 weeks gestation if at least one glucose value was met (fasting plasma glucose ≥5.1 mmol/L, 1 hr OGTT ≥10 mmol/L or 2 hr OGTT ≥8.5 mmol/L), according to the International Diabetes and Pregnancy Study Group (IADPSG) criteria (IADPSG panel, 2010). Anthropometric measurements were obtained according to standard procedures and demographic and socio-economic data were obtained in the form of a standardised questionnaire (Adam & Rheeder, 2017). HIV testing were offered to all pregnant women using rapid HIV kits, and results were confirmed with a different kit according to the guidelines of the SA Department of Health (National Department of Health South Africa, 2015). HIV

positive women were treated with Atripla™; a fixed-dose coformulation of three anti-HIV drugs, efavirenz, emtricitabine and tenofovir given once-daily (National Department of Health South Africa, 2015). Blood samples were collected after an overnight fast, and C-reactive protein (CRP) and fasting glucose and insulin concentrations were measured in an accredited laboratory (Vermaak and Partners/Pathcare laboratories, South Africa). The homeostatic model assessment (HOMA), a measure of insulin resistance was calculated using the equation: (fasting plasma glucose x fasting serum insulin)/22.5. Serum adiponectin concentrations were measured using the human adiponectin enzyme-linked immunosorbent assay (ELISA) (Merck, Dermstadt, Germany). Blood were stored at -80 ^{o}C for DNA methylation profiling. A subset of women between the ages of ≥18≤40 years, with (n=95) or without (n=191) GDM, who had serum adiponectin concentration measurements were selected for this cross-sectional study. These women were stratified according to HIV negative (n=63, GDM; n=118, non-GDM) and HIV positive (n=32, GDM; n=73 non-GDM) groups (Figure 8.1). Of the 105 HIV positive women, 36 were receiving antiretroviral therapy (ART) (GDM: n=12, non-GDM: n=24), 68 were ART naïve (GDM: n=19, non-GDM: n=49) and one had missing data, which was not included in ART analysis.

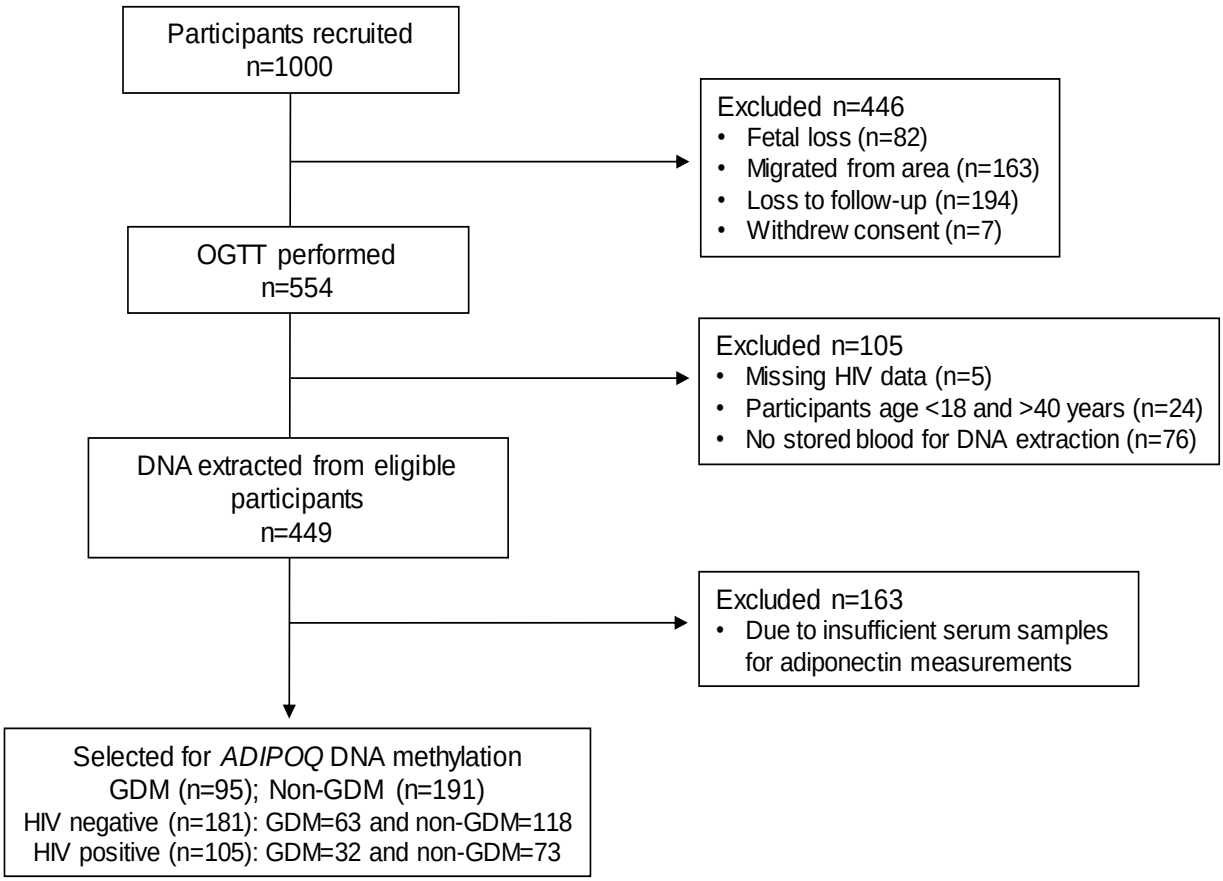


Figure 8.1. Flow diagram for study participants. A subset of women with (n=95) and without (n=191) GDM were selected for this cross sectional study and were stratified according to HIV negative and HIV positive groups. HIV: Human Immunodeficiency Virus

3.2. Peripheral Blood Collection and DNA Extraction

Peripheral blood was collected in Ethylenediaminetetraacetic acid (EDTA) tubes, and genomic DNA was extracted from 2 ml of whole blood using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA concentrations were measured using the Qubit Flourometer (Invitrogen, Carlsbad, USA) and the Quanti-iT dsDNA Broad Range assay kit (ThermoFisher, Massachusetts, USA).

3.3. Primers

Primers designed specifically to bisulfite modified regions were selected from publications that identified two regions important for *ADIPOQ* gene regulation (Bouchard et al., 2012; Houshmand-Oeregaard et al., 2017). Region 1 (R1) represents 4 CpGs at -3413, -3410, -3400 and -3372 (region C in Bouchard et al. 2012 (Bouchard et al., 2012)) and R2 represents 2 CpGs at -112 and -45 (Houshmand-Oeregaard et al. 2017 (Houshmand-Oeregaard et al., 2017)) upstream from the transcription start site (TSS). In addition, primers for R3 (2 CpGs at -473 and -415 upstream from the TSS) was designed by us, using the PyroMark Assay Design Software (version 2.0.2.5, Qiagen). A schematic representation of the eight CpG sites investigated in the promoter of *ADIPOQ* is illustrated in Figure 8.2. The PCR primer set included a forward and reverse primer, of which one was biotinylated, and a sequencing primer (Integrated DNA Technologies, Inc., South Africa). The amplicon length was restricted to <200 bp to ensure optimal sequencing. The primer and target sequences, chromosomal location and amplicon length are listed in Table S1.

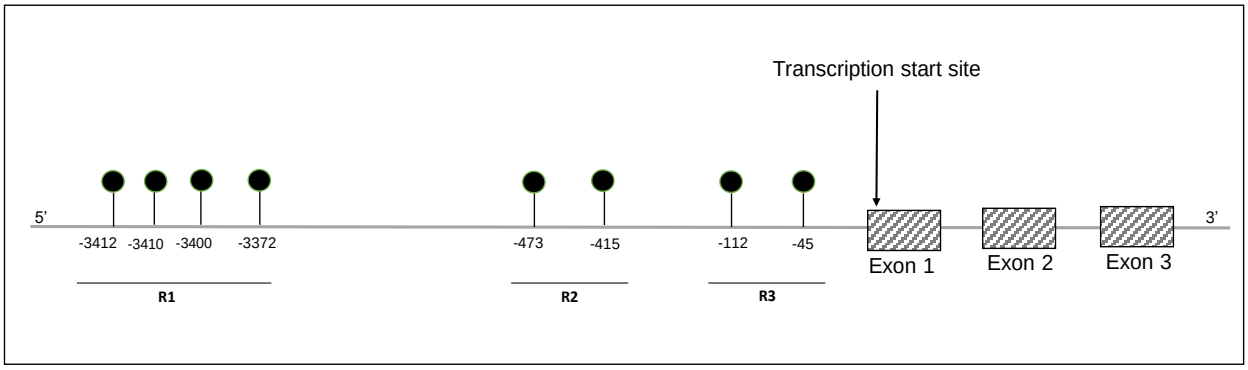


Figure 8.2. Schematic illustration of the Adiponectin gene locus, including three exons and the location of analysed CpG sites upstream of the transcription start site. Region 1 (R1) represents 4 CpGs at -3413, -3410, -3400 and -3372, R2 represents 2 CpGs at -112 and -45 and R3 represents 2 CpGs at -473 and -415

3.4. DNA Methylation Analysis

Bisulfite conversion was performed using the EpiTech Fast DNA Bisulfite Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Briefly, 500 ng of genomic DNA was mixed with 85 μl of sodium bisulfite solution, 15 μl of DNA protect buffer and variable RNase free water to a total volume of 140 μl. Thereafter, bisulfite conversion was performed in a thermal cycler as follows: 5 min at 95 °C, 20 min at 60 °C, 5 min at 95 °C and 20 min at 60 °C. For purification, bisulfite converted DNA was mixed with 310 μl of BL buffer and 250 μl of ethanol (96 – 100%) and loaded onto the EpiTech DNA spin column. DNA was washed using BW buffer, incubated with desulfonation BD buffer for 15 min at room temperature (15–25°C), and eluted by centrifugation at 15,000 g for 1 min, using 15 μl of EB buffer. PCR was performed using 20 ng of bisulfite converted DNA and 0.2 μM of specific primers containing CpG sites of interest, in a final volume of 25 μl using the Pyromark PCR kit (Qiagen), according to the manufacturer's instructions. Quality control for pyrosequencing was conducted using no template and bisulfite controls on the PyroMark Q96 MD pyrosequencing system, according to the manufacturer's instructions. Bisulfite conversion efficiency was tested by performing PCR and pyrosequencing using differing ratios of DNA methylated standards ranging from unmethylated to completely methylated (0%, 10%, 25%, 50%, 75%, 90% and 100%) (Qiagen). Methylation at each CpG site was associated with the approximate percentage of methylation for each standard, with R1 (CpGs at -3413, -3410, -3400 and -3372) showing good correlation (Figure S1). Next, 15 μl of PCR product was used for pyrosequencing with the PyroMark Gold Q96 reagent kit (Qiagen), and the percentage of methylation was quantified for all CpG sites using Pyromark Q96 Assay Design software (V2.0). Bisulfite conversion and pyrosequencing were repeated in randomly selected samples for validation.

3.5.In Silico Analysis

In silico analysis was conducted to identify transcription factors that bind to regions of altered CpG methylation. The sequence of the human *ADIPOQ* gene was retrieved from Ensemble (GRCh38) (https://www.ensembl.org/index.html), and the region between -3425 bp to -3383 bp (total 42 bp), overlapping CpG -3410 and -3400 was analysed to identify potential transcription factors using ALIBABA 2.1 (Grabe, 2000) and ALGGEN-PROMO software (Messeguer et al., 2002). CpG -3410 and -3400 showed high levels of methylation, thus, the resulting transcription factor predictions were cross-referenced using the MeDReader database to assess the likelihood of these transcription factors to bind to highly methylated regions (Wang, Luo, et al., 2018).

3.6.Statistical Analysis

Data were evaluated for normality using the Shapiro-Wilk tests in STATA 14 (StataCorp, College Station, USA). Data were not normally distributed and are expressed as the median and interquartile range (25^{th} – 75^{th} percentile) and categorical data are expressed as count (percentage). Comparisons between groups were analysed using the Mann-Whitney test, while categorical variables were analysed using the Chi-squared test. To investigate the association between GDM and differentially methylated CpGs, univariate or multivariate logistic regression adjusting for covariates were used. GDM was classified as the binary dependent variable and DNA methylation as the continuous independent variable. The association between DNA methylation and clinical characteristics were assessed using Spearman's correlation *(r)* coefficient. Graphs were drawn in Prism 7, Version 7.03 (GraphPad, La Jolla, USA). A p-value of ≤ 0.05 was considered statistically significant.

4. RESULTS

4.1.Participant Characteristics

Participant characteristics are presented in Table 8.1. Random ($p<0.001$), fasting ($p<0.001$), 1 hr OGTT ($p<0.001$) and 2 hr OGTT ($p<0.001$) glucose and HbA1c ($p=0.022$) concentrations, HOMA ($p<0.001$) and risk factors ($p=0.017$) were higher in women with GDM compared to women with normoglycemia, while serum adiponectin concentrations ($p=0.009$) were lower in women with GDM. When women were stratified according to HIV status, random ($p<0.001$), fasting ($p<0.001$), 1 hr OGTT ($p<0.001$) and 2 hr OGTT ($p<0.001$) glucose, HbA1c ($p=0.007$) and fasting insulin ($p=0.034$) concentrations, HOMA ($p<0.001$) and risk factors ($p=0.038$) were higher in HIV negative women with GDM compared to those with normoglycemia, while adiponectin concentrations ($p=0.009$) were lower in women with GDM. Most of these differences were ablated in HIV positive women, with only fasting ($p<0.001$), 1 hr OGTT ($p<0.029$) and 2 hr OGTT ($p<0.001$) glucose concentrations higher in HIV positive women with GDM compared to women with normoglycemia. Importantly, the percentage of women receiving ART compared to ART naïve women were not different between GDM and non-GDM groups in HIV positive women ($p=0.567$).

Table 8.1. Participant characteristics

	ALL			HIV negative			HIV positive		
Participant Characteristics	GDM	Non-GDM	p-value	GDM	Non-GDM	p-value	GDM	Non-GDM	p-value
Participants: n	95	191		63	118		32	73	
Age (years)	29 (24-32)	27 (23-31)	0.114	28 (24-32)	26 (23-30)	0.079	30 (25-32)	29 (25-33)	0.613
BMI (kg/m^2)	26.7 (23.3-31.2)	25.6 (22.5-28.6)	0.057	26.9 (22.9- 30.7)	25.6 (22.7-28.6)	0.232	26.6 (24.4-33.6)	25.8 (22.3-29.7)	0.085
Gestational Age (weeks)	25 (21-27)	25 (21-28)	0.399	25 (21-27)	25 (20-28)	0.754	25 (20.5-26.5)	26 (22-28)	0.306
Random glucose (mmol/L)	4.6 (4.2-5.0)	4.3 (4.0-4.8)	**<0.001**	4.6 (4.1-5.0)	4.3 (3.9-4.7)	**<0.001**	4.6 (4.3-5.2)	4.4 (4.1-4.9)	0.124
Fasting glucose (mmol/L)	5.6 (5.3-6.0)	4.4 (4.1-4.7)	**<0.001**	5.7 (5.3-6.0)	4.3 (4.0-4.6)	**<0.001**	5.4 (5.3-5.7)	4.5 (4.2-4.8)	**<0.001**
OGTT 1 hr (mmol/L)	6.2 (5.5-7.5)	5.4 (4.6-6.3)	**<0.001**	6.3 (5.6-8.3)	5.3 (4.5-6.3)	**<0.001**	5.9 (5.3-7.0)	5.5 (4.7-6.3)	**0.029**
OGTT 2 hr (mmol/L)	6.1 (5.1-7.2)	5.2 (4.6-5.7)	**<0.001**	6.1 (5.1-7.3)	5.2 (4.6-5.7)	**<0.001**	6.1 (5.1-7.1)	5.1 (4.3-5.8)	**<0.001**
HbA1c (%)	5.3 (5.0-5.5)	5.2 (4.9-5.4)	**0.022**	5.3 (5.0-5.5)	5.1 (4.8-5.3)	**0.007**	5.3 (5.1-5.5)	5.3 (5.1-5.5)	0.886
Fasting insulin (mIU/L)	5.9 (3.7 -8.1)	5.2 (3.6-7.0)	0.132	6.5 (4.5-8.5)	5.4 (3.9-7.6)	**0.034**	3.6 (2.8-5.6)	4.5 (3.1-5.8)	0.387
HOMA	1.6 (1.0-2.4)	1.0 (0.7-1.5)	**<0.001**	1.7 (1.2-2.1)	1.1 (0.8-1.6)	**<0.001**	0.8 (0.7-1.4)	0.9 (0.6-1.1)	0.473
C-reactive protein (mg/L)	7.1 (3.7-10.5)	6.1 (3.0-8.8)	0.174	6.9 (3.6-9.0)	5.3 (3.0-8.3)	0.367	10.3 (5.3-21.7)	7.8 (2.9-16.3)	0.169
Adiponectin (μg/ml)	9.2 (5.3-15.1)	10.5 (8.1-16.5)	**0.009**	7.6 (4.9-11.8)	9.7 (7.3-14.5)	**0.009**	14.0 (7.2-19.6)	14.4 (9.4-20.3)	0.427

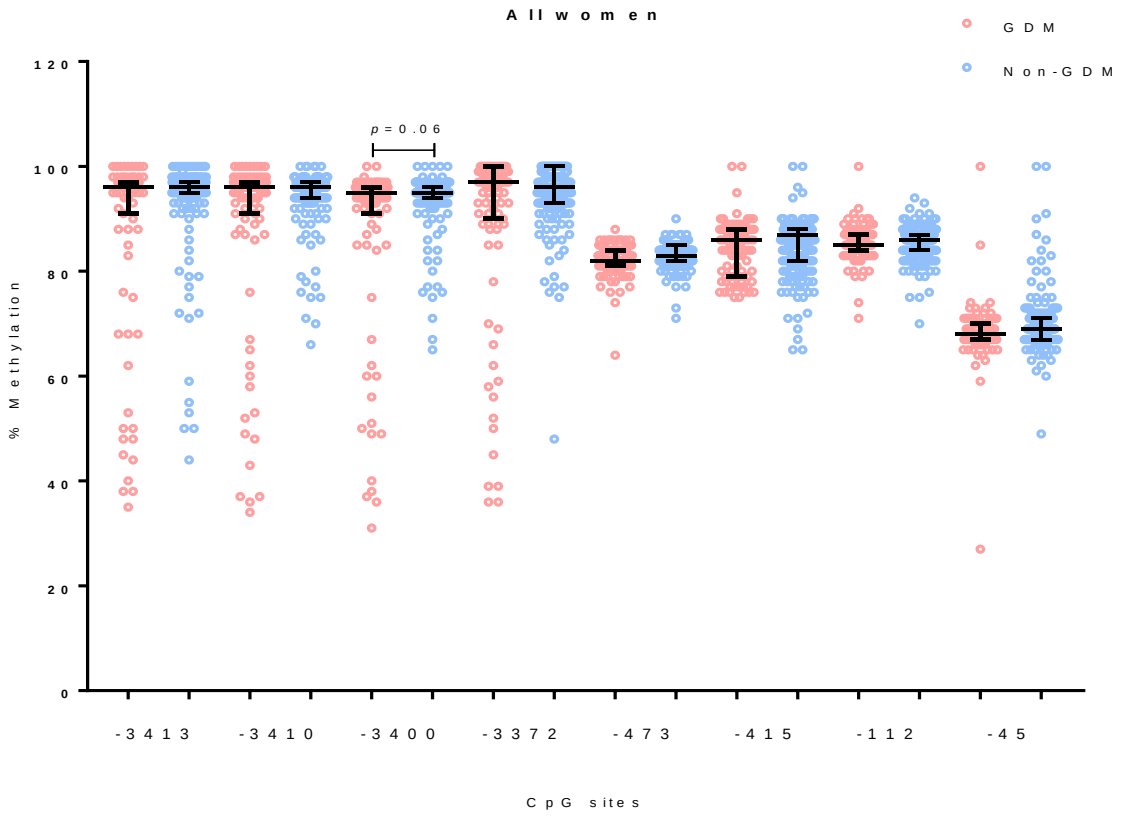
Education: n (%)[a]									
<grade 12	48 (51.1)	96 (52.5)	0.826	28 (45.2)	49 (43.4)	0.819	20 (62.5)	47 (67.1)	0.647
≥grade 12	46 (58.9)	87 (47.5)		34 (54.8)	64 (56.6)		12 (36.5)	23 (32.9)	
Risk Factors: n (%)[a]									
None	40 (42.1)	109 (57.1)	**0.017**	30 (37.6)	75 (63.6)	**0.038**	10 (31.3)	34 (46.6)	0.143
≥1 risk factor	55 (57.9)	82 (42.9)		33 (52.4)	43 (36.4)		22 (68.7)	39 (53.4)	
ART: n (%)[a]									
On treatment	-	-	**-**	-	-	**-**	12 (38.7)	24 (32.8)	0.567
ART naïve							19 (61.3)	49 (67.1)	

BMI: body mass index; OGTT: oral glucose tolerance test; HbA1c: glycated haemoglobin; HOMA: homeostatic model assessment; Risk factors: advanced maternal age (age > 35 years), obesity (BMI > 30 kg/m2), family history of diabetes mellitus, delivery of a previous baby more than four kilograms, glucosuria, previous recurrent pregnancy loss, stillbirth, or birth of a baby with congenital abnormalities; ART: antiretroviral treatment using Atripla™: a fixed-dose coformulation of three anti-HIV drugs, efavirenz, emtricitabine and tenofovir given once-daily. Data are expressed as the median and interquartile range (25th–75th percentiles) or as [a]count (percentage). Values in bold type indicate statistical significance

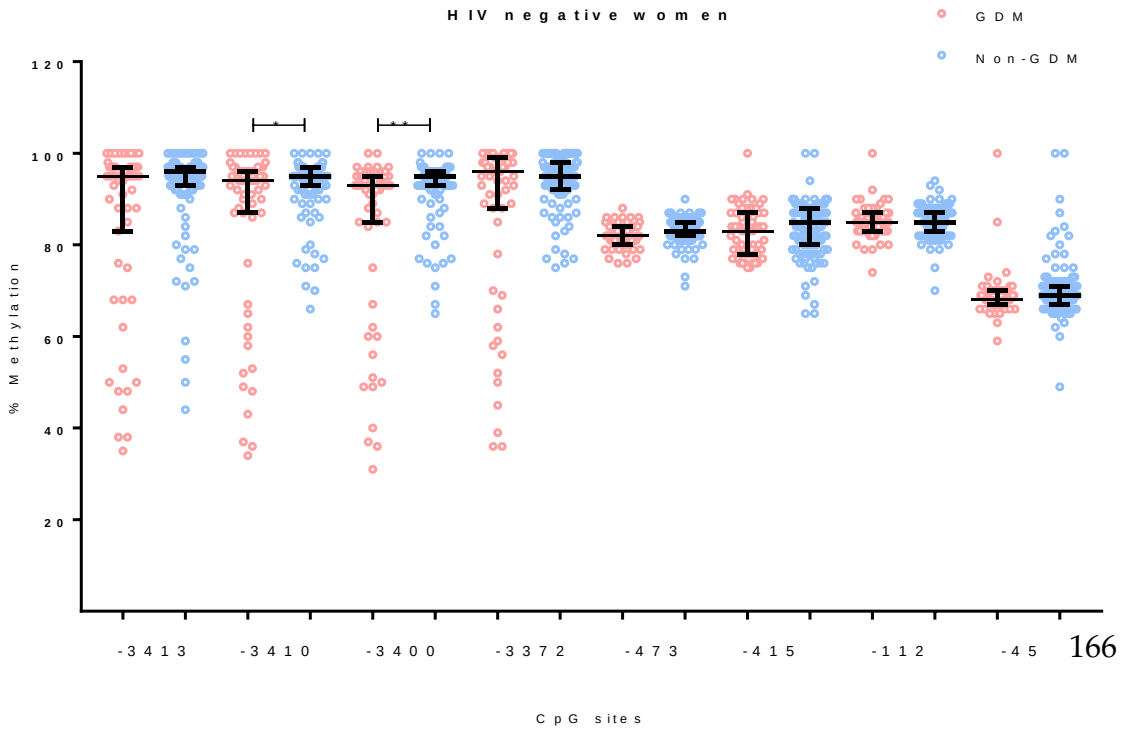
4.2. DNA Methylation

DNA methylation at CpG -3413, -3410, -3400 and -3372 in R1 was lower (hypomethylated) in women with GDM compared to normoglycemia, although only CpG -3400 showed a trend towards statistical significance ($p<0.06$) (Figure 8.3a). Stratification according to HIV status demonstrated hypomethylation of two CpG sites (-3410: $p=0.048$ and -3400, $p=0.004$) in HIV negative women with GDM compared to normoglycemia (Figure 8.3b). These GDM-associated differences were not observed in HIV positive women (Figure 8.3c). Logistic regression analysis showed that methylation at CpGs -3410 and -3400 were associated with GDM in HIV negative women, with the strength of association increasing slightly when methylation at both CpGs were included in the model (Table 8.2). The association between DNA methylation and GDM in HIV negative women remained significant after adjusting for age, BMI and gestational age. Regression analysis confirmed that methylation levels were not associated with GDM in HIV positive women. The percentage of women with methylation values in the upper quartile (between the median and 75th percentile) at CpGs -3410 and -3400 were significantly higher ($p<0.001$) in the HIV positive women compared to HIV negative women (Table S2). ART did not affect DNA methylation levels at CpG -3410 ($p=0.196$) and -3400 ($p=0.617$) in HIV positive women (Figure S2).

a)


All women
GDM
Non-GDM
p = 0.06
% Methylation
120
100
80
60
40
20
0
-3413
-3410
-3400
-3372
-473
-415
-112
-45
CpG sites

b)


HIV negative women
GDM
Non-GDM
*
**
% Methylation
120
100
80
60
40
20
-3413
-3410
-3400
-3372
-473
-415
-112
-45
CpG sites

c)

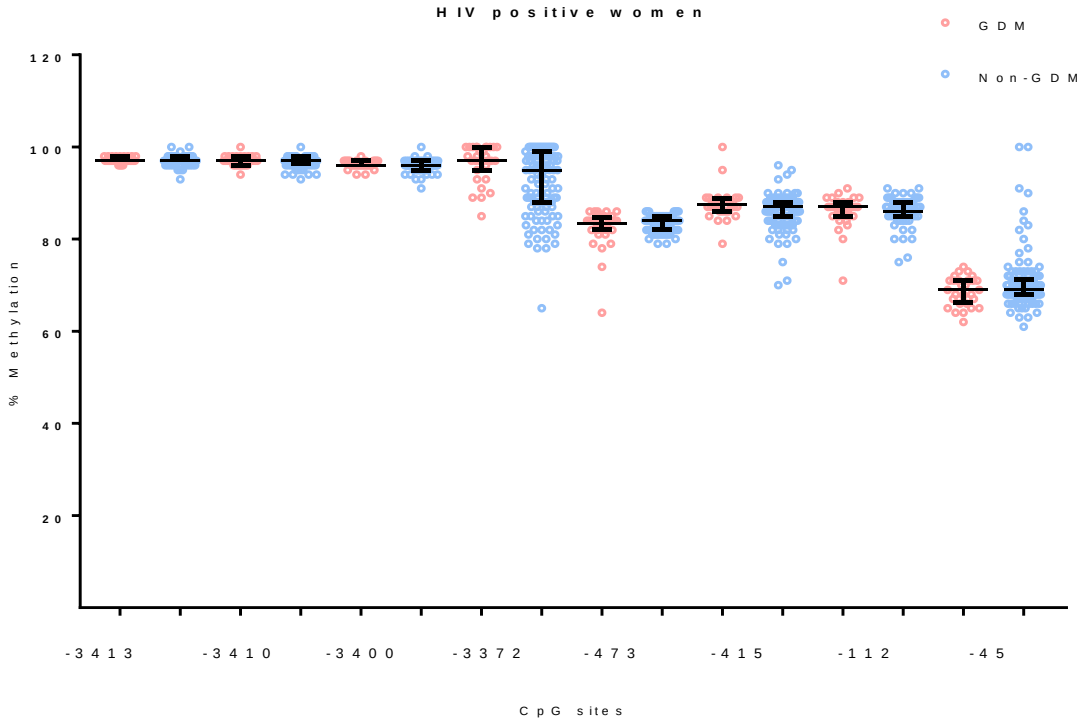


*Figure 8.3. DNA methylation of eight CpG sites in the promoter of the adiponectin gene. DNA methylation was measured in a) all women (n=95 GDM; n=191 non-GDM), b) HIV negative women (n=63 GDM; n=118 non-GDM) and c) HIV positive women (n=32 GDM; n=73 non-GDM). Data are represented as the median and interquartile range. *p<0.05, **p<0.01*

Table 8.2. Logistic regression analysis of ADIPOQ gene promoter DNA methylation at CpG -3410 and -3400 associated with GDM by HIV status

	[a]Univariate			[b]Multivariate		
GDM	β	95% CI	p-value	β	95% CI	p-value
			HIV negative			
-3410	-0.051	-0.079; -0.022	**0.001**	-0.043	-0.075; -0.011	**0.008**
-3400	-0.056	-0.087; -0.026	**<0.001**	-0.050	-0.084; -0.017	**0.003**
-3410 & -3400	-0.027	-0.042; -0.012	**<0.001**	-0.024	-0.040; -0.007	**0.005**
			HIV positive			
-3410	-0.129	-0.245; 0.504	0.498	0.170	-0.251; -0.592	0.428
-3400	0.246	-0.139; -0.630	0.211	0.245	-0.168; -0.658	0.245
-3410 & -3400	0.145	-0.096; -0.385	0.238	0.150	-0.104; -0.404	0.246

[a]Univariate linear regression: Association between CpG-specific methylation and GDM. [b]Multivariate linear regression: Adjusting for age (years), body mass index (kg/m2) and gestational age in HIV negative women and age (years), body mass index (kg/m^2), gestational age (weeks) and antiretroviral therapy in HIV positive women; β: beta coefficient; CI: Confidence interval. Values in bold type indicate statistical significance.

The correlation between methylation levels at CpG sites (-3410, -3400) and clinical characteristics was explored using Spearman's analysis (Table 8.3). An inverse relationship between DNA methylation and fasting insulin concentrations at CpG -3400 was observed (p=0.029), although the association was ablated when stratified according to HIV status. DNA methylation was negatively correlated with fasting glucose concentrations at CpG -3410 (p=0.015) and CpG -3400 (p=0.001) and with serum adiponectin concentrations at CpG -3410 (p=0.023) and CpG -3400 (p=0.013) in HIV negative women.

Table 8.3. Spearman's correlation between ADIPOQ gene promoter DNA methylation at CpG -3410 and -3400 and participant characteristics

Variable	All women		HIV negative		HIV positive	
	r	p-value	*r*	p-value	*r*	p-value
CpG -3410						
Fasting glucose (mmol/L)	-0.084	0.158	-0.180	**0.015**	-0.024	0.806
OGTT 2 hr (mmol/L)	-0.011	0.857	0.035	0.642	-0.034	0.733
Fasting insulin (mIU/L)	-0.118	0.089	-0.079	0.318	0.234	0.096
Adiponectin (μg/ml)	0.040	0.512	-0.170	**0.023**	-0.020	0.852
CpG -3400						
Fasting glucose (mmol/L)	-0.111	0.062	-0.238	**0.001**	0.130	0.186
OGTT 2 hr (mmol/L)	-0.062	0.303	-0.014	0.857	-0.050	0.615
Fasting insulin (mIU/L)	-0.149	**0.029**	-0.134	0.092	0.130	0.358
Adiponectin (μg/ml)	0.023	0.690	-0.180	**0.013**	-0.040	0.721

OGTT: oral glucose tolerance test; HIV: human immunodeficiency virus; ADIPOQ: Adiponectin gene; Rho: Spearman's correlation. Values in bold type indicate statistical significance: **p<0.01, *p<0.05

4.3.In Silico Analysis

Transcription factors that may bind to the region of altered DNA methylation in the ADIPOQ promoter are illustrated in Figure 8.4. Using ALIBABA software, binding sites for Specificity Protein 1 Transcription Factor (SP-1) and Odd-Skipped Related Transcription factor 1 (OSR1) were identified, while ALGGEN-PROMO software identified binding sites for glucocorticoid receptor alpha and beta (GRα and GRβ), X-Box Binding Protein 1 (XBP1) and General Transcription Factor IIi (GTF2I). Binding sites for Transcription Factor AP2-alpha (TFAP2A) were similarly identified by both ALIBABA and ALGGEN-PROMO software.

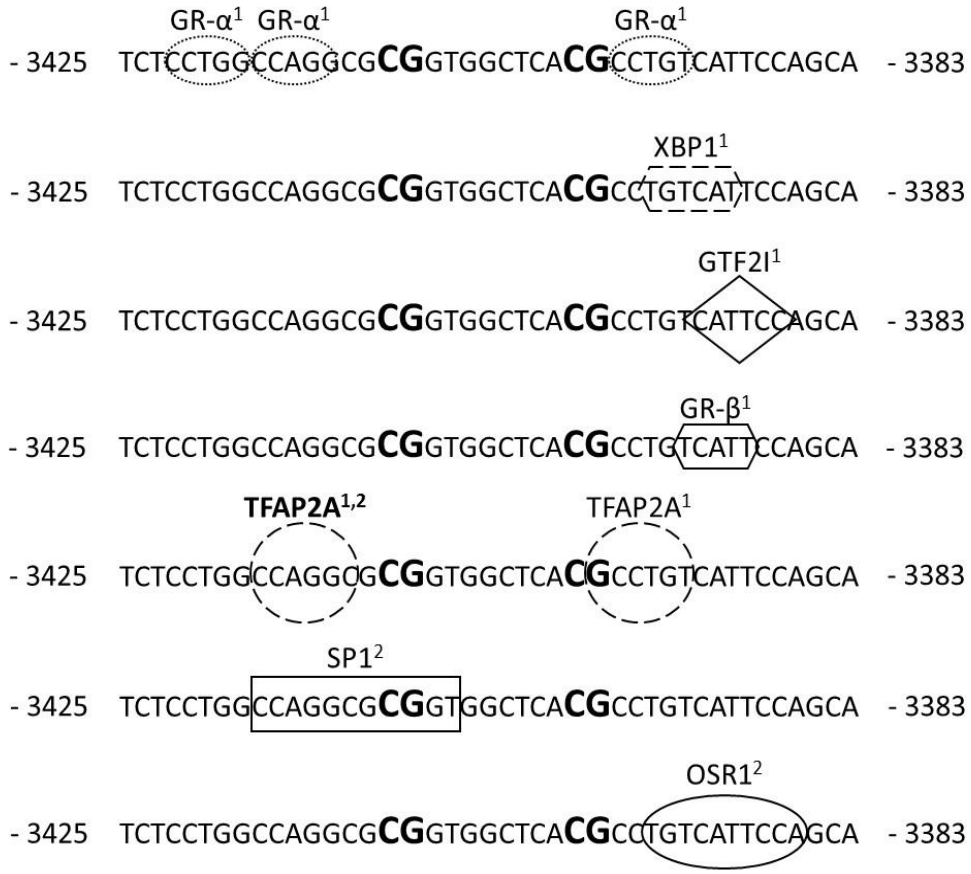


Figure 8.4. Schematic illustration showing potential transcription factors specific to the region of altered DNA methylation in the promoter of ADIPOQ. Potential transcription factor binding sites within the region -3425 to -3383 upstream of the ADIPOQ gene transcription start site were identified using ALGGEN-PROMO1 and ALIBABA 2.12 software. These transcription factors were cross-referenced with the MeDReader database to identify transcription factors capable of binding to highly methylated DNA. The following factors were identified: GRα - glucocorticoid receptor alpha; XBP1 - X-Box Binding Protein 1; GTF2I - General Transcription Factor Iii; SP-1 - Specificity Protein 1 Transcription Factor; OSR1 - Odd-Skipped Related Transcription factor 1; GRβ - glucocorticoid receptor beta and TFAP2A - Transcription Factor AP2-alpha. Bold-face nucleotides indicate differentially methylated CpG sites

5. DISCUSSION

The effect of HIV infection on DNA methylation during GDM is underexplored. This study demonstrates that HIV infection affects the association between *ADIPOQ* DNA methylation in peripheral blood and GDM in South African women. Two CpG sites (-3410 and -3400) in the proximal promoter of *ADIPOQ* was hypomethylated in HIV negative women with GDM compared to normoglycemia, while these changes were not observed in HIV positive women. Furthermore, methylation at these differentially methylated CpGs were negatively correlated with fasting glucose and adiponectin concentrations in HIV negative, but not in HIV positive pregnant women. In addition, *in silico* analysis identified two transcription factors, *SP-1* and *TFAP2A,* that binds to differentially methylated sites in the *ADIPOQ* promoter region at CpG -3410 and -3400, respectively.

Our findings are consistent with others who have similarly reported altered *ADIPOQ* methylation during GDM. Although the methylation differences between GDM and non-GDM groups were small, they were previously reported in other populations (Bouchard et al., 2012; Houshmand-Oeregaard et al., 2017; Ott et al., 2018), suggesting that these CpG sites may be important for *ADIPOQ* gene regulation. Ott et al. showed small, yet significant alterations in CpG-specific DNA methylation in paired subcutaneous and visceral adipose tissue and maternal blood between GDM and non-GDM obese women. In addition, the authors demonstrated a significant inverse correlation between differentially methylated CpG sites and *ADIPOQ* gene expression in adipose tissue, indicating functional relevance of altered CpG-specific DNA methylation. However, this study was conducted in a low HIV prevalence setting and did not investigate the effect of HIV. Similarly, other studies that have reported altered DNA methylation of the *ADIPOQ* gene were conducted in low HIV prevalence

populations and did not report HIV status (Bouchard et al., 2012; García-Cardona et al., 2014; Houshmand-Oeregaard et al., 2017; Nogues et al., 2019). Nogues et al. showed that placental hypomethylation of the *ADIPOQ* promoter is associated with maternal obesity in a French population consisting of 12 obese and 18 non-obese pregnant women (Nogues et al., 2019). Gacia-Cardona et al. reported altered *ADIPOQ* promoter methylation at two CpG sites in peripheral blood of 39 obese and 22 morbidly obese Mexican adolescents with or without insulin resistance (García-Cardona et al., 2014). Moreover, Houshmand-Oeregaard et al. reported an increase in *ADIPOQ* DNA methylation and a decrease in *ADIPOQ* gene expression in subcutaneous adipose tissue from adult offspring of Danish women with diet treated GDM compared to controls (Houshmand-Oeregaard et al., 2017).

Accumulating evidence suggests that HIV infection modifies DNA methylation to promote viral integration into the host cell and to increase the virus's ability to replicate, survive and establish latency (Harbers et al., 1981; Nelson et al., 2017). Furthermore, HIV infection has been shown to increase DNA methyltransferase expression, which is responsible for *de novo* methylation in $CD4^+$ T cells (Mikovits et al., 1998; Fang et al., 2001). Accordingly, DNA methylation differences between HIV negative and positive individuals have been reported, although not during GDM (Zhang et al., 2016). Using the HumanMethylation450 Beadchip array, Zhang et al. identified 20 CpG sites that were differentially methylated in peripheral blood of 261 HIV infected and 117 uninfected individuals. Of these, two CpGs in the promoter of the NLR family, CARD domain containing gene 5 (NLRC5), a key regulator of major histocompatibility complex class I gene expression, had significantly lower methylation in HIV-infected compared to uninfected subjects (Zhang et al., 2016). Pion et al. reported that HIV infection of regulatory T cells cultured from peripheral blood samples of healthy individuals induced downregulation of Forkhead box P3 *(FOXP3)*,

which was associated with increased methylation in the regulatory regions of *FOXP3* (Pion et al., 2013). In addition, Dye et al. identified 123 differentially methylated CpG sites in monocytes of HIV-infected individuals with varying levels of insulin sensitivity using the HumanMethylation450 BeadChip array. These CpGs were enriched at genes involved in pathways relating to immune activation, glucose metabolism and insulin signalling (Dye et al., 2019). Furthermore, a study investigating global DNA methylation in LINE-1 and AluYb8 repetitive elements showed decreased DNA methylation profiles in infants exposed to HIV infection and combined ART treatment *in utero* compared to unexposed infants (Marsit et al., 2015). In our study, DNA methylation at CpG -3410 and -3400 within the *ADIPOQ* gene were not different between women receiving ART or those who were ART naïve, which may suggest that HIV infection rather than ART is associated with altered *ADIPOQ* methylation in South African women. Alternatively, our study may have been underpowered to detect differences due to the small HIV positive sample size compared to previously reported (n=445) (Marsit et al., 2015).

DNA methylation at CpG -3410 and -3400 within the *ADIPOQ* gene was inversely correlated with fasting glucose and serum adiponectin concentrations, and is consistent with Bouchard et al. who similarly reported an association between *ADIPOQ* methylation and hyperglycaemia or circulating adiponectin concentrations during pregnancy (Bouchard et al., 2012). In contrast, we did not observe an association between *ADIPOQ* methylation and insulin resistance as reported by Bouchard et al. Possible reasons for differences observed between studies could be due to several factors including sample size, different ethnicity, biological material, timing of methylation analysis during pregnancy and treatment strategies. Bouchard et al. investigated DNA methylation in the placenta of 98 French-Canadian women at delivery, while DNA methylation in our study was conducted in peripheral blood of

286 South African women at <26 weeks of gestation. In addition, the authors reported that women with GDM were on diet or insulin treatment, whereas women in our study were not on any treatment at the time of blood collection.

Common risk factors (advanced maternal age, obesity, family history of diabetes mellitus, previous baby more than four kg, glucosuria, previous recurrent pregnancy loss, stillbirth, or birth of a baby with congenital abnormalities) were significantly different in HIV negative women with GDM compared to normoglycemia, while these changes were not observed in HIV positive women. It is possible that HIV infection may influence the risk of developing GDM, although epidemiological studies on HIV infection during GDM are limited. Recently, a meta-analysis exploring the relationship between GDM diagnosis, HIV infection and treatment reported no association between HIV infection and the development of GDM in four studies using combination ART, two of which were conducted in the USA and two in Africa (Soepnel et al., 2017). A study investigating the prevalence of GDM in South African pregnant women reported a higher HIV prevalence in GDM compared to non-GDM women, although this was not statistically significant (Adam & Rheeder, 2017). Evidence suggest that ART, particularly first-generation protease inhibitors induces insulin resistance in both pregnant and non-pregnant women (Jao et al., 2013). Furthermore, ARTs cause dysregulated glucose metabolism, which likely increases the risk of developing GDM (Soepnel et al., 2017). Although Atripla™, a newer generation ART used in this study have fewer effects on cellular function and metabolism than protease inhibitors, metabolic dysfunction has not completely been eliminated (Willig & Overton, 2016). Thus, the association between HIV, ART and the development of GDM warrants further investigation.

In silico analysis identified transcription factors that are capable of binding highly methylated DNA and whose binding may be affected by altered methylation of the CpG sites. These factors are involved in adipocyte differentiation, stress response, and glucose and lipid metabolism (O'Connor, Gilmour & Bonifer, 2016; Piperi, Adamopoulos & Papavassiliou, 2016; Weikum et al., 2017). Of the identified transcription factors, *SP-1* and *TFAP2A* had their core recognition sites directly over the altered CpG sites. SP-1, a ubiquitously expressed transcription factor involved in proliferation and differentiation (O'Connor, Gilmour & Bonifer, 2016) has previously been shown to bind to the *ADIPOQ* proximal promoter (Barth et al., 2004). *TFAP2A*, a member of the developmentally important AP2 transcription factor family is able to form a heterodimer with its paralogue *TFAP2B* and consequently may negatively regulate *ADIPOQ* expression (Ikeda et al., 2006). In addition, the high level of DNA methylation observed within this region suggests that other mechanisms such as histone modification and chromatin remodelling may also have an important function in regulating *ADIPOQ*. Taken together, these results suggest that altered CpG methylation in the *ADIPOQ* promoter could potentially play a role in regulating *ADIPOQ* gene expression during GDM, and that this may differ in HIV negative and positive women. A limitation of this analysis is the short region of DNA (42 bp) explored for transcription factor binding. DNA methylation of nearby cytosines may influence the binding of trans-acting elements through steric interactions or other mechanisms such as the binding of methyl-CpG-binding proteins (Kudo, 1998; Baubec & Schübeler, 2014), that may play an important role in *ADIPOQ* gene regulation located beyond the region investigated. In this study, *ADIPOQ* promoter methylation and serum adiponectin concentration levels were not inversely correlated, supporting the role of other epigenetic mechanisms.

Our study has several strengths. First, DNA methylation of the *ADIPOQ* promoter was quantified in both HIV negative and positive women with GDM, and to our knowledge is the first such analysis conducted globally. Second, our sample size was larger (n=286) than previous studies on DNA methylation and GDM (Bouchard et al. (n=98) and Ott et al. (n=55)) (Bouchard et al., 2012; Ott et al., 2018), and women were matched according to age, BMI and gestational age as far as possible. Third, pyrosequencing is a highly reproducible method that is able to accurately detect small methylation differences (Fakruddin & Chowdhury, 2012). However, our study also has limitations. The use of peripheral blood, which consists of a variety of different cell types, such as erythrocytes, lymphocytes and platelets (Reinius et al., 2012) may confound methylation analysis. Although, previously we showed no significant differences in cell type composition between GDM and non-GDM women in a subset of our sample (Dias, Adam, Rheeder, et al., 2019). In addition, adiponectin gene expression was not quantified, which may have provided more insight into the potential role of DNA methylation. We quantified DNA methylation levels of *ADIPOQ* in blood and analysing DNA methylation in adipose tissue may offer more insight into its regulation. Furthermore, in addition to disease state, other infections, as well environmental factors such as diet, physical activity, smoking and alcohol consumption could lead to similar DNA methylation changes (Joubert et al., 2012; Lim & Song, 2012; Pauwels et al., 2017; Miyake et al., 2018). Thus, the lack to account for environmental factors pose a significant limitation to our study. However, the study population was recruited from the same community, had similar lifestyle behaviours and education status, and had no significant intergroup differences in socioeconomic factors, suggesting that they were likely to have similar environmental exposures. Furthermore, our study revealed inter-individual heterogeneity in methylation levels, highlighting another caveat of DNA methylation analysis. Consideration of factors such as viral load, CD4+ count, immune status and HIV and ART duration, which

were not known for our study, are important to enable a better understanding of the effect of HIV infection on DNA methylation during GDM. Nevertheless, our study paves the way for future longitudinal studies to investigate the relationship between HIV and GDM.

6. CONCLUSION

Our results demonstrate that HIV infection affects the association between *ADIPOQ* DNA methylation in peripheral blood and GDM in South African women. This study highlights the complexities of DNA methylation profiling and emphasises the need for biomarker discovery in both HIV infected and uninfected individuals. This is particularly important in South Africa, where approximately one in three pregnancies are complicated by HIV. To our knowledge, this is the first study to investigate the effects of HIV infection on DNA methylation during GDM. Studies with larger sample sizes are needed to confirm that methylation differences between HIV negative and HIV positive women are not due to chance, but rather due to HIV infection. Furthermore, longitudinal studies in both HIV infected and uninfected individuals are required to elucidate the underlying mechanisms associated with methylation differences and the effect it may have on gene regulation during GDM.

CHAPTER 9

GENETIC VARIANTS OF THE ADIPONECTIN AND METHYLENETETRAHYDROFOLATE REDUCTASE GENES IN SOUTH AFRICAN WOMEN WITH GESTATIONAL DIABETES MELLITUS

This chapter will be submitted as a research article to the *Journal of Assisted Reproduction and Genetics*. Dias S, Adam S, Rheeder P, Pheiffer C.

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1. ABSTRACT

Introduction: Gestational diabetes mellitus (GDM) occurs due to both genetic and environmental factors which increases susceptibility to disease. Genetic risk variants for GDM have been identified in many genes, although limited studies have been conducted in South African populations, which warrants further investigation. The aim of this study was to determine whether *ADIPOQ* -11377C>G and -11391G>A, and *MTHFR* 677C>T are associated with GDM in South African women.

Methods: The genotype and allele frequencies of *ADIPOQ* rs266729 and rs17300539, and *MTHFR* rs1801133 were quantified in the peripheral blood of women with (n=118) and without GDM (n=331), using quantitative real-time PCR and validated with DNA sequencing.

Results: Genotype and allele frequencies of *ADIPOQ* rs266729 and rs17300539, and *MTHFR* rs1801133 were not associated with GDM in our population. In addition, no association between *ADIPOQ* rs266729 and rs17300539 and clinical characteristics were observed. However, women with the minor T allele at the *MTHFR* polymorphism had significantly lower fasting insulin and higher serum adiponectin concentrations compared to women with the C allele. No association between *MTHFR* polymorphisms and global DNA methylation was observed.

Conclusion: This study is the first to investigate the association between *ADIPOQ* (rs266729 and rs17300539) and *MTHFR (*rs1801133) polymorphisms and GDM in a South African population. The low minor allele frequency observed in this population for all SNPs suggests that these polymorphisms may not be associated with the risk of GDM in South African women. However, to confirm this, future studies in larger

sample sizes are required to determine whether these genetic polymorphisms are associated with GDM in our population. Furthermore, the high genetic variability within the South African population emphasises the need to explore African specific SNPs.

2. INTRODUCTION

GDM is defined as glucose intolerance that is first diagnosed during the latter half of pregnancy, with return to normoglycemia after birth (WHO, 2013). The prevalence of GDM is increasing worldwide, with approximately 14% of pregnancies affected by GDM (International Diabetes Federation, 2017). Although different diagnostic criteria could partly account for the increase in prevalence observed, the increasing rates of obesity and maternal age are contributing factors. Without appropriate glucose management, GDM is associated with adverse pregnancy outcomes (Hod et al., 1991; Hadar & Hod, 2013; Moore, 2018) and an increased risk of type 2 diabetes (T2D) and future metabolic syndrome (Kim, Newton & Knopp, 2002; Clausen et al., 2008; Bellamy et al., 2009; Zhao et al., 2016) in both mothers and offspring. It is becoming increasingly evident that both genetic and environmental factors play a role in the pathophysiology of GDM (Shaat & Groop, 2007). Evidence suggest that environmental factors such as diet and physical activity increase susceptibility to GDM (Khan et al., 2016; Mijatovic-Vukas et al., 2018). In addition, genetic risk variants in genes responsible for metabolic changes during pregnancy predispose to GDM (Dias et al., 2018). Thus, screening for SNPs may detect genetic susceptibility to GDM and may be useful as biomarkers.

Adiponectin is an adipose tissue-derived adipokine with insulin sensitising properties that regulates glucose and lipid homeostasis (Brochu-Gaudreau et al., 2010; Ghadge, Khaire & Kuvalekar, 2018). Decreased levels of adiponectin are observed during obesity and T2D (Daimon et al., 2003; Gariballa et al., 2019). Adiponectin concentrations progressively decline during pregnancy (Guelfi et al., 2017), with lower levels observed in women with GDM compared to those with normoglycemic pregnancies (Ranheim et al., 2004; Worda et al., 2004; Mohammadi & Paknahad, 2017;

Adam et al., 2018). Studies have suggested that single nucleotide polymorphisms (SNPs) in the adiponectin gene *(ADIPOQ)* could influence adiponectin concentrations and subsequently insulin sensitivity and glucose tolerance (Hara et al., 2002; González-Sánchez et al., 2005). The majority of these SNPs have been shown to be associated with obesity, insulin resistance and T2D (Kondo et al., 2002; Menzaghi et al., 2002; Stumvoll et al., 2002; Kang et al., 2005; Bouatia-Naji et al., 2006; Han et al., 2011; Fan et al., 2014; Lu et al., 2014), with only a few studies investigating *ADIPOQ* SNPs during GDM (Dias et al., 2018). The SNPs rs266729 (*-11377T>G*) in the promoter region (Beltcheva et al., 2014; Pawlik et al., 2017) and rs2241766 (45T>G) in exon 2 (Low et al., 2011; Beltcheva et al., 2014; Han et al., 2014; Takhshid & Zare, 2015) were reported to be associated with GDM in Polish, Bulgarian, Iranian, Chinese and Malaysian populations, while rs1501299 (276G>T) in intron 2 was not associated with GDM in a Polish and Bulgarian populations (Beltcheva et al., 2014; Pawlik et al., 2017).

DNA methylation is an epigenetic mechanism that plays a key role in gene regulation in response to environmental cues (Christensen & Marsit, 2011; Ling & Rönn, 2019). Altered DNA methylation has been reported during several metabolic diseases (Wang et al., 2010; Cash et al., 2011; Martín-Núñez et al., 2014; Chambers et al., 2015; Matsha et al., 2016; Huang et al., 2017; Willmer et al., 2018) including GDM (Enquobahrie et al., 2015; Kang et al., 2017; Wu et al., 2018). Recently, we conducted genome-wide methylation analysis using the MethylationEPIC bead chip array and demonstrated differential methylation of 1046 CpGs in South African women with GDM compared to women without GDM (Dias, Adam, Rheeder, et al., 2019). Several of these CpGs mapped to genes involved in glucose metabolism, insulin resistance and inflammation, suggesting that dysregulated DNA methylation could contribute to the development of GDM. Methylenetetrahydrofolate reductase *(MTHFR),* an enzyme in the transmethylation pathway, catalyses the conversion of homocysteine to

methionine in response to environmental cues (Goyette et al., 1994; Froese et al., 2016), thus affecting DNA methylation (Miranda & Jones, 2007; Tchantchou & Shea, 2008). Genetic variants in the *MTHFR* gene have been shown to impair enzyme function and consequently DNA methylation (Lievers et al., 2001), resulting in metabolic disease (Kang et al., 1988; Frosst et al., 1995; Wang et al., 2014, 2017). Two common functional polymorphisms, rs1801133 *(677C>T)* and rs1801131 (1298A>C) within the *MTHFR* gene were shown to decrease enzyme activity (Kang et al., 1988; Lievers et al., 2001). It is thus plausible to speculate that SNPs in *MTHFR* may play a role in the regulation of DNA methylation during GDM.

The molecular mechanisms that underlie the development of GDM are not fully elucidated. Our recent studies showed lower adiponectin expression (Adam et al., 2018) and altered DNA methylation (Dias, Adam, Rheeder, et al., 2019) in South African women with GDM compared to those with normoglycemic pregnancies. We hypothesised that SNPs in *ADIPOQ* and *MTHFR* underlie these differences and are thus associated with GDM. The aim of this study was to determine whether *ADIPOQ* -11377C>G and -11391G>A, and *MTHFR* 677C>T are associated with GDM in South African women. The genotype and allele frequencies of SNPs were determined in 118 women with GDM and 331 women with normoglycemic pregnancies using quantitative real-time PCR (qRT-PCR). In addition, we investigated the association between SNPs and clinical characteristics. Furthermore, the association between *MTHFR* 677C>T (rs1801133) polymorphism and global methylation was investigated. To our knowledge this is the first study to investigate the association between *ADIPOQ* and *MTHFR* polymorphisms during GDM in a South African population.

3. METHODOLOGY

3.1. Study Participants

Ethical approval for this study was granted by the University of Pretoria Health Sciences Ethics Committee (180/2012). The study was conducted according to the Declaration of Helsinki and all women gave written informed voluntary consent after the procedures had been fully explained in the language of their choice. One thousand participants were recruited from a primary care clinic in Johannesburg, South Africa. Black African women with singleton pregnancies, who were ≤26 weeks pregnant and who did not have pre-existing diabetes type 1 and type 2 diabetes (T1D and T2D) were enrolled in the study. At recruitment, demographic and socio-economic data were obtained in the form of a standardised questionnaire and risk factors for GDM were assessed (Adam & Rheeder, 2017). Women with random glucose and glycated haemoglobin (HbA1c) concentrations <11.1 mmol/L and 6.5%, respectively, were requested to return to the clinic within two weeks in a fasted state for GDM testing and blood collection. At this time, a 75 g oral glucose tolerance test (OGTT) was conducted, and GDM was diagnosed if at least one glucose value was met (fasting plasma glucose ≥5.1mmol/L, 1 hr OGTT ≥10 mmol/L or 2 hr OGTT ≥8.5 mmol/L), according to the International Association of Diabetes in Pregnancy Study Group (IADPSG) criteria (IADPSG panel, 2010). Four hundred and forty nine women were selected for this study, of which 118 were GDM and 331 non-GDM (Figure 9.1). Blood samples were collected after an overnight fast, and C-reactive protein (CRP) and fasting glucose and insulin concentrations were measured in an accredited laboratory (Vermaak and Partners/Pathcare laboratories, South Africa). The homeostatic model assessment (HOMA), a measure of insulin resistance was calculated using the equation: (fasting plasma glucose in mmol/L x fasting serum insulin in mIU/mL)/22.5.

Serum adiponectin concentrations were measured using the human adiponectin enzyme-linked immunosorbent assay (ELISA) (Merck, Dermstadt, Germany). Blood were stored at -80 ˚C for SNP genotyping analysis.

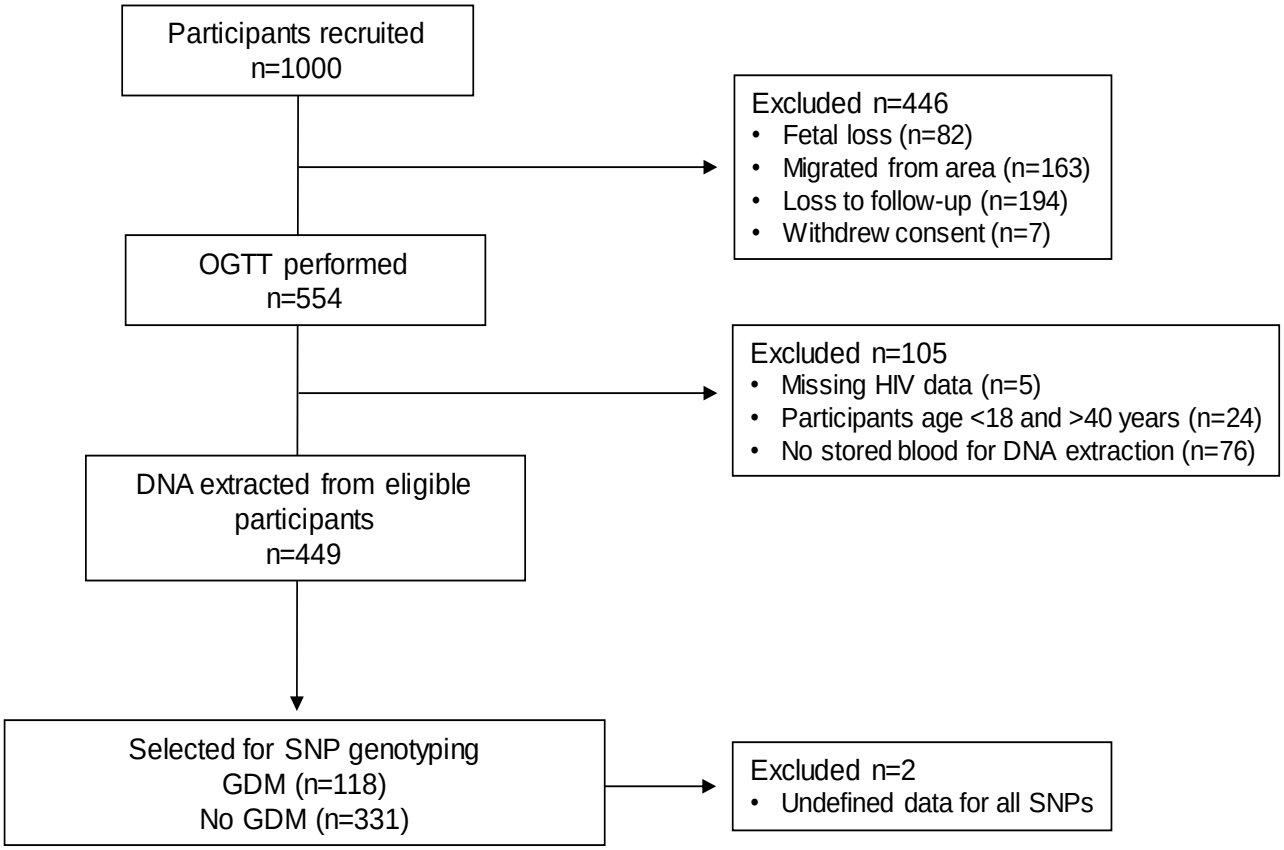

Figure 9.1. Flow diagram for study participants. For this study, women with (n=118) and without GDM (n=331) were selected from a larger prospective cohort study.

3.2.DNA Extraction and Genotyping

Genomic DNA was extracted from 2 ml of blood collected in Ethylenediaminetetraacetic acid (EDTA) tubes using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) as previously described (Dias, Adam, Wyk, et al., 2019). DNA concentration was measured using the Qubit Fluorometer (Invitrogen,

Carlsbad, USA) and the Quanti-iT dsDNA Broad Range assay kit (ThermoFisher, Massachusetts, USA). The *ADIPOQ* -11377C>G (rs266729) and 11391G>A (rs17300539) polymorphism, previously associated with GDM, overt diabetes and GDM, and *MTHFR* 677C>T (rs1801133) polymorphisms, previously associated with DNA methylation, were genotyped using qRT-PCR with Taqman genotyping assays (Table 9.1) (Applied Biosystems, Massachusetts, USA) on the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Massachusetts, USA). Briefly, qRT-PCR was performed using 9.5 ng of DNA, 5 μl of TaqPath ProAmp Master Mix and 0.25 μl of 40X TaqMan SNP Genotyping Assay in a total volume of 10 μl, according to manufacturer's instructions. The following PCR conditions were used: 10 min at 95 °C (initial denaturation/enzyme activation), 15 sec at 95 °C (denaturation) and 60 sec at 60 °C (annealing/extension) for 40 cycles. For quality control, 20% of samples were randomly selected and genotyped in duplicate. Positive and negative controls were included on all plates. Genotyping was validated by DNA sequencing (Central Analytical Facilities, Cape Town, South Africa). Details of sequencing primers are shown in Table 9.2. In total, nine samples for each SNP were randomly selected for validation. Primers for sequencing were designed on NCBI using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast.).

3.3. Statistical Analysis

Participant characteristics were tested for normality using the Shapiro-Wilk test in STATA 14 (StataCorp, College Station, USA). All data deviated from normality and were expressed as the median and interquartile range (25th and 75th percentiles). The Mann-Whitney test was used to compare variables across GDM and between genotypes and clinical parameters. The *ADIPOQ* rs266729 and rs17300539 and *MTHFR* rs1801133 genotype and allele frequencies in GDM and non-GDM groups

were compared using the Chi-squared (X^2) test or Fisher's exact test if the frequency was <5. A $p \leq 0.05$ was considered statistically significant. The Pearson's X^2 test was performed to determine whether the genotype frequencies at *ADIPOQ* rs266729 and rs17300539 and *MTHFR* rs1801133 were in Hardy-Weinberg Equilibrium (HWE) ($p > 0.05$).

Table 9.1. Details for ADIPOQ rs266729 and rs17300539 and MTHFR rs1801133 single nucleotide polymorphisms assays

Gene symbol	Assay ID	Sequence (5′-3′)	Global MAF
ADIPOQ	rs266729	TTGCAAGAACCGGCTCAGATCCTGC[C/G]CTTCAAAAACAAAACATGAGCGTGC	G=0.23 C=0.77
ADIPOQ	rs17300539	TCAGAATGTGTGGCTTGCAAGAACC[A/G]GCTCAGATCCTGCCCTTCAAAAACA	A=0.03 G=0.97
MTHFR	rs1801133	GAAAAGCTGCGTGATGATGAAATCG[G/A]CTCCCGCAGACACCTTCTCCTTCAA	T=0.25 C=0.75

SNP: Single nucleotide polymorphisms; ADIPOQ: Adiponectin ; MTHFR: methylenetetrahydrofolate reductase; MAF: Minor allele frequency

Table 9.2. Sequencing primers for SNP validation

rs number/ gene symbol	Primer sequence (5′-3′)	Template strand	Length	Start	Stop	Tm	GC%	Product Length (bp)
rs266729/ *(ADIPOQ)*	Fwd: TGGTGCTGGCATCCTAAGC	Plus	20	416	435	60.68	55	
	Rev: CCTTGGACTTTCTTGGCACG	Minus	20	541	522	59.41	55	126
rs17300539/ *(ADIPOQ)*	Fwd: TTGGTGCTGGCATCCTAAGC	Plus	20	180	199	60.68	55	
	Rev: GGACTTTCTTGGCACGCTCA	Minus	20	301	282	60.88	55	122
rs1801133/ *(MTHFR)*	Fwd: CTGTCATCCCTATTGGCAGGT	Plus	21	404	424	59.51	52	
	Rev: CATGCCTTCACAAAGCGGAA	Minus	20	564	545	59.4	50	161

SNP: Single nucleotide polymorphism; ADIPOQ: Adiponectin gene; MTHFR: Methylenetetrahydrofolate reductase; Fwd: forward primer; Rev: Reverse Primer; 5′: five prime; 3′: three prime.

4. RESULTS

4.1. Clinical Characteristics of Study Participants

The clinical characteristics of the study participants are shown in Table 9.3. BMI ($p=0.012$) and random ($p<0.001$), fasting ($p<0.001$), 1 hr OGTT ($p<0.001$) and 2 hr OGTT ($p<0.001$) glucose and fasting insulin ($p=0.03$) concentrations, glycated haemoglobin (HbA1c) ($p=0.005$) and HOMA ($p<0.001$) were higher in women with GDM compared to women with normoglycemia, while gestational age ($p=0.007$) and serum adiponectin concentrations ($p=0.013$) were lower in women with GDM.

4.2. Association Between ADIPOQ Genotypes, Gestational Diabetes Mellitus and Metabolic Characteristics

No differences in genotype and allele frequency distribution were observed for *ADIPOQ* rs266729 and *ADIPOQ* rs17300539 polymorphisms in GDM and compared to normoglycemia (Table 9.4). The genotype frequency distribution of the *ADIPOQ* rs17300539 polymorphism was in accordance with HWE ($p=0.92$), while the *ADIPOQ* rs266729 polymorphism deviated from HWE ($p<0.001$). Moreover, no differences in BMI, fasting glucose, 1 hr OGTT, 2 hr OGTT, HbA1c, fasting insulin and serum adiponectin concentrations were observed by genotype (Table 9.5).

Table 9.3. Participant characteristics according to GDM status

Participant Characteristics	Non-GDM	GDM	*p*-value
Participants: n	331	116	
Age (years)	27.0 (23.0 – 31.0)	29.0 (24.0 – 32.0)	0.083
BMI (kg/m^2)	25.6 (22.7 – 29.8)	27.1 (23.6 – 31.2)	**0.012**
Gestational age (weeks)	26.0 (23.0 – 28.0)	25.0 (21.0 – 27.0)	**0.007**
Random glucose (mmol/L)	4.4 (4.0 – 4.8)	4.7 (4.3 – 5.1)	**<0.001**
Fasting glucose (mmol/L)	4.4 (4.0 – 4.6)	5.5 (5.3 – 6.0)	**<0.001**
OGTT 1 hr (mmol/L)	5.5 (4.7 – 6.4)	6.3 (5.4 – 7.5)	**<0.001**
OGTT 2 hr (mmol/L)	5.2 (4.5 – 5.8)	6.0 (5.1 – 7.2)	**<0.001**
HbA1c (%)	5.2 (4.9 – 5.4)	5.3 (5.1 – 5.5)	**0.005**
Fasting insulin (mIU/L)	5.2 (3.3 – 7.5)	5.9 (3.9 – 8.8)	**0.030**
HOMA	1.0 (0.7 – 1.5)	1.5 (0.9 – 2.2)	**<0.001**
C-reactive protein (mg/L)	5.7 (3.1 – 8.8)	7.0 (3.7 – 10.5)	0.125
Adiponectin (μg/ml)	10.4 (8.0 – 16.5)	9.1 (5.6 – 15.1)	**0.013**

BMI: body mass index; OGTT: oral glucose tolerance test; HbA1c: glycated haemoglobin; HOMA: homeostatic model assessment. Data are expressed as the median and interquartile range (25th–75th percentiles). Bold-type values indicate statistical significance between GDM and non-GDM groups.

Table 9.4. Genotype frequency and allele distribution of ADIPOQ rs266729 and rs17300539 polymorphisms between GDM and non-GDM groups

ADIPOQ	Genotype frequency (n (%))				Allele frequency (n (%))			
Variant	Genotype	Non-GDM	GDM	p-value	Allele	Non-GDM	GDM	p-value
rs266729	CC	258 (77.9)	90 (77.6)	0.936	C	576 (87.0)	199 (85.8)	0.634
	CG + GG	73 (22.1)	26 (22.4)		**G**	86 (13.0)	33 (14.2)	
rs17300539	GG	327 (98.8)	116 (100.0)	0.234	G	654 (99.4)	232 (100.0)	0.234
	GA + AA	4 (1.2)	0 (0.0)		**A**	4 (0.6)	0 (0.0)	

ADIPOQ: Adiponectin gene; GDM: gestational diabetes mellitus. Bold-type letters indicate the risk allele and genotype.

Table 9.5. Participant characteristics according to ADIPOQ (rs266729 and rs17300539) genotype

Participant characteristics	*rs266729*			*rs17300539*		
	CC n=348	CG + GG n=99	*p*-value	GG n=443	GA + AA n=4	*p*-value
BMI (kg/m^2)	26.1 (22.7 – 29.4)	25.9 (23.2 – 30.8)	0.796	26.2 (22.8 – 29.8)	22.7 (22.5 – 25.3)	0.226
Fasting glucose (mmol/L)	4.6 (4.2 – 5.3)	4.7 (4.4 – 5.3)	0.386	4.6 (4.3 – 5.3)	4 (3.9 – 4.8)	0.161
1 hr OGTT (mmol/L)	5.7 (4.8 – 6.7)	5.7 (4.7 – 6.4)	0.661	5.7 (4.8 – 6.7)	6.4 (4.4 – 6.6)	0.844
2 hr OGTT (mmol/L)	5.5 (4.8 – 6.3)	5.3 (4.6 – 6.1)	0.499	5.4 (4.7 – 6.2)	5.5 (4.6 – 6.2)	0.973
HbA1c (%)	5.2 (5.0 – 5.5)	5.2 (5.0 – 5.4)	0.949	5.2 (5.0 – 5.4)	5.0 (4.8 – 5.2)	0.241
Fasting insulin (mIU/L)	5.3 (3.4 – 7.4)	5.7 (3.1 – 9.0)	0.423	5.4 (3.4 – 7.5)	2.7 (2.7 – 2.7)	0.176
Adiponectin (μg/ml)	10.0 (6.8 – 15.3)	11.9 (7.9 – 17.5)	0.181	10.2 (6.8 – 15.4)	19.6 (8.5 – 31.4)	0.178

BMI: body mass index; OGTT: oral glucose tolerance test; HbA1c: glycated haemoglobin; Data are expressed as the median and interquartile range (25th–75th percentile)

4.3. Association Between MTHFR Genotype, Gestational Diabetes Mellitus and Metabolic Characteristics

No difference in the genotype or allele frequency of *MTHFR* rs1801133 was observed between women with or without GDM (Table 9.6). The genotype frequency distribution of the *MTHFR* rs1801133 polymorphism deviated from HWE ($p<0.001$). Fasting insulin concentrations were lower ($p=0.058$) and serum adiponectin concentrations were higher ($p=0.013$) in women with the T allele compared to the C allele, while no significant differences were observed for BMI and fasting, 1 hr OGTT and 2 hr OGTT glucose and HbA1c concentrations (Table 9.7).

Table 9.6. Genotype frequency and allele distribution of MTHFR rs1801133 polymorphisms between GDM and non-GDM groups

MTHFR	Genotype frequency (n (%))				Allele frequency (n (%))			
Variant	Genotype	Non-GDM	GDM	p-value	Allele	Non-GDM	GDM	p-value
rs1801133	CC	295 (89.1)	106 (91.4)	0.491	C	617 (93.2)	218 (93.9)	0.687
	CT + TT	36 (10.9)	10 (8.6)		**T**	45 (6.8)	14 (6.1)	

MTHFR: Methylenetetrahydrofolate reductase gene; GDM: gestational diabetes mellitus. Bold-type letters indicate the risk allele and genotype.

Table 9.7. Participant characteristics according to MTHFR rs1801133 genotype status

	rs1801133		
Participant characteristics	CC	CT + TT	p-value
BMI (kg/m^2)	26.1 (22.8 – 29.8)	26.2 (22.1 – 29.7)	0.096
Fasting glucose (mmol/L)	4.7 (4.3 – 5.3)	4.6 (4.2 – 5.3)	0.781
1 hr OGTT (mmol/L)	5.7 (4.8 – 6.6)	5.9 (4.8 – 6.7)	0.737
2 hr OGTT (mmol/L)	5.4 (4.7 – 6.2)	5.3 (4.7 – 6.3)	0.903
HbA1c (%)	5.2 (5.0 – 5.4)	5.1 (4.9 – 5.5)	0.817
Fasting insulin (mIU/L)	5.5 (3.5 – 7.6)	4.5 (2.9 – 6.1)	0.058
Adiponectin (μg/ml)	10.1 (6.7 – 15.1)	15.3 (8.3 – 19.8)	**0.013**
Global DNA methylation (%)	102.8 (75.4 – 149.8)	109.4 (76.1 – 152.5)	0.829

BMI: body mass index; OGTT: oral glucose tolerance test; HbA1c: glycated haemoglobin. Data are expressed as the median and interquartile range (25th–75th percentiles). Bold-type values indicate statistical significance between GDM and non-GDM groups.

5. DISCUSSION

Genetic factors are known to play a role in the development of GDM (Martin et al., 1985), however, relatively few studies have investigated the association between genetic variants and GDM. Results show no association between the investigated *ADIPOQ* and *MTHFR* gene polymorphisms and GDM in our population. However, we found that women with the minor T allele at the *MTHFR* polymorphisms had lower fasting insulin and higher serum adiponectin concentrations compared to women with the C allele.

In this study, no difference between genotype and allele frequencies were observed for *ADIPOQ* rs17300539 and rs266729 in women with GDM compared to normoglycemia in our population. These results are consistent with Gueuvoghlanian-Silva et al., who reported no differences in genotype or allele frequencies for the *ADIPOQ* rs266729 polymorphism in 79 women with GDM compared 169 normoglycemic controls in a Brazilian population (Gueuvoghlanian-Silva et al., 2012). Contrary to our findings, four studies investigating the *ADIPOQ* rs266729 polymorphism reported significant differences in genotype and allele frequencies between GDM and non-GDM women in various populations. Liang et al, reported that a higher frequency of the minor risk allele G (CG and GG genotypes) of rs266729 in 50 Chinese women with GDM compared to 80 controls at 38–39 weeks of gestation. Furthermore, the authors showed that the minor risk allele G remained associated with GDM after validation using a high throughput gene chip technique in 24 randomly selected Chinese women with GDM compared to controls (Liang et al., 2010). Among Polish women, Pawlik et al. reported a higher prevalence of the G allele (GG and CG genotypes) in GDM (n=204) compared to controls (n=207), which remained significant after adjusting for age, pre-pregnancy BMI and GDM in past

pregnancies (Pawlik et al., 2017). In addition, Nezamzadeh et al. showed that the G risk allele was significantly associated with GDM in Iranian women, with approximately 51 (61.4%) of the 83 GDM patients carrying the GG genotype (Nezamzadeh et al., 2019). Conversely, although Beltcheva et al. demonstrated a significant difference in genotype and allele frequency between GDM and controls, the authors reported a significant association between the minor risk allele G and controls, while the more common C allele was associated with GDM in a Bulgarian population (Beltcheva et al., 2014). In addition to ethnicity, discrepancies between our study and those that showed a significant association may be due to sample size and the frequency of the risk allele within our population. For example, the risk allele G of rs266729 occurs at a much lower frequency (9.5%) in Africans compared to the Europeans (28.1%), East Asians (27.6%) and Americans (24%), suggesting that a larger sample size is needed to predict disease risk in African populations.

To the best of our knowledge, no study has investigated the association between *ADIPOQ* rs17300539 polymorphisms and GDM. However, several studies have investigated the association between rs17300539 polymorphism and obesity, insulin resistance and T2D, although, with conflicting results. In a black South African population, Olckers et al. reported that the minor risk allele A was associated with normoglycemia, while the more common G allele was associated with T2D (Olckers et al., 2007). In contrast, a higher prevalence of the minor risk allele A was shown to be associated with T2D in French (Vasseur et al., 2002) and German (Schwarz et al., 2006) Caucasian populations. However, studies investigating the association between the rs17300539 polymorphism and T2D in a Japanese, Pakistani (Nadeem et al., 2017) and an African American population (Bostrom et al., 2009), did not report a significant difference in genotype frequencies between T2D and normoglycemia. Differences observed between these studies and ours may be due to the different degrees of

hyperglycaemia between GDM and T2D participants, different ethnicities, exposure to environmental factors and risk of disease. SNP polymorphism caused by somatic mutations are induced by environmental factors which are associated with changes in the expression of genes involved in disease development (Hollman, Tchounwou & Huang, 2016). The interaction between inherited genetics and environmental influences between different ethnicities and populations, confer different susceptibilities to disease outcome. Given the high level of genetic variation in the African population, as a result of the population migratory history and the dramatic variations in climate, diet and exposure to infectious diseases (Cabrera et al., 2018), the mechanisms that influences the risk of developing GDM or T2D in African populations may be different to non-African populations (Popova et al., 2017).

Dysregulated serum adiponectin concentrations are increasingly being implicated in the pathogenesis of GDM, although, the molecular mechanism underlying these changes remain unclear. Studies investigating the association between *ADIPOQ* rs266729 and rs17300539 polymorphisms, which are both located in the promoter region of *ADIPOQ*, and GDM are limited in South Africa. We found no association between rs266729 and rs17300539 polymorphisms and serum adiponectin levels, nor did we see an association between these SNPs and GDM related-metabolic traits such as BMI, glucose and insulin concentrations. Zemlin et al. showed no association between these SNPs and high molecular weight adiponectin levels in hyperglycaemic and normoglycemic individuals in a mixed ancestry South African population (Zemlin et al., 2016). Although our study investigated total adiponectin levels, while Zemlin et al. investigated high molecular weight adiponectin levels, similar results were observed, suggesting that these polymorphisms do not vary between different forms of adiponectin measurements within the South African population regardless of ethnicity. Conversely, Gueuvoghlanian-Silva et al. showed that serum adiponectin

levels at 28-36 weeks of gestation were significantly higher in Brazilian pregnant women, carrying the CC genotype for rs266729 (Gueuvoghlanian-Silva et al., 2012). Adiponectin levels have a strong genetic component (Comuzzie et al., 2001), suggesting that differences observed between studies may be due to genetic and ethnic backgrounds of the study populations. In addition, environmental factors such as diet and physical activity have also been shown contribute to variability observed in adiponectin concentrations (Mantzoros et al., 2006; Yu et al., 2009), which may play an important role in gene-environmental interactions.

In this study, no difference between genotype and allele frequencies were observed for *MTHFR* rs1801133 in women with or without GDM. However, women with the CT+TT genotype had lower fasting insulin and higher serum adiponectin concentrations compared to women with the CC genotype. In line with our findings Khan et al. showed no significant difference in allele and genotype frequencies of rs1801133 between GDM and non-GDM women in a South Indian population (Khan et al., 2015). Conversely, Kheradmand et al., reported that HOMA and insulin levels were significantly higher in non-pregnant Iranian women carrying the minor risk T allele compared to those with the CC genotype (Kheradmand et al., 2017). In addition, Chen et al. reported a higher prevalence of the T allele in T2D individuals with or without metabolic syndrome compared to controls in a Han Chinese population (Chen et al., 2010). Differences between these studies and ours may be due to age, gender, ethnicity, environmental influences and disease state. In addition, supplementation of folic acid and vitamin B12, which is generally taken during pregnancy, helps to overcome the negative health effect of SNPs in the *MTHFR* gene (Hiraoka & Kagawa, 2017). Unfortunately, data for folic acid and vitamin B12 intake is not known for these participants and therefore presents a limitation of our study.

In this study, we did not observe an association between *MTHFR* polymorphisms and DNA methylation. In line with our findings, Matsha et al., showed no association between the genotype distribution of *MTHFR* across quarters of global DNA methylation in diabetic (n=158), prediabetic (n=119) and normoglycemic (n=287) subjects in a mixed ethnic ancestry South African population (Matsha et al., 2016). In addition, Gobbo et al. showed no association between the risk T allele and DNA methylation in the placenta of uncomplicated (n=179) and complicated pregnancies (n=124) in a Canadian population, even though the risk T allele was increased in pregnancies affected by pre-eclampsia and intrauterine growth restriction (Del Gobbo et al., 2018). Moreover, in a recent meta-analysis of 10 studies across various populations, Wang et al. showed no association between the risk allele and global DNA methylation in various tissue samples affected by neural tube defects (Wang et al., 2016). *MTHFR* encodes a key enzyme in the folate and homocysteine metabolism and is essential for providing methyl groups for DNA methylation. Genetic mutations such as rs1801133 in the *MTHFR* gene, decreases enzyme activity and alters DNA methylation patterns (Fox & Stover, 2008). These results suggest the *MTHFR* polymorphisms may not play a role in regulating DNA methylation in diabetes-related complications. Although, further studies should be conducted to identify and understand methylation regulation as a result of *MTHFR* polymorphisms.

In contrast to most of the studies described previously, a strength of our study is that genotyping results were validated by DNA sequencing, thereby confirming all genotypes of randomly selected samples. While our sample size was relatively larger than most previously reported studies, differences in allele frequencies and disease aetiology between ethnicities, may explain our failure to detect statistically significant associations. Allele frequencies of rs266729 and rs1801133 deviated from HWE, suggesting that the SNPs investigated may be under possible selection pressure, or

that our sample size may be too small to detect an association between the risk allele an GDM. In addition, GDM diagnosis is not standardised internationally; thus, different diagnostic criteria could have contributed to the discordant results observed between studies. Importantly, GDM is a multifactorial disease, resulting from complex interactions between genetic and environmental factors (Shaat & Groop, 2007). Thus, the lack to account for factors such as diet, physical activity, smoking and alcohol consumption, which have been found to contribute substantially to the risk of GDM, pose a significant limitation to our study (Bao et al., 2016; Khan et al., 2016; Mijatovic-Vukas et al., 2018). In addition, environmental factors are known to influence DNA methylation patterns and may have impacted our analysis (He, Zhang, et al., 2017). Furthermore, our analysis may have overlooked the possibility of SNP-SNP and SNP-environment interaction, as well as linkage disequilibrium (Hinds et al., 2006; Zhang et al., 2019). Thus, in future, it would be worth investigating the combined effects of these polymorphisms and other variants that may affect the pathophysiology of GDM and are specific to the African population. African populations are considered the most genetically diverse worldwide, yet genomic research on the continent is limited. In an attempt to understand the genetic variation that underly susceptibility to disease, particularly in admixed African ancestry populations SNPs on the H3A array, which is specific for the African population, should be explored in this population (Mulder et al., 2016).

6. CONCLUSION

This study is the first to investigate the association between *ADIPOQ* (rs266729 and rs17300539) and *MTHFR (*rs1801133) polymorphisms and GDM in a South African population. The low minor allele frequency observed in this population for all SNPs suggests that these polymorphisms may not be associated with the risk of GDM in

South African women. Future studies in larger sample sizes are required to determine whether these genetic polymorphisms are associated with GDM in our population. Furthermore, the high genetic variability within the South African population emphasises the need to explore SNPs that are more specific to the African population.

DISCUSSION

TABLE OF CONTENTS

1. SUMMARY

In the current study we explored the potential of DNA methylation and SNPs to serve as molecular biomarkers for GDM in a South African population. Global, genome-wide and gene-specific methylation of *ADIPOQ* were measured in peripheral blood to assess methylation differences between women with or without GDM. In addition, SNPs within the *ADIPOQ* and *MTHFR* genes were examined to investigate whether these polymorphisms are associated with GDM in our population. Results of each chapter are briefly summarised below, followed by an integration and synthesis of the overall thesis findings, highlighting the significance and novelty of the study and how the study findings contribute to existing knowledge both locally and globally. Lastly, we discuss the potential impact of biomarkers on health systems, the strengths and limitations of the study and recommendations for future research.

CHAPTER 3 provides an overview of GDM focusing on risk factors, prevalence and treatment. All studies on GDM prevalence conducted between 1969 and 2018 in South Africa were reviewed and showed that the prevalence of GDM has increased over the years. The escalating prevalence may partly be due to the less stringent diagnostic criteria used in some studies (IADPSG panel, 2010), however the rising obesogenic environment, spurred by urbanisation, unhealthy diets and physical inactivity, undeniably plays an important role in the increased prevalence of GDM. Our recommendation is that intervention strategies targeting obesity may have the greatest impact on decreasing the prevalence of GDM.

CHAPTER 4 discusses the major screening and diagnostic strategies used worldwide and in South Africa, including the novel screening and diagnostic methods that are being explored. GDM screening and diagnostic tests have evolved over the years, with

different criteria used between and within countries. Although most international bodies advocate the IADPSG criteria based on findings from the HAPO study, concerns about over-diagnosis and increased burden on the health system, has led to decreased implementation, particularly in resource limited settings. In SA, the four most common diagnostic criteria are the IADPSG, NICE, American College of Obstetricians and Gynaecologists (ACOG) and WHO 1999 criteria. Lack of uniformity in diagnostic methods may negatively impact universal health care for all South Africans, while also resulting in inaccurate epidemiological data on prevalence, which is required to inform health planning and policy. This chapter highlights the need for novel screening and diagnostic tests that are simple, sensitive, specific and cost-effective alternatives to the current strategies employed, thus potentially being more amenable to widespread implementation.

CHAPTER 5 provides an overview of published literature on the use of DNA methylation and SNPs as molecular biomarkers of GDM. Several studies support the use of these molecular biomarkers as diagnostic tools for GDM. Despite their potential, we highlight the many challenges that need to be addressed before molecular biomarkers can become clinically applicable. Variation in analytical methods and lack of standardisation, together with population differences hamper the accuracy of these tests. We recommend the establishment of an international body to standardise analytical conditions for molecular biomarkers and screening biomarkers in large prospective cohort studies in different populations.

CHAPTER 6 investigated the association between global DNA methylation and GDM in our population. Results showed no differences in global DNA methylation between GDM and non-GDM groups, in contrast to studies in other populations, albeit in different biological samples (Nomura et al., 2014; Reichetzeder et al., 2016).

Interestingly, global DNA was associated with obesity, suggesting that maternal BMI rather than GDM may influences global methylation during pregnancy. Importantly, the diagnostic criteria used may have contributed to our failure to see differences. The IADPSG diagnosed GDM based on modest hyperglycaemia, resulting in small glucose differences between GDM and non-GDM groups. Furthermore, the lack of association between GDM and global DNA methylation, a crude measure of overall genomic methylation, may suggest that a more targeted approach profiling gene or locus specific methylation may be required.

CHAPTER 7 quantified genome-wide methylation in 801,236 CpG sites across the genome. Results showed differential methylation at 1046 CpG sites (associated with 939 genes), which mapped to pathways key to metabolic regulation, in women with GDM compared to women with normoglycemia. Of these, differential methylation of 15 genes were consistent with findings from a study conducted in Chinese women, using the same Illumina Infinium Human MethylationEPIC Bead Chip array (Kang et al., 2017). Other studies using the previous version of the bead chip array similarly reported DNA methylation differences during GDM in Non-Hispanic Caucasian American and Caucasian English populations (Enquobahrie et al., 2015; Wu et al., 2018), suggesting that GDM influences genome-wide methylation across different populations. Among the top five CpG sites identified in our study, one CpG site mapped to the *CAMTA1* gene, shown to regulate insulin production and secretion in previous studies (Mollet et al., 2016), which may offer potential as an epigenetic biomarker in our population.

CHAPTER 8 investigated whether DNA methylation levels at eight CpG sites within the *ADIPOQ* promoter are associated with GDM in HIV negative and HIV positive women. Results showed that two (CpG -3410 and -3400) of the eight CpG sites within

the *ADIPOQ* promoter were significantly hypomethylated during GDM in HIV negative, but not in HIV positive women. Although the methylation differences between GDM and non-GDM groups were small, they are consistent with findings in other low HIV prevalence settings (Bouchard et al., 2012; Houshmand-Oeregaard et al., 2017; Ott et al., 2018), suggesting that these CpG sites may be important for *ADIPOQ* gene regulation. Bioinformatic analysis identified transcription factor binding sites across this region, supporting their role in gene regulation. Our findings suggest that HIV infection may modify DNA methylation of *ADIPOQ*, potentially affecting gene expression and adiponectin's candidacy as a biomarker for GDM in South African women.

CHAPTER 9 investigated the association between *ADIPOQ* and *MTHFR* polymorphisms and GDM in our population. Results showed no association between the *ADIPOQ* (rs266729 and rs17300539) and *MTHFR (*rs1801133) polymorphisms and GDM in our population, in contrast to studies in other populations that reported an association between *ADIPOQ* (rs266729) and GDM (Liang et al., 2010; Pawlik et al., 2017; Nezamzadeh et al., 2019) and between *ADIPOQ* (rs17300539) and *MTHFR (*rs1801133) with T2D and diabetes-related metabolic traits (Vasseur et al., 2002; Schwarz et al., 2006; Kheradmand et al., 2017). These findings suggest that these SNPs are population specific and may not be associated with the risk of GDM in South African women. Alternatively, our sample size may have been too small. The minor allele frequency of these SNPs are lower in our population compared to non-African populations, which may explain the discrepancies between our study and those that showed a significant association. Interestingly, the minor allele of the *MTHFR* polymorphism was associated with lower fasting insulin and higher serum adiponectin concentrations. These results suggest that the *MTHFR* polymorphism

may be a useful marker to identify women at risk of developing insulin resistance during pregnancy, although further studies are required to explore this hypothesis.

2. INTEGRATION AND SYNTHESIS

GDM is becoming a growing public health concern both globally and in South Africa. Without appropriate screening and treatment, GDM affects maternal and child health, and has a negative impact on the health system. The lack of uniformity in diagnosis has been shown to hamper the detection of GDM, thus, research efforts to identify simple and efficient strategies to detect GDM has become a major focus. In this regard, studies have explored the use of molecular biomarkers as screening and diagnostic tools that could potentially aid in the accurate detection of GDM in future.

Collectively, experimental findings suggest that gene-specific, but not global methylation nor SNPs rs266729, rs17300539 and rs1801133, may offer potential as molecular biomarkers of GDM in this population. Quantification of global DNA may be too crude to detect small methylation differences during GDM, while the allele frequency of genetic variants, rs266729 and rs17300539 in *ADIPOQ* and rs1801133 in *MTHFR* may be too low to detect the risk of GDM in black South African women. Our findings show that HIV may affect DNA methylation, which has important ramifications for biomarker discovery in our population, given the high HIV prevalence. Overall, this study highlights the strengths and challenges of DNA methylation profiling and SNP genotyping, and emphasises the need for further studies to explore the candidacy of molecular biomarkers for GDM in South Africa and globally.

3. NOVELTY AND SIGNIFICANCE OF THE STUDY

To our knowledge, this is the first study to investigate the association between DNA methylation or *ADIPOQ* and *MTHFR* polymorphisms and GDM in a South African population. Molecular biomarkers are affected by both ethnicity and environmental factors, which emphasises the need to explore their candidacy as biomarkers in different populations. Globally, genetic variants rs17300539 in *ADIPOQ* and rs1801133 in *MTHFR* were investigated for the first time during GDM in our population, thus, adding new knowledge to the growing field of biomarker discovery for GDM. In addition, genetic variant rs266729 in *ADIPOQ* was found to be associated with GDM in different populations, but not in ours, suggesting that it might be population specific, and may not be associated with the risk of developing GDM in South African women. We showed differences in gene-specific methylation despite the low glucose levels differences observed between groups. These methylation differences suggest that molecular changes may occur despite modest hyperglycaemia, which may be associated with an increased risk of adverse health outcomes in mothers and offspring. Unfortunately, we did not have information on the pregnancy outcomes in mothers and offspring in this study.

Molecular biomarkers indicative of epigenetic changes with functional impact may offer potential as additional risk factors for GDM. This could facilitate earlier detection, initiation of treatment and management of GDM leading to improved health outcomes in the South African population. The use of biomarkers is a more acceptable GDM screening and diagnostic strategy for pregnant women and clinicians, as it is less time consuming than the OGTT, is not associated with nausea and vomiting, and does not require fasting or multiple blood draws. In addition, DNA methylation and SNPs can be detected in buccal cells from saliva (McMichael et al.,

2009; Van Dongen et al., 2018) and could provide a non-invasive method for detecting biomarkers, thereby obviating the need for blood collection. In populations with a high prevalence of HIV such as ours, blood collection is a safety concern due to the risk of HIV transmission. Moreover, extraction of DNA from buccal swabs provides an inexpensive, non-invasive and simple method to investigate these biomarkers in the offspring of mothers with GDM, which may facilitate intervention strategies to decrease adverse long-term health outcomes in these babies. This provides an opportunity to decrease the growing burden of non-communicable diseases, which would positively impact the overall disease trajectory.

4. RECOMMENDATIONS AND FUTURE WORK

Future work focusing on community-based, longitudinal studies in larger sample sizes that include both HIV negative and positive pregnant women are required to explore the candidacy of *CAMT1* as a biomarker for GDM in this population. There is a need to identify early biomarkers to facilitate earlier intervention strategies and possibly decrease adverse effects. In SA, many women seek antenatal care late during their pregnancies, thus community-based studies offer a pragmatic approach to identify women earlier during pregnancy. To increase the sensitivity, specificity and predictive power of molecular biomarkers, future studies should consider using a combination of these markers alone or together with other traditional risk factors in risk stratification models for predicting GDM risk. DNA methylation is affected by gene-environment interactions (He, Zhang, et al., 2017), thus we recommend that potential biomarkers be screened in different populations and countries to identify robust markers that are globally applicable. Lastly, we propose that future studies should investigate the use of non-invasive methods to collect DNA for analysis. Several studies have demonstrated the potential of using buccal cells (Teschendorff et

al., 2015; Van Dongen et al., 2018), which would be more clinically acceptable, yet would also allow an opportunity to assess DNA methylation in offspring exposed to GDM. Lastly, we recommend that genome-wide SNP genotyping using the H3A array (Mulder et al., 2016) should be conducted in our population to identify African specific SNPs, given the low allele frequency of non-African SNPs in our population.

5. IMPACT ON THE PUBLIC HEALTH SYSTEM

Our study adds to the growing body of evidence supporting the use of SNPs and DNA methylation as biomarkers for GDM. However, further validation in larger sample sizes are required to confirm their clinical candidacy as screening and diagnostic biomarkers of GDM. Despite their potential, these molecular biomarkers face several challenges, including the current expensive cost and complexity of analysis (McDermott et al., 2013), which negatively impacts their successful introduction into the clinical setting. The recent rapid advancement in molecular biology and laboratory technologies raise hope that technically advanced biomarkers may be made easier and become clinically feasible (Mayeux, 2004), leading to the development of a quick, cost-effective, point-of-care test, that could accurately identify women at high risk for GDM. Successful integration of these molecular biomarkers into the clinical setting will make the diagnosis of GDM easier compared to the challenges associated with risk-factor based strategies and the OGTT, as many health systems are already overburdened and under-resourced. In addition, use of biomarkers may allow earlier management and treatment of GDM, which is beneficial for patient outcomes. In this regard, biomarker research may have the potential to improve the performance of health systems and health equity in underserved communities. However, although promising new biomarkers are continuously being proposed, the reluctance of health

systems to move away from routine procedures, hinder translation and implementation of these markers into the clinical setting (Frangogiannis, 2012). Thus, the integration and implementation of potential biomarkers into the clinical setting requires close co-disciplinary, collaborative efforts between researchers, clinicians and health care personnel.

6. STRENGTHS AND LIMITATIONS

A strength of our study is the pragmatic study approach. Pregnant women from surrounding communities attending the local clinic, were recruited under standard routine clinical practices in a primary health care setting, thus, increasing the potential of biomarker discovery in realistic situations. In addition, women were not on any form of medication at the time of blood collection, thereby precluding any impact medication may have on DNA methylation profiling (García-Calzón et al., 2017). In this study, a relatively larger sample size compared to other studies on GDM was used, and confounding factors such as age, BMI and gestational age were adjusted for in our analysis. It is widely reported that obesity and maternal age affect DNA methylation patterns. Thus, to minimise any influence these factors may have on DNA methylation patterns, women were matched according to maternal age, BMI and gestational age as far as possible in each chapter. Moreover, GDM was diagnosed using the widely recommended IADPSG criteria, suggesting that even modest glucose differences may affect DNA methylation.

A number of limitations should be taken into account when interpreting the results of the current study. Of the 1000 participants recruited, only 554 women were scheduled for the 75g 2 hr OGTT at 24-28 weeks of gestation due to foetal loss, migration, loss to follow up and withdrawal of consent, which is a large drop-out rate in comparison to

other studies. Pregnant women from rural communities travel long distances, thus seldom attend the antenatal clinic in a fasted state, nor can they afford repeated visits in the event where a trained phlebotomist may not be available to conduct an OGTT at their given appointment. Other factors such as the lack of education about GDM and its adverse effects may have contributed to the high drop-out rates. Conducting community-based studies rather than clinic-based studies may lead to higher retention rates. Moreover, due to the migratory nature of this population, we were unable to measure post-partum OGTT to confirm the diagnosis of GDM. Thus, there is a possibility that some participants may have had pre-existing or undiagnosed diabetes. However, women in this study had low glucose levels, which are not considered diagnostic of T1D or T2D according to WHO (WHO, 2013). In addition, pre-pregnancy BMI was not known for these women, which may have influenced DNA methylation differences between groups. Another limitation of our study is the use of peripheral blood cells, which consist of a mixture of different cell types that may confound methylation analysis (Reinius et al., 2012). Statistical analysis of cell type composition in a subset of women did not show significant differences between GDM and non-GDM groups. However, the use of whole blood is robust, and does not require cell type isolation. Furthermore, the method of quantification could hinder reproducibility of findings across studies. Thus, standardisation of analytical methods is critical when profiling molecular biomarkers. Lastly, physical activity, diet, alcohol consumption and smoking, which are widely reported to influence DNA methylation patterns (Joubert et al., 2012; Lim & Song, 2012; Ling & Rönn, 2014; Pauwels et al., 2017; Miyake et al., 2018), are not known for our study and could confound our analysis. However, women in our study were recruited from the same community and had similar lifestyle behaviours, education and employment status, suggesting that they had roughly similar environmental influences. Importantly, the aim of the

study was to identify a robust marker that is not affected by these environmental factors, thus making it applicable in different settings.

APPENDICES

APPENDIX 1: STUDY OUTCOMES

Publications

1. Dias, S., Pheiffer, C., Abrahams, Y., Rheeder, P. & Adam, S. 2018. Molecular Biomarkers for Gestational Diabetes Mellitus. International Journal of Molecular Sciences. 19(10). DOI: 10.3390/ijms19102926.
2. Dias, S., Adam, S., Van Wyk, N., Rheeder, P., Louw, J. & Pheiffer, C. 2019. Global DNA methylation profiling in peripheral blood cells of South African women with gestational diabetes mellitus. Biomarkers. 24(3):225–231. DOI: 10.1080/1354750X.2018.1539770.
3. Dias, S., Pheiffer, C., Rheeder, P. & Adam, S. 2019. Screening and diagnosis of gestational diabetes mellitus in South Africa: What we know so far. South African Medical Journal. 109(7):457-462–462. DOI: 10.7196/SAMJ.2019.v109i7.14064.
4. Dias, S., Adam, S., Rheeder, P. & Pheiffer, C. 2019. Prevalence of and risk factors for gestational diabetes mellitus in South Africa. South African Medical Journal. 109(7):463-467–467. DOI: 10.7196/SAMJ.2019.v109i7.14127.
5. Dias, S., Adam, S., Rheeder, P., Louw, J. & Pheiffer, C. 2019. Altered Genome-Wide DNA Methylation in Peripheral Blood of South African Women with Gestational Diabetes Mellitus. International Journal of Molecular Sciences. 20(23):5828. DOI: 10.3390/ijms20235828.

Conferences

Poster presentation - DNA methylation profiling during Gestational Diabetes Mellitus. Society of Endocrinology, Metabolism and Diabetes South Africa (SEMDSA), 2017.

1. Oral presentation - Investigating molecular biomarkers during gestational diabetes mellitus. South African Medical Research Council Symposium, 2018 & 2019.

Funding

1. Thuthuka Grant (Unique grant no. 99391), National Research Foundation, 2016, 2017 & 2018

APPENDIX 2: ETHICS APPROVAL AND AMENDMENTS

Ethical approval certificate

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 28 August 2018.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

3/10/2016

Approval Certificate
New Application

Ethics Reference No.: 191/2016

Title: Investigating circulating microRNAs as predictive biomarkers for gestational diabetes.

Dear Stephanie Dias

The **New Application** as supported by documents specified in your cover letter dated 27/06/2016 for your research received on the 27/06/2016, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 3/10/2016.

Please note the following about your ethics approval:
- Ethics Approval is valid for 3 years.
- Please remember to use your protocol number (**191/2016**) 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, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- 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); MPharMed,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).

☎ 012 356 3084 deepeka.behari@up.ac.za http://www.up.ac.za/healthethics
✉ Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 60, Gezina, Pretoria

Ethics Amendment 1

Permission to change site of recruitment and include existing blood samples from a previous study (Ethics no: protocol 180/2012).

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.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

28/04/2017

Approval Certificate
Amendment
(to be read in conjunction with the main approval certificate)

Ethics Reference No.: 191/2016

Title: The effect of gestational hyperglycemia on maternal and fetal epigenetic programming

Dear Stephanie Dias

The **Amendment** as described in your documents specified in your cover letter dated 20/04/2017 received on 24/04/2017 was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 26/04/2017.

Please note the following about your ethics amendment:
- Please remember to use your protocol number (**191/2016**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committe may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics amendment is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- 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

*** Kindly collect your original signed approval certificate from our offices, Faculty of Health Sciences, Research Ethics Committee, Tswelopele Building, Level 4-60*

Dr R Sommers; MBChB; MMed (Int); MPharMed; 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).

☎ 012 356 3084 deepeka.behari@up.ac.za / fhsethics@up.ac.za http://www.up.ac.za/healthethics
✉ Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 60, Gezina, Pretoria

Ethics Amendment 2

Permission to screen for genetic variants in all samples

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 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

30-Jul-2018

Approval Certificate

New Application

Ethics Reference No.: 191/2016

Title: Investigating circulating microRNAs as predictive biomarkers for gestational diabetes

Dear Stephanie Dias

The **New Application** as supported by documents specified in your cover letter for your research received on the , was approved by the Faculty of Health Sciences Research Ethics Committee on the 27-Jun-2018.

Please note the following about your ethics approval:

- Ethics Approval is valid from to .
- Please remember to use your protocol number (191/2016) 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, or monitor the conduct of your research.

Ethics approval is subject to the following:

- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- 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) MPharMed
Deputy Chairperson: Faculty of Health Sciences Research Ethics Committee

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 2004 (Department of Health).

(012 3563084 - deepeka.behari@up.ac.za - http://www.up.ac.za/healthethics
Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 4-60 (opposite the BMW Building), Dr Savage Road, Gezina, Pretoria

Ethics Amendment 3

Permission to screen for biomarkers in samples from protocol 180/2012.

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 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

25/02/2016

Approval Certificate
Amendment
(to be read in conjunction with the main approval certificate)

Ethics Reference No.: 180/2012

Title: Comparing screening strategies for gestational diabetes in a South African population

Dear Sumaiya Adam

The **Amendment** as described in your documents specified in your cover letter dated 8/02/2016 received on 9/02/2016 was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 24/02/2016.

Please note the following about your ethics amendment:
- Please remember to use your protocol number (**180/2012**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committe may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics amendment is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- 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

*** Kindly collect your original signed approval certificate from our offices, Faculty of Health Sciences, Research Ethics Committee, H W Snyman South Building, Room 2.33 / 2.34.*

Professor Werdie (CW) Van Staden
MBChB MMed(Psych) MD FCPsych FTCL UPLM
Chairperson: Faculty of Health Sciences Research Ethics Committee

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 2004 (Department of Health).

♦ Tel:012-3541330 ♦ Fax:012-3541367 Fax2Email: 0866515924 ♦ E-Mail: fhsethics@up.ac.za
♦ Web: http://www.up.ac.za/healthethics ♦ H W Snyman Bld (South) Level 2-34 ♦ Private Bag x 323, Arcadia, Pta, S.A., 0007

Title amendment

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

30 August 2019

Dr C Pheiffer and Prof S Adam
Department of Reproductive Biology
Faculty of Health Sciences

Dear Dr C Pheiffer and Prof S Adam

STUDENT: DIAS SC (PhD REPRODUCTIVE BIOLOGY)

TITLE: Investigating molecular biomarkers during gestational diabetes mellitus

The above-mentioned student's protocol title amendment has been approved by the PhD committee.

We wish the student all the best with her studies.

Kind regards

PROF V STEENKAMP
CHAIR: PhD COMMITTEE

APPENDIX 3: CONSENT FORM

PARTICIPANT'S INFORMATION LEAFLET AND INFORMED CONSENT

TITLE OF STUDY:

NAME OF RESEARCHER:

Dear Miss/Mrs _____

Date: ___/___/20___

Invitation:

You are invited to volunteer for a research study. This information leaflet is to help you to decide whether you would like to participate. Before you agree to take part in this study you should fully understand what is involved. Please take your time to read the following information carefully and discuss it with others if you wish. If you have any questions which are not fully explained in this leaflet, do not hesitate to ask. You should not agree to take part unless you are completely happy about all the procedures involved. Thank you for reading this.

What is the purpose of the study?

The aim of this study is to develop a simple, easy method of diagnosing diabetes mellitus in pregnancy. We will also evaluate how common diabetes mellitus in pregnancy is in South Africa.

Why have I been chosen?

You have been chosen as you are at an early stage in your pregnancy.

Procedures to be followed:

This study involves answering some questions regarding your past pregnancies and your family history, an examination and blood and urine tests. This is part of your routine ante-natal care. In addition, we will test your blood for glucose. We will also collect a tube of your blood that will be frozen and used for tests related to gestational diabetes at a later stage. In a couple of weeks, you will be asked to come to the clinic so we can do a glucose tolerance test. For this test you will be asked to drink a glucose/sugar solution and your blood will be tested for glucose thereafter.

You will also be asked to return at six weeks after delivery. At this visit, your baby will be immunized and we will ask you questions regarding the birth of your baby.

None of these procedures is harmful to you or your baby.

Risk and discomfort involved:

There may be slight bruising after taking blood. The glucose solution may make you feel nauseous. There are no risks involved with any of the tests.

Possible benefits of this study:

Many of the questions asked and tests are done routinely in pregnancy. If any of the test results are abnormal, you will be referred for appropriate care.

I understand that if I do not want to participate in this study, I will still receive standard treatment for my illness. I may withdraw from this study at any time.

Has the study received ethical approval?

This protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki which deals with the recommendations guiding doctors in biomedical research involving humans. A copy of the Declaration may be obtained from the investigator should you wish to review it.

If you have any question concerning this study, you should contact:

Dr. Sumaiya Adam

Department of Obstetrics and Gynaecology

Steve Biko Academic Hospital

Pretoria

012 354 3715 or 084 951 1773

Confidentiality:

All records obtained whilst in this study will be regarded as confidential. Results will be published in such a fashion that patients remain unidentifiable.

Consent to participate in this study:

I have read the above information, or it has been read to me in a language that I understand. I understand the above information before signing the consent form. The content and meaning of this information have been explained to me. I have been given the opportunity to ask questions and am satisfied that they have been answered satisfactorily. I understand that if I do not participate it will not alter my management of this pregnancy in any way. I hereby volunteer to take part in this study.

I have received a signed copy of this informed consent agreement.

_____ ___/___/20___

Participant's Signature Date

_____ ___/___/20___

Person obtaining informed consent Date

_____ ___/___/20___

Witness Date

Verbal participant informed consent (if person cannot read or write):

I, the undersigned _____, have read and have explained fully to the participant, named

_____ and/or to her relative, the patient information leaflet, which has indicated the nature and purpose of the study in which I have asked the person to participate. The explanation I have given has mentioned both the possible risks and benefits of the study. The participant indicated that she understands that she will be free to withdraw from the study at any time for any reason without jeopardizing the further care of her pregnancy.

I hereby certify that the patient has agreed to participate in this study.

Participant's name: _____

Investigator's name: _____

Investigator's signature: _____

Witness's Name: _____ Witness's signature: _____

Date: ___/___/20___

APPENDIX 4: SUPPLEMENTARY FILES FOR CHAPTER 7

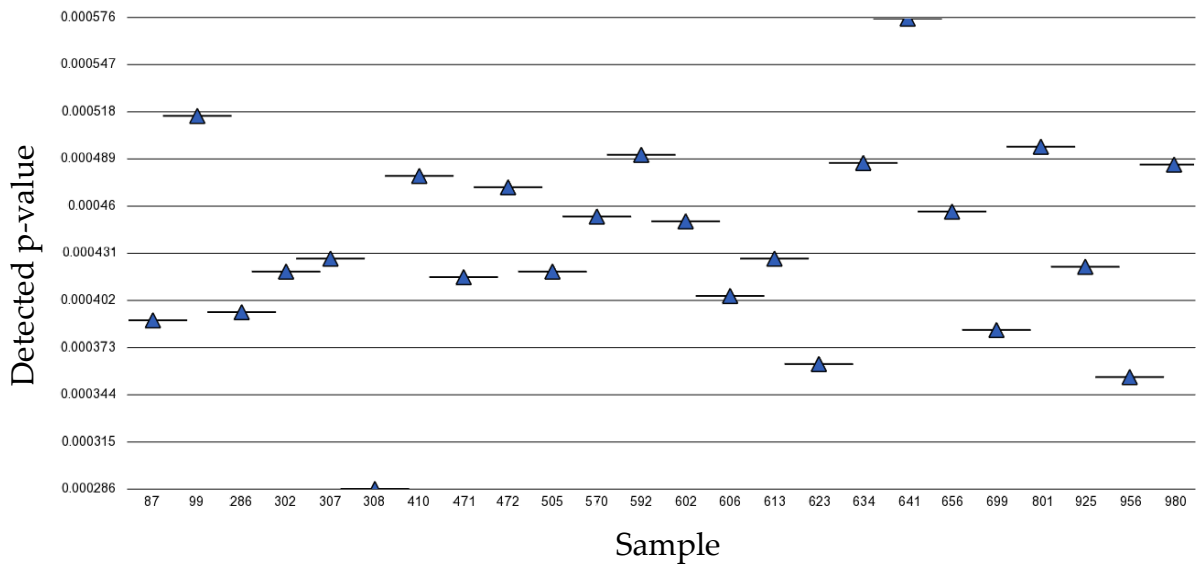

Figure S1. Average detection p-values per sample for all probes. All samples passed the quality control test. P<0.01.

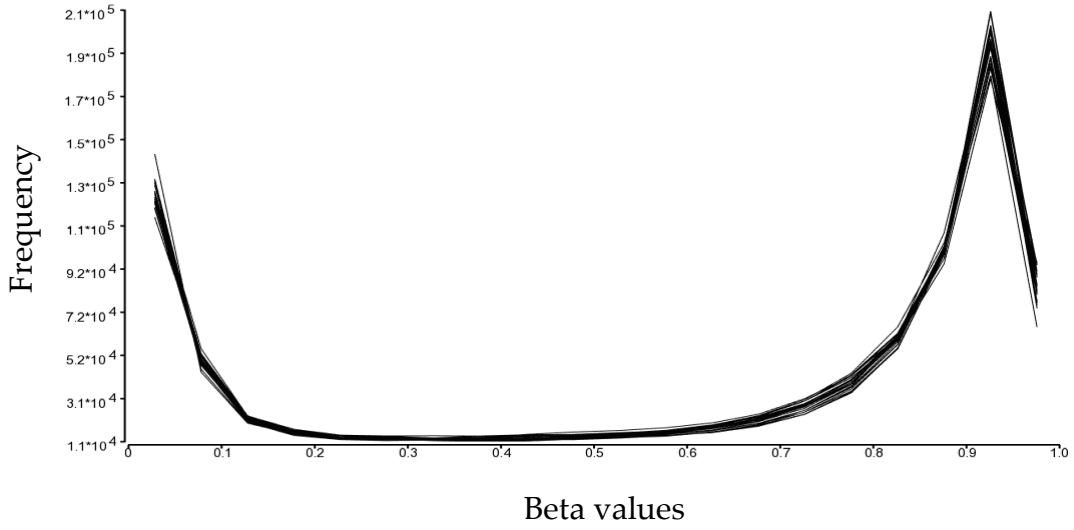

Figure S2. Histogram of β-values showing the frequency distribution of CpG methylation across all samples.

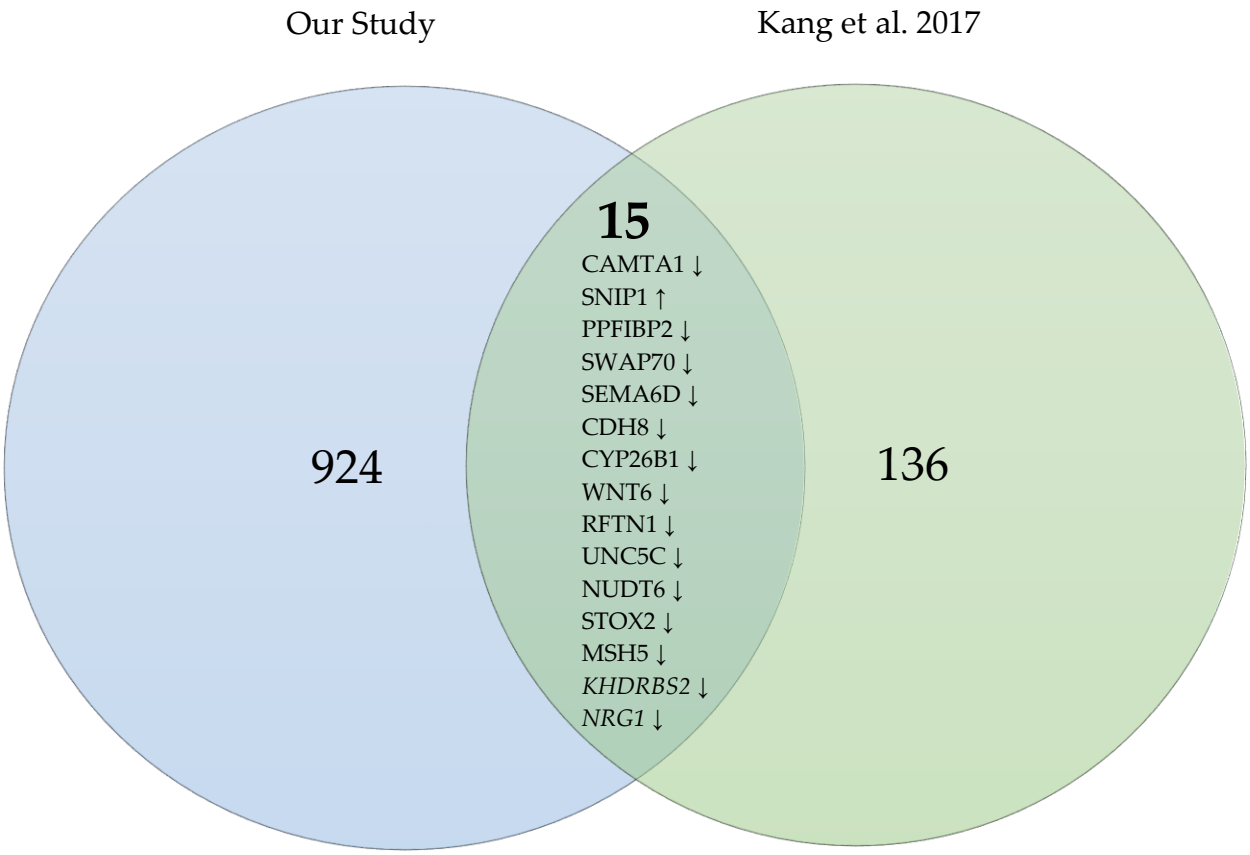

Figure S3. Venn diagram illustrating comparison of genes differentially methylated in GDM between our study (left) and those identified by Kang et al. 2017 (right) in maternal blood. Left: Identification of 939 differentially methylated genes in our study. Right: Identification of 151 differentially methylated genes by Kang et al. Centre: 15 differentially methylated genes that were common in our study compared to Kang et al. Arrows illustrate up or down regulation in GDM vs. non-GDM in our study. Information for Kang et al. was not available. Calmodulin Binding Transcription Activator 1 (CAMTA1), Smad Nuclear Interacting Protein 1 (SNIP1), Protein-Tyrosine Phosphatase, Receptor-Type, F Polypeptide-Interacting Protein-Binding Protein 2 (PPFIBP2), Switching B Cell Complex Subunit SWAP70 *(SWAP70), Semiphorin 6D (SEMA6D), Cadherin 8 (CDH8), Cytochrome P450 Family 26 Subfamily B Member 1 (CYP26B1), Wnt Family Member 6 (WNT6), Raftlin, Lipid Raft Linker 1 (RFTN1), Unc-5 Netrin Receptor C (UNC5C), Nucleoside Diphosphate-Linked Moiety X Motif 6 (NUDT6), Storkhead Box (STOX2), MutS Protein Homolog 5 (MSH5), KH RNA Binding Domain Containing, Signal Transduction Associated 2 (KHDRBS2), and Neuregulin 1 (NRG1).*

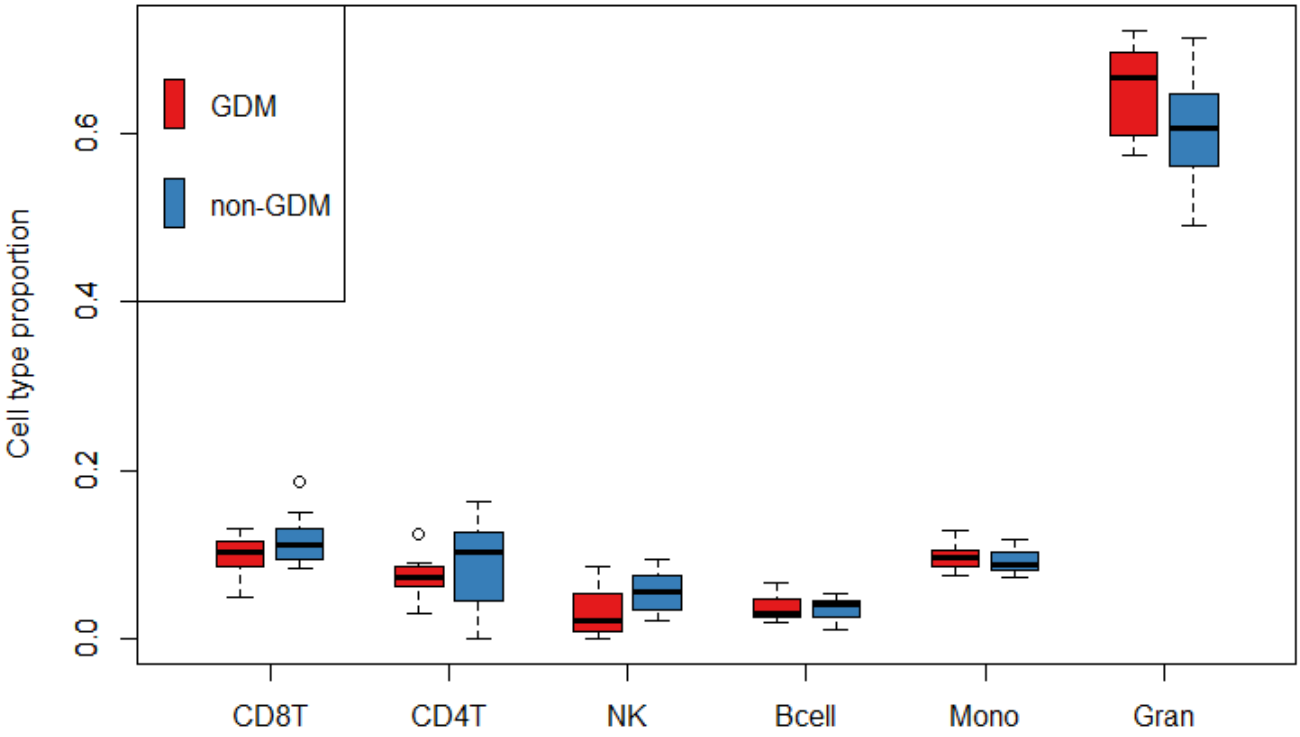

Figure S4. Comparison of six major peripheral blood cell components in GDM and non-GDM women. No significant difference was observed between cells types. All Data points are presented as means with standard deviation. p<0.01 is considered significant. CD8T and CD4T: T lymphocytes; NK: Natural killer cells; Bcell: B lymphocytes; Mono: Monocytes; Gran: Granulocytes.

Table S3: The association between the significantly differentially methylated CpG sites with fasting glucose, 1 hr OGTT and fasting insulin, using linear regression adjusting for gestational diabetes mellitus.

	[a]Univariate			[b]Multivariate		
CpG site	Coefficient	95% CI	*p*-value	Coefficient	95% CI	*p*-value
Fasting glucose (mmol/L)						
cg22985016	0.019	0.012; 0.027	**<0.001**	0.003	-0.010; 0.016	0.669
cg21910650	-0.059	-0.084; -0.033	**<0.001**	-0.006	-0.052; 0.040	0.805
cg23643951	-0.037	-0.051; -0.022	**<0.001**	0.001	-0.022; 0.024	0.934
cg16306629	0.193	0.116; 0.269	**<0.001**	0.049	-0.092; 0.192	0.492
cg07966372	-0.010	-0.018; -0.002	**0.017**	-0.001	-0.018; 0.015	0.867
1 hr OGTT (mmol/L)						
cg22985016	0.006	0.002; 0.010	**0.006**	0.002	-0.001; 0.005	0.283
cg16306629	0.066	0.025; 0.108	**0.002**	0.029	-0.005; 0.063	0.095
Fasting insulin (mIU/L)						
cg07966372	-0.001	-0.002; -0.0001	**0.027**	-0.001	-0.002; -0.0003	**0.043**

[a]Univariate linear regression: Association between CpG-specific methylation and fasting glucose, 1 hr OGTT or fasting insulin. [b]Multivariate linear regression: Adjusting for gestational diabetes mellitus; CI – Confidence interval. $p<0.001$, $p<0.01$, $p<0.05$ OGTT: Oral glucose tolerance test; CI: Confidence interval; GDM: Gestational diabetes mellitus

*Supplementary tables for chapter 7 are available on request as they are too large to be included in this document:

Table S1: Genome-wide DNA methylation profiling identified 1046 differentially methylated CpG

Table S2: Differentially methylated CpG sites annotated to 939 unique genes

Table S4: Functional enrichment analysis identified 261 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways

Table S5: Statistically significant KEGG pathways associated with GDM

Table S6: GO terms enriched by differentially methylated genes, categorized into 1181 biological processes, 167 molecular functions and 85 cellular components.

APPENDIX 5: SUPPLEMENTARY FILES FOR CHAPTER 8

Table S1. Primer design for CpG sites in the adiponectin gene (ADIPOQ)

Name	Target sequence*	Chromosomal region	PCR and pyrosequencing primer*	Target sequence after NaBis treatment*	Amplicon length
Assay 1	CGCGGTGGCTCAC GCCTGTCATTCCA GCACTTTGGGAGG CCG	Chromosome 3: 186,839,297-186,839,338 Positive strand	Fwd1: GGTGGTAGGAGGTGATAGTTTAA Rev1: ACTCCCCACCTCAAATAATCCAC Seq1: GAAATGTTTTTTTGGTTAGG	YGYGGTGGTTTAYGTTTG TTATTTTAGTATTTTGGG AGGTYGAGGGGGTGGA T TATTTGAGGT	199 (4CpGs)
Assay 2	CTGAACGTACAC AGTCTCAGACTTA ATCATGCACAGTG AGCAAGACTGTG GTGTGATAATTGG CGTCCCTGAC	Chromosome 3: 186,842,231-186,842,303 Positive strand	Fwd3: TTAGGTTAGAGAGTGGAGGATGTG Rev3: TCCCCCTCCCATAAATTTACC Seq3: CTCCCATAAATTTACCCTAATA	AATCAAAAACRCCAATT ATCACACCACAATCTTA CTCACTATACATAATTA AATCTAAAACTATATAC RTTCAAACAATAAATAC TTCAAAAAAAAACA	186 (2CpGs)
Assay 3	TTTGTTTATCGGTT TTTGGTTTTTATTG AGTTGGTTAATGG GAAATGATAATTG TGAGGTGGGGATT GTTTGTTTTCGTG AG	Chromosome 3: 186,842,599-186,842,672 Positive strand	Fwd2: GTGGGTAATTGTTAGGGATATGT Rev2: AAAAAATAACCCAACCTCAACAAC Seq2: GTAATTGTTAGGGATATGTG	TTTGTTTATYGGTTTTTG GTTTTTATTGAGTTGGTT AATGGGAAATGATAATT GTGAGGTGGGGATTGTT TGTTTTYGTGAGTATTAG GTTGTTGAGGTTGGGTT AT	136 (2CpGs)

5′ to 3′ orientation; NaBis - Sodium Bisulfite; Red bold type letters indicated CpG sites investigated on the adiponectin gene (ADIPOQ) promoter region.

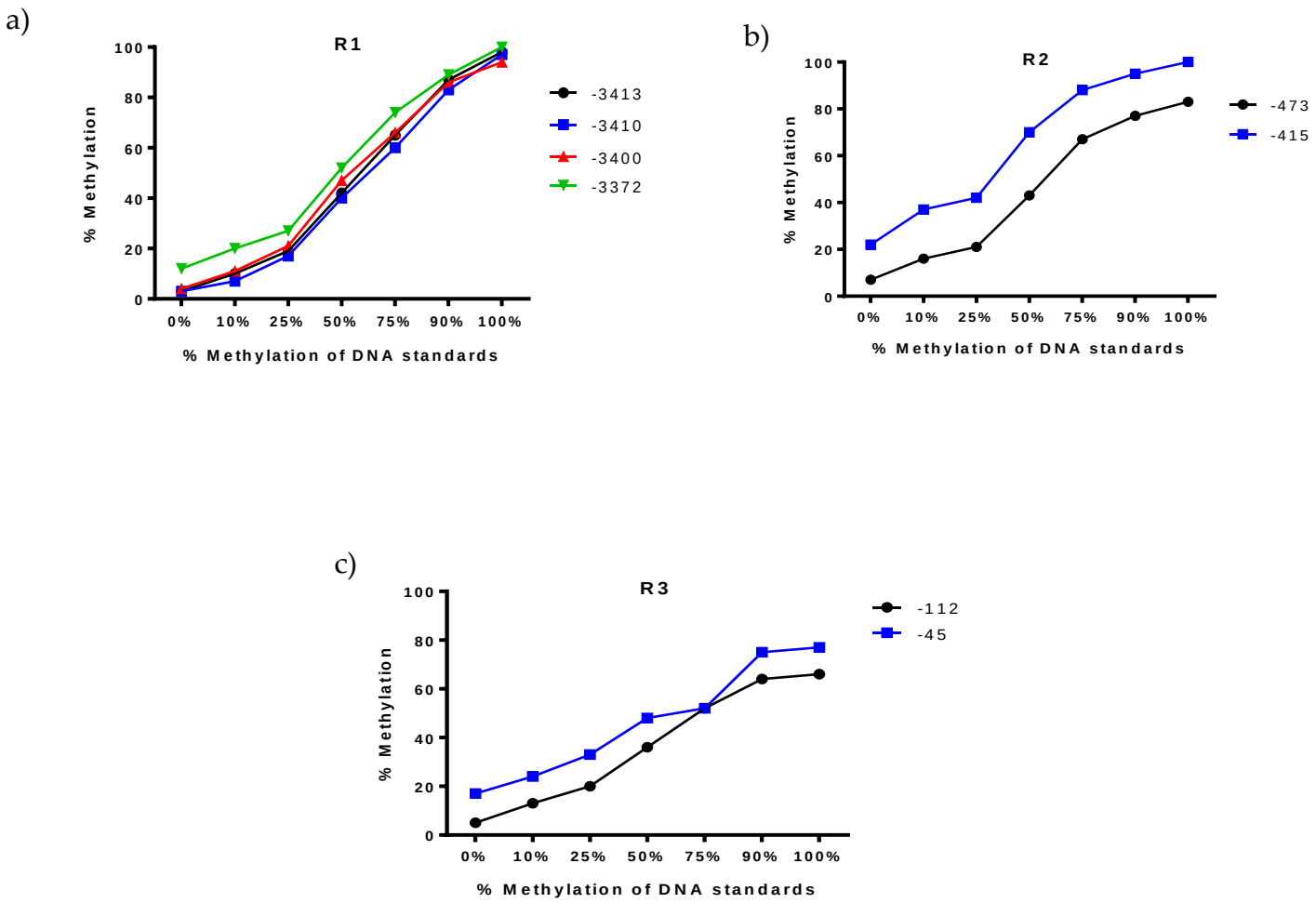

Figure S1. Primer sensitivity using known methylated standards (x-axis) for each pyrosequencing probe. The percentage (%) of methylation as determine (y-axis) for a) 4 CpGs (-3412, -3410, -3400, -3372) in R1, b) 2 CpGs (-473, -415) in R2 and c) 2 CpGs (-112, -45) in R3.

Table S2. Frequency distribution of methylation at CpG -3410 and -3400 in HIV negative and HIV positive women.

CpG sites	HIV negative	HIV positive	p-value
n	181	104	
CpG -3410			
Upper quartile	104 (57.5)	98 (94.3)	<0.001
Lower quartile	77 (42.5)	6 (5.7)	
CpG -3400			
Upper quartile	84 (46.4)	94 (90.4)	<0.001
Lower quartile	97 (53.6)	10 (9.6)	

Upper quartile: methylation between the median and 75th percentile; Lower quartile: methylation between the median and 25th percentile.

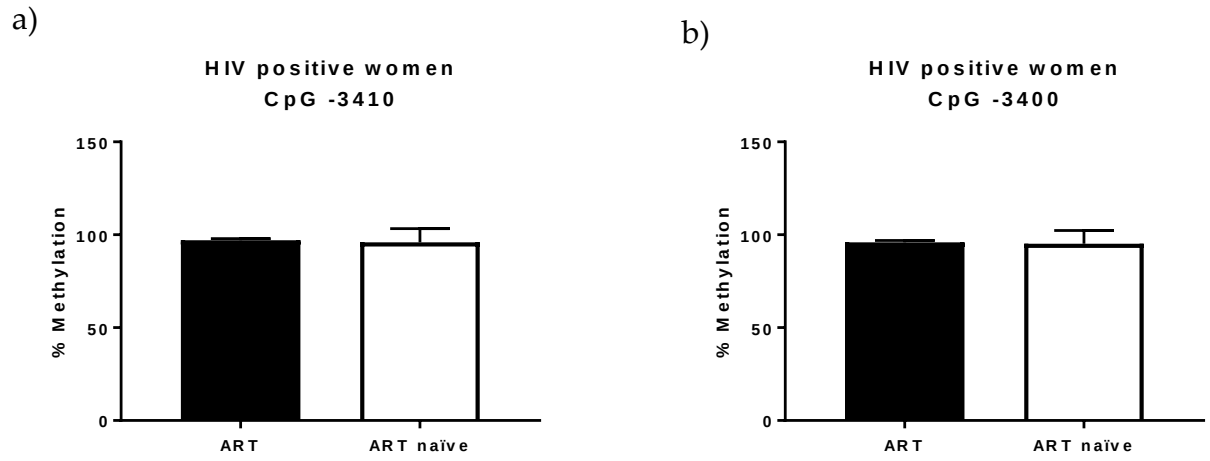

Figure S2. DNA methylation levels in HIV positive women receiving antiretroviral therapy (ART) (n=36) and those who were ART naïve (n=69). DNA methylation levels at a) CpG -3410 and b) CpG -3400.

APPENDIX 6: PUBLISHED RESEARCH ARTICLES

Biomarkers

Global DNA methylation profiling in peripheral blood of South African women with gestational diabetes mellitus

Journal:	*Biomarkers*
Manuscript ID	TBMK-2018-OR-0281.R1
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Global DNA methylation profiling in South African women with gestational diabetes mellitus

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Abstract

Background/Objective: Recently, several studies have reported that placental global DNA methylation is associated with gestational diabetes mellitus (GDM), sparking interest in the potential use of global DNA methylation as a biomarker for this disorder. This study investigated whether global DNA methylation is associated with GDM in South African women.

Methods: Global DNA methylation was quantified in the peripheral blood of women with (n=63) or without (n=138) GDM using the MDQ1 Imprint® DNA Quantification Kit.

Results: Global DNA methylation levels were not different between women with or without GDM and were not associated with fasting glucose nor insulin concentrations. However, levels were 18% (p=0.012) higher in obese compared to non-obese pregnant women, and inversely correlated with serum adiponectin concentrations (p=0.005).

Discussion: Contrary to our hypothesis, global DNA methylation was not associated with GDM in our population. Our findings suggest that despite being a robust marker of overall genomic methylation that offers opportunities as a biomarker, global DNA methylation profiling may not offer the resolution required to detect methylation differences in the peripheral blood of women with GDM. Further studies are required to explore the candidacy of a more targeted approach using gene-specific methylation as a biomarker for GDM in our population.

Words: 200

Key words: Gestational diabetes mellitus; global DNA methylation; biomarker; peripheral blood; South Africa, obesity

Clinical Significance

- The prevalence of gestational diabetes mellitus (GDM) is rapidly increasing globally
- The 75 g 2 hr oral glucose tolerance test remains the gold standard for GDM diagnosis, however, several challenges hamper its use
- Risk factor-based screening has poor sensitivity for detecting GDM
- Thus, a more robust, non-invasive, simple and cost effective screening tool is needed
- Altered DNA methylation patterns in peripheral blood holds potential as a biomarker for GDM screening

Introduction

Gestational diabetes mellitus (GDM), defined as glucose intolerance that is first diagnosed during pregnancy, is a significant source of morbidity and mortality. In South Africa, a middle-income country, the prevalence of GDM has rapidly increased over the last few years (Mamabolo *et al.* 2007, Adam and Rheeder 2017, Macaulay *et al.* 2018), paralleling the rising obesity epidemic. Recently, it was reported that 68% of South African women over the age of 15 years were either overweight or obese (Statistics South Africa 2017), a serious cause for concern since obesity is a major risk factor for the development of GDM. GDM is associated with adverse perinatal outcomes (Jensen *et al.* 2000) and increases susceptibility to future metabolic disorders in both mothers and their offspring (Damm 2009), thus posing a significant burden to the already struggling and over-burdened South African health system.

The oral glucose tolerance test (OGTT), conducted between 24-28 weeks of gestation is the gold standard for the diagnosis of GDM (WHO 1999). However, the test is cumbersome to conduct and has several challenges including high costs, requirement for fasting, multiple blood draws, and is associated with nausea and vomiting. Currently, universal screening for GDM is recommended for all pregnant women (Hod *et al.* 2015). However, due to limited

resources, selective screening based on traditional GDM risk factors such as obesity (body mass index (BMI) ≥ 30 kg/m^2), advanced maternal age (> 35 years), family history of diabetes, history of GDM, previous macrosomic (baby weighing ≥ 4000 g) pregnancy, glycosuria, or previous adverse pregnancy outcomes (congenital abnormalities, unexplained still birth or recurrent pregnancy loss) is often performed in low and middle income countries. Unfortunately, these risk factors have poor sensitivity for detecting GDM in our population (Adam and Rheeder 2017), resulting in a large number of GDM cases being missed. The identification of simple and cost effective biomarkers to detect women with GDM could offer an alternative to the OGTT. Although a number of circulating biomarkers such as adiponectin, sex hormone globulin, C-reactive protein (CRP) and glycosylated fibronectin have been explored as biomarkers for GDM, none have yet achieved clinical applicability (Smirnakis *et al.* 2007, Nanda *et al.* 2011, Rasanen *et al.* 2013, Adam *et al.* 2018).

Epigenetics reflect gene-environment interactions and is increasingly being implicated in the pathophysiology of metabolic diseases (Gu *et al.* 2013, Martín-Núñez *et al.* 2014). DNA methylation, the most widely studied and best characterized epigenetic mechanism, refers to the addition of a methyl group to the fifth carbon position of a cytosine residue within CpG dinucleotides, often leading to transcriptional repression (Lim and Maher 2010). The process is reversible thus offering opportunities for risk stratification and intervention, and has accordingly received considerable interest as biomarkers of disease. Although both gene-specific and global DNA methylation profiling have been explored, global DNA methylation, which gives an estimate of overall genomic methylation can be quantified using non-invasive, inexpensive and simple methods, thus making it an attractive target for biomarker discovery. Several studies have reported that global DNA methylation is altered during hyperglycemia (Matsha *et al.* 2016, Pinzón-Cortés *et al.* 2017) and in placental tissue of women with GDM (Nomura *et al.* 2014, Reichetzeder *et al.* 2016). We hypothesized that global DNA methylation

in the peripheral blood of black South African women is altered during GDM, and accordingly has potential as a biomarker for GDM in our population.

Materials and methods

Participants

The study was approved by the Health Sciences Ethics Committee of the University of Pretoria (180/2012). Pregnant women were recruited at a primary care clinic in Johannesburg, South Africa (Adam and Rheeder 2017). Written informed consent was obtained from all participants. Women of black ethnicity, who were less than 26 weeks pregnant and who had a singleton pregnancy were included in this study. Women with pre-existing diabetes, random glucose level >11.1 mmol/L, measured with a glucometer (Roche Diagnostics, Mannheim, Germany) or glycated hemoglobin (HbA1c) level > 6.5%, measured with the point-of-care Afinion system (Alere Technologies, Oslo, Norway) were excluded. Of the 1000 women who were recruited, the 75 g 2 hr OGTT was conducted on 554 women (Figure 1). Human Immunodeficiency Virus (HIV) positive women and those with an unknown HIV status were excluded from the current study. For this case control study, 63 women with GDM and 138 women without GDM were matched individually according to age, BMI and gestational age as far as possible, and were selected.

Clinical and biochemical characteristics

Demographic information was obtained from a standardized questionnaire and anthropometric measurements were assessed according to standard procedures (Adam *et al.* 2018). The OGTT was conducted according to the International Association of Diabetes in Pregnancy Study Group (IADPSG) criteria (Metzger *et al.* 2010). Briefly, women were given a 75 g glucose drink to ingest, and blood was collected for glucose measurements at 0 hr, 1 hr and 2 hr. At the time of OGTT, HbA1c concentrations were measured again by an accredited laboratory (Vermaak and Partners, Pretoria, South Africa). For comparative analysis, GDM was classified using the National Institute for Health and Care Excellence (NICE) and the World Health

Organization (WHO) 1999 criteria (Table 1) (WHO 1999, NICE guidelines 2015). Fasting insulin and CRP concentrations were measured in stored serum samples (Pathcare laboratories, Cape Town, South Africa). Serum adiponectin concentrations were quantified using the human adiponectin enzyme-linked immunosorbent assay (ELISA) (Merck, Darmstadt, Germany). The homeostatic model assessment (HOMA), a measure of insulin resistance was calculated using the equation: (glucose x insulin)/22.5, using fasting plasma glucose and fasting serum insulin concentrations. Whole Blood for DNA methylation analysis was stored at -80° C.

Global DNA methylation

DNA was extracted from 2 ml of stored whole blood in Ethylenediaminetetraacetic acid (EDTA) tubes using the QIAmp DNA Blood Midi Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany), and concentrations were measured using the Qubit Flourometer dsDNA Broad Range Assay Kit (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Global DNA methylation was quantified using the MDQ1 Imprint® Methylated DNA Quantification Kit according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, USA) as previously described (Pheiffer *et al.* 2014). Briefly, 100 ng of DNA was allowed to bind to the ELISA plate, where after the methylated fraction of DNA was detected using a 5-methylcytosine monoclonal antibody, and the absorbance was measured at 450 nm on a BioTek® ELX 800 plate reader (BioTek Instruments Inc., Winooski, USA). Global DNA methylation levels were calculated relative to the methylated positive control which was included in the kit. All samples were analysed in duplicate.

Statistical analysis

Statistical analysis was conducted using STATA 14 (StataCorp, Texas, USA). Data were expressed as the mean ± standard error of the mean (SEM), or as the median and interquartile range (25th and 75th percentiles) for data that were not normally distributed. Categorical data

were expressed as count (n) and percentage (%). The Shapiro-Wilk test was used to test for normality. The unpaired student t test or the Mann-Whitney test was used to compare variables across GDM groups, and the Chi-square test was used to analyze categorical variables. Spearman's rank correlation (r_s) was used to evaluate the relationship between global DNA methylation and serum adiponectin concentrations. A $p \leq 0.05$ was considered statistically significant.

Results

The clinical characteristics of participants are presented in Table 2. As expected, fasting blood glucose ($p<0.001$), 1 hr OGTT ($p<0.001$), 2 hr OGTT ($p<0.001$) and HbA1c ($p=0.008$) concentrations were significantly higher in women with GDM compared to women without GDM. Similarly, fasting insulin ($p=0.067$) and HOMA ($p<0.001$) levels were increased in women with GDM. In contrast, women with GDM had lower concentrations of serum adiponectin than women without GDM ($p=0.018$).

Since the extent of hyperglycemia may influence the association between global DNA methylation and GDM, GDM was classified using IADPSG, WHO and NICE criteria. Glucose concentrations differed significantly between women with GDM compared to women without GDM, using all three diagnostic criteria (Table 3). Fasting plasma glucose values were significantly lower in women with GDM using the WHO criteria compared to the IADPSG ($p=0.014$) and NICE ($p=0.005$) criteria, while the 2 hr OGTT values were significantly higher in women with GDM using the WHO criteria compared to the IADPSG criteria and NICE criteria ($p<0.001$) (Table 3). However, no difference in global DNA methylation levels between women with or without GDM were observed when the different diagnostic criteria were used ($p>0.05$) (Figure 2A-C). Global DNA methylation levels were 18% ($p=0.012$) higher in obese compared to non-obese pregnant women (Figure 3) and were inversely correlated with serum adiponectin concentrations ($r_s=-0.243$, $p=0.005$) (Figure 4).

Discussion

This study investigated whether global DNA methylation profiling has potential as a screening tool for GDM in black South African women. Contrary to our hypothesis, global DNA methylation levels in the peripheral blood of black South African women were not associated with GDM. The IADPSG criteria is stringent, and the failure to observe differences in methylation between women with or without GDM may be due to small glucose concentration differences between groups. However, no difference in methylation was observed when GDM was classified according to NICE and WHO criteria, where glucose concentration differences between women with or without GDM were more pronounced. Previous studies have reported that global DNA methylation is associated with GDM (Nomura *et al.* 2014, Reichetzeder *et al.* 2016), however, these were conducted on placental tissue and used different methods to quantify global DNA methylation, possibly accounting for the discrepancies observed. Biological source affects global DNA methylation (Reinius *et al.* 2012), thus our failure to observe an association between GDM and global DNA methylation could be due to the use of peripheral blood rather than placenta. Furthermore, using liquid chromatography–mass spectrometry Reichetzeder *et al.* demonstrated that placental DNA methylation was increased during GDM (Reichetzeder *et al.* 2016), while using a luminometric methylation assay Nomura *et al.* reported that placental DNA methylation is decreased during GDM (Nomura *et al.* 2014), illustrating that method of quantification influences results. The ELISA, as used in this study, offers several advantages over other methods of quantifying global DNA methylation. It is cost-effective and does not require specialized equipment and expertise, making it more amenable for screening in low-and middle income countries (Kurdyukov and Bullock 2016). Several studies have reported that the ELISA is able to detect aberrant global DNA methylation patterns during disease

(Nakano *et al.* 2012, Keller *et al.* 2014, Kagohara *et al.* 2015, Ramos *et al.* 2016), and in response to environmental factors (Guerrero-Preston *et al.* 2010, Tellez-Plaza *et al.* 2014, Ivorra *et al.* 2015, Sánchez *et al.* 2015).

Global DNA methylation levels were higher in obese compared to non-obese pregnant women. It has been widely reported that global DNA methylation is associated with obesity (Cash *et al.* 2011, Jintaridth *et al.* 2013, Piyathilake *et al.* 2013, Na *et al.* 2014), however studies in pregnant women are limited (Herbstman *et al.* 2013, Nomura *et al.* 2014). Consistent with our results, Nomura *et al.* reported that global DNA methylation was higher in obese compared to non-obese pregnant women (Nomura *et al.* 2014). However, in contrast to our findings, Herbstman *et al.* reported that global DNA methylation is decreased during pre-pregnancy obesity, while Michels *et al.* failed to see an association between global DNA methylation and obesity (Michels *et al.* 2011, Herbstman *et al.* 2013). These findings confirm the variability in assessing global DNA methylation levels according to biological source and methods of quantification.

Intriguingly, global DNA methylation was inversely correlated with serum adiponectin concentrations. Adiponectin is an adipokine with insulin-sensitizing properties, which is dysregulated during obesity and metabolic disease (Cao 2014). Similar to our findings, several studies have reported that adiponectin concentrations are decreased during GDM (Lacroix *et al.* 2013, Pala *et al.* 2015, Adam *et al.* 2018). Recently, it was reported that altered methylation at the adiponectin gene *(ADIPOQ)* locus is inversely correlated with circulating adiponectin concentrations during pregnancy (Bouchard *et al.* 2012), and is associated with decreased *ADIPOQ* gene expression levels in adult offspring of women with GDM (Houshmand-Oeregaard *et al.* 2017). To further explore the significance of the association between global DNA

methylation and adiponectin, pyrosequencing of *ADIPOQ* is currently being conducted in our laboratory.

Although quantification of global DNA methylation is a robust method to assess overall genomic DNA methylation, and has potential as a biomarker to facilitate risk stratification and intervention (Ramos *et al.* 2016), it may not offer the resolution required to detect subtle methylation differences in women with or without GDM. Another limitation of the study is the use of peripheral blood, which consists of a mixture of different cell types such as erythrocytes, lymphocytes and platelets which may confound methylation analysis (Reinius *et al.* 2012). Future studies should consider purification of blood cell populations to separate specific cell types (Reinius *et al.* 2012). It has been widely reported that DNA methylation is affected by environmental factors such as diet, smoking, alcohol consumption and physical activity (Joubert *et al.* 2012, Lim and Song 2012, Ling and Rönn 2014, Pauwels *et al.* 2017, Miyake *et al.* 2018). Thus, the lack to account for these environmental factors, pose a significant limitation to our study. However, women were recruited from the same community with similar life experiences, suggesting that they were likely to have similar environmental exposures.

Conclusion

Contrary to our hypothesis, global DNA methylation was not associated with GDM in our population. Our findings suggest that despite being a robust marker of overall genomic methylation that offers opportunities as a biomarker, global DNA methylation profiling may not offer the resolution required to detect subtle methylation differences in the peripheral blood of women with GDM. Further studies are required to explore the candidacy of a more targeted approach using gene-specific methylation as a biomarker for GDM in our population. To our knowledge, this is the first study to investigate the association between global DNA methylation and GDM in South Africa.

Disclosure of statement

No potential conflict of interest was reported by the authors.

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Appendices

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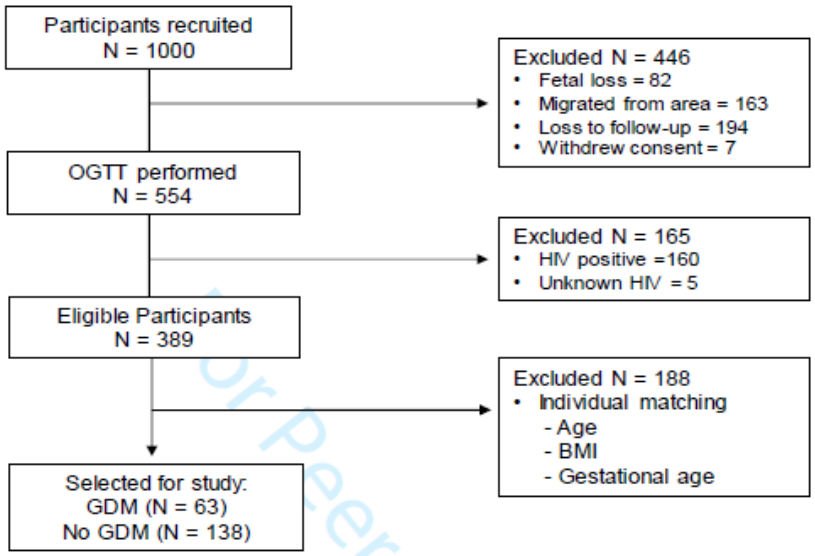

Figure 1. Flow diagram for study participants. For the current study, women with (n=63) or without (n=138) GDM were selected from a prospective cohort study in which 1000 pregnant women were recruited.

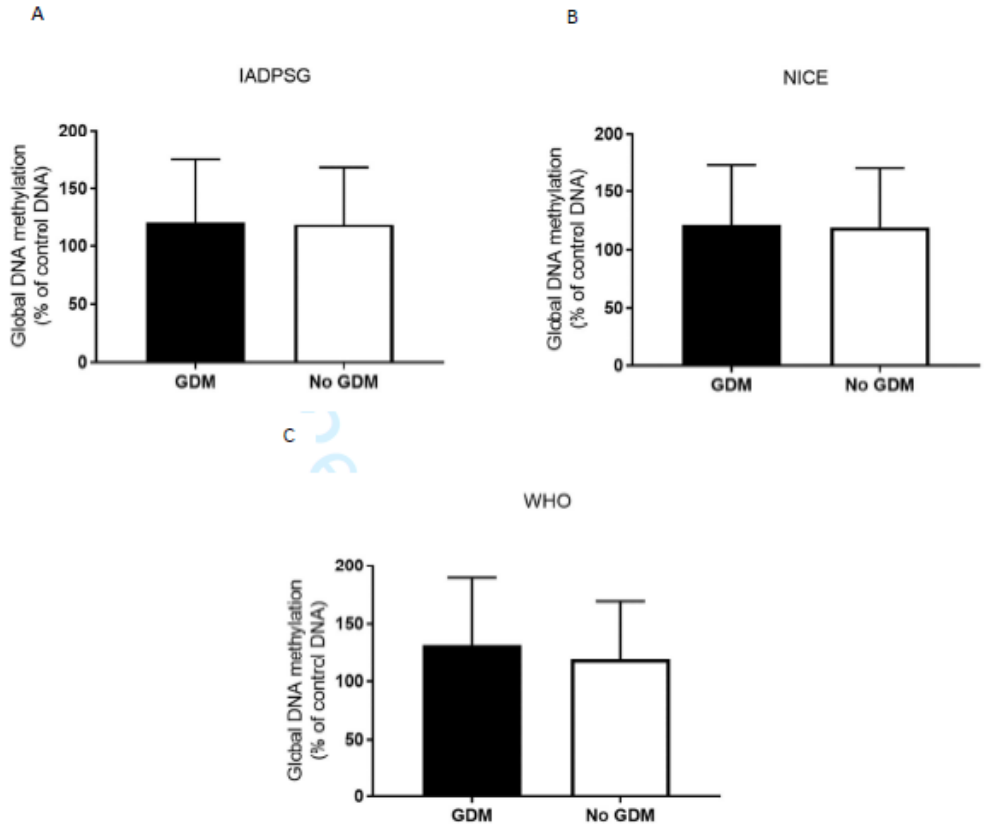

Figure 2. Global DNA methylation levels according to GDM status. Global DNA methylation was measured in the peripheral blood of women with or without GDM according to the IADPSG (A), the NICE (B) and the WHO (C) criteria, using the MDQ1 Imprint® Methylated DNA Quantification Kit. Global DNA methylation levels were calculated relative to the methylated positive control which was included in the kit. Data are represented as the mean ± standard error of mean (SEM).

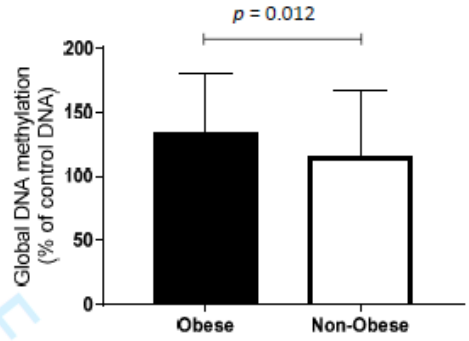

Figure 3. Global DNA methylation levels varies according to obesity status. Global DNA methylation was measured in the peripheral blood of obese (n=51) and non-obese (n=138) pregnant women using the MDQ1 Imprint® Methylated DNA Quantification Kit. Global DNA methylation levels were calculated relative to the methylated positive control which was included in the kit. Data are represented as the mean ± standard error of mean (SEM).

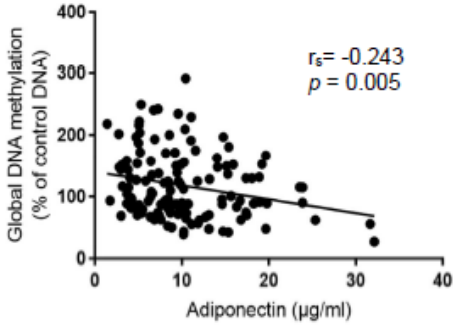

Figure 4. Global DNA methylation is inversely correlated with serum adiponectin concentrations. Each data point represents an individual (n=139) and indicates global DNA methylation levels relative to a methylated positive control and adiponectin concentration.

Table 1: GDM diagnostic criteria commonly used in South Africa

	Glucose concentration (mmol/L)		
Time*	IADPSG	NICE	WHO 1999
0 hr OGTT	5.1	5.6	7.0
1 hr OGTT	10	-	-
2 hr OGTT	8.5	7.8	7.8

*Time after ingesting 75 g glucose drink; OGTT: oral glucose tolerance test; hr: hour; GDM: gestational diabetes mellitus; IADPSG: International Association of Diabetes in Pregnancy Study Group; NICE: National Institute for Health and Care Excellence; WHO: World Health organization (WHO 1999, Metzger *et al.* 2010, NICE guidelines 2015)

Table 2: Clinical characteristics of the study population stratified according to GDM using the IADPSG criteria

Variable	GDM (n=63)	No GDM (n=138)	*p*-value
Age (years)[a]	28.0 (24.0-32.0)	28.0 (24.0-32.0)	0.810
BMI (kg/m^2)[a]	27.4 (23.4-31.2)	25.8 (23.5-29.8)	0.180
Fasting glucose (mmol/L)[a]	5.5 (5.2-5.9)	4.3 (4.0-4.6)	< 0.001
OGTT 1 hr (mmol/L)[a]	6.5 (5.5-8.3)	5.5 (4.7-6.5)	< 0.001
OGTT 2 hr (mmol/L)[a]	6.1 (5.2-7.2)	5.2 (4.6-5.9)	< 0.001
HbA1c (%)[b]	5.2 (0.4)	5.1 (0.3)	0.008
Fasting insulin (mIU/L)[a]	6.5 (4.8-9.4)	5.7 (3.8-8.0)	0.067
HOMA[a]	1.6 (1.2-2.4)	1.1 (0.8-1.7)	< 0.001
C-reactive protein (mg/L)[a]	6.9 (3.7-9.9)	5.4 (3.1-8.5)	0.209
Adiponectin (µg/ml)[a]	7.6 (4.9-11.8)	9.8 (6.6-14.7)	0.018
Education: n (%)[c]			
<grade 12	29.0 (46.7)	66.0 (49.6)	0.769
≥grade 12	33.0 (53.3)	67.0 (50.4)	
Risk factors: n (%)[c]			
None	27.0 (42.8)	79.0 (57.3)	0.143
≥1 Risk factors n (%)	36.0 (57.2)	59.0 (42.7)	

BMI: body mass index; OGTT: oral glucose tolerance test; HbA1c: glycated hemoglobin; HOMA: homeostatic model assessment.

Data are expressed as the [a]median (25th–75th percentiles); [b]mean ± standard error of the mean or as [c]count (percentage).

P-values for continuous data were calculated using the Mann-Whitney or the unpaired student *t* test.

P-values for categorical data were calculated using the Chi-square test.

Table 3: GDM defined using the IADPSG, NICE and WHO 1999 diagnostic criteria

Glucose concentration (mmol/L)									
	IADPSG			NICE			WHO 1999		
Time*	GDM	No GDM	*p*-value‡	GDM	No GDM	*p*-value‡	GDM	No GDM	*p*-value‡
0 hr OGTT	5.5 (5.2-5.9)[a]	4.3 (4.0-4.6)	< 0.001	5.8 (5.5-6.0)[b]	4.4 (4.0-4.8)	< 0.001	5.2 (4.7-5.4)[a,b]	4.5 (4.1-5.1)	<0.05
1 hr OGTT	6.5 (5.5-8.3)	5.5 (4.7-6.5)	< 0.001	-	-		-	-	
2 hr OGTT	6.1 (5.2-7.2)[c]	5.2 (4.6-5.9)	< 0.001	6.6 (5.6-8.0)[d]	5.3 (4.7-5.9)	< 0.001	8.6 (8.0-9.7)[c,d]	5.3 (4.7-6.0)	< 0.001

*Time after ingesting 75 g glucose drink; ‡significant difference between women with or without GDM in each diagnostic criteria; similar superscripts indicate significant difference between groups; OGTT: oral glucose tolerance test; hr: hour; GDM: gestational diabetes mellitus; IADPSG: International Association of Diabetes in Pregnancy Study Group; NICE: National Institute for Health and Care Excellence; WHO: World Health Organization (WHO 1999, Metzger *et al.* 2010, NICE guidelines 2015).

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Altered Genome-Wide DNA Methylation in Peripheral Blood of South African Women with Gestational Diabetes Mellitus

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Abstract: Increasing evidence implicate altered DNA methylation in the pathophysiology of gestational diabetes mellitus (GDM). This exploratory study probed the association between GDM and peripheral blood DNA methylation patterns in South African women. Genome-wide DNA methylation profiling was conducted in women with (n = 12) or without (n = 12) GDM using the Illumina Infinium HumanMethylationEPIC BeadChip array. Functional analysis of differentially methylated genes was conducted using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. A total of 1046 CpG sites (associated with 939 genes) were differentially methylated between GDM and non-GDM groups. Enriched pathways included GDM-related pathways such as insulin resistance, glucose metabolism and inflammation. DNA methylation of the top five CpG loci showed distinct methylation patterns in GDM and non-GDM groups and was correlated with glucose concentrations. Of these, one CpG site mapped to the calmodulin-binding transcription activator 1 (*CAMTA1*) gene, which have been shown to regulate insulin production and secretion and may offer potential as an epigenetic biomarker in our population. Further validation using pyrosequencing and conducting longitudinal studies in large sample sizes and in different populations are required to investigate their candidacy as biomarkers of GDM.

Keywords: gestational diabetes mellitus; molecular biomarkers; DNA methylation; MethylationEPIC Bead Chip Array; South Africa

1. Introduction

Gestational diabetes mellitus (GDM) is defined as glucose intolerance that arises during pregnancy, and usually resolves postpartum. The prevalence of GDM is increasing, affecting approximately 14% of pregnancies globally [1], although rates vary between <1% and 28% according to the diagnostic criteria employed and population studied [2]. GDM is associated with maternal (preeclampsia, caesarean section and birth injuries), fetal (macrosomia, shoulder dystocia, hyperinsulinemia, hypoglycemia, hyperbilirubinemia) and perinatal (respiratory distress syndrome, metabolic derangements and jaundice) complications [3–5], while both mothers and their offspring are at an increased risk of developing metabolic disease in later life [6–8]. Current estimates indicate that more than 50% of women with GDM develop type 2 diabetes (T2D) within 10 years, making GDM a strong predictor of T2D [6,9]. The identification of women with GDM who are at risk of developing T2D allows the introduction of timely measures to prevent or better manage disease progression.

Epigenetic mechanisms are increasingly being implicated in the pathophysiology of metabolic diseases, including GDM [10]. DNA methylation, the most widely studied and best characterized epigenetic marker, is a reversible process that refers to the addition of a methyl group to the fifth carbon position of a cytosine residue within a cytosine-phosphate-guanine (CpG) dinucleotide, and regulates gene expression through transcriptional mechanisms [11]. Altered global and gene-specific DNA methylation are observed in the placenta of women with GDM [12,13]. DNA methylation is a tissue-specific process, although recent evidence suggests that peripheral blood reflects DNA methylation in tissue [14], while several studies report that maternal blood reflects pregnancy-associated DNA methylation changes [15–17], supporting its potential as epigenetic biomarkers for GDM.

DNA methylation during GDM has been studied using various techniques such as enzyme-linked immunosorbent assays, whole-genome bisulfite sequencing, methylated DNA immunoprecipitation sequencing, liquid chromatography coupled with mass spectrometry, pyrosequencing, bead chip arrays and methyl light polymerase chain reaction (PCR) [12,15,17–20]. Due to its comparatively low cost compared to sequencing, reproducibility and high sample throughput, bead chip arrays are currently the most widely used technique for genome-wide DNA methylation profiling [21,22]. The current bead chip array version, the HumanMethylationEPIC, allows the interrogation of >850,000 CpG sites across the genome, enriched for promoters and enhancer sequences, covering 99% of RefSeq genes [23]. Previous versions, the HumanMethylation450 and HumanMethylation27, measured >480,000 and >27,000 CpG sites, respectively across the genome [21].

In South Africa, the prevalence of GDM has increased from about 1.6–25.8% in recent years [24,25]. The possible increase in future T2D cases will place a major burden on the already overburdened health system and creates an urgent need to identify preventative strategies. DNA methylation has attracted considerable interest as biomarkers that could facilitate risk stratification and offer opportunities for intervention strategies to prevent or delay the development of T2D after pregnancy [26]. The aim of this study is to explore the potential of DNA methylation to serve as biomarkers of GDM in black South African women. Genome-wide DNA methylation profiling was conducted in the peripheral blood of women with (n = 12) or without (n = 12) GDM using the Illumina methylationEPIC Bead Chip array. Functional analysis of differentially methylated genes was conducted to identify pathways associated with GDM in the South African population.

2. Results

2.1. Study Participants

Participant characteristics are presented in Table 1. As expected, no difference in age, gestational age and body mass index (BMI) was observed between women with or without GDM. Women with GDM had significantly higher fasting ($p < 0.001$) and 1 h oral glucose tolerance test (OGTT) ($p < 0.01$) glucose concentrations compared to women without GDM, while 2 h OGTT ($p = 0.07$) glucose concentrations showed a trend towards significance. In addition, fasting insulin concentrations, homeostatic model of assessment (HOMA), and c-reactive protein (CRP) levels were higher in women with GDM compared to women without GDM, although these were not statistically significant. No difference between groups were observed for HbA1c and adiponectin concentrations, nor for common risk factors (advanced maternal age (age ≥ 35 years), obesity (BMI ≥ 30 kg/m^2), family history of diabetes mellitus, delivery of a previous baby more than four kg, glucosuria, previous recurrent pregnancy loss, stillbirth, or birth of a baby with congenital abnormalities), as well as education and employment status.

Table 1. Participant characteristics.

Variables		Non-GDM (*n* = 12)	GDM (*n* = 12)	*p*-Value
Age (years) [a]		27.3 (0.3)	27.3 (0.3)	1.00
Gestational age (weeks) [a]		19.3 (1.5)	19.3 (2.0)	1.00
BMI (kg/m^2) [a]		27.1 (1.3)	27.6 (1.1)	0.77
Fasting glucose (mmol/L) [a]		4.3 (0.1)	5.5 (0.1)	<0.001
1hr OGTT (mmol/L) [a]		5.2 (0.3)	6.6 (0.4)	0.01
2hr OGTT (mmol/L) [a]		5.2 (0.3)	5.8 (0.3)	0.07
HbA1c (%) [a]		5.1 (0.1)	5.1 (0.1)	0.85
Fasting insulin (mIU/L) [b]		8 (7.5-9.0)	10.2 (6.3-12.7)	0.65
HOMA [b]		1.6 (1.6-1.8)	2.6 (1.5-2.9)	0.31
Adiponectin (μg/mL) [b]		10.4 (7.3-23.8)	9.7 (4.7-12.0)	0.28
C-reactive protein (mg/L) [a]		7.1 (1.2)	7.7 (1.1)	0.75
Risk factors: *n* (%) [c]	None	10 (83.3)	7 (58.3)	0.37
	≥1 risk factor	2 (16.7)	5 (41.8)	
* Education: *n* (%) [c]	<grade 12	7 (63.6)	5 (41.7)	0.29

	≥grade 12	4 (36.4)	7 (58.3)	
Employment: *n* (%) [c]	None	8 (66.7)	7 (58.3)	1.00
	Formal/informal employment	4 (33.3)	5 (41.7)	

GDM: gestational diabetes mellitus; BMI: body mass index; OGTT: oral glucose tolerance test; HbA1c: glycated hemoglobin; HOMA: homeostatic model assessment calculated according to the formula: fasting insulin (mIUL) × fasting glucose (mmol/L)/22.5; Risk factors: advanced maternal age (age > 35 years), obesity (BMI > 30 kg/m^2), family history of diabetes mellitus, delivery of a previous baby more than four kilograms, glucosuria, previous recurrent pregnancy loss, stillbirth, or birth of a baby with congenital abnormalities. * One participant had missing data for education. Data are expressed as the [a] mean ± standard error of the mean, as [b] median (25th–75th percentiles) or as [c] count (percentage). *p*-values for continuous data were calculated using the Mann–Whitney or the unpaired Student *t* test. *p*-values for categorical data were calculated using chi-square test or Fisher's exact test if frequency was <5.

2.2. Genome-Wide DNA Methylation Profiling

The average detection *p*-values for all probes were calculated for each sample and are presented in supplementary Figure S1. Each sample showed *p*-values below the usual cut-off of 0.01, indicating that all samples passed the quality control. In addition, box and whisker plots showed concordance across samples without any outliers, suggesting good quality and consistency of samples (Figure 1). Median β-values ranged between 0.79 and 0.83 across the 24 samples. A histogram of β-values showing the frequency distribution of CpG methylation across all samples is illustrated in Figure S2. A clear separation between GDM and non-GDM groups is evident in the principal component analysis (PCA) score plot, with characteristic DNA methylation profiles aggregating together within the same group (Figure 2). The first three PCAs explain 27.6% of the variance observed. The β-values were then converted to M-values for statistical analysis. To identify differentially methylated CpG sites between GDM and non-GDM pregnancies, data were filtered using the criteria shown in Figure 3. An M-value cut-off threshold between >0.4 and >0.6 was explored in this study, which is within the threshold range suggested by Du et al. [27]. In the first filtering step a M-value difference of >0.4 or <–0.4 and unadjusted $p < 0.01$ was used, to permit comparison between differentially methylated probes. Further filtering steps including M-values which ranged between >0.5 or <–0.5 and >0.6 or <–0.6 with unadjusted $p < 0.01$ were assessed. We identified 1046 differentially methylated CpG loci with M-value differences of >0.6 or <–0.6 and unadjusted $p < 0.01$ (Table S1). To facilitate a more stringent analysis, a false discovery rate (FDR) <0.1 was added, which did not identify any significant probes. Hierarchical clustering was performed to determine whether these methylation patterns could distinguish between women with or without GDM. The heatmap in Figure 4 illustrates that there are distinct methylation patterns between the GDM and non-GDM groups.

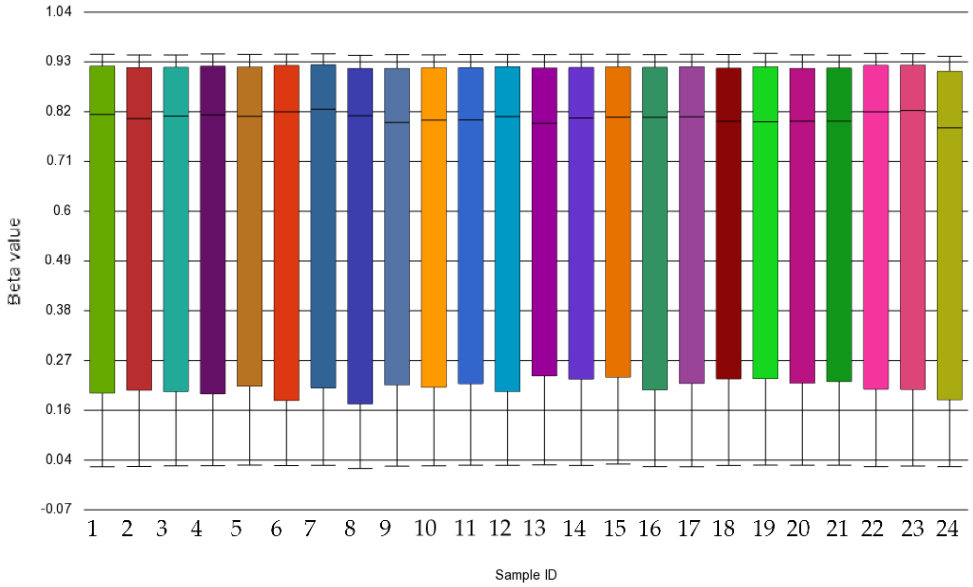

Figure 1. Box and whisker plots of β-values. Each box represents a sample (n = 24) which is illustrated by a different color bar. The median β-value is 0.042 with a minimum and maximum range of 0.785 and 0.827.

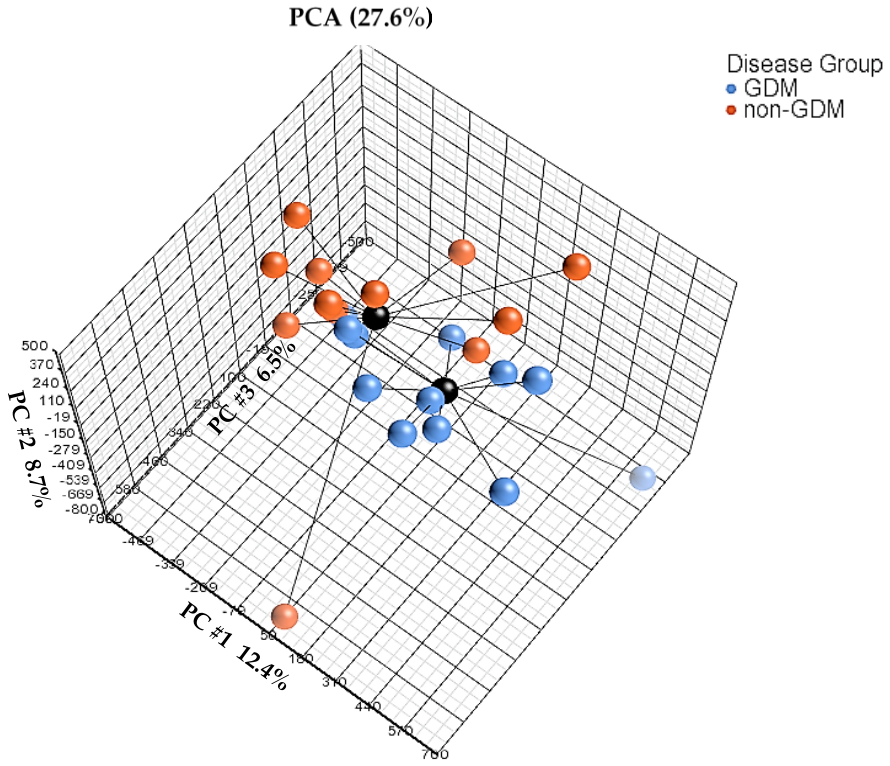

Figure 2. Principal component analysis (PCA) between GDM and non-GDM groups. Each dot represents a sample. Centroids (black) connect samples from the respective GDM (blue) or non-GDM (red) group and indicate the center of distribution, while the black bars indicate the distance between samples and centroids. The first three PCAs explain 27.6% of the variance.

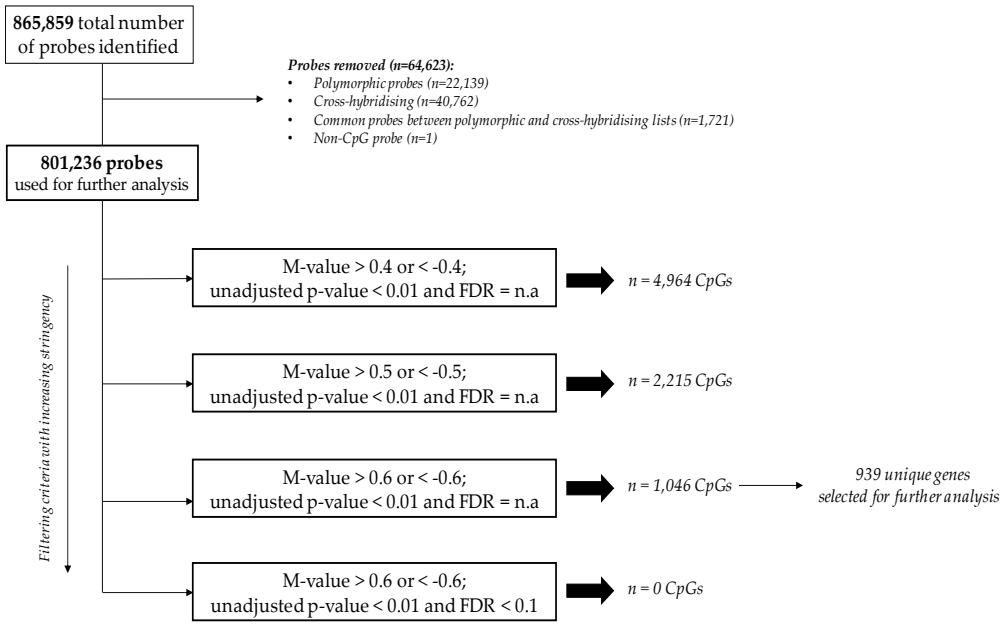

Figure 3. Filtering criteria for the identification of CpGs differentially methylated between GDM and non-GDM groups. A total of 801,236 probes, derived through the removal of polymorphic, cross-hybridising and non-CpG probes were used for analysis. FDR: false discovery rate; M-values closest to 0 indicate similar methylation intensities between probes.

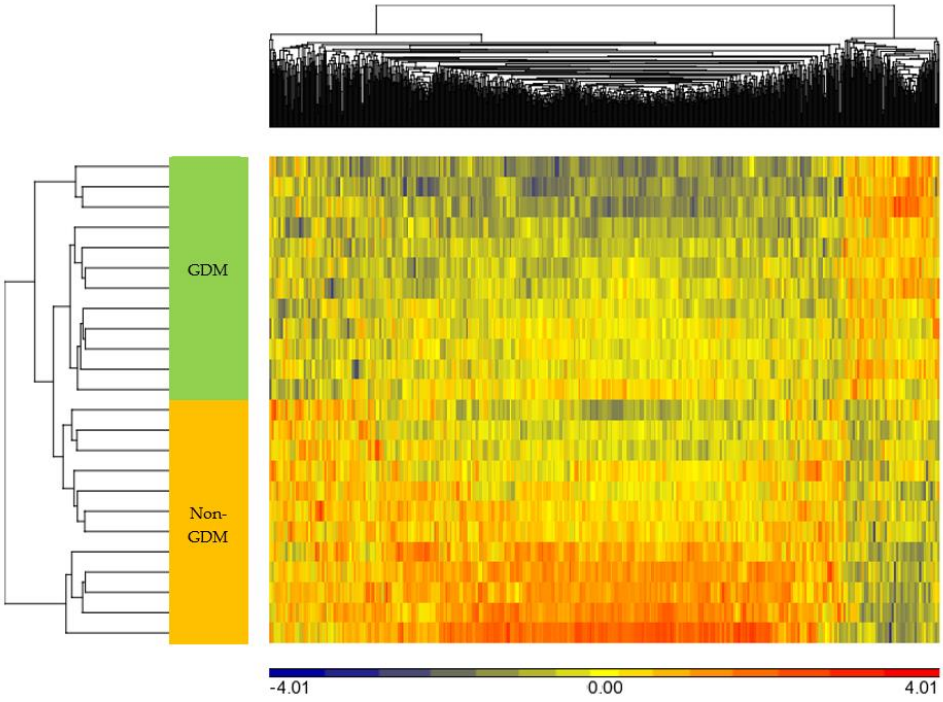

Figure 4. Heatmap showing methylation signatures of 1046 CpG sites in women with/without GDM. DNA methylation across 1046 CpG sites in each sample was analyzed using Euclidean distance for both rows (observations) and columns (features) and average linkage criteria. Samples are shown in rows and are clustered in GDM (green) and non-GDM (orange) groups. Standardized M-values are depicted using a blue (hypomethylation in GDM) to red (hypermethylation in GDM) methylation gradient.

Of the 1046 differentially methylated CpG loci, 148 CpG sites (14.2%) were hypermethylated and 898 CpG sites (85.8%) were hypomethylated in women with GDM compared to women without GDM. To increase the likelihood of identifying differentially methylated promoters, probes located 5 kbp upstream or up to 3 kbp downstream of the transcription start site were also included as promoter regions. The frequency of all CpG sites analysed and differentially methylated CpG sites in relation to their genomic location is shown in Figure 5. Of the differentially methylated CpGs, 16.3% were associated with 5′-untranslated regions (UTR), 49.7% with promoters, 6.2% with coding domain sequences (CDS), 19.1% with introns, 4.0% with non-coding regions, 2.1% with 3′-UTRs and 4.6% with intergenic regions. Differentially methylated CpG sites were annotated to 939 unique genes using RefSeq build 87 (Table S2). The top five significantly differentially methylated CpG sites selected for further analysis, were associated with four unique genes, including Solute Carrier Family 9 Member A3 *(SLC9A3)*, Male-Enhanced Antigen 1; Kelch domain-containing protein 3 *(MEA1;KLHDC3)*, Calmodulin Binding Transcription Activator 1 *(CAMTA1)* and RAS P21 Protein Activator 3 *(RASA3)*, and one unknown gene. The probe ID, location, gene region and direction of methylation (GDM vs. non-GDM), as well as the nearest gene/regulatory region for the unknown gene is shown in Table 2. Of the differentially methylated CpG sites, cg22985016 and cg16306629 was shown to be significantly hypermethylated, while cg21910650, cg23643951 and cg07966372 was significantly hypomethylated in GDM compared to non-GDM groups. The association between GDM and the top five CpG sites remained significant for each CpG after linear regression adjusting for age

BMI and gestational age (Table 3). To examine the degree to which DNA methylation levels at these CpGs are associated with the clinical characteristics of GDM, Pearson's correlation analysis was performed (Table 4). For cg22985016 and cg16306629, a positive correlation between DNA methylation and fasting glucose concentrations was observed, while methylation at cg21910650, g23643951 and cg07966372 was inversely correlated with glucose concentrations. Furthermore, DNA methylation at cg22985016 and cg16306629 was correlated with 1 h glucose, while methylation at cg07966372 was negatively correlated with fasting insulin concentrations. When adjusting for GDM, the association between the five CpGs and fasting glucose concentrations and between cg22985016 and cg16306629 and 1 h OGTT was no longer significant, while the association between cg07966372 and fasting insulin remained significant (Table S3).

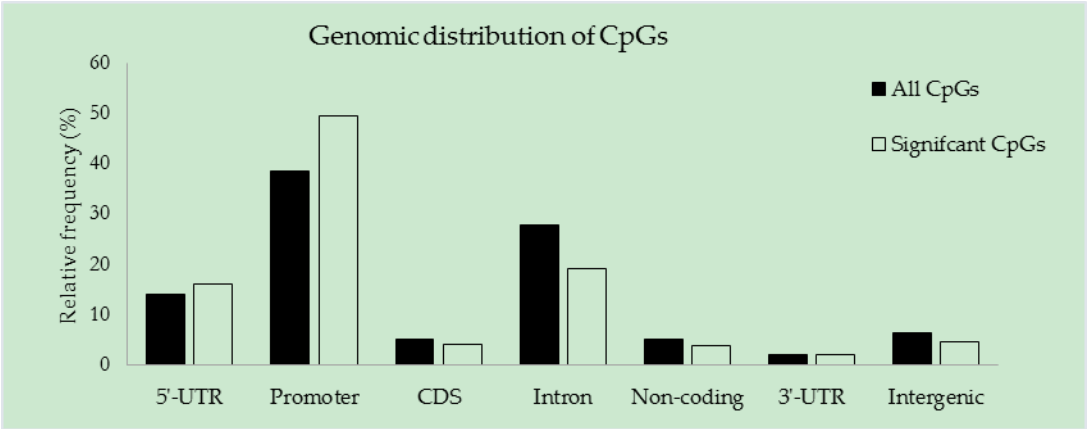

Figure 5. Relative frequency of all CpGs analysed (black bars) and differentially methylated CpGs identified in our study (white bars) in relation to genomic location across the genome. UTR: untranslated region; CDS: coding domain sequence.

Table 2. The top five significantly differentially methylated CpG sites between GDM and non-GDM groups.

Probe ID	Location	Gene Symbol	Gene Name	Region	*p*-Value	Methylation
cg22985016	Chr5:492187–524227	*SLC9A3*	Solute Carrier Family 9 Member A3	Intron	1.84×10^{-7}	↑
cg21910650	Chr6:42976841–42986722	*MEA1*; *KLHDC3*	Male-Enhanced Antigen 1; Kelch domain-containing protein 3	Promoter/5′UTR	3.23×10^{-6}	↓
g23643951	Chr1:7151432–7309551	*CAMTA1*	Calmodulin Binding Transcription Activator 1	Intron	4.46×10^{-6}	↓
cg16306629	Chr8:119121060–119129059	*COLECT10* *	Collectin Subfamily member 10*	Enhancer *	9.22×10^{-6}	↑
07966372	Chr13:114782770–114898099	*RASA3*	RAS P21 Protein Activator 3	5′UTR/Intron	9.75×10^{-6}	↓

* Nearest gene/regulatory region of cg16306629. ↑: hypermethylation and ↓: hypomethylation between GDM vs. non-GDM groups. Significance is shown as $p < 0.05$.

Table 3. Linear regression analysis of gestational diabetes mellitus and the top five significantly differentially methylated CpG sites, adjusting for age, body mass index and gestational age.

CpG Site	[a] Univariate			[b] Multivariate		
	Coefficient	95% CI	*p*-Value	Coefficient	95% CI	*p*-Value
cg22985016 *(SLC93A)*	0.028	0.019; 0.037	<0.001	0.028	0.019; 0.037	<0.001
cg21910650 *(MEA1;KLHDC3)*	−0.088	−0.117; −0.058	<0.001	−0.087	−0.118; −0.056	<0.001
cg23643951 *(CAMTA1)*	−0.056	−0.070; −0.042	<0.001	−0.056	−0.071; −0.042	<0.001
cg16306629 *(Unknown)*	0.274	0.183; 0.366	<0.001	0.275	0.192; 0.359	<0.001
cg07966372 *(RASA3)*	−0.015	−0.025; −0.004	0.006	−0.015	−0.026; −0.004	0.008

[a] Univariate linear regression: association between CpG-specific methylation and GDM. [b] Multivariate linear regression: adjusting for age (years), body mass index (kg/m^2) and gestational age (weeks); CI: Confidence interval. Significance is shown as $p < 0.05$.

Table 4. Correlation analysis showing the association between DNA methylation and fasting plasma, 1 h OGTT, 2 h OGTT and fasting insulin for the top five differentially methylated CpG sites.

Variable	cg22985016 (SLC93A)		cg21910650 (MEA1; KLHDC3)		cg23643951 (CAMTA1)		cg16306629 (Unknown)		cg07966372 (RASA3)	
	Rho	*p*-Value	Rho	*p*-Value	Rho	*p*-Value	Rho	*p*-Value	Rho	*p*-Value
Fasting glucose (mmol/L)	0.728	<0.001	−0.694	<0.001	−0.735	<0.001	0.724	<0.001	−0.452	0.026
1 h OGTT (mmol/L)	0.502	0.012	−0.377	0.069	−0.399	0.053	0.559	0.004	0.016	0.939
2 h OGTT (mmol/L)	0.297	0.168	−0.249	0.250	−0.338	0.115	0.266	0.219	0.098	0.658
Fasting insulin (mIU/L)	−0.037	0.888	−0.103	0.691	−0.204	0.433	0.109	0.674	−0.495	0.043

OGTT: oral glucose tolerance test; SLC93A: Solute Carrier Family 9 Member A3; MEA1; KLHDC3: Male-Enhanced Antigen 1; Kelch domain-containing protein 3; CAMTA1: Calmodulin Binding Transcription Activator 1; Unknown: gene nearest to this region is called Collectin Subfamily member 10; RASA3: RAS P21 Protein Activator 3. Pearson's correlation coefficient (rho) is shown with significance at $p < 0.05$.

2.3. Functional Enrichment Analysis

Differentially methylated CpG sites (1046), annotated to 939 unique genes using M-values >0.6 and <–06 with unadjusted $p < 0.01$ threshold criteria, were selected for functional enrichment analysis. Functional enrichment analysis identified 261 *Kyoto Encyclopedia of Genes and Genomes* (KEGG) pathways, including pathways for T2D and insulin signaling (Table S4). Only 50 KEGG pathways were statistically significantly different between GDM and non-GDM groups (Table S5). Statistically significant pathways included cancer, brain signaling, cell growth, proliferation, viability and inflammation pathways. The most significant KEGG pathway was 'Signaling pathways regulating pluripotency of stem cells' with an enrichment score of 10.496, a p-value = 2.76×10^{-5} and 19 differentially methylated associated genes. In addition, Gene Ontology (GO) terms were enriched by differentially methylated genes, categorized into 1181 biological processes, 167 molecular functions and 85 cellular components with a p-value < 0.05 (Table S6). The top 10 GO terms categorized into biological processes, molecular functions and cellular components are illustrated in Figure 6. Of these, homophilic cell adhesion via plasma membrane adhesion molecules (biological process), calcium ion binding (molecular function) and integral component of plasma membrane (cellular component) have the highest ranked enrichment score and p-value < 0.001.

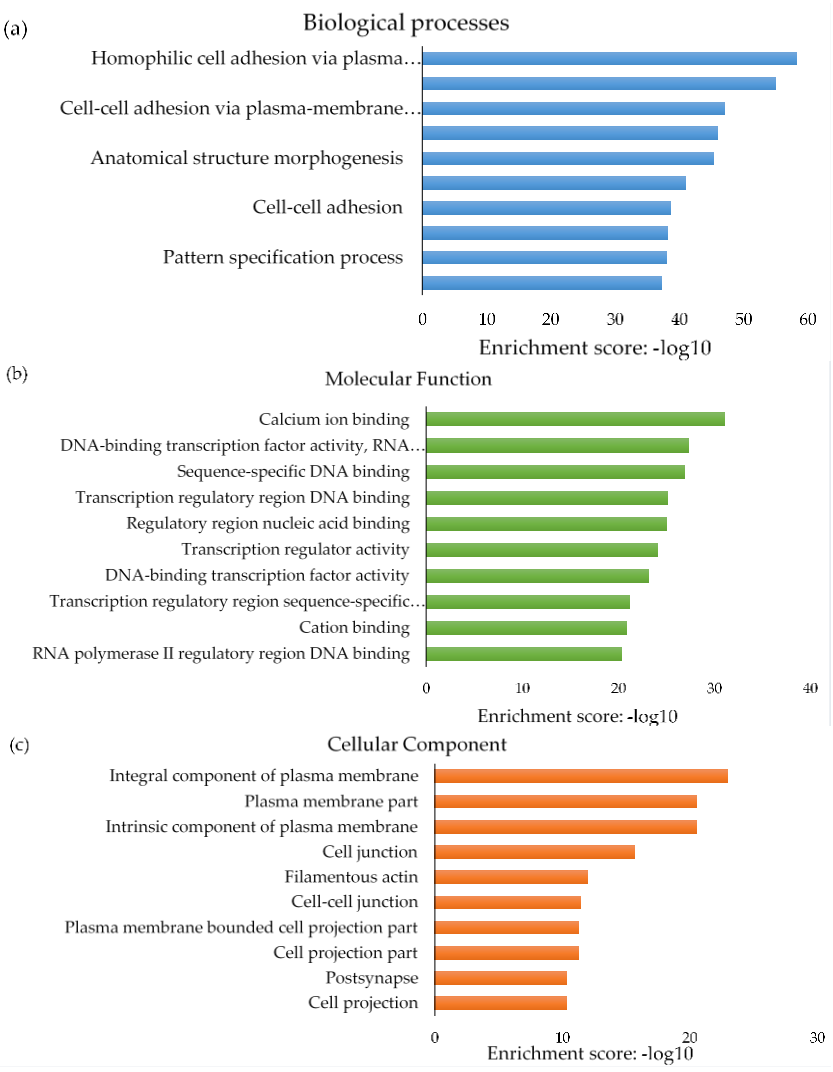

Figure 6. Top 10 Gene Ontology (GO) terms enriched by differentially methylated genes in GDM and non-GDM groups. Enriched GO terms were categorized into (**a**) biological processes, (**b**) molecular function and (**c**) cellular components. Data are presented as enriched scores expressed as −log10 (*p* value). Fisher $p \leq 0.001$.

3. Discussion

We report the differential methylation of 1046 CpG sites in the peripheral blood of black South African women with GDM compared to women with normoglycemic pregnancies. Functional analysis mapped these CpGs to genes in pathways key to metabolic regulation. Furthermore, differential methylation of the five CpG loci, within *SLC93A* was positively correlated with fasting and 1 h glucose, while CpGs within *CAMTA, MEA1;KLHDC3* and *RASA3* was inversely correlated to fasting glucose, with distinct methylation profiles in GDM and non-GDM groups. *CAMTA1* is a transcriptional activator that was previously shown to regulate insulin production and secretion [28]. These results support the plausibility of the observed DNA methylation differences in GDM pathophysiology and potential as diagnostic biomarkers of GDM.

Genome-wide DNA methylation differences during GDM have been demonstrated in other populations. Kang et al. used the Illumina Infinium Human MethylationEPIC Bead Chip array to investigate DNA methylation in Chinese women with GDM, and showed that the top 200 differentially methylated loci mapped to 151 genes [15]. Of these, 15 genes, *CAMTA1,* Smad Nuclear Interacting Protein 1 *(SNIP1)*, Protein-Tyrosine Phosphatase, Receptor-Type, F Polypeptide-Interacting Protein-Binding Protein 2 *(PPFIBP2),* Switching B Cell Complex Subunit *SWAP70 (SWAP70)*, Semiphorin 6D *(SEMA6D)*, Cadherin 8 *(CDH8)*, Cytochrome P450 Family 26 Subfamily B Member 1 *(CYP26B1),* Wnt Family Member 6 *(WNT6)*, Raftlin, Lipid Raft Linker 1 *(RFTN1),* Unc-5 Netrin Receptor C *(UNC5C),* Nucleoside Diphosphate-Linked Moiety X Motif 6 *(NUDT6),* Storkhead Box *(STOX2)*, MutS Protein Homolog 5 *(MSH5),* KH RNA Binding Domain Containing, Signal Transduction Associated 2 *(KHDRBS2),* and Neuregulin 1 *(NRG1)* were similarly shown to be differentially methylated in our study, and has been illustrated in a venn diagram (Figure S3). Disparities in the number of differentially methylated CpG sites identified between studies could be due to population differences such as ethnicity, age and stage of pregnancy, and the data filtering criteria used. Although M-values were used to measure methylation differences in both studies, Kang et al. used a more stringent FDR adjusted p-value < 0.05 for their analysis whereas we used an unadjusted p-value < 0.01, since an FDR of <0.05 did not identify any significantly differentially methylated loci in our analysis. Despite using a higher FDR than Kang et al., the differential methylation of 15 genes were similar between studies [15]. Other technical differences between studies which may affect methylation levels include sample preparation, loading during hybridization and batch effect bias [21,29]. Soriano-Tárraga et al. reported that the method of DNA extraction affects global DNA methylation levels [29]. Thus, standardization of analytical methods across laboratories is essential to enable comparison of DNA methylation patterns between studies. Other studies that used previous versions of the bead chip array similarly reported DNA methylation differences during GDM in Non-Hispanic Caucasian American and Caucasian English populations [16,17]. As reported in these studies [15–17,30], the majority of CpG differences in our study were hypomethylated in women with GDM compared to women without GDM. However, in contradiction, in our study most of the 1046 differentially methylated CpG sites occurred in promoter regions, whereas previous studies identified most of the differentially methylated CpGs in gene body regions [30,31]. Differences could be due to the method of analysis used. Our analysis included additional CpGs located 5 kbp upstream and 3 kbp downstream of the transcription start site to increase the probability of detecting differentially methylated promoter regions. Altered DNA methylation in promoter regions influences the expression of specific genes [32–34], which may enable the identification of genes/pathways involved in metabolic processes during GDM.

Recently, we demonstrated that global DNA methylation is not associated with GDM in South African women [19]. We hypothesized that the failure to detect DNA methylation differences was due to technical limitations and that gene-specific methylation analysis would be able to identify GDM-associated methylation differences. Global DNA methylation quantification is a crude marker of overall genomic methylation and does not have the resolution to detect gene-specific differences, as observed in the current study. Similar findings were reported by Matsha et al., who showed no difference in global DNA methylation between 61 diabetic individuals on treatment and 287 normoglycemic subjects in a mixed ethnic ancestry South African population [35]. In addition, no difference in global DNA methylation was observed in peripheral blood mononuclear cells of a Danish population with obesity or T2D compared to controls [36].

The diagnosis of GDM is contentious and varies across countries and health institutions. Currently the International Association of Diabetes in Pregnancy Study Group (IADPSG) criteria are advocated by several international bodies and endorsed by the World Health Organisation (WHO) [37]. However, concerns that the high costs and increased workload of IADPSG criteria outweigh the clinical effects of small glucose differences has hampered its universal use. We were able to see altered DNA methylation patterns despite small glucose differences between women with or without GDM, suggesting that epigenetic programming is evident even during mild hyperglycemia. Kang et al. also demonstrated altered DNA methylation in women diagnosed with GDM according to IADPSG diagnostic criteria [15]. These findings support The Hyperglycemia and Adverse Pregnancy Outcomes (HAPO) study, which showed that even mild hyperglycemia is associated with adverse pregnancy outcomes and requires treatment [38]. Furthermore, several clinical trials have confirmed that treatment of mild hyperglycemia decreases maternal morbidity and adverse perinatal outcomes [39].

Functional analysis of differentially methylated CpG sites identified canonical pathways related to signal transduction, cell growth, proliferation, differentiation and apoptosis, insulin resistance, glucose metabolism, inflammation, neurological signaling, and oncogenesis. Altered DNA methylation of two signaling pathways, mitogen-activated protein kinase *(MAPK)* and phosphoinositide 3-kinase *(PI3K)*, which play a role in cell growth and differentiation, and the metabolic action of insulin [40], have previously been reported during GDM in other populations [15], identifying these CpG sites as likely biomarkers for the development of GDM. Our results demonstrated that pathways associated with cancer are differentially methylated in women with GDM compared to controls. Several studies have reported a link between GDM and cancer, particularly breast cancer [41–43], identifying GDM as a potential risk factor for the development of cancer in later life , Nine of the top 10 GO terms enriched for biological processes were associated with structural organization and developmental processes, supporting the influence of GDM on in utero programming of fetal growth and development [44]. As expected, all 10 GO terms enriched for molecular functions were associated with regulatory or binding activities and offer insight into functions influenced by altered methylation at a molecular level during GDM.

A strength of our study is that women were matched for age, gestational age and BMI, to ensure that results were comparable between groups. In addition, DNA methylation analysis was conducted using the most comprehensive MethylationEPIC Bead Chip array currently available, which is considered a high-throughput method, that has a lower cost compared to sequencing, and is reproducible and time-efficient [21,22]. Our study has a number of limitations. The sample size (n = 24) is small, although, it is larger than previously reported [15–17]. No CpG sites reached FDR cut-off, suggesting that the study might have been underpowered. However, 15 of the differentially methylated genes identified in our study were amongst the top 151 identified by Kang et al. Peripheral blood cells consist of a mixture of different cell types [45], which may confound methylation analysis. In our study, cell type composition did not differ significantly between GDM and non-GDM groups and therefore was not adjusted for in further analysis due to the small sample size. Thus, methylation differences

between cell types could have confounded our analysis. Furthermore, physical activity, diet, smoking and alcohol consumption, which are known to influence DNA methylation patterns, are not known, and could confound our analysis. However, women in our study were recruited from the same community and had similar lifestyle behaviours, education and employment status, suggesting that they had roughly similar environmental influences.

To our knowledge, this exploratory study is the first to profile genome-wide DNA methylation levels in the peripheral blood of South African women with GDM. We have identified five CpGs which are associated with GDM and offer potential as epigenetic biomarkers in our population. Further validation using pyrosequencing and conducting longitudinal studies in large sample sizes and in different populations are required to investigate their candidacy as biomarkers of GDM

4. Materials and Methods

4.1. Study Participants

Ethical approval for this study was granted by the University of Pretoria Health Sciences Ethics Committee (180/2012: approved on the 26/09/2012). The study was conducted according to the Declaration of Helsinki and all women gave written informed voluntary consent after the procedures had been fully explained in the language of their choice. One thousand pregnant women attending a primary care clinic in Johannesburg, South Africa were enrolled in the study. At recruitment, demographic and socio-economic data were obtained in the form of a standardized questionnaire and risk factors for GDM, i.e. advanced maternal age (age ≥ 35 years), obesity (BMI ≥ 30 kg/m^2), family history of diabetes mellitus, delivery of a previous baby more than four kilograms, glucosuria, previous recurrent pregnancy loss, stillbirth, or birth of a baby with congenital abnormalities) were assessed [25]. Patients with pre-existing diabetes mellitus (Type 1 diabetes (T1D) and T2D) and those who were more than 26 weeks pregnant were excluded. At their first visit, random glucose and glycated hemoglobin (HbA1c) concentrations were measured. Women with random glucose and HbA1c concentrations less than 11.1 mmol/L and 6.5 %, respectively, were requested to fast overnight and return to the clinic within two weeks. At this time, a 75 g oral glucose tolerance test (OGTT) was conducted, and GDM was diagnosed if at least one glucose value was met (fasting plasma glucose > 5.1 mmol/L, 1 h OGTT > 10 mmol/L or 2 h OGTT > 8.5 mmol/L), according to the IADPSG criteria [46]. Blood for measurement of cytokines and DNA methylation was collected and stored at –80 °C. For this sub-study, a subset of women with (n = 12) and without (n = 12) GDM were selected for genome-wide DNA methylation analysis. The inclusion criteria were pregnant women ≥18≤40 years of age, black ethnicity, human immunodeficiency virus (HIV) negative and women with a singleton pregnancy. All women were matched according to age, BMI and gestational age as far as possible.

4.2. DNA Extraction

Genomic DNA was extracted from 2 ml of peripheral blood collected in Ethylenediaminetetraacetic acid (EDTA) tubes using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, North Rine-Westphalia, Germany), as previously described [19]. Briefly, white blood cells were lysed and loaded onto the QIAamp Midi column, bound DNA was washed and then eluted from the column membrane using 300 µl of elution buffer and centrifuged at 4500× g for 2 mins. DNA concentration was measured using the Qubit Fluorometer (Invitrogen, Carlsbad, California, USA) and the Quanti-iT dsDNA Broad Range assay kit (ThermoFisher, Waltham, Massachusetts, USA). One microgram of DNA in a volume of 45 µl was frozen and shipped on dry ice, as instructed by the University of Southern California Molecular Genomics Core for genome-wide DNA methylation

analysis using the Illumina Infinium HumanMethylationEPIC BeadChip (USC Molecular Genomics Core, Los Angeles, California, USA).

4.3. Genome-Wide DNA Methylation Profiling

Genome-wide DNA methylation profiling was conducted using the Illumina's Infinium HumanMethylationEPIC Bead Chip (HumanMethylationEPIC, Illumina inc., San Diego, California, USA) according to manufacturer's instructions. Bisulfite conversion of 500 ng genomic DNA was performed using the Illumina-specific EZ DNA methylation kit (D5001, Zymo Research, Orange city, Florida, USA), and quality control was conducted by quantitative real-time polymerase chain reaction (PCR) and melt curve analysis. Bisulfite converted DNA was amplified up to 1000-fold with DNA polymerase during the incubation step in the Illumina hybridization oven at 37 °C. Amplicons were then fragmented to 300–600 bp products, precipitated with isopropanol and loaded onto Illumina Infinium HumanMethylationEPIC Bead Chips prepared for hybridization in the capillary flow-through chamber (Human MethylationEPIC, Illumina Inc.), according to the Infinium protocol [47]. After annealing to locus-specific 50-mer probes, a single base extension occurs at the base immediately adjacent to the interrogated CpG site. Products were fluorescently labelled with either dinitrophenol-labelled ddATP/ddTTP or biotin-labelled ddCTP/ddGTP, depending on the methylation state of the interrogated CpG site. Fluorescence intensity was measured with the Illumina iScan system (iScan Control Software v.3.3.28) and was based on the ratio of methylated probe intensities and the overall intensity (sum of methylated and unmethylated probe intensities). The methylation scores were represented as raw beta (β)-values and were exported as 48 intensity data files (IDAT).

4.4. Processing and Analysis of the Human Methylation EPIC Bead Chip Array

Data analysis was conducted by Partek (Partek, St. Louis, Missouri, USA). IDAT files were imported to Partek (R) Genomics Suite (R) v.7.18.0803 software. Functional normalization with normal-exponential out-of-band (NOOB) background correction and dye correction was used [48]. Quality control was performed across all imported probes (865,859) for each sample. All samples passed the quality control, and those with detection $p < 0.01$ were included in the analysis. Thereafter, β-values for imported probes were plotted and no outliers were detected, indicating that the data were technically sound. In addition, a histogram was used to illustrate distribution of methylation β-values across all CpG sites in each sample. Data filtering was conducted to remove polymorphic probes (n = 22,139), cross-hybridising probes (n = 40,762), non-CpG probes (n = 1) and probes overlapping both the polymorphic and cross-hybridising probe lists (n = 1,721) (Figure 3), according to McCartney et al. [23]. The clean data set consisted of 801,236 probes (referred to as CpG sites). Exploratory analysis was performed using PCA. Cell count estimation was performed empirically using methylation data from sorted blood cells using the 'Estimate Cell Count' function in the minfi package in R [49]. The function is based on a modification of the original method by Houseman et al. [50] and the R package FlowSorted.Blood.450k [51]. No differences in cell composition were identified, and cell composition was deemed unlikely to be a confounder (Figure S4). Therefore, cell composition was not corrected for in further analysis.

Following data processing, β-values were converted to M-values ($\log_2$ ratio [methylated signal intensity/unmethylated signal intensity]) to account for heteroscedasticity and allow for analyses assuming a Gaussian distribution [27]. M-values have a range of $-\infty$ to $+\infty$, with a value close to 0 indicating similar intensities between methylated and unmethylated probes. Positive M-values represent hyper-, while negative M-values represent hypo-methylation. M-values were then

standardized (converted to Z-scores) to perform hierarchical clustering, using Euclidean distance and average linkage criteria for visualization of methylation signatures.

4.5. Functional Enrichment Analysis

All differentially methylated CpG sites were annotated to genes using the reference sequence database (RefSeq) build 87 and were subjected to functional analysis using KEGG pathway analysis and GO grouping categories (biological process, cellular component, and molecular function). The results of enriched pathways were ranked by enrichment scores to identify overrepresented pathways and then sorted by factor score to consider those pathways with the most significant *p*-value. A high enrichment score indicates that a significant number of the differentially methylated genes within a pathway are present, while factor score enables comparison of pathways with similar enrichment scores between GDM and non-GDM groups.

4.6. Statistical Analysis

Participant characteristics were tested for normality using the Shapiro-Wilk test in STATA 14 (StataCorp, College Station, USA). Normally distributed data are expressed as the mean ± standard error of the mean (SEM), or as the median and interquartile range (25th and 75th percentiles) for data that were not normally distributed. An unpaired *t*-test or the Mann–Whitney test was used to compare variables across GDM groups. Categorical variables were analysed using the chi-square test or the Fisher's exact test if the frequency was <5. A $p \leq 0.05$ was considered statistically significant. Due to the matched case control study design, a two-way analysis of variance (ANOVA, one factor was the GDM status and the other was the pairing ID), was used to identify differentially methylated sites. To investigate the association between GDM and differentially methylated CpGs, univariate and multivariate generalised linear regression models were tested and adjust for confounding factors. Pearson's rank correlation (r) was used to evaluate the relationship between specific CpG DNA methylation (β-values; 0–1, as a percentage of methylated to unmethylated) states and clinical parameters. Pathway enrichment was based on the current publicly available human database, GRCh38, and statistical significance was calculated using Fisher's exact test. An enrichment score ≥3 was considered significant ($p < 0.05$).

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/s1: Figure S1: Average detection *p*-values per sample, Figure S2: Histogram of β-values showing frequency distribution, Figure S3: Venn diagram illustrating comparison of differentially methylated genes, Figure S4: Comparison of six major peripheral blood cell components in GDM and non-GDM women, Table S1: Genome-wide DNA methylation profiling identified 1046 differentially methylated CpG loci, Table S2: Differentially methylated CpG sites annotated to 939 unique genes, Table S3: Univariate and multivariate linear regression analysis, Table S4: Functional enrichment analysis identified 261 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, Table S5: Statistically significant KEGG pathways associated with GDM, Table S6: GO terms enriched by differentially methylated genes, categorized into 1181 biological processes, 167 molecular functions and 85 cellular components.

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Abbreviations

GDM	Gestational diabetes mellitus
CAMTA1	Calmodulin binding transcription activator 1
MAPK	Mitogen activated protein kinase
PI3K	Phosphoinositide 3-kinase
T2D	Type 2 diabetes
CpG	Cytosine-phosphate-guanine
OGTT	Oral glucose tolerance test
HIV	Human immunodeficiency virus
BMI	Body mass index
HOMA	Homeostatic model of assessment
CRP	c-Reactive protein
HbA1c	Glycated hemoglobin

PCA	Principal component analysis
FDR	False discovery rate
UTR	Untranslated regions
CDS	Coding domain sequences
KEGG	*Kyoto Encyclopedia of Genes and Genomes*
GO	Gene Ontology
NRG1	Neuregulin 1
SNIP1	smad Nuclear Interacting Protein 1
PPFIBP2	Protein-tyrosine phosphatase, receptor-type, f polypeptide-interacting protein-binding protein 2
SWAP70	Switching b cell complex subunit *swap70*
SEMA6D	Semiphorin 6d
CDH8	Cadherin 8
WNT6	Wnt family member 6
RFTN1	Raftlin, lipid raft linker 1
UNC5C	Unc-5 netrin receptor c
NUDT6	Nucleoside diphosphate-linked moiety x motif 6
STOX2	Storkhead box
MSH5	Muts protein homolog 5
KHDRBS2	KH RNA binding domain containing, signal transduction associated 2
NRG1	Neuregulin 1
SLC9A3	Solute carrier family 9 member a3
MEA1	Male-enhanced antigen 1
KLHDC3	Kelch domain-containing protein 3
RASA3	RAS p21 protein activator 3
CYP26B1	Cytochrome p450 family 26 subfamily b member 1

IADPSG	International association of diabetes in pregnancy study group
WHO	World Health Organisation
HAPO	Hyperglycemia and adverse pregnancy outcomes
T1D	Type 1 diabetes
EDTA	Ethylenediaminetetraacetic acid
NOOB	Normal-exponential out-of-band
SEM	Standard error of the mean
ANOVA	One-way analysis of variance

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