Cape Peninsula University of Technology

DNA METHYLATION: A RISK FACTOR FOR TYPE 2 DIABETES MELLITUS

By

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Declaration

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

Signed **Date**

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Dedication

To my parents, Mr A.G.M Mutize and Mrs R. Mutize, I count you twice on my blessings list.

Abstract

Background: The early detection of individuals who are at risk of developing type 2 diabetes mellitus (T2DM) would decrease the morbidity and mortality associated with this disease. DNA methylation, the most widely studied epigenetic mechanism, offers unique opportunities in this regard. Aberrant DNA methylation is associated with disease pathogenesis and is observed during the asymptomatic stage of disease. DNA methylation has therefore attracted increasing attention as a potential biomarker for identifying individuals who have an increased risk of developing T2DM. The identification of high risk biomarkers for T2DM could facilitate risk stratification and lifestyle interventions, which could ultimately lead to better ways to prevent, manage and control the T2DM epidemic that is rampant worldwide. The aim of the study was to investigate global DNA methylation as a potential risk factor for T2DM by studying the association between the global DNA methylation levels and hyperglycaemic states.

Methods: A cross-sectional, quantitative study design, involving 564 individuals of mixed ancestry descent, residing in Bellville South, South Africa was used. Participants were classified as normal, pre-diabetic (impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT)) or diabetic (screen detected diabetic and known diabetics) according to WHO criteria of 1998. DNA was extracted from whole blood using the salt extraction method. The percentage global DNA methylation was measured by an enzyme-linked immunosorbent assay (ELISA). The association between global DNA methylation and hyperglycaemia, as well as other biochemical markers of T2DM was tested in a robust linear regression analysis adjusted for age, gender and smoking.

Results: Of the 564 participants, median age (53.0 (43.0 – 62.0); [median 25Q – 75Q]), 126 (22.1 %) were male and 438 (77.9 %) were female. Global DNA methylation was increased in subjects with diabetes ($p = 0.0115$) and pre-diabetes ($p = 0.0328$) compared to individuals with normoglycaemia. Furthermore, the percent global DNA methylation was significantly increased in subjects with screen-detected diabetes when compared to subjects with known diabetes and normoglycaemia ($p = 0.0003$). However, global DNA methylation did not differ between subjects with known diabetes and normoglycaemia ($p = 1.0000$).

In the overall sample, global DNA methylation correlated positively with fasting plasma glucose (FPG) ($R = 0.1256$, $p = 0.0029$), 2-hour plasma glucose (2h-PG) ($R = 0.1674$, $p = 0.0002$), body mass index (BMI) ($r = 0.0898$, $p = 0.0336$), and waist circumference (WC) ($r = 0.0900$, $p = 0.0330$). The correlation between global DNA methylation with blood glucose levels and obesity indices was driven by females who also showed correlation with FPG ($r = 0.1175$, $p = 0.0141$), 2-h PG ($r = 0.2001$, $p = 0.0001$), weight ($r = 0.1142$, $p = 0.0169$) and

WC (r = 0.1079, p = 0.0241). In men, global DNA methylation showed a negative correlation with high density lipoproteins (HDL) (r = -0.1848, p = 0.0391).

In a linear regression analysis adjusted for age, gender and smoking, diabetes was associated with global DNA methylation (β = 0.621, p = 0.036). When participants with diabetes were classified as either screen-detected diabetics or known diabetics, the association remained significant only for screen-detected diabetes (β = 1.069, p = 0.004) but not for known diabetes.

Discussion: Global DNA methylation was higher in individuals with hyperglycaemia, particularly screen-detected diabetes, compared to those with normoglycaemia. The observation of global DNA methylation changes in pre-diabetes, the asymptomatic stage of T2DM, and the identification of more pronounced changes in individuals with diabetes suggests that these epigenetic changes could be involved in the progression of T2DM, and holds potential as a biomarker for risk stratification of T2DM. Global DNA methylation was not different in known diabetes compared to individuals with normoglycaemia, suggesting that treatment reverses DNA methylation patterns induced by disease. Contrary to other studies, no association between global DNA methylation and gender or age was observed in this study.

Conclusion: In conclusion, this study adds to the growing body of evidence that posits that global DNA methylation is a potential risk factor of T2DM. To our knowledge, this is the first study to investigate global DNA methylation as a risk factor in South Africa, and paves the way for future studies in Africa.

Keywords

DNA methylation, early detection, epigenetics, pre-diabetes, risk factor, type 2 diabetes.

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List of Abbreviations

ADA	American Diabetes Association
BMI	Body mass index
CpG	Cytosine-Guanine dinucleotide
CRP	C-reactive protein
CTGF	Connective tissue growth factor
DBP	Diastolic blood pressure
DCCT	Diabetes Control and Complications Trial
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferases
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic reticulum
FPG	Fasting plasma glucose
FBI	Fasting blood insulin
FTO	Fat mass and obesity associated
GDM	Gestational diabetes mellitus
GGT	Gamma glutamyl-transferase
HbA1c	Glycosylated haemoglobin, type A1C
HDL	High density lipoproteins
HiF3A	Hypoxia inducible factor 3, alpha subunit
HNSCC	Head and neck squamous cell carcinoma
HP	Average hip circumference
HPLC	High performance liquid chromatography
IDDM	Insulin-dependent diabetes mellitus
IDF	International Diabetes Federation
IEC	International Expert Committee
IFG	Impaired fasting glucose
IGFBP-1	Insulin-like growth factor binding protein-1
IGT	Impaired glucose tolerance
IRS1	Insulin receptor substrate 1
LADA	Latent autoimmune diabetes of adults
LDL	Low density lipoproteins
LINE-1	Long interspersed nucleotide element-1
LUMA	Luminometric methylation assay
MBDs	Methyl binding domains
MeDIP	Methylated DNA immunoprecipitation
MODY	Maturity Onset Diabetes of the Young

NDDG	National Diabetes Data Group
NGSP	National Glycohemoglobin Standardization Program
OGTT	Oral glucose tolerance test
PBLs	Peripheral blood leukocytes
POC	Point of care
PRKCZ	Protein kinase C epsilon zeta
2h-PG	2-hour plasma glucose
2h-SI	2-hour Serum Insulin
PPAR-γ	Peroxisome proliferator-activated receptor gamma
RISC	RNA Induced Silencing Complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SADHSR	South African Demographic and Health Survey Report
SAM	S-adenosyl-L-methionine
SBP	Systolic blood pressure
SDS	Sodium dodecyl sulfate
SIN3A	SIN3 transcription regulator family member A
SEMDSA	Society for Endocrinology, Metabolism and Diabetes of South Africa
SLE	Systemic lupus erythematosus
SSc	Systemic sclerosis
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCF7L2	Transcription factor 7-like 2
TET	Ten-eleven translocation
TLC	Thin layer chromatography
Trigs	Triglycerides
WC	Waist circumference
WHR	Waist hip ratio

Definitions

- **Biomarker**: a naturally occurring molecule, gene or characteristic by which a particular pathological or physiological process or disease can be identified.
- **CpG islands**: regions on the genome which are rich in cytosine and guanine content (greater than 55 %) and about 500 base pairs long, usually found in the promoter regions of genes.
- **DNA methylation**: the covalent modification when methyl groups are added to the hydrogen on position 5 of cytosine nucleotides, primarily in CpG islands, in the promoter regions of genes.
- **Epigenetics**: heritable changes in gene function which do not involve nucleotide sequence changes.
- **Epigenome**: the overall epigenetic state of an organism.
- **Genome**: the overall gene collection of an organism.
- **Pre-diabetes**: a condition whereby a person's blood glucose levels are higher than normal but not high enough to be classified as diabetic. The condition includes IGT and IFG.
- **Risk factor**: A factor that is associated with the development of disease.

CHAPTER 1: LITERATURE REVIEW

1.1 DIABETES MELLITUS

Diabetes mellitus (DM), a group of metabolic diseases characterised by high blood glucose levels (hyperglycaemia), is a major cause of morbidity and mortality worldwide. It was estimated that the global prevalence of DM in 2014 was 9 % (WHO, 2014a), and 1.5 million deaths were directly related to DM in 2012 (WHO, 2014b). The three most common forms are type 1 diabetes (T1DM), type 2 diabetes (T2DM) and gestational diabetes (GDM), accounting for approximately 5 %, 90 % and 3 % of all DM cases, respectively (ADA, 2010; ADA, 2014).

1.1.1 Type 1 diabetes (T1DM)

Type 1 diabetes, also referred to as insulin dependent diabetes mellitus (IDDM) or juvenile diabetes because it is usually diagnosed in young people, is an autoimmune disorder characterised by hyperglycaemia due to the inability of the pancreas to secrete insulin (ADA, 2014). Insulin producing beta (β) cells of the islets of Langerhans in the pancreas are destroyed by a T-cell-mediated autoimmune attack resulting in insulin deficiency and hyperglycaemia. Exogenous administration of insulin, usually by an injection, and more recently via an insulin pump, a device which mimics the action of the pancreas by supplying insulin to an individual, is required to maintain normoglycaemia (Afrand et al., 2012). Recently, an insulin patch has been developed for the treatment of T1DM. The insulin patch is an 'artificial pancreas' modelled device, which is fully automated, closed-loop and has the ability to monitor insulin levels without the patient's input (Anhalt & Bohannon, 2010).

Although the majority of T1DM cases occur due to autoimmunity, in some cases, the disease arises spontaneously with no definite cause (Seino et al., 2010). In certain cases, T1DM presents in adults, and is referred to as latent autoimmune diabetes of adults (LADA). Latent autoimmune diabetes of adults is often misdiagnosed as T2DM because physicians base their diagnosis on patient age rather than on aetiology (Afrand et al., 2012).

1.1.2 Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is first diagnosed during pregnancy (Seino et al., 2010). The mechanisms underlying GDM are not yet fully elucidated, although progressive insulin resistance during pregnancy has been implicated. About 20 - 50 % of women with GDM develop T2DM later in life (Afrand et al., 2012).

Although transient in nature, GDM can adversely affect the health of the foetus and mother. Risks to the baby include macrosomia (high birth weight), congenital cardiac and nervous system abnormalities, skeletal muscle malformations and increased foetal insulin levels, which inhibits foetal surfactant production, possibly resulting in respiratory distress syndrome. In severe cases, perinatal death may occur, most commonly as a result of poor placental perfusion due to vascular system impairment (Afrand et al., 2012).

1.1.3 Other types of diabetes

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

1.1.4 Type 2 diabetes

1.1.4.1 Epidemiology in South Africa

As discussed previously, T2DM is the major contributor to the DM epidemic accounting for more than 90 % of DM cases globally (ADA, 2014). Recently, Gwebu reported that about 3.5 million South Africans (6 % of the population) have T2DM, while about 5 million South Africans have pre-diabetes, a condition whereby the blood glucose levels are higher than normal, but not yet high enough to be classified as T2DM (Gwebu, 2013). Pre-diabetes precedes T2DM with about 5 - 10 % people with pre-diabetes usually progressing to DM annually (Forouhi et al., 2007; Nathan et al., 2007). A prevalence survey conducted between 2008 - 2009 in the Bellville South community, in the Western Cape province of South Africa reported an age-adjusted T2DM prevalence of 26.3 % in individuals of mixed ethnic ancestry in this setting (Erasmus et al., 2012). A study conducted in the Cape Town suburbs of Langa, Gugulethu, Crossroads, Nyanga and Khayelitsha between 2008 and 2009 reported a prevalence of 13.1 % in black South Africans which is almost half of that reported for individuals of mixed ethnic ancestry in the Bellville South setting during the same period (Peer et al., 2012; Erasmus et al., 2012).

1.1.4.2 Aetiology

The aetiology of T2DM involves a complex interplay between environmental and genetic factors (Tripathy & Chavez, 2010). Studies have reported that only about 5 - 10 % of T2DM

cases can be explained by genetics (Voight et al., 2010), thus environmental factors have received increased attention as major mediators of T2DM progression (Hirst & Marra, 2009; Barrès et al., 2009; Drong et al., 2012; Keating & El-Osta, 2013). In most cases a diabetogenic lifestyle and a susceptible genotype is required for disease development. A diabetogenic lifestyle includes excessive caloric intake, inadequate caloric expenditure (such as physical inactivity) and obesity (Nolan et al., 2011).

1.1.4.3 Pathophysiology

The hormone insulin is the primary mediator of glucose homeostasis (Leahy, 2005). After the ingestion of food, β cells in the pancreas secrete insulin to increase glucose uptake in insulin-responsive tissues such as skeletal muscle and adipocytes, and to decrease glucose production from the liver so as to restore normoglycemia. However, insulin resistance, defined as the condition where insulin responsive tissues fail to increase glucose uptake in response to physiological concentrations of insulin (Yarbrough et al., 1998; Reaven, 2005) leads to persistent hyperglycaemia. Insulin resistance is characterized by high levels of circulating insulin, due to increased insulin secretion by β cells as a compensatory mechanism to counteract insulin resistance. A number of factors including insulin resistance, as described above, genetic predisposition, glucotoxicity, lipotoxicity, increased reactive oxygen species (ROS), endoplasmic reticulum (ER) stress and elevated intracellular calcium (Poitout & Robertson, 2008) contribute to β cell dysfunction, which is a prerequisite for the development of T2DM (Tripathy & Chavez, 2010; Cerf, 2013).

Although the exact mechanisms underlying the development of T2DM is not yet completely understood, insulin resistance in peripheral tissue, and decreased insulin secretion due to pancreatic β cell dysfunction is accepted to play a major role (Tripathy & Chavez, 2010). Initially β cells compensate for insulin resistance by increasing insulin secretion, however, they subsequently become exhausted and dysfunctional. In severe cases of T2DM, β cells are unable to secrete insulin, requiring the administration of exogenous insulin. As depicted in Figure 1.1, T2DM pathophysiology is thought to commence about 10 years prior to clinical diagnosis, at which time insulin resistance is high and β cell function is already significantly impaired. Moreover, T2DM related microvascular and macrovascular complications are thought to occur about 5 to 10 years before diagnosis

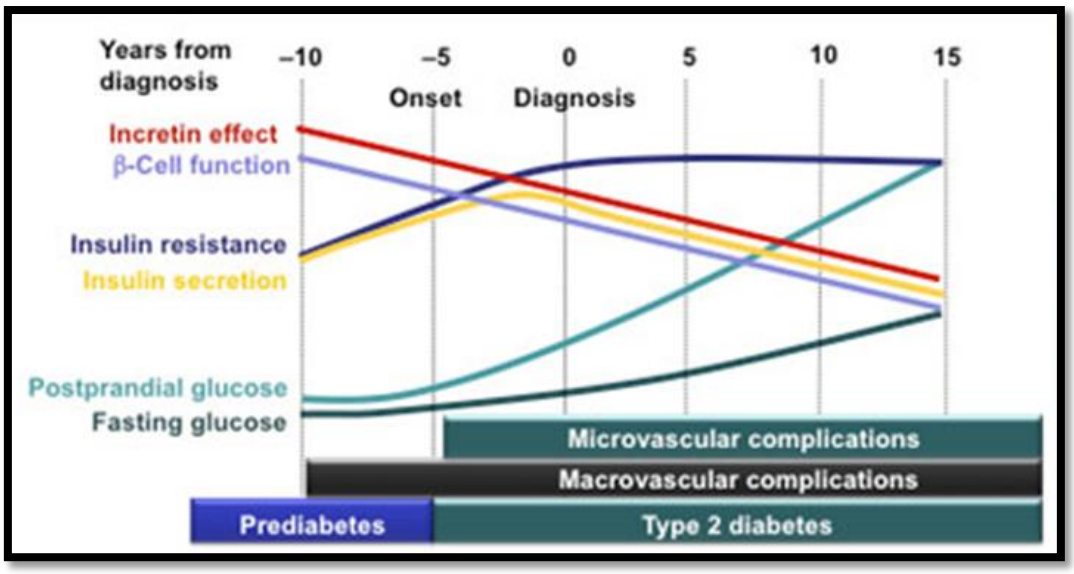

Figure 1.1 Diagrammatic representation of type 2 diabetes pathogenesis. (Adapted from (Holman, 1998; Nathan, 2002).

1.1.4.4 Diabetes complications

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

The major long term macrovascular complications associated with DM relate to blood vessel damage, with DM doubling the risk of cardiovascular disease. Cardiovascular disease is the most common cause of death and disability in diabetic patients (Aguiree et al., 2013). The main macrovascular diseases (related to atherosclerosis of larger arteries) in diabetes are ischemic heart disease (angina and myocardial infarction), stroke and peripheral vascular disease. Capillary damage may also occur, causing macro and micro-angiopathy (Boussageon et al., 2011). Macroangiopathy refers to an angiopathy (blood vessel disease) which affects large and medium size blood vessels. Microangiopathy refers to an angiopathy that affects small blood vessels in the body.

One of the major diabetes complications during T1DM and the late stages of T2DM is diabetic ketoacidosis (DKA). Diabetic ketoacidosis is defined as a state of high anion gap metabolic acidosis due to an excessive concentration of ketone bodies (keto-anions) in the bloodstream (Kitabchi et al., 2009). Due to the body's inability to utilize glucose as an energy source in these individuals, fat is metabolized as an alternative source of energy. Fat or lipid metabolism produces ketone bodies as by-products. Ketone bodies (acetoacetate, beta-hydroxybutyrate and acetone) are released into the bloodstream where they dissociate into ketone anions and hydrogen ions (H^+). In an attempt to maintain extracellular pH and ionic balance, H^+ ions are coupled with bicarbonate ions (HCO_3^-), which depletes the body's alkali reserves. The end result is acidosis and failure to regulate the ionic balance, which can result in fatal consequences such as a coma or even death (Fowler, 2009; Kitabchi et al., 2009).

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

1.1.4.5 Current methods of diagnosis

In 1979, the National Diabetes Data Group (NDDG) issued the first unified classification of diabetes (NDDG, 1979). This was followed by the World Health Organisation's (WHO) classification in 1980 (WHO, 1980). These two recommendations ushered a more standardized approach to the diagnosis of diabetes (Alberti & Zimmet, 1998). The WHO criterion was modified in 1985, and again in 1998 (Alberti & Zimmet, 1998; WHO, 1999). The latest recommendations worldwide are based on the 2006 WHO recommendations (WHO, 2006) modified in 2011 to include glycosylated haemoglobin type A1c (HbA1c) (WHO, 2011) and the American Diabetes Association (ADA) criteria of 2003 modified in 2009 to include HbA1c (ADA, 2004; ADA, 2010). These are summarised in **Table 1.1**.

Table 1.1: The WHO and ADA diagnostic criteria (Adapted from (WHO, 2006; ADA, 2010; WHO, 2011).

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

Footnotes:

- *Criteria adopted by a WHO expert committee, only if stringent quality assurance tests are in place and assays are standardized to criteria aligned to international reference values. Not suitable for point of care (POC) assays.
- [†]Conducted in a laboratory that is National Glycohemoglobin Standardization Program (NGSP) certified and standardized to the Diabetes Control and Complications Trial (DCCT) assay.
- [¥]Together with classic symptoms of hyperglycaemia or a hyperglycaemic crisis.
- FPG = fasting plasma glucose: a test which measures blood glucose concentrations after an overnight fast (about eight hours without food but may have water).
- 2h-PG = 2h-plasma glucose: a test which assesses glucose metabolism and clearance from the bloodstream, conducted by ingesting 75 g of glucose after an overnight fast of about eight hours and taking serial blood samples immediately before and two hours after the glucose drink.
- RPG = random plasma glucose: when blood glucose levels are tested without a period of fasting.

A random plasma (RPG) test is conducted when blood glucose levels are tested without a period of fasting. A value of more than 11.1 mmol/L, together with the classic symptoms of diabetes (polyuria, polyphagia, polydipsia, unexplained weight loss and fatigue) is considered to indicate diabetes, a diagnostic criteria common for both the WHO and ADA (WHO, 2006; ADA, 2010). A RPG value less than 11.1 mmol/L does not exclude diabetes and has to be followed up with another RPG or other diagnostic tests such as fasting plasma glucose (FPG), oral glucose tolerance test (OGTT) and HbA1c. The RPG obviates the requirement for fasting, thus is particularly useful for rapid diagnosis.

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

The oral glucose tolerance test (OGTT) is also known as the glucose tolerance test (GTT) or the 2-hour plasma glucose (2h-PG) test. An OGTT is conducted by ingesting 75 g of glucose after an overnight fast of about eight hours and taking serial blood samples immediately before and two hours after the glucose drink. The OGTT assesses glucose metabolism and clearance of glucose from the bloodstream (Seino et al., 2010). Limitations of the OGTT include poor reproducibility and the requirement for fasting (Ko et al., 1998; Patel & Macerollo, 2010). However, besides the limitations, the OGTT is considered as the gold standard of diabetes diagnosis (Ko et al., 1998).

The HbA1c test measures the percentage of glycosylated haemoglobin. This test assesses glycaemic control over a period of about three months, the lifespan of red blood cells (Franco, 2012). During the lifespan of a red blood cell, glucose molecules bind to haemoglobin molecules through a process called glycosylation. The process, though gradual, is believed to be directly proportional to the amount of glucose in the bloodstream (Makris & Spanou, 2011). Therefore the test reflects average glucose control over a period of about three months.

The HbA1c test has several advantages over FPG and OGTT, such as obviating the need for fasting; no fluctuations due to recent meals (HbA1c is a three month estimate) thus free of the 12 - 15 % day to day variance, and decreased susceptibility to measurement variation

due to conditions such as stress and illness (Saudek et al., 2008; ADA, 2014). However, limitations of HbA1c testing include greater cost, incomplete correlation between HbA1c and glucose concentrations in some individuals, limited availability and standardization in certain regions of the developing world, possible disparities due to race/ethnicity and interference by anaemia, as well as some medications (Saudek et al., 2008; IEC, 2009; Ziemer et al., 2010; Kumar et al., 2010; ADA, 2014). Conditions which shorten red blood cell survival, such as recovery from acute blood loss, pregnancy, blood transfusion and haemolytic anaemia will falsely decrease HbA1c results (Makris & Spanou, 2011; ADA, 2014). It is therefore apparent that, like other current methods of diagnosis, HbA1c testing has limitations.

The criteria recommended by WHO and ADA differ mainly on the lower limit of the FPG's normal levels (WHO: < 6.1 mmol/L and ADA: < 5.6 mmol/L) (WHO, 2006; ADA, 2010). In South Africa, the WHO criterion of 1998 is adopted and the recommendations by the Society for Endocrinology, Metabolism and Diabetes of South Africa (SEMDSA) also endorse the WHO criteria (WHO, 1999; Amod et al., 2012). However, the SEMDSA recommendations do not replace professional judgement, experience and appropriate referral where applicable (Amod et al., 2012).

Taken together, it is apparent that the current methods of diabetes diagnosis are not without limitations, creating the need for the identification of other methods of diagnosis. Epigenetic changes, in particular DNA methylation offers an option as a high risk marker for diabetes, and also as a promising diagnostic and prognostic marker of T2DM (How Kit et al., 2012; Levenson & Melnikov, 2012; Zou et al., 2013; Maghbooli et al., 2014).

1.2 EPIGENETICS

1.2.1 Definition of epigenetics

The prefix “Epi” in Greek refers to, “in addition to” or “on top of”, thus epigenetic traits exist on top of, or in addition to the traditional molecular genetic basis of inheritance. Epigenetics means “in addition to changes in genetic sequence” (Weinhold, 2006) and refers to heritable and non-heritable changes in cells and organisms which do not involve changes in the underlying DNA sequence (Bird, 2007; Christensen & Marsit, 2011).

1.2.2 Molecular mechanisms of epigenetics

Epigenetic mechanisms include DNA methylation, lysine methylation, histone methylation, histone phosphorylation, RNA interference (RNAi) and genomic imprinting (Tchurikov, 2005; Szyf, 2009a; Sharma et al., 2009). Lysine methylation refers to a process whereby enzymes called lysine methyltransferases catalyse the addition of one or more methyl groups from S-adenosyl-L-methionine to the ε-amino group of a lysine residue (Qian & Zhou, 2006). Histone methylation occurs when a methyl group is attached to the amino acids of histone proteins on nucleosomes (Szyf, 2009a).

Histone phosphorylation refers to the addition of a phosphate group to histone proteins and it is a key process which regulates chromatin structure (Rossetto et al., 2012). RNA interference (RNAi) is an epigenetic mechanism involved in gene expression control and its mechanisms include RNA induced silencing complex (RISC), small interfering RNA (siRNA), long non-coding RNAs and microRNAs (Tchurikov, 2005; Alshatwi & Shafi, 2012; Fatica & Bozzoni, 2014). Genomic imprinting is an epigenetic mechanism in which the expression of a gene is restricted to one of the parental alleles (Sharma et al., 2009). DNA methylation is the most studied epigenetic mechanism and because it is the focus of this research project, it will be discussed in more detail below.

1.3 DNA METHYLATION

DNA methylation refers to the covalent modification when a methyl group is added to the carbon at position 5 of cytosine nucleotides (H5), primarily in CpG islands in the promoter regions of genes. The process is catalysed by enzymes called DNA methyltransferases (DNMTs), with S-adenosyl-methionine (SAM-CH_3) acting as the methyl donor as shown in Figure 1.2 (Bird, 2002; Miranda & Jones, 2007). Generally, it is believed that DNA hypermethylation (increased DNA methylation) causes gene silencing whereas DNA hypomethylation (decreased DNA methylation) is associated with gene activation (Zou et al., 2013). Aberrant DNA methylation has been observed in cancers where CpG islands of

tumour suppressor genes undergo hypermethylation and CpG poor regions become hypomethylated (Zou et al., 2013).

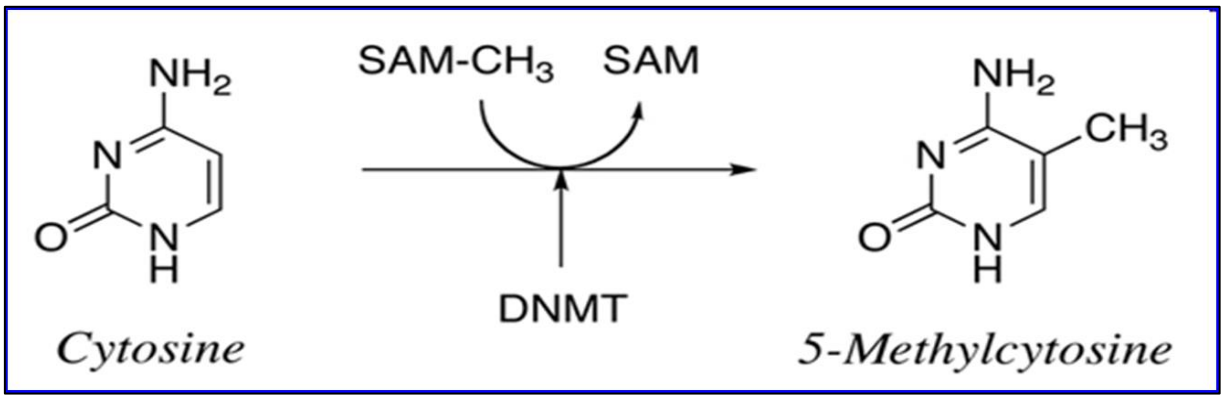

Figure 1.2 Diagrammatic illustration of the process of DNA methylation (Downloaded from: http://www-medchem.ch.cam.ac.uk/lab_rotations/murrell.php).

1.3.1 DNA methyltransferases

Three major groups of DNMTs have been identified in mammals: DNMT1, DNMT3a and DNMT3b (Kareta et al., 2006). DNMT1 is regarded as the maintenance DNA methyl transferase active during replication, while DNMT3a and DNMT3b are regarded as *de novo* DNA methyl transferases that function early during development and establish methylation states (Chen & Li, 2006). However, the distinctions between *de novo* and maintenance roles do not always apply. Both classes of enzymes can participate in both *de novo* and maintenance methylation with the eventual target being DNA methylation (Grandjean et al., 2007; Szyf, 2009a).

1.3.2 CpG Islands and CpG poor regions

DNA methylation occurs at CpG sites, which are regions of DNA where a cytosine nucleotide occurs next to a guanine nucleotide in the linear sequence of DNA. The p denotes the phosphodiester bond which links the cytosine and guanine nucleotides (Takai & Jones, 2002). In the context of DNA methylation, there are two distinct groups of sequences in the genome, namely CpG poor regions and CpG islands, which are regions with a large CpG content (greater than 55 %), a length of about five hundred base pairs (500 bp), and usually occurs in the promoter regions of genes (Jones & Baylin, 2002; Vinson & Chatterjee, 2012). The rest of the genome (exonic and intronic regions) are thought to be CpG poor regions (Takai & Jones, 2002). In healthy cells, CpG poor regions are usually methylated whereas CpG islands are generally unmethylated, with a few exceptions, such as the inactive X-chromosome (Miranda & Jones, 2007). Methylation of CpG islands is thought to be involved

in gene expression control (Jones & Baylin, 2002). Methylation or demethylation of CpG islands can affect health due to silencing or activating genes, resulting in phenotypic change (Gibney & Nolan, 2010). The process of CpG methylation can be transmitted from a parent to a daughter cell (Reik & Dean, 2001). DNA methylation patterning, first established during embroyonic development by the *de novo* DNA methyltransferases DNMT3a/b is maintained in subsequent cell divisions by the maintenance methyltransferase DNMT1 (Reik & Dean, 2001). In the human genome, approximately 3 % of all cytosines are methylated (Nafee et al., 2008) while roughly 70 - 90 % of cytosines in CpG islands are methylated (Jones & Baylin, 2002; Miranda & Jones, 2007).

1.3.3 Mechanisms of gene silencing

DNA methylation has been observed to be a highly effective mechanism of silencing gene expression, both in vertebrates and plants (Szyf, 2009a). DNA methylation alters protein binding to target sites on DNA, leading to transcriptional silencing on genes and interference with heterochromatin formation. The silenced states of the genes can be inherited throughout cellular divisions and eventually affect the phenotype, hence the development of disease (Miranda & Jones, 2007; Heyn & Esteller, 2012).

Several models have been proposed as to how the process occurs. One model suggests that DNA methylation directly blocks the binding of transcriptional factors to their target sites, thus impeding transcription. Other proposed mechanisms are based on the idea that methylation of the CpG sequences can attract methyl DNA binding domains (MBDs) proteins such as methyl CpG phosphate guanine binding protein 2 (MeCP2) (Szyf, 2009a). MeCP2 is associated with a closed chromatin configuration and gene silencing because it recruits proteins such as SIN3 transcription regulator family member A (SIN3A) and histone modifying enzymes which lead to a closed chromatin configuration (Szyf, 2009a). Gene silencing through chromatin structure interference usually occurs within the promoter regions of genes (Miranda & Jones, 2007).

1.3.4 DNA methylation and disease

The epigenome has more plasticity than the genome and epigenetic changes are considered to be important contributors to human disease (Hirst & Marra, 2009). Evidence suggests that epigenetic changes such as DNA methylation (both gene specific and global) are important factors involved in the initiation and progression of diabetes, and could give insight into disease pathogenesis (Hirst & Marra, 2009; Zou et al., 2013). Epigenetics has been implicated in the development of a number of diseases including cancer (Christoph et al., 2008), cardiovascular disease (Stenvinkel et al., 2007), hypertension (Smolarek et al., 2010),

autoimmune diseases and neurological diseases such as Parkinson's and Alzheimer's diseases (Gräff & Mansuy, 2008; Liu et al., 2011).

DNA methylation is an epigenetic mechanism that results due to interplay between environmental and genetic factors, and is therefore thought to reflect T2DM aetiology (Barrès et al., 2009; Heyn & Esteller, 2012; Drong et al., 2012). Kuroda *et al.* showed that CpG sites in both the mouse and human insulin promoters were demethylated in maturing insulin producing pancreatic β cells, suggesting that insulin promoter CpG demethylation plays an important role in β cell maturation, as well as in tissue-specific insulin gene expression (Kuroda et al., 2009).

1.3.5 DNA methylation and T2DM

DNA methylation is one of the pathogenic factors involved in T2DM, both in the initiation and progression of disease (Mair and Olek, 2002; Zou et al., 2013). Gene loci associated with T2DM account for only about 10% of its heritability, and modification of the epigenome in response to environmental factors has been identified as a key mechanism that underpins the development of metabolic diseases such as T2DM. Some of the genes involved in the pathogenesis of T2DM are shown in **Table 1.2**.

Table 1.2: Table 1.2: DNA methylation modified genes involved in T2DM

Gene	**Findings**
Peroxisome proliferator-activated receptor gamma (*PPAR-γ*)	Hypermethylation of the promoter region of the *PPAR-γ* gene inhibited gene expression in T2DM (Fujiki et al., 2009).
Peroxisome proliferator activated receptor gamma coactivator 1 alpha (*PPARGC1A*)	Increased methylation of the *PPARGC1A* gene promoter during T2DM.
Insulin	Insulin expression is regulated by methylation of the promoter region (Kuroda et al., 2009).
Glucose transporter protein 2 (*GLUT 2*)	Hypermethylation of the *GLUT 2* promoter suppresses gene expression, leading to decreased glucose uptake (Ban et al., 2002).

Protein kinase C epsilon zeta (*PRKCZ*)	The *PRKCZ* promoter in peripheral blood leukocytes was shown to be hypermethylated during T2DM (Zou et al., 2013).
Transcription factor A, mitochondrial (*TFAM*)	Methylation changes have also been seen in the *TFAM* gene promoter in peripheral white blood cells and this has been associated with insulin resistance in adolescents (Gemma et al., 2010).
Solute carrier family 30 member 8 (*SLC30A8*)	Hypermethylation of the *SLC30A8* gene was associated with T2DM in a Malay population (Seman et al., 2015).
Insulin-like growth factor binding protein-1 (*IGFBP-1*)	Hypermethylation of the IGFBP-1 gene was associated with T2DM. Both newly diagnosed and treated T2DM patients (with mean diabetes duration of 3 years) had 19.8 % and 20.2 % respectively as compared to 16.9 % for normal glucose tolerance subjects ($p < 0.001$ for both) (Gu et al., 2013).
Connective tissue growth factor (*CTGF*)	Hypomethylation of the *CTGF* gene was reported in patients with DM, particularly those with diabetic nephropathy, and was suggested to be involved in the pathogenesis of nephropathy (Zhang et al., 2014).
Hypoxia inducible factor 3 alpha gene (*HIF3A*)	Site-specific DNA hypermethylation at the *HIF3A* locus was suggested to be caused by increased BMI (Dick et al., 2014).
Transcription factor 7-like 2 (*TCF7L2*)	Methylation in the *TCF7L2* promoter was seen to be correlated with fasting glucose in peripheral blood DNA, which is suggestive of some role by epigenetic regulation (DNA methylation) of the gene in T2DM. The *TCF7L2* gene has been identified as the one with the strongest effect on T2DM susceptibility (Canivell et al., 2014).

1.3.5 Gene specific DNA methylation

Gene-specific DNA methylation refers to the analysis of the methylation status of specific genes. Gene-specific DNA methylation is increasingly reported in various diseases such as bladder, kidney and testicular cancer; systemic lupus erythematosus (SLE) and systemic sclerosis (SSc) (Christoph et al., 2008; Matatiele et al., 2015). In T2DM, a number of genes have been reported to be differentially methylated during disease progression, including the

peroxisome proliferator-activated receptor gamma (PPAR-γ) (Fujiki et al., 2009), insulin (Kuroda et al., 2009), glucose transporter protein 2 (GLUT 2) (Ban et al., 2002), insulin-like growth factor binding protein-1 (IGFBP-1) (Gu et al., 2013), the connective tissue growth factor (CTGF) and the transcription factor 7-like 2 (TCF7L2) genes (Canivell et al., 2014).

Bisulfite conversion and pyrosequencing is considered the 'gold standard' to identify DNA methylation at single nucleotide resolution (Patterson et al., 2011). During this process, DNA is treated with sodium bisulfite which converts free cytosines to uracil, while methylated cytosines are unaffected. Thereafter, pyrosequencing is conducted to identify methylated and unmethylated cytosines (Patterson et al., 2011; Li & Tollefsbol, 2011).

Other methods used to measure gene-specific DNA methylation include high performance liquid chromatography (HPLC), DNA array methods such as the Infinium Human Methylation450 BeadChip, PCR based methods such as the luminometric methylation assay (LUMA) (Karimi et al., 2006), methylation sensitive PCR (MSP) and methylated DNA immunoprecipitation (MeDIP) sequencing. Although MSP is rapid, sensitive, and can be used for DNA samples of limited quantity and quality (Herman et al., 1996), it is not quantitative. MethyLight is one of the variations of MSP modified using real-time PCR for quantitative methylation detection (Eads et al., 2000; Zeschnigk et al., 2004).

The Infinium Human Methylation450 BeadChip (Infinium Methylation 450K; Illumina, Inc. CA, USA), a DNA methylation array developed from the previous Infinium Human Methylation27 Beadchip (Illumina, Inc. CA, USA) has been used for genome-wide DNA methylation analysis by many studies (Dedeurwaerder et al., 2011; Touleimat & Tost, 2012; Dayeh et al., 2014; Florath et al., 2016). This method has the capability to detect up to 480 000 cytosine sites and up to 14 495 genes in the human genome (Dedeurwaerder et al., 2011). Lower cost and easier experimental procedures, compared to sequencing and PCR based methods (Wu et al., 2013), makes this method amenable to high throughput screening. The Infinium Human Methylation450 BeadChip method, however, may introduce bias to a study if not properly applied and interpreted. This is due to its technique of utilising two different assay chemistries (Infinium I and Infinium II) on the same array, causing a signal shift in the methylation profiles of the same sample. The bias may also be compounded by signal bias due to 'noise detection', a problem in all microarray data samples (Touleimat & Tost, 2012).

Although gene-specific DNA methylation analysis is powerful enough to provide information at a single base pair resolution, its initial stage of bisulfite conversion fails to differentiate between 5-methylcytosine (5mC) and 5-hydroxy-methylcytosine (5hmC). This is because the 5hmC sites are not converted to uracil by bisulfite treatment (Huang et al., 2010). The 5hmC sites are converted from 5mC by enzymes called ten-eleven translocation (TET), a family of

5mC-hydroxylases which add a hydroxyl group to the methyl on cytosine to give 5hmC (Xu et al., 2011). Both 5mC and 5hmC have been reported to affect DNA expression (Guo et al., 2011; Xu et al., 2011).

None of the methods for the measurement of DNA methylation is suitable for every application, and investigators should choose the best method for their specific research needs and to address their research question. Factors such as the potential for bias, cost, throughput, specificity and reproducibility should be considered (Shen & Waterland, 2007).

1.3.6 Global DNA methylation

Global DNA methylation refers to the average methylation status that occurs across the genome (Zhao et al., 2012). Various studies have reported aberrant global DNA methylation patterns during diseases such as hypertension (Smolarek et al., 2010), systemic lupus erythematosus (SLE) (Liu et al., 2011) and cancers such as the head and neck squamous cell carcinoma (HNSCC) (Hsiung et al., 2007), and breast cancer (McCullough et al., 2015). In T2DM, many studies have reported the effect of global DNA methylation on the disease (Kim et al., 2010a; Zhao et al., 2012; Luttmer et al., 2012; Maghbooli et al., 2014).

Global DNA methylation quantifies the average methylation across the genome, without identifying the CpG sites where they occur. Transposable genetic elements such as long interspersed nucleotide element-1 (LINE-1) and Alu repeat elements constitute approximately 50 % of the whole genome (Treangen & Salzberg, 2013) and their methylation status is often used to estimate global DNA methylation. Alu elements, for example, are the most abundant of the family of repetitive sequences with more than one million copies per haploid genome and representing more than about 10 % of the whole human genome. Therefore, Alu elements have been used as surrogate markers for estimating global DNA methylation levels due to their high copy numbers on the genome (Yang et al., 2004). Techniques used to measure global DNA methylation include enzyme linked immunosorbent assays (ELISAs), bisulfite sequencing (Zhao et al., 2012), thin layer chromatography (TLC) (Smolarek et al., 2010) and PCR-pyrosequencing (Bollati et al., 2009). Of these, ELISA has received increased interest since it is suitable for routine analysis. It does not require sophisticated instruments and is cost-effective compared to the other methods mentioned previously.

1.3.8 Factors affecting DNA methylation

DNA methylation has been reported to be affected by many factors including heavy metals, pesticides, tobacco smoke, polycyclic aromatic hydrocarbons, hormones, radioactive substances, viruses, bacteria (Weinhold, 2006), basic nutrients, the social environment and maternal care (Delage & Dashwood, 2009; Szyf et al., 2007; Szyf, 2009b), age, gender and disease state (Heyn & Esteller, 2012). Some of these factors are listed in **Table 1.3**. The conflicting results of the studies could be attributed to different study populations and methods. For example for the association between global DNA methylation and age, Fuke et al (2004) used health individual individuals and high performance liquid chromatography (HPLC), whereas Moore et al. (2008) used bladder cancer patients and high performance capillary electrophoresis (HPCE).

Table 1.3: Factors associated with DNA methylation

Factor	Findings
Age	DNA methylation decreased with age (Fuke et al., 2004), while another reported an increase with age (Moore et al., 2008).
Diet	Methyl groups used in DNA methylation are derived from methionine, serine, folate, biotin and choline from the diet. Foods containing these components can avail methyl groups to DNA and histones through S-adenylmethionine (SAM) and influence the expression of many genes (Zeisel, 2009; Mckay & Mathers, 2011; Park et al., 2012; Milagro et al., 2013).
Gender	Various studies have shown that DNA methylation is increased in males compared to females (Fuke et al., 2004; Hsiung et al., 2007; El-Maarri et al., 2007; Wilhelm et al., 2010; El-Maarri et al., 2011; Zhang et al., 2011; Zhu et al., 2012). Decreased DNA methylation in females is thought to be due to decreased folate, the key supplier of methyl groups which is also involved in erythrocyte formation and development. Since menstruation regularly depletes the supply of erythrocytes, females tend to have lower levels of circulating folate (Terry et al., 2011). On the other hand, other studies have reported an increase in methylation status in females than males (Moore et al., 2008; Kim et al., 2010) while another study reported no difference between the two (Maghbooli et al., 2014).
Race and Ethnicity	Few studies have explored the association between race/ethnicity and DNA methylation. However, Terry et al reported more DNA methylation in the white blood cells of Hispanic Americans compared to white Americans, with the lowest level of DNA methylation occurring in black Americans (Terry et al., 2008).
Chemicals	An association between benzene and decreased DNA methylation was observed (Bollati et al., 2007; Fustinoni et al., 2012).
Smoking	DNA methylation was reported to be associated with smoking (Breitling et al., 2011; Zeilinger et al., 2013; Tsaprouni et al., 2014; Dogan et al., 2014; Zaghlool et al., 2015). Hillemacher *et al.* reported that there was association between smoking and global DNA methylation but no association in gene specific methylation (Hillemacher et al., 2008). However, a number of other studies saw no association between smoking and DNA methylation (Moore et al., 2008; Choi et al., 2009).
Alcohol	Alcoholism is associated with 8 - 10 % increased DNA methylation (Bönsch et al., 2004; Bleich et al., 2006; Philibert et al., 2012).
Body mass index (BMI)	An association was reported between BMI and DNA hypermethylation in the hypoxia inducible factor 3, alpha subunit (*HiF3A*) gene in blood cells and adipose tissue (Dick et al., 2014). BMI and waist circumference were positively associated with DNA methylation status at the adiponectin (ADIPOQ) gene locus in subcutaneous tissues (Houde et al., 2015). A negative association was also reported between BMI and DNA methylation of the leptin (*LEP*) gene in blood cells (Houde et al., 2015). Rönn et al., reported that many genes including the fat mass and obesity associated (*FTO*) gene, insulin receptor substrate 1 (*IRS1*) and *HiF3A*; had their DNA methylation status and expression correlated with BMI (Rönn et al., 2015). However, Zhu *et al.* reported no correlation between BMI and DNA methylation (Zhu et al., 2012).
Physical activity	Physical activity has been reported to be associated with DNA methylation (Zhang et al., 2011; Rönn et al., 2013; Xi et al., 2014).
Infection	Various infections have been reported to be associated with aberrant DNA methylation. Examples include virus induced-hepatitis (Kiran et al., 2009), gastritis induced by *Helicobacter pylori* (Nakajima et al., 2009), and cervical dysplasia caused by human papilloma infection (Flatley et al., 2009).

1.4 MOTIVATION FOR THIS STUDY

1.4.1 Problem Statement

Type 2 diabetes mellitus, a complex, multifactorial metabolic disorder that is caused by genetic and environmental factors, is a major source of morbidity and mortality worldwide. Intensive research is aimed at improved diagnosis and management of the disease. The mixed ancestry population of South Africa, commonly referred to as 'coloureds' has a high prevalence of diabetes (Erasmus et al., 2012). Furthermore, this population has also been reported to have high rates of obesity and metabolic syndrome, and a high risk of developing CVD (Matsha et al., 2012). Follow-up studies have shown that the accelerated deterioration of glucose tolerance status over time is not explained by the known determinants of diabetes occurrence (Matsha et al., 2013). Emerging data from around the world support the pivotal role of epigenetics, in particular DNA methylation (Barrès et al., 2009; Heyn & Esteller, 2012; Drong et al., 2012) in the development of T2DM, and suggests that these epigenetic changes could be used as risk factors in this population.

1.4.2 Rationale

The identification of individuals who are at risk of developing T2DM could facilitate intervention strategies to delay or prevent their progression to disease, thus minimising the burden of T2DM. Interventions to protect against insulin resistance and β cell dysfunction by lifestyle modifications or therapeutic drugs may reverse the disease phenotype and prevent the progression to T2DM. Identifying individuals with an increased risk of developing T2DM, before the clinical onset of these multiple malfunctions and complications is a major priority in diabetes research.

1.4.3 Hypothesis

We hypothesize that during T2DM progression, there is observable differential global DNA methylation, before the clinical onset of the disease.

1.4.4 Aim

The aim of this study was to probe the association between global DNA methylation and T2DM.

1.4.4.1 Objective

To investigate the variation in global DNA methylation profiles for normal, pre-diabetic and T2DM individuals of mixed ancestry descent.

CHAPTER 2: RESEARCH DESIGN AND METHODOLOGY

2.1 RESEARCH DESIGN

A cross sectional, quantitative methodology was employed to investigate the association between global DNA methylation and T2DM in the Bellville South community, Cape Town, South Africa. The dependent variable, global DNA methylation status, was compared to the independent variable, disease status (diabetic, pre-diabetic and normoglycaemic). Comparisons between these variables were extrapolated to other factors such as gender, age, anthropometric and biochemical measurements, and smoking. This study forms a sub-section of a larger study investigating risk factors for T2DM in which random sampling and voluntary participation were accommodated (Zemlin et al., 2011; Matsha et al., 2012). DNA extraction, measurement of global DNA methylation, and statistical analyses was conducted in this study.

A random sampling approach was used as follows: A list of streets from the Bellville South community were classified as short, medium and long, based on the number of houses in the streets. Streets having 22 or less houses were classified as short; streets having 23 - 40 houses were classified as medium, while those with more than 40 houses were classified as long streets (Erasmus et al., 2012). A total of 16 short streets (approximately 190 houses), 15 medium streets (approximately 410 houses) and 12 long streets (approximately 400 houses), were randomly selected across the different strata. All household members from the selected streets who met the selection criteria were eligible to participate. In addition, subjects who did not form part of the random sampling strategy but who volunteered to participate in the study were allowed to participate on the request of community authorities. Voluntary subjects were also assigned a different study code number (Erasmus et al., 2012).

2.2 STUDY SETTING

Bellville South is located within the northern suburbs of Cape Town, Western Cape, South Africa. The township was formed in the late 1950s and is mainly inhabited by coloureds (88 %), a term used to define individuals of mixed ethnic origin in South Africa. According to 2011 census data, it is estimated that 24,642 individuals, representing 6,045 households with an average family size of 4.08 are resident in Bellville South. Gender distribution is approximately equal, with about 54 % females and 46 % males (City of Cape Town Census, 2013).

Afrikaans is the predominant language, although other languages such as English and Xhosa are also spoken. Most of the residents have lived in the community for over five years

while others have been there for their entire lives. According to a study by Erasmus *et al.,* the crude prevalence of T2DM was 28.2 % (age-adjusted 26.3, 95 % confidence interval (CI) 22.0 - 30.3) (Erasmus et al., 2012). A 4.4 % prevalence (age-adjusted 3.2 %, 95 % CI 1.6 - 4.9) was reported for IFG and a 15.3 % prevalence (age-adjusted 15.0 %; 95 % CI 11.4 - 18.6) for IGT. The prevalence of undiagnosed T2DM was 18.1 % (age-adjusted 16.8 %, 95 % CI 13.3 - 20.4) (Erasmus et al., 2012). The prevalence of T2DM in this community is higher than the prevalence in a different coloured community where a crude prevalence of 7.1 % for T2DM and 8.0 % for IGT was reported. The age-adjusted prevalence was 10.8 % for T2DM (95 % CI 8.2 - 13.5) and 10.2 % for IGT (95 % CI 7.7 - 12.8 %) (Levitt et al., 1999). Therefore, this community presents an ideal study setting to investigate the association between global DNA methylation and T2DM.

2.3 STUDY POPULATION

The enrolment of participants for this study was conducted from January 2008 to March 2009. A total of 956 subjects were enrolled; 642 subjects (age range 35 - 65 years) were enrolled as part of the random sampling strategy, while 304 subjects (age range 16 - 95 years) volunteered to participate in the study. The inclusion criteria were: all coloured participants who enrolled in the study on a voluntary basis and gave informed consent. The exclusion criteria were: subjects less than 16 or older than 95 years of age, pregnant women, those who did not volunteer to participate in the study, and acutely ill participants. Of the 956 subjects initially enrolled in the study, 10 participants were excluded because they were not coloured and only 946 subjects were finally enrolled in the study. Of these, only 615 participants were selected for this study based on the availability of blood samples for DNA extraction.

Ethical approval for the larger study (CPUT/HW-REC 2008/002 and CPUT/HW-REC 2010) and for this sub-study (REC-230408-014-RA Level 01H07) was granted by the Cape Peninsula University of Technology Faculty of Health and Wellness Sciences Research Ethics Committee. All participants signed informed written consent for genetic testing, and were informed about their rights, and their involvement in the research. Furthermore, all study procedures were explained to participants in their language of choice. Participants were also informed of their right to freely withdraw from the study whenever they wanted to. Information about the participants and aspects of the study were kept confidential. The study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.4 DATA COLLECTION

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

2.4.1 Clinical data

2.4.1.1 Questionnaire

The questionnaire requested information about the participant's demographics such as gender, age, previous medical history of diabetes, dietary, as well as lifestyle aspects. The four components of the questionnaire were: i) personal demographics, ii) family health history, iii) diet and iv) cigarette smoking. The questionnaire was adopted from other standard and recognized sources such as the CAGE Questionnaire (Ewing, 1984), the South African Demographic and Health Survey Report by the South African Medical Research Council (SADHSR) (Bradshaw et al., 1995; Goedecke et al., 2005). The SADHSR questionnaire focuses on chronic and lifestyle diseases by addressing the diabetic status, nutritional status, hypertension and physical activity of the participants. The CAGE questionnaire is mainly used in alcohol related studies.

The questionnaire used for this study was also adopted from a previous study to measure the prevalence of DM amongst learners attending schools in the Belhar, Delft and Mfuleni suburbs in the Cape Town metropole (Somers et al., 2006). The family health history section of the questionnaire covered the presence or previous treatment of family members, up to third degree relatives, for related inheritable diseases. The section of the questionnaire on cigarette smoking focused on: a) the particular habit, b) duration of the habit, c) quantity or amounts consumed, and d) when the habit was stopped. A detailed medication history was obtained by asking participants, as well as examining their clinic cards, and recording the drugs which they brought to the study site.

2.4.1.2 Clinical measurements

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

2.4.1.2.1 Height

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

2.4.1.2.2 Weight

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

2.4.1.2.3 Waist circumference

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

2.4.1.2.4 Hip circumference

The hip circumference (HP) was measured at the maximal circumference over the buttocks. A non-elastic tape was also used for this measurement. The investigator would squat before the subject with the tape placed around the buttocks on the widest area over the horizontal

plane without pressing tightly against the skin, and the measurement taken. When the hip circumference exceeded the tape size, the measurement was not recorded, but noted (Tolonen et al., 2002).

2.4.1.2.5 Blood pressure measurements

Measurements used to assess the blood pressure were systolic blood pressure and diastolic blood pressure. Systolic blood pressure (SBP) refers to the highest arterial pressure as a result of the exertion of the blood upon the walls of the blood vessels (arteries) immediately after the pumping action of the left ventricle of the heart. Diastolic blood pressure, in contrast, refers to the lowest arterial blood pressure when the heart muscles contract after a systolic event (Pickering et al., 2005). Blood pressure is expressed as SBP over DBP, with the ratio (as a fraction) taken as the blood pressure and the units are millimeters of mercury (mm Hg) (Pickering et al., 2005).

Blood pressure measurements were done according to WHO guidelines (WHO, 1999). Blood pressure measurements were taken using a calibrated baumanometer (Rossamax; Berneck, Switzerland) with the participant in a relaxed sitting position. Participants were allowed to sit with their back supported on the chair backrest, while their arms were exposed and rested on the table at the same level as their heart. The correct adult cuff size was placed 2 cm above the elbow joint to ensure accurate readings (Pickering et al., 2005). Three readings, at one minute intervals, were taken and the lowest reading was chosen as the participant's blood pressure. During the time of taking the measurement, the participants were not allowed to speak as this has been found to affect the readings (Adams et al., 2002; Panchón et al., 2004).

2.4.1.2.6 Blood collection

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

Metropolis Private Pathology Laboratory (Century City, Cape Town). The purple tube for DNA extraction was stored at -20 ^{0}C.

2.4.1.2.7 The oral glucose tolerance test (OGTT)

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

2.4.2 Biochemical data

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

2.4.2.1 Diabetes status classification

Participants were classified according to the revised WHO criteria of 1999 as shown in Table 1.2 (WHO, 1999). Participants were categorized as diabetes, pre-diabetes (IGT and/or IFG) or normoglycemic according to their history of diabetes, as well as their fasting, and postprandial glucose concentrations.

2.4.3 Quality control procedures

The study was conducted adhering to strict standard operating procedures (SOPs). Field workers and nurses involved in the study were briefed on how to take blood pressure, blood collection, anthropometric measurements and questionnaire administration. Researchers were required to sign off on questionnaires, blood pressure and collection, as well as anthropometric measurements that they conducted in order to trace and verify measurements and data entries. Spot field checks were also conducted by project supervisors in order to verify the accuracy of measurements and enhance quality control (Adams et al., 2002).

Equipment was routinely calibrated according to the manufacturer's instructions. Scales were routinely checked for accuracy using standard weights. The work-load of all staff involved in the study was kept within acceptable limits in order to avoid false measurements due to staff fatigue. Each team had a supervisor who was responsible for monitoring performance and who was also responsible for equipment calibration. Weekly meetings were held to assess progress, problem-solving, and retraining if necessary.

2.5 DNA EXTRACTION

Genomic DNA was extracted from whole blood collected in EDTA blood tubes and stored at -20 °C. DNA was extracted from 1 - 2 ml of blood using the salt extraction method (www.genomics.liv.ac.uk, 2001). Briefly, blood cells were lysed by adding a five times volume (~5 - 10 ml) of lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA; pH 7.4) to the blood. Thereafter, the tubes were vigorously vortexed for about 10 - 15 seconds and then placed on ice for 5 minutes. This step was repeated three times. The tubes were then centrifuged at 1,500 rpm for 10 minutes using a Beckman General Purpose centrifuge (Beckman Coulter Inc., CA, USA). The pellets obtained were washed with 10 ml of phosphate buffered saline (PBS; 2.68 mM KCl, 136 mM NaCl, 1.47 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.4) and centrifuged at 1,500 rpm for 10 minutes, twice. Thereafter 3 ml of nucleic lysis buffer (10 mM Tris, 400 mM NaCl, 2 mM EDTA; pH 8.2) and 300 µl of 10 % (w/v) sodium dodecyl sulfate (SDS) was added and the pellet was dissolved by vortexing. Thirty microliters of 10 mg/ml proteinase K was added, mixed well, and the tubes were incubated at 55 °C overnight to digest proteins.

The following day, 1 ml of a saturated solution of 6 M NaCl was added to the tube, vortexed, and centrifuged at 2,500 rpm for 10 minutes. The supernatant was carefully transferred to a clean tube, taking care not to transfer the white protein precipitate. The supernatant was re-centrifuged and the DNA was precipitated by adding two volumes (~ 9 ml) of 100 % ethanol.

DNA is insoluble in ethanol and will thus form a string like precipitate. The tubes were centrifuged at 8,000 rpm for 30 minutes and the supernatant was discarded.

The DNA pellet was washed with 1 ml of 70 % (v/v) ethanol; whereafter the DNA-ethanol mixture was transferred to a 1.5 ml eppendorf tube. To ensure that DNA was not lost during this step, the tube was gently tapped at the bottom to ensure that the DNA pellet was dissolved. The transfer of the DNA pellet to the eppendorf tube was confirmed by visual inspection. Eppendorf tubes were centrifuged in a microcentrifuge (Beckman Coulter Inc., CA, USA) at 13,800 rpm for 30 minutes. The supernatant was discarded, and the DNA pellet was dried by inverting the tube on a paper towel and thereafter air dried for 15 minutes. The DNA pellet was dissolved in 100 - 200 µl of nuclease free water, depending on the size of the pellet. Tubes were placed on a mixer (Elmi Intelli-Mixer RM-2, Elmi Ltd, Riga, Latvia) overnight to aid suspension of the pellet.

The concentration (A_{260}) and purity (A_{260}/A_{280} and A_{260}/A_{230}) of DNA was measured by nanodrop spectrophotometry (Nanodrop Technologies, Wilmington, DE, USA). An $^{A260}/_{A280}$ ratio between 1.8 and 2.0 was indicative of pure DNA. A lower ratio is suggestive of the presence of protein, phenol or other contaminants which absorb strongly at or near 280 nm and 230 nm, respectively. The optical density (OD) ratio $^{A260}/_{A230}$ was used as a secondary measure of nucleic acid purity and a range of 2.0 - 2.2 is commonly accepted as pure.

2.6 GLOBAL DNA METHYLATION ANALYSIS

Global DNA methylation was measured with the 5mC DNA ELISA kit according to the manufacturer's instructions (Zymo Research Coorp., Irvine, CA, USA). The 5mC DNA ELISA kit directly quantitates DNA methylation using a sandwich ELISA where DNA binds to an anti-methylcytosine antibody, and is detected using a horseradish peroxidise (HRP)-conjugated secondary antibody. DNA samples diluted to 100 ng/µl in coating buffer (supplied with the kit) and controls representing 0 %, 5 %, 10 %, 25 %, 50 %, 75 % and 100 % methylation were denatured at 98 ^{0}C for 5 minutes using an AccuBlock Digital Dry Bath (Labnet International Inc., Edison, New Jersey, USA), and then immediately transferred to ice for 10 minutes. Thereafter, 100 µl of DNA or controls were added to the wells of the ELISA plate ensuring that the solution covered the bottom of the well, by tilting the plate gently.

The ELISA plate was covered with foil and incubated at 37 ^{0}C for 60 minutes to allow DNA binding. Thereafter, wells were emptied by inverting plates and tapping onto paper towel. Wells were washed by adding 200 µl of 5-mC ELISA buffer and discarding the wash buffer,

three times. DNA was blocked by adding 200 µl of 5-mC ELISA buffer to each well. The plate was covered with foil and incubated at 37 ^{0}C for 30 minutes.

Thereafter, a 1: 2000 dilution of anti-5-methylcytosine antibody, a 1: 1000 dilution of secondary antibody and 5-mC ELISA buffer, in a total volume of 100 µl was added to each well. The plate was covered with foil and incubated at 37 ^{0}C for 60 minutes. The wells were then emptied and washed three times with 5-mC ELISA buffer as described before. One hundred microliters of HRP was added to each well and plates were incubated for 10 - 60 minutes at room temperature, protected from light, to allow colour development. Results read between 45 and 60 minutes were finally chosen from the multiple readings done for each run. Absorbance was measured at 450 nm on an ELISA Reader (Dasitaly, Polambara Sabina, Rome, Italy).

Readings obtained from the ELISA reader were manually entered into Microsoft Excel (Microsoft Office 2010). All data entries were verified by an independent researcher. A standard curve was constructed by plotting absorbance (y-axis) against the percentage methylation (x-axis) of the controls, and the percentage methylation (5-mC) in a DNA sample was quantified by extrapolation from the standard curve, using the equation % 5-mC = $e^{\{(abs - y\text{-intercept})/slope\}}$, derived from the logarithmic second order regression. All samples were assayed in duplicate according to the manufacturer's recommendation and to ensure accurate global DNA methylation detection and quantitation.

2.7 STATISTICAL ANALYSIS

2.7.1 Descriptive statistics for general characteristics of participants

Statistical analysis of global DNA methylation data was performed using STATISTICA 12 (StatSoft Inc., Tulsa, USA). The normality of data distribution was tested using Shapiro-Wilk's W test. Due to the skewed distribution of data, the median and interquartile range (25Q - 75Q) was used to summarize the general characteristics of participants' quantitative data and count percentage was used for categorical data. The Mann-Whitney U test was used for group comparisons of quantitative variables (normoglycaemic, pre-diabetic and diabetic groups), and the Bonferroni test was used to compare multiple groups of categorized parameters. Correlations were done using the Spearman Rank Order Correlations for non-parametrics (r and p-value).

2.7.2 Global DNA methylation Association analysis (linear regression analysis)

The association between the percentage of global DNA methylation with hyperglycaemia and other biochemical markers of T2DM were tested in a robust linear regression analysis adjusted for age, gender, status for hyperglycaemia and smoking. Monotonous trends in the distribution of characteristics across quarters of global DNA methylation were assessed using the Cochran-Armitage trend test for proportions and Jonckheere-Terpstra trend test for medians. Robust regressions were then used to assess the effect of various traits on global DNA methylation in models accounting for age, gender, smoking and glucose tolerance status. Results corresponding to p-values below 5 % are described as significant. Adjustment for multiple testing was conducted using Bonferroni methods. Analyses used the statistical software R (version 3.2.2 [2015-08-14], The R Foundation for statistical computing, Vienna, Austria).

CHAPTER 3: RESULTS

3.1 DNA EXTRACTION

DNA was successfully extracted from 564 of the 615 blood samples included in the study, and these participants were used for further analysis. Concentrations varied between 20 ng/µl and 180 ng/µl, yielding between 50 µg and 100 µg DNA. The average purity of samples was 1.8 for the 260/280 OD and 2.0 for the 260/230 OD.

3.2 CHARACTERISTICS OF PARTICIPANTS

The clinical characteristics of study participants and other biochemical parameters associated with T2DM (Korat et al., 2014) according to gender and glycaemic status are summarized in **Tables 3.1 and 3.2,** respectively. The Shapiro-Wilks test for normality showed that data was not normally distributed ($p < 0.05$). Therefore, the median and interquartile range (IQR), and the Mann-Whitney U test for comparative statistical analysis was used in this study.

3.2.1 Clinical characteristics according to gender

Of the 564 participants, 126 (22.1 %) were male and 438 (77.9 %) were female (**Table 3.1**). Females had higher blood glucose with an increase in 2h-PG ($p = 0.0036$), insulin (FBI ($p < 0.0001$) and 2h-SI ($p < 0.0001$)) concentrations; therefore a higher glucose/insulin ratio ($p < 0.0001$) compared to males. Moreover, higher levels of CRP ($p = 0.0158$), a marker of inflammation, lipids (cholesterol ($p = 0.0005$); HDL ($p = 0.0012$) and LDL ($p = 0.0002$)) and bodyweight (BMI ($p < 0.0001$), HP ($p < 0.0001$) and WC ($p = 0.0019$)) were observed in females versus males.

However, waist hip ratio (WHR) ($p < 0.0001$) was higher in males than in females. Blood pressure (SBP ($p = 0.0111$) and DBP (0.0054), GGT ($p = 0.0011$) concentrations and height ($p < 0.0001$) were increased in males compared to females. None of the other parameters tested (age, FPG, HbA1c, Cotinine and Trigs) were significantly different between females and males. A detailed presentation of the results is shown in **Table 3.1**.

Table 3.1 Biochemical and anthropometric measurements according to gender

	Shapiro-Wilks test	**Total (n = 564)**	**Male (n = 126)**	**Female (n = 438)**	**P-value Male v female**
Age (years)	p = 0.0006	53.00 (43.00 - 62.00)	54.50 (43.00 - 64.00)	52.00 (43.00 - 62.00)	0.2155
Fasting plasma glucose (mmol/L)	p < 0.0001	5.60 (5.00 - 6.4)	5.30 (5.00 - 6.10)	5.65 (5.00 - 6.40)	0.1218
2hour-Plasma glucose (mmo/L)	p < 0.0001	7.00 (5.70 - 8.90)	6.30 (5.30 - 8.40)	7.10 (5.80 - 9.00)	**0.0036**
Fasting blood insulin (uU/mL)	p < 0.0001	7.10 (2.90 -12.75)	4.10 (1.60 - 8.30)	7.80 (3.30 - 13.35)	**< 0.0001**
2-hour Serum insulin (µU/mL)	p < 0.0001	40.0 (19.2 - 72.8)	26.7 (9.6 - 45.1)	44.0 (22.5 - 81.3)	**< 0.0001**
Glucose/Insulin ratio	p < 0.0001	0.84 (0.49 - 1.93)	1.38 (0.74 - 3.30)	0.72 (0.44 - 1.65)	**< 0.0001**
Glycated haemoglobin type A1c (%)	p < 0.0001	5.90 (5.50 - 6.30)	5.80 (5.50 - 6.20)	5.90 (5.50 - 6.30)	0.2221
C-reactive protein (mg/L)	p < 0.0001	4.20 (1.20 - 9.90)	2.80 (0.70 - 6.90)	4.80 (1.40 - 10.40)	**0.0158**
Cotinine (ng/mL)	p < 0.0001	10.00 (9.00 - 276.50)	10.00 (9.00 - 295.00)	10.00 (9.00 - 276.00)	0.2728
Gamma glutamyl transferase (IU/L)	p < 0.0001	28.0 (19.0 - 42.0)	32.0 (22.0 - 48.0)	27.0 (18.0 - 40.0)	**0.0011**
Triglycerides (mmol/L)	p < 0.0001	1.29 (0.95 - 1.81)	1.30 (0.95 - 1.82)	1.28 (0.95 - 1.79)	0.8390
High density lipoproteins (mmol/L)	p < 0.0001	1.23 (1.02 - 1.45)	1.13 (0.96 - 1.33)	1.25 (1.03 - 1.47)	**0.0012**
Low density lipoproteins (mmo/L)	p < 0.0001	3.55 (2.88 - 4.22)	3.23 (2.54 - 3.92)	3.65 (2.95 - 4.26)	**0.0002**
Cholesterol (mmol/L)	p < 0.0001	5.53 (4.73 - 6.29)	5.17 (4.40 - 5.90)	5.59 (4.77 - 6.39)	**0.0005**
Height (m)	p = 0.0009	1.58 (1.53 - 1.64)	1.69 (1.64 - 1.73)	1.56 (1.52 - 1.60)	**< 0.0001**
Weight (kg)	p < 0.0001	74.7 (62.5 - 85.7)	72.0 (61.2 - 85.6)	75.3 (63.4 - 85.8)	0.1536
Body mass index	p < 0.0001	29.5 (24.5 - 34.4)	25.1 (21.9 - 29.1)	31.0 (26.4 - 35.0)	**< 0.0001**
Hip circumference (cm)	p < 0.0001	108.5 (100.0 - 118.0)	100.0 (93.5 - 107.0)	112.0 (103.0 - 121.5)	**< 0.0001**
Waist circumference (cm)	p = 0.0024	97.0 (86.5 - 107.0)	92.0 (81.3 - 104.0)	98.5 (87.8 - 107.3)	**0.0019**
Waist hip ratio	p = 0.0012	0.87 (0.83 - 0.93)	0.92 (0.86 - 0.98)	0.87 (0.82 - 0.91)	**< 0.0001**
Systolic blood pressure (mmHg)	p < 0.0001	120.0 (109.0 - 133.0)	124.0 (112.0 - 136.0)	119.0 (108.0 - 132.0)	**0.0111**
Diastolic blood pressure (mmHg)	p = 0.0063	74.0 (67.0 - 82.0)	76.0 (69.0 - 84.5)	73.0 (66.0 - 81.0)	**0.0054**

Footnotes:

- Results are reported as the median (25Q - 75Q). Mann-Whitney U test for non-parametrics (p-value).
- Bold values indicate statistical significance (p < 0.05).

3.2.2 Clinical characteristics according to glycaemic status

Participants were classified as normal, pre-diabetic (IFG and/or IGT) or diabetic (screen-detected diabetes and known diabetes) according to WHO criteria **(Table 3.2)**. Screen-detected diabetes refers to diabetic individuals identified during screening for diabetes, and who were not on anti-diabetic treatment. Thirty seven (29.4 %), 21 (16.7 %) and 68 (54.0 %) males participants were classified as diabetic, pre-diabetic or normal, respectively. Of the female participants, 121 (27.8 %) were diabetic, 99 (22.7 %) were pre-diabetic and 216 (49.5 %) were normal.

Two participants were omitted from this analysis because their diabetic status was unknown due to missing data. Clinical parameters varied significantly according to glycaemic status. Significant median differences for diabetic status between normal and pre-diabetes were for age ($p = 0.0058$), FPG ($p = 0.0039$), 2h-PG $p < 0.0001$), 2h-SI ($p < 0.001$), glucose/insulin ratio ($p = 0.0282$), weight ($p = 0.0165$), BMI ($p = 0.0048$), WC ($p = 0.0005$). Significant median differences between normals and diabetes were for age ($p < 0.0001$), FPG ($p < 0.0001$), PostBG ($p < 0.0001$), FBI ($p = 0.0151$), HbA1C ($p < 0.0001$), triglycerides (Trigs) ($p < 0.0001$), HDL ($p = 0.023$), cholesterol ($p = 0.0143$), weight ($p = 0.0001$), WHR ($p < 0.0001$), SBP ($p < 0.0001$). Significant median differences between pre-diabetes and diabetes groups were in: age ($p = 0.0311$), FPG ($p < 0.0001$), PostBG ($p < 0.0001$), FBI ($p = 0.0151$), HbA1C ($p < 0.0001$), Trigs ($p = 0.0299$) and systolic blood pressure (SBP) ($p = 0.0168$). A detailed representation of the results is shown in **Table 3.2**.

Table 3.2: Biochemical and anthropometric measurements according to glycaemic status

	Normal[a] (n = 284)	**Pre-diabetes[b] (n = 120)**	**Diabetes[c] (n = 158)**	**P-value**
Age (years)	48.0 (41.0 - 59.0)	53.5 (44.0 - 62.5)	59.0 (48.0 - 65.0)	**0.0058[ab]** **< 0.0001[ac]** **0.0311[bc]**
Fasting plasma glucose (mmol/L)	5.00 (4.70 - 5.50)	5.90 (5.20 - 6.10)	8.00 (7.00 - 11.40)	**0.0039[ab]** **< 0.0001[ac]** **< 0.0001[bc]**
2hour-Plasma glucose (mmo/L)	5.90 (5.10 - 6.70)	8.60 (8.00 - 9.20)	12.50 (10.10 - 15.80)	**< 0.0001 [ab]** **< 0.0001 [ac]** **< 0.0001 [bc]**
Fasting blood insulin (µU/mL)	6.20 (2.90 - 10.60)	7.20 (1.90 - 12.70)	8.75 (3.55 - 14.70)	1.0000[ab] **0.0151[ac]** **0.0151[bc]**
2-hour Serum Insulin (µU/mL)	33.4 (17.1 - 54.5)	57.8 (26.1 - 97.9)	55.8 (21.2 - 108.6)	**< 0.001[ab]** **< 0.001[ac]** 1.000[bc]
Glucose/Insulin ratio	0.78 (0.44 - 1.67)	0.80 (0.46 - 2.90)	1.00 (0.57 - 2.30)	**0.0282[ab]** 0.1547[ac] 1.0000[bc]
Glycated haemoglobin type A1c (%)	5.60 (5.40 - 6.00)	5.80 (5.60 - 6.10)	6.95 (6.20 - 8.65)	0.6560[ab] **< 0.0001[ac]** **< 0.0001[bc]**
C-reactive protein (mg/L)	2.80 (0.70 - 7.10)	6.45 (1.80 - 12.60)	5.10 (1.80 - 10.30)	0.0034[ab] 0.0043[ac] 1.0000[bc]
Cotinine (ng/mL)	10.00 (9.00 – 308.0)	10.00 (9.00 - 279.5)	10.00 (9.00 - 156.0)	1.0000[ab] 0.1922[ac] 1.0000[bc]
Gamma glutamyl transferase (IU/L)	25.0 (17.0 - 36.0)	30.0 (22.0 - 42.5)	32.5 (23.0 - 52.0)	**0.0827[ab]** 1.0000[ac] 0.6802[bc]
Triglycerides (mmol/L)	1.10 (0.82 - 1.45)	1.40 (1.10 - 1.82)	1.57 (1.17 - 2.11)	0.1206[ab] **< 0.0001[ac]** **0.0299[bc]**
High density lipoproteins (mmol/L)	1.25 (1.02 - 1.51)	1.23 (1.05 - 1.44)	1.19 (0.99 - 1.38)	1.0000[ab] **0.0233[ac]** 0.1377[bc]
Low density	3.48 (2.91 - 4.14)	3.65 (2.83 - 4.28)	3.69 (2.88 - 4.59)	1.0000[ab]

lipoproteins (mmo/L)				0.1300[ac] 0.5395[bc]
Cholesterol (mmol/L)	5.45 (4.71 - 6.06)	5.58 (4.75 - 6.31)	5.75 (4.82 - 6.55)	1.0000[ab] **0.0143[ac]** 0.4299[bc]
Height (m)	1.58 (1.53 - 1.65)	1.57 (1.52 - 1.63)	1.58 (1.53 - 1.63)	0.9341[ab] 1.0000[ac] 1.0000[bc]
Weight (kg)	70.2 (59.5 - 82.4)	77.6 (66.5 - 88.6)	77.5 (66.4 - 89.5)	**0.0165[ab]** **0.0001[ac]** 1.0000[bc]
Body mass index	28.2 (23.1 - 33.2)	31.2 (26.9 - 35.2)	30.7 (26.7 - 36.5)	**0.0048[ab]** **0.0001[ac]** 1.0000[bc]
Hip circumference (cm)	107.0 (97.8 - 116.8)	111.0 (103.0 - 121.0)	110.0 (101.5 - 122.0)	0.1702[ab] **0.0213[ac]** 1.0000[bc]
Waist circumference (cm)	92.4 (82.0 - 102.6)	100.3 (91.0 - 109.5)	101.0 (93.0 - 110.0)	**0.0005[ab]** **< 0.0001[ac]** 0.4554[bc]
Waist hip ratio	0.86 (0.81 - 0.91)	0.88 (0.83 - 0.94)	0.91 (0.86 - 0.97)	**0.0010[ab]** **< 0.0001[ac]** 0.3260[bc]
Systolic blood pressure (mmHg)	116.0 (106.0 - 130.0)	121.0 (112.0 - 130.0)	127.5 (113.0 - 140.0)	0.2309[ab] **< 0.0001[ac]** **0.0168[bc]**
Diastolic blood pressure (mmHg)	73.0 (65.5 - 81.0)	74.0 (67.0 - 81.0)	75.0 (67.0 - 84.0)	1.0000[ab] 0.1960[ac] 1.0000[bc]

Footnotes:

- Results are reported as Median (25Q - 75Q). Mann-Whitney U test for non-parametrics (P-value).
- Bold values indicate statistical significance ($p < 0.05$).
- [a] Normal.
- [b] Pre-diabetes.
- [c] Diabetes

3.3 GLOBAL DNA METHYLATION IS INCREASED DURING HYPERGLYCAEMIA

To assess whether the percentage of global DNA methylation correlates with glycaemia, participants were classified as screen-detected diabetic, known diabetic, pre-diabetic (IFG, IGT or both) or normal, according to WHO criteria. Two participants were omitted from this analysis because their diabetic status was unknown. Global DNA methylation was significantly higher in screen-detected diabetes compared to those with normoglycaemia: (4.62 (1.71 - 6.77 % vs. 2.81 (0.68 - 4.62) %, p = 0.0003). Global DNA methylation was also significantly higher in diabetes (screen-detected) than known diabetes: (4.62 (1.71 - 6.77) %, p = 0.0188 vs. 3.07 (0.80 - 4.45) %). Individuals with pre-diabetes similarly had increased global DNA methylation compared to those with normoglycaemia, although the difference was not statistically significant: (3.40 (1.53 - 5.45) % vs. 2.81 (0.68 - 4.62) %, p = 0.0623). No difference in global DNA methylation was observed between individuals with known diabetes and those with normoglycaemia: (3.07 (0.80 - 4.45) % vs. 2.81 (0.68 - 4.62) %, p = 1.0000), or the screen-detected diabetes and pre-diabetes groups: (4.62 (1.71 - 6.77 % vs. 3.40 (1.53 - 5.45) %, p = 0.8931). A diagrammatic representation is shown in **Figure 3.1**.

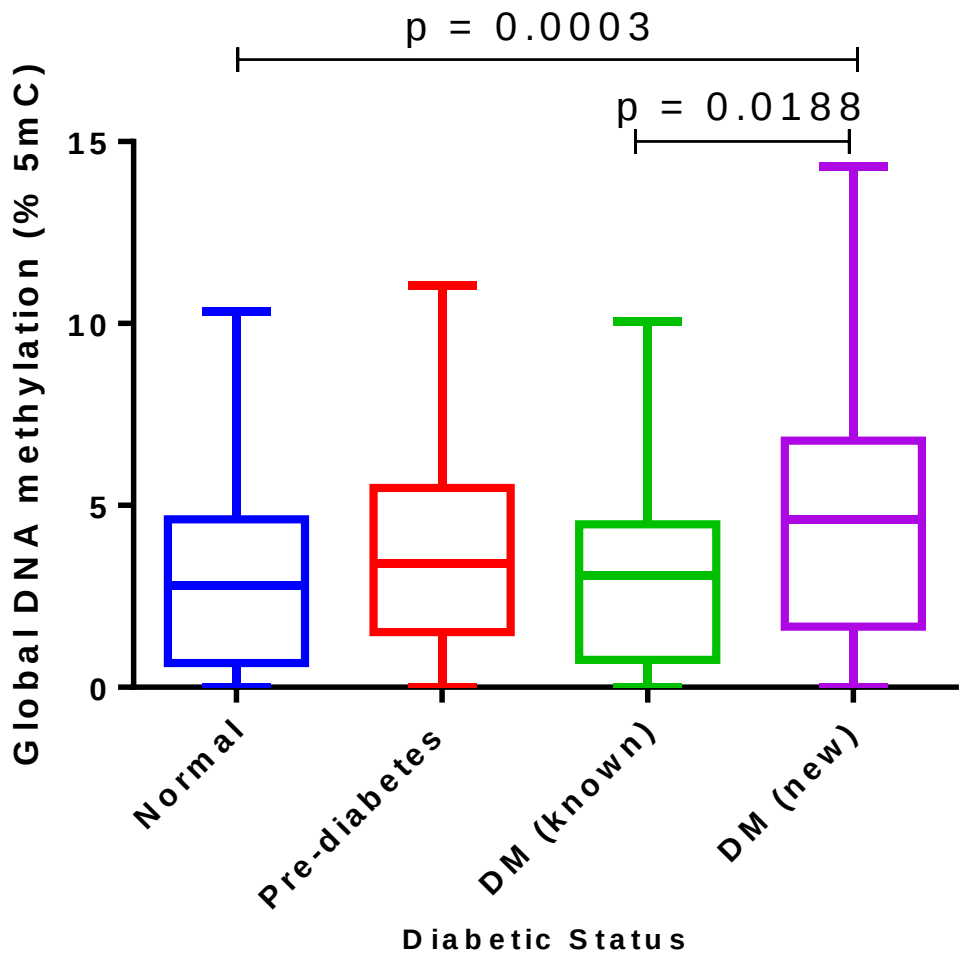

Figure 3.1: Global DNA methylation levels in Normal (n = 284), Pre-diabetes (n = 120), Known diabetes (n = 61) and Diabetes (Screen-detected) (n = 97) individuals. Results are represented as the median (25Q – 75Q). (Bonferroni test/Post hoc).

Key:

- DM (known) – Known diabetes
- DM (new) – Screen-detected diabetes

3.3.1 Correlation between global DNA methylation and clinical parameters according to gender

Global DNA methylation was positively correlated with FPG (r = 0.1256; p = 0.0029), 2h-PG (r = 0.1674; p = 0.0002), BMI (r = 0.0898, p = 0.0336) and WC (r = 0.0900; p = 0.0330). To determine whether gender affected the correlation between global DNA methylation and clinical parameters, participants were stratified according to gender. Global DNA methylation was positively correlated with FPG (r = 0.1175, p = 0.0141), 2h-PG (r = 0.2001, p = 0.0001), weight (r = 0.1142, p = 0.0169) and WC (r = 0.1079, p = 0.0241) in females but not in males. In contrast, HDL was inversely associated with global DNA methylation in males but not in females. A detailed presentation of the results is shown in **Table 3.3**.

Table 3.3: Correlation between global DNA methylation and clinical parameters according to gender

	Total (n = 564)		Male (n = 126)		Female (n = 438)	
	R	**P-value**	**R**	**P-value**	**R**	**P-value**
Age (years)	0.0362	0.3914	0.0630	0.4834	0.0362	0.4499
Fasting plasma glucose (mmol/L)	**0.1256**	**0.0029**	0.1634	0.0687	**0.1175**	**0.0141**
2h-Plasma glucose (mmo/L)	**0.1674**	**0.0002**	0.0157	0.8735	**0.2001**	**0.0001**
Fasting blood insulin (uU/mL)	-0.0054	0.8997	-0.0711	0.4326	-0.0039	0.9362
2-hour Serum Insulin (µU/mL)	0.0701	0.1200	0.0592	0.5488	0.0449	0.3768
Glucose/Insulin ratio	0.0359	0.3986	0.1275	0.1581	0.0256	0.5958
Glycated haemoglobin type A1c (%)	0.0566	0.1810	0.0257	0.7766	0.0638	0.1834
C-reactive protein (mg/L)	0.0797	0.0591	-0.0060	0.9471	0.0891	0.0627
Cotinine (ng/mL)	-0.0594	0.1603	-0.0603	0.5039	-0.0567	0.2375
Gamma glutamyl transferase (IU/L)	0.0005	0.9904	-0.0100	0.9123	0.0261	0.5863
Triglycerides (mmol/L)	0.0793	0.0609	0.0859	0.3427	0.0873	0.0685
High density lipoproteins (mmol/L)	-0.0809	0.0555	**-0.1848**	**0.0391**	-0.0730	0.1281
Low density lipoproteins (mmo/L)	0.0788	0.0621	0.0819	0.3642	0.0669	0.1635
Cholesterol (mmol/L)	0.0614	0.1468	0.0320	0.7246	0.0583	0.2244
Height (m)	-0.0340	0.4222	-0.1120	0.2176	0.0423	0.3775
Weight (kg)	0.0787	0.0627	-0.0552	0.5439	**0.1142**	**0.0169**
Body mass index	**0.0898**	**0.0336**	-0.0140	0.8781	0.0901	0.0600
Hip circumference (cm)	0.0828	0.0503	-0.0257	0.7781	0.0769	0.1084
Waist circumference (cm)	**0.0900**	**0.0330**	-0.0131	0.8854	**0.1079**	**0.0241**
Waist hip ratio	0.0306	0.4700	0.0188	0.8366	0.0739	0.1229
Systolic blood pressure (mmHg)	-0.0249	0.5566	-0.0989	0.2744	0.0128	0.7906
Diastolic blood pressure (mmHg)	-0.0390	0.3567	-0.1327	0.1419	0.0005	0.9919

Footnotes:

- Spearman Rank Order Correlations for non-parametrics (R and p-value).
- Values highlighted as bold indicate significant values ($p < 0.05$)

3.3.2 Correlation between global DNA methylation and clinical parameters according to glycaemic status

To determine whether glycaemic status affected the correlation between global DNA methylation and clinical parameters, participants were stratified as normoglycaemic, pre-diabetic, screen-detected diabetes or known diabetes. An inverse and positive correlation between global DNA methylation and HDL ($r = -0.2116$, $p = 0.0203$) and HP ($r = 0.1937$ and $p = 0.0348$), respectively was observed in pre-diabetes. Global DNA methylation was inversely associated with DBP in individuals with normoglycaemia ($r = 0.1230$ and $p = 0.0391$). A detailed representation of the results is shown in **Table 3.4**.

Table 3.4: Correlation between percentage global DNA methylation and glycaemic status

	Normal (n = 284)		Pre-Diabetes (n = 120)		New Diabetes (n = 97)		Known Diabetes (n = 61)	
	R	**P-value**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**
Age (years)	0.0372	0.5326	-0.0209	0.8204	0.0182	0.8597	-0.0965	0.4593
Fasting plasma glucose (mmol/L)	0.0279	0.6400	-0.0261	0.7771	0.0069	0.9468	0.1057	0.4214
2-hour Plasma glucose (mmo/L)	0.0009	0.9874	-0.0082	0.9294	-0.0944	0.3787	0.0690	0.6004
Fasting blood insulin (uU/mL)	-0.0017	0.9774	-0.0951	0.3059	-0.0568	0.5822	N/A	N/A
2-hour Serum Insulin (µU/mL)	0.0494	0.4079	-0.0129	0.8890	0.0422	0.6944	0.0388	0.7702
Glucose/Insulin ratio	0.0159	0.7899	0.0831	0.3710	0.0520	0.6151	0.0474	0.7189
Glycated haemoglobin type A1c (%)	0.0248	0.6771	0.0152	0.8695	-0.1582	0.1236	0.1829	0.1619
C-reactive protein (mg/L)	0.0098	0.8695	0.0604	0.5123	0.0109	0.9152	0.0050	0.9698
Cotinine (ng/mL)	-0.0894	0.1328	0.0455	0.6228	-0.0983	0.3380	0.2015	0.1226
Gamma glutamyl transferase (IU/L)	-0.0400	0.5020	-0.1663	0.0695	0.0153	0.8821	0.0766	0.5609
Triglycerides (mmol/L)	0.0499	0.4031	0.0477	0.6045	-0.0390	0.7044	-0.0966	0.4628
High density lipoproteins (mmol/L)	-0.0119	0.8418	**-0.2116**	**0.0203**	-0.1303	0.2032	0.0650	0.6219
Low density lipoproteins (mmo/L)	0.0938	0.1148	-0.1130	0.2192	0.0901	0.3799	0.1104	0.4012
Cholesterol (mmol/L)	0.0606	0.3097	-0.1500	0.1019	0.0663	0.5186	-0.1349	0.3041
Height (m)	-0.0095	0.8735	-0.0243	0.7928	-0.0384	0.7090	0.0117	0.9300
Weight (kg)	0.0718	0.2286	0.0982	0.2882	-0.0636	0.5362	-0.0289	0.8282
Body mass index	0.0621	0.2977	0.1410	0.1260	-0.0226	0.8258	0.0011	0.9933
Hip circumference (cm)	0.0715	0.2304	**0.1937**	**0.0348**	-0.0434	0.6732	-0.0681	0.6085
Waist circumference (cm)	0.1034	0.0826	0.0952	0.3030	-0.1135	0.2681	-0.0074	0.9550
Waist hip ratio	0.0682	0.2525	-0.1083	0.2411	-0.1451	0.1563	0.1296	0.3279
Systolic blood pressure (mmHg)	-0.1028	0.0847	-0.0218	0.8143	0.0235	0.8200	-0.0424	0.7455
Diastolic blood pressure (mmHg)	**-0.1230**	**0.0391**	-0.0175	0.8500	0.0341	0.7413	0.0308	0.8139

Footnotes:

- Total*: Total number of participants was 562 due to two samples which had no diagnosis in the database (missing data).
- Spearman Rank Order Correlations for non-parametrics (r and p-value).
- Bold values indicate statistical significance ($p < 0.05$).

3.4 GLOBAL DNA METHYLATION ASSOCIATION STUDIES

The baseline characteristics of participants across quarters of global DNA methylation are summarized in **Table 3.5.** The proportion of participants with any diabetes increased across increasing quarters of DNA methylation (p = 0.028) and in a linear fashion (p = 0.008 for linear trend). In robust linear regression analysis adjusted for age, gender and smoking; any diabetes (β = 0.621, p = 0.036) was associated with global DNA methylation. When participants with diabetes were distinguished into those with screen-detected diabetes or known diabetes, the association remained significant only for screen-detected diabetes (β = 1.069, p = 0.004) but not for known diabetes.

Table 3.5: Baseline characteristics across quarters of global DNA methylation

Characteristic	Global DNA methylation				β (p-value)***
	Q1	Q2	Q3	Q4	
n	141	141	140	142	N/A
Global DNA methylation (%)					
Range	0.00 - 1.11	1.12 - 3.12	3.13 - 5.35	5.36 - 30.38	N/A
Median	0.14	2.14	3.97	7.27	N/A
Women, n (%)	100 (70.9)	109 (77.3)	115 (82.1)	114 (80.3)	0.543 (0.063)
Any diabetes (n = 158)	31 (22.0)	38 (26.9)	36 (25.7)	53 (37.3)	**0.621 (0.036)**
Screen-detected (n = 97)	15 (10.6)	23 (16.3)	17 (12.1)	42 (29.6)	**1.069 (0.004)**
Known diabetes (n = 61)	16 (11.3)	15 (10.6)	19 (13.6)	11 (7.7)	-0.026 (0.947)
Current smoking, n (%)	55 (39.0)	60 (42.5)	59 (42.1)	50 (35.2)	-0.162 (0.505)
Age (years)	51 (41 - 64)	52 (44 - 60)	53 (44 - 62)	53 (44 - 64)	0.007 (0.443)

Footnotes:

- P-values from Kruskal-Wallis and chi square tests for the differences across quarters of global DNA methylation.
- ** Spearman correlation coefficients and p-values for the continuous associations of global DNA methylation with covariates.
- *** Beta coefficients and p-values from age, gender and status for hyperglycaemia and smoking adjusted robust linear regressions for the prediction of global DNA methylation by various traits.
- Bold values indicate statistical significance ($p < 0.05$).

CHAPTER 4: DISCUSSION

4.1 INTRODUCTION

DNA methylation is receiving increased attention as a potential biomarker to identify those who are at risk for developing T2DM (Kim et al., 2010; Levenson, 2010; Terry et al., 2011; Almén et al., 2012). Moreover, a number of studies have showed that measurement of global DNA methylation, although a crude estimation, offers potential as a rapid and cost-effective high risk marker for T2DM, thus facilitating intervention strategies to prevent or delay disease progression (Smolarek et al., 2010; Zhao et al., 2012; Luttmer et al., 2012; Ronn & Ling, 2015; Maghbooli et al., 2014). Despite growing support for DNA methylation as a high risk biomarker for T2DM globally, such evidence is lacking in South Africa. Previous studies from our laboratory have shown that the prevalence of T2DM in the mixed ancestry cohort of Bellville South, South Africa is increasing rapidly (Erasmus et al., 2012). In this study we explored the potential of DNA methylation as a high risk marker for T2DM by measuring global DNA methylation status in 562 individuals with varying degrees of dysglycemia.

4.2 GLOBAL DNA METHYLATION IS ASSOCIATED WITH HYPERGLYCAEMIA

DNA methylation is affected by a number of factors including age (Fraga et al., 2005; Moore et al., 2008), gender (Zhu et al., 2012; Zhang et al., 2014) and smoking (Dogan et al., 2014; Zaghlool et al., 2015), therefore these factors were adjusted for in a linear regression analysis of DNA methylation and hyperglycaemia. Global DNA methylation was positively correlated with both FPG and 2h-PG. Moreover, global DNA methylation was significantly higher in individuals with screen-detected diabetes compared to those with known diabetes (on treatment) and normoglycaemia. A similar trend was observed in pre-diabetes, although the increase was not statistically significant.

The identification of global DNA methylation changes in pre-diabetes, the asymptomatic stage of T2DM, and the identification of more pronounced changes in T2DM, suggests that these global DNA methylation changes are involved in the progression of T2DM. These findings are consistent with other studies that have also reported that global DNA methylation is increased in pre-diabetes and diabetes, and might offer new opportunities for risk stratification and prevention (Yang et al., 2011; Chambers et al., 2015; Maghbooli et al., 2014; VanderJagt et al., 2015).

The study by Yang and associates showed an increased DNA methylation in T2DM patients as compared to non-diabetic individuals (Yang et al., 2011). Though the sample source was pancreatic islet cells and not peripheral blood as in this study, the trend of an increase in individuals with diabetes as compared to non-diabetic individuals was also demonstrated in our study. Furthermore, the approach used by Yang *et al.,* was gene-specific DNA methylation (Yang

et al., 2011), different from the global DNA methylation method used for this study, however the trend of an increase in individuals with diabetes as compared to non-diabetic individuals was also demonstrated in our study. Again, several studies have reported gene-specific DNA hypermethylation in T2DM (Ling et al., 2008; Fujiki et al., 2009; Zou et al., 2013; Gu et al., 2013; Dick et al., 2014; Seman et al., 2015). Increased (hypermethylation) gene-specific DNA methylation can be used as a proxy for increased global DNA methylation since the sum of gene-specific DNA methylation contribute to overall global DNA methylation. It should, however be noted that these two mechanisms are different and may not always correlate.

VanderJagt and associates reported differential methylation (hypermethylation for some CpG sites and hypomethylation for some CpG sites) using the Infinium 27K Methylation array (Illumina Infinium; HumanMethylation27 BeadChip, Illumina, San Diego, CA), a genome-wide DNA methylation analysis approach (VanderJagt et al., 2015). The differential DNA methylation states were observed at the time of transition from the pre-diabetes stage to the diabetes stage (VanderJagt et al., 2015). Another difference with our study is that, their study was a longitudinal study in white males whereas ours was a cross-sectional study in mixed-ancestry population of both genders. Maghbooli *et al.,* also reported that global DNA methylation is modulated during or even possibly before the primary stage of diabetes (Maghbooli et al., 2014). Their study also used peripheral blood as the source of DNA, though the methodology used was reversed-phase high-pressure liquid chromatography.

Our study provides support for the use of global DNA methylation as a rapid and cost-effective screening tool for T2DM in populations where traditional risk factors for T2DM have poor predictive value. Thus, future longitudinal prospective studies to investigate the potential of DNA methylation as a high risk biomarker in South Africa are warranted. Global DNA hypomethylation has also been shown to be associated with a number of chronic diseases including diabetes, bipolar disorders, cancer, obesity and schizophrenia (Pogribny & Beland, 2009; Wilson et al., 2007). Kim and associates showed that global DNA hypermethylation in peripheral blood leucocytes (PBLs) is positively associated with the prevalence of cardiovascular disease (CVD) (myocardial infarction and stroke)/ predisposing conditions (hypertension and diabetes) and obesity (Kim et al., 2010). The study assessed global DNA methylation as represented by Alu and Satellite 2 (AS) repetitive elements using the MethyLight technique, a precise and highly reproducible technique (Kim et al., 2010b). Although a target section of the genome (Alu and satellite 2 (AS) repetitive elements) were used as surrogate markers for determining global DNA methylation in their study, whereas our study which looked at the whole genome, the comparison between these studies is valid because these surrogate markers have been reported to be representative of the whole genome due to their high copy numbers (Yang et al., 2004).

Our study showed that global DNA methylation is associated with increased glycaemic status, consistent with other studies (Zhao et al., 2012; Luttmer et al., 2012; Maghbooli et al., 2014; Chambers et al., 2015; VanderJagt et al., 2015). Insulin resistance is a known hallmark of T2DM, however, the mechanisms behind the pathogenesis has not been fully elucidated (Cerf, 2013). Zhao and associates provided the first evidence of an association between altered global DNA methylation and increased risk of insulin resistance, independent of established risk factors (Zhao et al., 2012). Global DNA methylation was measured using Alu elements and quantitative bisulfite sequencing (Zhao et al., 2012).

As mentioned earlier, global DNA methylation was higher in screen-detected diabetes individuals, as compared to normoglycaemic individuals, but not in known diabetes who were on diabetes treatment. DNA methylation is a reversible process, and the reversal of T2DM induced methylation changes by anti-diabetic therapy is a plausible suggestion. The modulation of DNA methylation by anti-diabetic treatment has previously been suggested (Nilsson et al., 2015; Volkmar et al., 2012). We therefore speculate that anti-diabetic drugs could be involved in a DNA methylation pathway resulting in affecting gene expression mechanisms, a sentiment also shared by Volkmar and associates (Volkmar et al., 2012).

Contrary to our findings of increased global DNA methylation during T2DM, Smolarek *et al.,* reported decreased global DNA methylation in patients with hypertension, one of the main risk factors of T2DM (Smolarek et al., 2010). The methylation status was correlated with the stage of hypertension (Smolarek et al., 2010), which is suggestive of an epigenetic change of clinical significance, thus suggesting that global DNA methylation is a possible biomarker of hypertension, hence T2DM. Differences in methodologies used between these studies could account for the difference in the direction of methylation (hyper- and hypomethylation) observed. For example, Smolarek *et al.,* used thin layer chromatography (TLC) to asses global DNA methylation, whereas an ELISA based method was used in our study. Moreover, Nilsson *et al.,* reported genome-wide DNA hypomethylation in human liver cells of T2DM individuals (Nilsson et al., 2015), another finding inconsistent with ours. The different methylation results between these studies could be due to various factors such as different techniques used, different source of samples used, as well as sample sizes for the two studies. Our study used an ELISA based method and peripheral blood DNA, whereas Nilsson *et al.* (2015) used a genome-wide approach using the Infinium HumanMethylation450 BeadChip and DNA from liver cells. Finally, our study had a larger sample size (404 non-diabetics and 158 diabetics) while theirs had a relatively smaller sample size (60 non-diabetics and 35 diabetic individuals).

Our study found no difference in global DNA methylation between females and males. However, the association between global DNA methylation with FPG and 2h-PG, was only observed in females. Since there were more females enrolled in the study as compared to males (438 females

as to 126 males), this could have masked the effect of and/or drown the possible significance of the gender related methylation status. A study by Maghbooli and associates similarly reported no gender-specific differences in DNA methylation (Maghbooli et al., 2014). This study also used peripheral blood leucocytes as a sample source, although they used reversed-phase high-pressure liquid chromatography to determine global DNA methylation status.

Most studies which compared gender specific DNA methylation status reported an increase in males as compared to females (Fuke et al., 2004; Hsiung et al., 2007; El-Maarri et al., 2007; Wilhelm et al., 2010; El-Maarri et al., 2011; Zhang et al., 2014). The low methylation levels in females could be due to menstruation in females which continuously depletes circulating folate levels when blood has to be made, since folate is needed in red blood cell (erythrocyte) formation and development (Terry et al., 2011). Folate is also the key supplier of methyl groups which are used in methylation reactions, therefore its depletion in females could create a methyl group availability imbalance which could result in hypomethylation (Terry et al., 2011). However, other studies have also reported an increase in methylation status in females than males (Moore et al., 2008; Kim et al., 2010).

Global DNA methylation was not associated with BMI, though an association was found in the total population, with a trend observed in females only. Similar to our findings, Zhu *et al.,* also found no correlation between BMI and global DNA methylation (Zhu et al., 2012). Another study by Dick and associates also found no association between BMI and genome-wide DNA methylation in a meta-analysis study (Dick et al., 2014). Contrary to our findings, another study reported a correlation between BMI and global DNA methylation, using the same methodology as in our study (Na et al., 2014). However, the discrepancy could be attributed to the difference in the study populations. Our study had males and females whereas the study by Na *et al.,* had healthy females only. As mentioned earlier, our study also had a trend observed in the females only.

Global DNA methylation was also associated with WC in females in our study. Similarly, another study reported that BMI and WC were positively associated with gene-specific DNA methylation status at the adiponectin (ADIPOQ) gene locus in subcutaneous tissue (Houde et al., 2015). This finding also points to the methodology difference in our study and these studies which used gen-specific methylation as compared to our method which was global DNA methylation through ELISA. The ELISA method and global DNA methylation has less specificity due to its inability to pinpoint genes involved. Another gene-specific study also reported that many genes including the fat mass and obesity associated (FTO) gene, insulin receptor substrate 1 (IRS1) and HiF3A; had their DNA methylation status and expression correlated with BMI (Rönn et al., 2015)

Waist circumference has been reported to be better associated with metabolic risk, in this case T2DM, than BMI (Dalton et al., 2003; Zhu et al., 2004), though both parameters are essential all

depending on the study population. A limitation of BMI is that it could overestimate or underestimate body fat levels in an individual (Hall & Cole, 2006; Ross & Janiszewski, 2008). This is due to its inability to differentiate muscle weight from fat weight in an individual probably resulting in muscular people being classified as obese. Weight circumference is therefore a better indicator of obesity since it takes into account where the individual's fat is stored (Ross & Janiszewski, 2008).

4.3 OTHER FINDINGS

A negative correlation between global DNA methylation and HDL was observed in male participants. Similar to our findings, other studies have also reported the association between low levels of HDL cholesterol with an increased risk of T2DM (Schmidt et al., 2005; Wilson et al., 2007). Described as good cholesterol, HDL plays a role in protecting against atherosclerosis development thereby reducing the risk of cardiovascular disease (Toth, 2005). This is achieved via a process called 'reverse cholesterol transport' in which HDL molecules pick excess cholesterol from the bloodstream to the liver for disposal or reprocessing (Toth, 2005).

Decreased HDL (dyslipidaemia) is a known risk factor for T2DM, detectable even during the asymptomatic disease stage (Fukui et al., 2011). In our study, HDL was significantly decreased during disease progression from normoglycaemia to T2DM, suggesting that the relationship between global DNA methylation and HDL may be a pathogenic mechanism in disease progression. This leads to speculation that the differential DNA methylation changes are a pathogenic aspect of clinical significance in disease progression and it would be important to investigate the actual pathways involved in disease pathogenesis. Furthermore, our results also showed that there were significant correlations between global DNA methylation and high density lipoproteins (HDL) in the pre-diabetic group but not in the non-diabetic and diabetic groups. This is suggestive of an involvement of DNA methylation in a pathogenic pathway or mechanism in T2DM during the initial stages of disease progression. A pathogenic signal or risk factor detectable during an asymptomatic stage is of utmost importance because it would have the potential to be used as an early detection diagnostic biomarker for the disease.

No association between global DNA methylation and other factors previously reported to affect DNA methylation was observed in this study. For example, our results showed that age was not correlated with global DNA methylation. These findings are in contrast to others who have reported that age is a major confounder in DNA methylation studies (Fuke et al., 2004; Fraga et al., 2005; Moore et al., 2008). Longitudinal studies would be better suited to probe age related DNA methylation changes over time. However, using a longitudinal study design, Bjornsson and

colleagues also failed to observe an association between DNA methylation and age (Bjornsson et al., 2008). Therefore, the link between DNA methylation and age remains to be elucidated.

4.4. STRENGTHS OF THE STUDY

In this study an ELISA was used to measure global DNA methylation. This is a cost-effective and inexpensive method that does not require sophisticated instruments, thus making it a practical tool in routine laboratories, particularly those in low-and middle income countries with limited resources. Moreover, the ELISA method obviates the need for bisulphite conversion which poses many challenges to DNA methylation studies (Patterson et al., 2011). Gene-specific DNA methylation is able to quantify methylation at specific CpG sites and is considered superior to global DNA methylation analysis that lacks the resolution to identify individual genes or pathways differentially methylated during T2DM (Zhao et al., 2012). However, the requirement for bisulfite conversion where unmethylated cytosines are converted to uracils makes gene-specific DNA methylation analysis labour intensive and time consuming (Patterson et al., 2011; Li & Tollefsbol, 2011). Methylated cytosines are identified using complex procedures such as pyrosequencing and mass spectrometry, processes that require expensive equipment that are often not available in low income settings. Although these assays are more expensive than routine measurement of glucose concentrations, further improvement of such methods could lead to the development of a more sensitive, specific and cost-effective alternative to assessing glucose concentrations.

In this study whole blood was used to assess global DNA methylation. Although DNA methylation exerts its function in body tissues, a number of studies have reported that DNA methylation is stably expressed across different tissue types including blood (Pidsley & Mill, 2011; Terry et al., 2011; Dick et al., 2014), therefore making blood a suitable candidate for assessing global DNA methylation changes (Pidsley & Mill, 2011). Blood based biomarkers are less-invasive that tissue samples, and can be measured repeatedly over time, thus enabling epidemiologic studies to understanding disease in longitudinal studies (Terry et al., 2011). Blood based biomarkers have been successfully developed for prostate cancer (Liong et al., 2012).

4.5 LIMITATIONS OF THE STUDY

This study used a cross-sectional study design, which is able to demonstrate association, but not causality. Although the idea that biomarkers should be causative is debatable, prospective longitudinal studies are the ideal study design to identify risk factors. However, the high costs, time and challenges required to conduct such studies limit their use. Therefore, cross-sectional studies could be used to identify potential targets and proof-of-principle, which could be validated in these complex studies.

As discussed previously, DNA methylation is affected by a number of factors, which are possible confounders, and may affect the interpretation of the results. Although some of these (age, gender and smoking) were adjusted for in this study, other factors such folate consumption and presence of some disease states such as cancer were unknown and therefore unaccounted for, and could have affected the interpretation of the results. Therefore, study design and sampling strategy are critical aspects to consider when conducting DNA methylation studies. In this study a random sampling strategy was employed, therefore minimizing bias.

Folate is a major supplier of methyl groups during DNA methylation (Nilsson et al., 2015). Therefore, different folate concentrations in individuals could influence DNA methylation and consequently affect gene expression (Crider et al., 2012). The lack of information about folate levels in study participants poses a major limitation of the study. Another limitation of the study is the decreased number of male participants compared to female participants, thus possibly affecting the statistical power.

Another limitation of the study is the use of whole blood. Whole blood consists of a number of different blood cells including the leukocytes such as monocytes, neutrophils, eosinophils and lymphocytes. These different cell types may have different DNA methylation profiles all contributing to the final global DNA methylation (Wu et al., 2011; Reinius et al., 2012). However, the use of whole blood is a rapid and convenient method to prepare DNA, and obviates the use of complex procedures such as density gradient centrifugation to separate blood cells. Individuals of mixed ancestry ethnicity were investigated in this study. A number of studies have reported that genetics affect DNA methylation (Terry et al., 2008), thus genetic heterogeneity within the study population could have masked DNA methylation changes.

4.6 FUTURE PERSPECTIVES

Although this study showed that global DNA methylation was associated with pre-diabetes and diabetes, prospective, longitudinal studies in different populations are required to confirm its potential as a risk factor for T2DM. Longitudinal studies have the ability to determine when DNA methylation changes develop in the progression of disease. Moreover, incorporation of global DNA methylation status into predictive algorithms, together with other T2DM risk factors, may produce a risk model with higher sensitivity and specific than the currently available risk factors.

CHAPTER 5: CONCLUSION

Despite the limitations of the study, which have been alluded to before, our results show that global DNA methylation levels are associated with T2DM, and to a lesser extent, pre-diabetes, in individuals of mixed ancestry descent. Our results add to the growing body of evidence that global DNA methylation is associated with T2DM and can be detected during pre-diabetes, the asymptomatic stage of T2DM. These findings support the use of global DNA methylation as a risk factor and early detection biomarker of T2DM. The failure of traditional risk factors such as obesity makes this an attractive avenue to explore for T2DM risk stratification and lifestyle interventions, which could ultimately lead to better ways to prevent, manage and control the T2DM epidemic that is rampant worldwide. To our knowledge, this is the first study to assess DNA methylation as a risk factor for T2DM in South Africa and Africa at large.

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Appendix A

Salt Extraction method (Laboratory Protocol 2014) (adapted from http://www.liv.ac.uk/~kempsj/IsolationofDNA.pdf).

Day 1:	**Day 2:**
Work on ice Add to 50 ml tubes: • 1 - 5 ml blood • 10 ml lysis buffer at 4°C • [OR 10 ml blood + 30 ml lysis buffer] Vortex, put on ice for 5 min, repeat 3x Spin 1,500 rpm, 10 min at 4 °C Discard supernatant **KEEP PELLET** **Add to pellet:** • 10 ml PBS (to rinse) Vortex Spin 1,500 rpm, 10 min at 4 °C Discard supernatant *If pellet is still red, repeat:* Add 10 ml PBS Vortex Spin 1,500 rpm, 10 min at 4 °C Discard supernatant **KEEP PELLET** **Dissolve pellet in:** • 3 ml nucleic lysis buffer • 30 µl proteinase K (10 mg/ml) • 300 µl 10 % SDS Vortex Incubate overnight at 55°C (waterbath full)	Don't work on ice Add to volume in tube: • 1 ml 6 M NaCl Vortex well, but not too vigorously Spin 2500 rpm for 30 min **KEEP SUPERNATANT FLUID** Shake well for 15 sec Spin 2500 rpm for 30 min **KEEP SUPERNATANT FLUID** To precipitate DNA add to supernatant: • two volumes 100 % ethanol at -20 °C (~ 5 ml supernatant + 10 ml ETOH) Tilt to precipitate DNA Take out DNA and put into tube with 1ml 70 % ETOH at -20 °C Spin at max speed for 30 min (to get rid of last salt; supernatant will have salt in if any) Discard supernatant **KEEP PELLET** Tip tube dry on paper towel **Dissolve DNA pellet in:**: • 100 - 200 µl distilled water (depending on size of pellet) Put overnight on turning apparatus to dissolve
	Day 3
	Read ODs/Nanodrop OD 260/280 ratio Aliquot and freeze at -20 °C **Keep record:** Volume blood used Volume of final DNA sample Concentration of final DNA sample/µl DNA concentration per starting material

Salt extraction method (Buffers and reagents needed)

Buffers/Solutions needed	**Nuclear lysis buffer**	**PBS**
1. Preliminary buffers 2. Lysis buffer 3. Nuclear lysis buffer 4. 6 M NaCl (70.13 g/200 mL) 5. 10 % SDS (10 g/100mL) 6. PBS 7. Proteinase K	10 mM Tris (1 ml from 1 M stock) 400 mM NaCl (2.3 g NaCl) 2 mM EDTA (2 ml from 100 mM stock) Make up 100 ml pH to 8.2	0.2 g KCl (2.68 mM) 8.0 g NaCl (136 mM) 0.2 g KH_2PO_4 (1.47 mM) 1.15 g Na_2HPO_4 (8.1 mM) Add components one at a time to 900 ml of distilled water Stir to dissolve pH to 7.4 Make up to 1 L with distilled water
Preliminary buffers	**6 M NaCl**	**Proteinase K**
1 M stock NH_4Cl 1 M stock $KHCO_3$ 100 mM stock EDTA	70.13 g/200 ml/saturated	10 mg/ml proteinase K
Lysis buffer	**10 % SDS**	
155 mM NH_4Cl (15.5 ml from 1 M stock) 10 mM $KHCO_3$ (1 ml from 1 M stock) 0.1 mM EDTA (100 µl from 100 mM stock) Make up to 100 ml pH to 7.4	10 g/100ml	

Appendix B

CONSENT FORM

THE BELLVILLE, SOUTH AFRICA CARDIOVASCULAR STUDY CONSENT FORM

Principal Investigators: Prof. Tandi Matsha (CPUT)

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Dear Participant,

You are invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied and that you clearly understand what this research entails and how you could be involved. Also, your participation is entirely voluntary and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do initially agree to take part.

This study has been approved by the Health and Wellness Sciences Research Ethics Committee at the Cape University of Technology and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

Worldwide new causes of certain diseases or conditions are continuously being discovered by research on the cells and molecules of the body. For research to be carried out on certain diseases it is necessary to first establish the incidence and prevalence of the disease. This project

aims to determine the incidence, prevalence as well as factors which are predictive of cardiovascular disease. When a large group of patients with similar diseases has been collected, meaningful research into the disease processes may become possible.

Why have you been invited to participate?

The prevalence of cardiovascular disease in South Africa is not well documented and few studies have been conducted since 1994. Many subjects with cardiovascular disease are unknown to the health service, often because they are not yet diagnosed. In order to assess the magnitude of the problem, you have been approached to participate in this project to determine the incidence of cardiovascular disease amongst our adult population.

You have been selected since you previously participated in a diabetes study and that you were aged between 35 - 65 years at the time. You also meet other criteria used to select participants into this study.

What will your responsibilities be?

The participant will be requested to provide information about his/her medical history with specific emphasis on cardiovascular disease. You will also be asked to complete a questionnaire which will take no longer than 10 minutes. A painless procedure known as electrocardiography (ECG) will then be performed to check the health of your heart. No pharmaceutical agents (medication) will be tested in the study.

Will you benefit from taking part in this research?

You will be notified of your cardiovascular status by the medical nurse or doctor. Thereafter, you will be referred to your local health center or general practitioner for further investigations and treatment if necessary.

In the unlikely event that the research may lead to the development of commercial applications, the participant or the participant's heirs will not receive any compensation, but profits will be reinvested into supporting the cause of further research which may bring benefits to me/the participant's family and to the community, such as health screening, medical treatment, educational promotions, etc.

Are there any risks involved in my taking part in this research?

There are no risks involved when you take part in this study since it will not involve any invasive procedure.

Who will have access to your medical records?

The participant's identity will be kept confidential throughout. Information will not be associated with the participant's name. The research staff will use only a coded number, access will be limited to authorized scientists and any scientific publications, lectures or reports resulting from the study will not identify me/*the participant.

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

Will you or your child be paid to take part in this study and are there any costs involved?

You will not be paid to take part in the study, but your transport, if required will be covered for each study visit. There will be no costs involved for you if you take part in the project.

Is there anything else that you should know or do?

You should inform your family practitioner or usual doctor that you are taking part in a research study.
You can contact Prof Erasmus at Tel: 021 938 4107 or rte@sun.ac.za, if you have any further queries or encounter any problems.
You can also contact the chairperson of Health and Wellness Sciences Research Ethics Committee of the Cape Peninsula University of Technology at 021 442 6162 or engelhillssp@cput.ac.za, if you have any concerns or complaints that have not been adequately addressed by the research staff.

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

DECLARATION BY PARTICIPANT:

I declare that:

- I have read or had read to me this information and consent form and that it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is voluntary and I have not been pressurized to take part.
- I may choose to withdraw from the study at any time and will not be penalized or prejudiced in any way.

- I may be asked to leave the study before it has finished if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan as agreed to.
- I also consent that my information may be:

i Used and kept for future research studies

ii Used and discarded

Signed at (place) .....…..…….. On (date) ……...…….. 20....

Signature of participant---------- Signature of witness----------

DECLARATION BY THE INVESTIGATOR

I (name)……..…… declare that: I explained the information in this document to (Names of
Participant)…….……
I encouraged him/her to ask questions and took adequate time to answer them.
I am satisfied that he/she adequately understands all aspects of the research, as discussed above.

Signed at (place) .....…..…….. On (date) ……...…….. 20....

Signature of investigator----------Signature of witness----------