

# **THE EFFECT OF FRUCTOSAMINE 3 KINASE (FN3K) GENOTYPES ON THE GLYCATION GAP IN TYPE 2 DIABETIC AND NON-DIABETIC MIXED ANCESTRY POPULATION OF SOUTH AFRICA**

**BY**

**Dipuo Dephney Motshwari**

Thesis presented in fulfilment of the requirements for the  
degree of Masters of Science (Chemical Pathology) at the  
University of Stellenbosch



UNIVERSITEIT  
iYUNIVESITHI  
STELLENBOSCH  
UNIVERSITY

**100**  
**Supervisor**  
1918 · 2018

**Professor Rajiv T Erasmus**

**Co-supervisors**

**Professor Annalise E Zemlin**

**Professor Tandi E Matsha**

**December 2018**

## Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Signature:

Date:

Copyright © 2018 Stellenbosch University  
All rights reserved

# Abstract

## Introduction

In 2017 the International Diabetes Federation (IDF) reported that approximately 425 million adults aged 20-79 years were estimated to have diabetes mellitus (DM) worldwide. The non-enzymatic glycation reactions of proteins such as haemoglobin have been associated with the development of diabetic related complications. These reactions were believed to be irreversible until the discovery of a protein repair enzyme fructosamine 3 kinase (FN3K). This enzyme deglycates glycated haemoglobin (HbA1c) in erythrocytes and other glycated proteins in other tissues. Animal model studies found that the activity of this enzyme varies between individuals leading to differences in HbA1c levels. This results in discrepancies between HbA1c and other glycaemic measures which is termed the glycation gap. The glycation gap is consistent over time within individuals and is associated with diabetic complications. Genetic variants in the FN3K gene have been associated with altered enzyme activity. Therefore, the aim of this study was to examine the role of FN3K genotypes on the glycation gap

## Methods

A total of 1412 subjects (925 normal, 216 pre-diabetic and 271 type 2 diabetics), with 339 males and 1073 females aged  $\geq 20$  years of mixed ancestry descent, residing in Bellville South, South Africa were included in this study. The diabetics were diagnosed using the oral glucose tolerance test. The glycation gap was determined according to a formula: Glycation gap = HbA1c - FHbA1c, (FHbA1c =  $\{[(\text{fructosamine} - \text{mean fructosamine}) / \text{SD fructosamine}] \times \text{SD HbA1c}\} + \text{mean HbA1c}$ ). DNA was extracted from whole blood using the salt extraction method. FN3K single nucleotide polymorphisms (SNPs) were genotyped with the Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System 96 well fast from Thermo Fisher Scientific. HbA1c was measured using HPLC (Biorad Variant Turbo) and fructosamine was measured using a colorimetric test nitro-blue-tetrazolium (NBT).

## Results

SNP c. -232A/T deviated from Hardy Weinberg Equilibrium (HWE) and was left out for the rest of the statistical analysis. The polymorphism G900C followed the Hardy-Weinberg Equilibrium and was therefore studied. The genotype frequencies for SNP G900C in the

glycaemic sub-groups were as follows, GG: 45.9 %, GC: 43.7 %, CC: 10.4 % in normal subjects; GG: 48.6 %, GC: 41.7 %, CC: 9.7% in pre-diabetics and GG: 41.7 %, GC: 46.5 %, CC: 11.8 % in diabetics, and they followed the Hardy-Weinberg equilibrium. There were no significant differences in the SNP G900C genotype frequencies between the glycaemic sub-groups. The glycation gap significantly decreased across the GG, GC and CC genotype variants in males, mean  $\pm$  SD were  $-0.13\pm 0.86$ ,  $-0.25\pm 0.72$  and  $-0.80\pm 1.04$  respectively, ( $P=0.0239$ ). However the difference was not observed in females. Moreover the glycation gap showed a positive correlation with non glycaemic factors including body mass index (BMI) ( $r=0.3694$ ,  $p<0.0001$ ), waist circumference (waistC) ( $r=0.3749$ ,  $p<0.0001$ ), hip circumference (hipC) ( $r=0.3151$ ,  $p<0.0001$ ), triglycerides ( $r=0.2540$ ,  $p<0.0001$ ) and a negative correlation with high density lipoprotein cholesterol (HDL-Chol) ( $r=-0.2031$ ,  $p<0.0001$ ).

## **Conclusion**

In conclusion the present study found that the glycation gap might be influenced by genetic active mechanisms in the intracellular erythrocyte compartment. Identification of the G900C polymorphism in an early stage of diabetes could be useful especially in therapeutic decisions and prediction of improved prognosis. However, there are other confounding factors influencing the glycation gap and future studies are required to confirm these findings.

# Dedication

To the one who made it possible, Thank you Lord!!!  
This thesis is dedicated to my parents and siblings who have always supported me and encouraged me. I love you all.

## Acknowledgement

Firstly, I would like to express my sincere gratitude to my supervisor Prof. Rajiv T Erasmus for the continuous support during my MSc. study and research. I appreciate his patience, motivation, enthusiasm, immense knowledge as well as financial help. I could not have imagined having a better advisor and mentor for my MSc. study. I would also like to express all my gratitude to my co-supervisors Prof. Annalise E Zemlin and Prof. Tandi E Matsha for their passionate participation, insightful comments, motivation and support throughout the project data analyses and thesis write up. I couldn't have made it without you.

My sincere gratitude also to Dr. Gloudina Hon- it wouldn't have been possible without her help. I appreciate the hard work she puts during the course of this study and assistance with the laboratory work, data analyses and thesis write up. I would like to thank Mrs. Soraya Chalklen and Ms. Saraah Davids as well as my laboratory mates Ms. Desiree Lem, Mr. Setjie Maepa, Mr. Cecil Weale, Mr. Lwando Mampunye, Mr. Abisola Okunola, Ms. Kelebogile Moremi and Mr. Don Matshazi for the stimulating discussions, for the sleepless nights where we worked together, for the support and for all the fun we have had in the last three years.

Last but not least, a special thank you to my mother Salphinah Motshwari. You are an amazing woman and I thank you for being there with me every step of the way. My father Thomas Motshwari you are my hero and I thank you. My siblings Tumiso Motshwari, Lehlogonolo Motshwari and Lebo Motshwari your love and support kept me going thank you. And to the rest of my family, your unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

TO GOD BE THE GLORY!!!

# Table of Contents

Declaration.....	i
Abstract.....	ii
Dedication.....	iv
Acknowledgement .....	v
Table of Contents .....	vi
List of Scientific conference output and research visit .....	viii
List of figures .....	ix
List of tables.....	x
List of abbreviations.....	xi
Chapter 1: Literature review.....	1
1.1. Diabetes Mellitus .....	1
1.2. Classification of DM.....	1
1.2.1. Type 1 diabetes mellitus (Type 1 DM).....	2
1.2.2. Type 2 diabetes mellitus (Type 2 DM).....	3
1.2.3. Gestational diabetes mellitus (GDM).....	3
1.2.4. Latent autoimmune diabetes of Adults (LADA) .....	4
1.2.5. Other types of diabetes mellitus .....	5
1.2.6. Prediabetes .....	6
1.3. Aetiology.....	7
1.4. Epidemiology .....	7
1.5. Diabetes diagnostic criteria.....	9
1.6. Non-enzymatic glycation of proteins.....	13
1.6.1. Glycated haemoglobin (HbA1c) .....	14
1.6.2. Fructosamines.....	16
1.6.3. Advanced glycation end products (AGEs).....	17
1.7. Diabetic complications .....	19
1.8. Glycation gap.....	21
1.9. Deglycation.....	23
1.9.1. Discovery of Fructosamine 3 Kinase (FN3K) .....	23
1.9.2. FN3K specificity, properties and role in deglycation.....	24
1.9.3. FN3K substrate and tissue distribution.....	26
1.9.4. Variability of FN3K activity.....	26
1.9.5. Other Repair Enzymes.....	27
Purpose of the study.....	28

Chapter 2: Methodology.....	31
2.1. Study design.....	31
2.2. Study setting.....	31
2.3. Sample size.....	31
2.4. Study population.....	32
2.4.1. Inclusion criteria.....	32
2.4.2. Exclusion criteria.....	32
2.5. Data collection.....	32
2.5.1. Clinical data.....	32
2.5.2. Biochemical data.....	35
2.5.3. Genotyping.....	36
2.6. Statistical analysis.....	39
2.7. Ethical consideration.....	40
Chapter 3: Results.....	41
3.1. The general characteristics of the study population.....	41
3.2. The Hardy-Weinberg Equilibrium (HWE) results.....	44
3.3. The general characteristics of the study population categorized by gender and SNP G900C genotypes.....	46
3.4. Characteristics of the total study population categorized according to the Glycation-gap and glycaemic sub-groups.....	49
3.5. Correlation analysis.....	52
Chapter 4: Discussion.....	55
4.1. Introduction.....	55
4.2. FN3K genotypes and the glycation gap.....	57
4.3. Glycation gap correlation analysis.....	60
Chapter 5: Conclusion.....	63
5.1. Limitations of the study.....	63
5.2. Clinical implications.....	64
5.3. Recommendation for future studies.....	64
Reference.....	66
Appendices.....	80
Appendix A: Ethics Approval.....	81
Appendix B: Salt Extraction method (Laboratory Protocol 2014).....	83
Appendix C: Consent Form.....	85



## List of Scientific conference output and research visit

### Conference Poster presentation

- International Federation of Clinical Chemistry and Laboratory Medicine WorldLab 2017, Durban, South Africa 22<sup>nd</sup> – 25<sup>th</sup> October 2017. Poster title: “Glycation gap in newly diagnosed and treated South African mixed ancestry individuals with diabetes”
- 1<sup>st</sup> World Congress on Migration, Ethnicity, Race and Health. 17-19 May 2018, EICC, Edinburgh. Poster title: “The effect of metabolic syndrome on the glycation gap in diabetic mixed ancestry population from South Africa”

### Conference oral presentation

- 56th International FSASP Congress Stellenbosch South Africa 16<sup>th</sup> -18<sup>th</sup> August 2018. “The CC variant of c.900G/C polymorphism of fructosamine-3- kinase (FN3K) gene is associated with lower levels of the glycation gap”.

### List of Research visits

- Research visit to the Clinical Chemistry Laboratory at the Ghent University Hospital in Belgium, Europe, for training of a colorimetric assay for FN3K enzyme activity. 13<sup>th</sup> August 2017- 24<sup>th</sup> August 2017

# List of figures

## Chapter 1

Figure 1.1: Change in diagnostic value for FPG

Figure 1.2. Initial steps of the Maillard reaction between lysine and glucose

Figure 1.3. Increased production of AGE precursors and its pathologic consequences

Figure 1.4. Proposed role of FN3K as a catalyst in the decomposition of FL.

## Chapter 3

Figure 3.1. The glycation-gap in females and males

Figure 3.2. The glycation-gap and the FN3K SNP G900C (rs1056534) genotypes in males

Figure 3.3. The glycation-gap correlation with the BMI ( $\text{kg/m}^2$ ) in the total group of subjects

## Chapter 4

Figure 4.1 Summarized role of FN3K SNP G900C genotypes (CC, GC and GG)

## List of tables

### Chapter 1

Table 1.1: World Health Organization (WHO) Classification of Hyperglycaemia in pregnancy

Table 1.2: Classification of MODY

Table 1.3: Criteria for normoglycaemia, prediabetes and the diagnosis of diabetes

### Chapter 2

Table 2.1: The reaction master mix used in PCR amplification

Table 2.2: Preparation of reaction for 96-well plate for PCR amplification

### Chapter 3

Table 3.1. Characteristics of the study population categorized by gender

Table 3.2. Genotype distributions, minor allele frequencies

Table 3.3. The general characteristics of the study population categorized by gender and SNP G900C genotypes

Table 3.4. Characteristics of the total study population categorized according to the Glycation-gap and glycaemic sub-groups

Table 3.5. Correlation between the general characteristics of the study population and the Glycation-gap, categorized by gender

## List of abbreviations

AACC	American Association of Clinical Chemistry
ADA	American Diabetes Association
ADAG	A1c-derived Average Glucose study group
AGEs	Advanced glycation end products
AIDS	Acquired immunodeficiency syndrome
ATP	Adenosine triphosphate
$\beta$ cells	Beta cells
BMI	Body mass index
Chol	Cholesterol
cm	Centimeter
CML	Ne - (carboxymethyl) lysine
CRP	C-reactive protein
CVDs	Cardiovascular diseases
DBP	Diastolic blood pressure
DCCT	Diabetes Control and Complications Trial
DG	Deoxyglucosone
DMF	Deoxymorpholino fructose
DCCT	Diabetes Control and Complications Trial
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
eAG	estimated average glucose
EDTA	Ethylene diamine tetra-acetic acid
FHbA1c	Fructosamine derived glycated haemoglobin
FL	Fructose lysine
FL3P	Fructoselysine-3-phosphate
FPG	Fasting plasma glucose
FN3K	Fructosamine 3- kinase
GA	Glycated albumin
GAD	Anti-glutamic acid decarboxylase
GDM	Gestational diabetes mellitus
GFR	Glomerular filtration rate

GLUT	Glucose transporter
HbA1c	Glycated haemoglobin
HbS	Hemoglobin S
HDL	High density lipoprotein
HGI	Haemoglobin glycation index
HipC	Hip Circumference
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HNF	Hepatocyte nuclear factor
HPLC	High performance liquid chromatography
HPLC-CE	High performance liquid chromatography-Capillary Electrophoresis
HPLC-MS	High performance liquid chromatography-Mass Spectrophotometry
HWE	Hardy-Weinberg Equilibrium
IDDM	Insulin-dependent diabetes mellitus
IDF	International Diabetes Federation
IEC	International Expert Committee
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IPF	Insulin promoter factor
JDS	Japanese Diabetes Society
KAPT	Adenosine triphosphate sensitive potassium channel
kg	Kilogram
LDL	Low Density Lipoprotein
m	Meters
mIU/L	Mill international units Per Litre
mmol/L	Mill moles Per Litre
mmHg	Millimetre of mercury
MODY	Maturity Onset Diabetes of the Young
NBT	Nitro blue tetrazolium
NCDs	Non communicable diseases
ng/mL	Nano grams Per Mililitre
NGPS	National Glycohaemoglobin Standardization Program
NHANES	National Health and Nutrition examination Survey

NeuroD	Neurogenetic differentiation
OGTT	Oral glucose tolerance test
PCR	Polymerase Chain reaction
POC	Point of care
2h-PG	2-hour post glucose
2h-PI	2-hour post insulin
RAGE	Receptors of advanced glycation end products
RBC	Red Blood Cell
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SADHSR	South African Demographic and Health Survey Report
SBP	Systolic blood pressure
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
SSA	Sub Saharan Africa
SUR1	Sulfonylurea receptor 1
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TB	Tuberculosis
WaistC	Waist circumference
WHR	Waist hip ratio
WHO	World Health Organization

# Chapter 1: Literature review

## 1.1. Diabetes Mellitus

Diabetes Mellitus (DM) is defined as a disorder of glucose metabolism characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from the inability of the pancreas to produce enough insulin, or when the body cannot effectively use the insulin it produces or both (Diabetes care 1997). Insulin is a hormone produced in the pancreas by the beta cells in the islets of Langerhans and it stimulates the cells in the body to take up glucose from the blood thereby regulating carbohydrate, lipid and protein metabolism. Insulin achieves this by binding to cell receptors and promoting glucose uptake by mobilizing glucose transporter-4 (GLUT-4) to the surface of muscle and adipose tissue. Furthermore, it increases glycogen storage in liver and muscle, fatty acids synthesis, and reduces glucose output by the liver (Cartee 2015). Therefore, failure to produce insulin or to respond to it can lead to DM and hyperglycaemia. Over time, the resulting high glucose levels in the blood damages many tissues in the body, leading to the development of microvascular complications involving small vessels (retinopathy, nephropathy and neuropathy) and macrovascular complications (myocardial infarction, stroke, and arterial disease of the lower extremities) as a result of acceleration and exacerbation of atherosclerosis (Seino et al. 2010).

DM accounted for 10.7 % of global all-cause mortality in 2017 among people in this age group (IDF 2017). This number was found to be higher than the combined number of deaths from infectious diseases (1.1 million deaths from Human Immunodeficiency Virus / Acquired Immunodeficiency Syndrome (HIV/AIDS) (WHO 2015), 1.8 million from tuberculosis (WHO 2016) and 0.4 million from malaria in 2015 (WHO 2015). The African region had the highest proportion of people who died from diabetes before the age of 60 in 2017 at 77.0 % (0.23 million death) (IDF 2017).

## 1.2. Classification of DM

Although all forms of DM are characterized by hyperglycaemia, the mechanism by which this develops differs. The classification of DM is based on the cause and the extent of the underlying disease process based on the degree of insulin action deficiency. These disorders are classified into five groups: the three main types which are type 1 diabetes

mellitus (type 1 DM), type 2 diabetes mellitus (type 2 DM) and gestational diabetes mellitus (GDM) and less common ones including latent autoimmune diabetes of the adults (LADA) and DM due to other specific mechanisms or diseases. There is also secondary DM which arise as a consequent of other diseases such as Cushing's disease, pancreatitis or due to drugs such as corticosteroids, pancreatic cancer is another reason to have diabetes when followed by total pancreatectomy and such patients will rely on insulin for the rest of their life (IDF 2017). Patients with any form of DM may require insulin therapy; for this reason, the previously used terms insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) have been eliminated.

### 1.2.1. Type 1 diabetes mellitus (Type 1 DM)

Type 1 DM is less common accounting for only 5 – 10% of cases. It is caused by lack of insulin due to pancreatic  $\beta$ -cell destruction as a result of an autoimmune reaction triggered by different factors (Imagawa et al. 2003). Markers which identify the autoimmune process leading to  $\beta$ -cell destruction include anti-glutamic acid decarboxylase (GAD), islet cell autoantibodies (ICA) and / or autoantibodies to insulin (ADA 2006). Therefore, type 1 DM can further be classified as autoimmune or idiopathic. When the autoantibodies are identified in the early phase, it is referred to as autoimmune type 1 DM and where they are not identified in the early stage it is referred to as idiopathic type 1 DM (Imagawa et al. 2003). The development of type 1 DM is associated with certain hereditary factors, such as human leucocyte antigen ((HLA) alleles in which the pancreatic  $\beta$ -cells do not secrete adequate or no insulin (Knip & Siljander 2008) and environmental factors, such as a viral infection which results in the body's immune system attacking and destroying the  $\beta$ -cells of the pancreas (Imagawa 2004).

The rate of  $\beta$ -cell destruction is variable, being rapid in some individuals (mainly infants and children) and slow in others (mainly adults) (Kobayashi et al. 1993). Type 1 DM often develops suddenly and presents with symptoms such as excessive thirst (polydipsia) and a dry mouth, frequent urination (polyuria), lack of energy, extreme tiredness, constant hunger, sudden weight loss and blurred vision (IDF, 2015). Some patients, particularly children and adolescents, may present with ketoacidosis as the first manifestation of the disease. Ketoacidosis is a pathological metabolic acidosis associated with high concentration of ketone bodies, caused the breakdown of fatty acids and the deamination of amino acids due



to lack of circulating insulin (Dillard-cannon 2014). In severe cases, ketoacidosis can be fatal. Ketoacidosis can also develop in T2DM patients requiring insulin therapy for diabetic control, but is more common in T1DM (Dillard-cannon 2014).

### 1.2.2. Type 2 diabetes mellitus (Type 2 DM)

This is the most common type of DM accounting for 90–95% of cases and is characterized by decreased insulin sensitivity (insulin resistance) resulting in relative but not absolute insulin deficiency (Hancock et al. 2008). Although the onset of type 2 DM used to be common in middle age or later, it is being increasingly described in children and young adults, most likely due to an increase in obesity (Hirata et al. 1997). Type 2 DM frequently goes undiagnosed for many years as hyperglycaemia develops gradually and in earlier stages is often not severe enough for the patient to notice any of the classic symptoms. The symptoms include frequent urination within short intervals, excessive thirst, excessive urge to eat (polyphagia), weight loss, blurred vision and increased susceptibility to infections (IDF , 2015). Hence type 2 DM patients are at increased risk of developing macrovascular and microvascular complications. In some instances, the diagnosis of type 2 DM is made at an advanced stage when the individual has already developed complications which are irreversible or when they are in a coma (Costa et al. 2007).

### 1.2.3. Gestational diabetes mellitus (GDM)

GDM is defined as any degree of glucose intolerance that is initially discovered or develops during pregnancy. The definition applies regardless of whether insulin or only diet modification is necessary for treatment or whether the condition persists after pregnancy (Diabetes 2010). Pregnancy triggers the manifestation of a glucose metabolism disorder, hence the diagnosis and control of GDM require special considerations, as even a comparatively mild disorder in glucose metabolism during pregnancy can exert significant influence on the infant and mother (Diabetes care 2010). It may be difficult to distinguish GDM from normal pregnancy symptoms, as they may include increased thirst and frequent urination (IDF 2015). Screening by means of an oral glucose tolerance test (OGTT) is therefore recommended as summarized in table 1.1. This should be conducted early in pregnancy for high risk woman (women who are > 25 years of age, overweight or obese, have family history of DM, certain ethnicities, previous delivery of a large baby or previous

unexplained miscarriage or stillbirth) and between the 24th and 28th week of pregnancy in all other women (Rohlfing et al. 2002). Although GDM normally disappears after delivery, these women are at higher risk of developing GDM in subsequent pregnancies and type 2 DM later in life. Babies born to mothers with GDM also have a higher risk of developing type 2 DM in their teens or early adulthood (Fetita 2006).

Table 1.1: World Health Organization (WHO) Classification of Hyperglycaemia in Pregnancy (WHO 2013)

Fasting plasma glucose (FPG) 5.1-6.9 mmol/L
Or
One-hour plasma glucose $\geq$ 10.0 mmol/L following a 75g oral glucose load
Or
Two-hour plasma glucose 8.5-11.0 mmol/L following a 75g oral glucose load

#### 1.2.4. Latent autoimmune diabetes of Adults (LADA)

LADA is a type of DM that develops slowly in adults and is positive for an autoantibody to GAD or ICA but does not require insulin-therapy at the time of diagnosis (Zimmet 1995). However, unlike the classic type 2 DM patients who are negative for islet autoantibodies, LADA patients rapidly become insulin dependent due to the insulin cell attack by their body. LADA has been considered as intermediate diabetes due to the fact that it shares immunological and genetic aspects with type 1 DM and it affects an age group that is typically affected by type 2 DM (Palmer 2003). The Immunology of Diabetes Society established the diagnoses of LADA using three main criteria: adult age of onset (>30 years); presence of any ICA and absence of insulin requirement for at least 6 months after diagnosis (Gottsa et al. 2005). The clinical features of LADA patients include weight loss, susceptibility to ketosis, unstable blood glucose levels and extremely diminished C-peptide reserve (Kobayashi et al. 1993).

LADA is not as rare as previously reported, and may be more prevalent than type 1 DM but less frequently than type 2 DM (Hawa et al. 2013). LADA may account for 2 - 12 % of all cases of DM in adults (Kobayashi et al. 1993; Zimmet 1995; Juneja et al. 2001). The United Kingdom Prospective Study of Diabetes (UKPDS) demonstrated that about 10% of adults with suspected type 2 DM at the time of diagnosis had evidence of islet autoimmunity in the

form of circulating ICA or GAD antibodies and most progressed to dependence on insulin in 6 years (Turner et al. 1997). Although both LADA and type 1 DM are autoimmune it has been demonstrated that anti-GAD and ICA are much more common than insulin autoantibodies (IAA), anti-tyrosine phosphatase (IA-2A), and zinc transporter (ZnT8) antibodies in LADA compared to type 1 DM (Juneja et al. 2001; Wenzlau et al. 2008). HLA studies may be of value in differentiating between type 1 DM and LADA. LADA has a higher frequency of HLA characteristic of type 1 DM: HLA-DR3 (28% of patients), DR4 (27%) and DR 3/4 (22%), in comparison to the general population (Cervin et al. 2008).

### 1.2.5. Other types of diabetes mellitus

There are several heredity forms of DM that are associated with monogenetic defects in  $\beta$  cell function. They are frequently characterized by onset of hyperglycaemia at an early age (generally before 25 years) and are referred to as maturity onset diabetes of the young (MODY). They present with impaired insulin secretion with minimal or no defects in insulin action. The development of MODY is due to a single abnormal gene - hence it is referred to as monogenic DM in order to distinguish it from type 1 DM and type 2 DM which are caused by multiple environmental and genetic factors (Yorifuji et al. 2004). MODY is caused by mutations in an autosomal dominant gene leading to disruption in insulin secretion (Goldstein & Müller-Wieland 2016). There are different types of MODY due to different genetic abnormalities as summarized in Table 1.2 (Doria et al. 1999).

Table 1.2: Classification of MODY

Types of MODY	Genetic abnormalities
MODY 1	Hepatocyte nuclear factor 4 (HNF-4a)
MODY 2	Glucokinase
MODY 3	HNF-1a
MODY 4	Insulin promoter factor-1 (IPF-1) (Pdx-1)
MODY 5	HNF-1b
MODY 6	Neurogenetic differentiation 1 (NeuroD1) / Beta 2 ( $\beta 2$ )
Neonatal diabetes	Kir6.2 and sulfonylurea receptor 1 (SUR1) (encode subunits of the adenosine triphosphate sensitive potassium channel (KATP) in $\beta$ -cells) (Babenko et al. 2006)

### 1.2.6. Prediabetes

This is a state where the blood glucose level is higher than normal but not high enough to be classified as DM and is a high-risk state for the development of DM (Nordwall et al. 2015). According to the World Health Organization (WHO), prediabetes is classified as one of two distinct states: impaired fasting glucose (IFG) with higher than normal glucose levels after a period of fasting, or impaired glucose tolerance (IGT) with higher than normal glucose levels following OGTT (Imagawa et al. 2003). Although not all prediabetic's develop type 2 DM, they are considered to be at high risk especially those with IGT (Shaw et al. 1999) and also have an increased cardiovascular risk (Perry 1999).

Prediabetics are often asymptomatic therefore it is important that the health care provider excludes prediabetes in high risk individuals such as those with increasing age, weight, family history of DM, certain ethnicities and history of GDM (ADA 2010). It has been reported that 5-10% of prediabetics develop DM annually (Nathan et al. 2007). Approximately 352.1 million people worldwide, namely 7.3% of adults aged 20 – 79 years were estimated to have IGT in 2017 (IDF 2017). The number of adults with IGT in Africa is expected to double to 154.3 million by 2045 with lack of interventions (IDF 2017). However, several studies have shown that the prediabetic state is reversible, with lifestyle and drug-based interventions

(Ramachandran et al. 2006 ; DPPR 2009). Hence it is important that prediabetics are followed up every one to two years and encouraged to undergo intensive lifestyle modification.

### 1.3. Aetiology

DM is due to multifactorial aetiologies. The causes of DM may be due to factors such as family history, ethnicity, genetic make-up, health and environmental factors. The causes of type 1 DM include autoimmunity and genetic factors. However, there are some risk factors which may trigger the development of type 1 DM including: viral or bacterial infection, chemical toxins within food and unidentified components causing autoimmune reaction by triggering the T cells to target the  $\beta$ -cells (Gavin et al. 1997 ; ADA 2010). Some environmental factors may expose the defects in genetics and it has been reported that an early introduction of supplementary milk feeding during infancy may increase the risk of type 1 DM in children carrying HLA class II genotypes than among those with low or decreased risk genotypes (Knip et al. 2005).

The causes of type 2 DM are usually multifactorial. The risk of developing type 2 DM is increased by both environmental and genetic factors. The driving factors for the epidemic of type 2 DM are mainly obesity which is fueled by sedentary lifestyle, smoking, alcohol consumption, diet, decreased exercise level and urbanization (WHO 1994). All these factors result in reduced sensitivity of cells to insulin. Furthermore studies have shown that obesity itself causes some degree of insulin resistance (ADA 2010) Abdominal obesity is associated with increased inflammation and the secretion of adipokines which may predispose an individual to DM (Kahn et al. 2006). Multiple genetic factors are also said to be associated with reduced insulin secretion or insulin resistance, although the genetics of type 2 DM are complex and not clearly defined (ADA 2010).

### 1.4. Epidemiology

In 2017 the International Diabetes Federation (IDF) reported that approximately 425 million people or 8.8% of adults 20-79 years worldwide are estimated to have DM with about 79% living in low and middle income countries (IDF 2017). The prevalence of DM has increased rapidly over the past 5 years, with a global DM prevalence of 382 million, 387 million, 415 million and 425 million in 2013, 2014, 2015 and 2017 respectively (IDF 2013; IDF 2014; IDF 2015; IDF 2017). This number is projected to rise beyond 629 million people aged 20 - 79

in 2045 if no action is taken (IDF 2017). DM is a major cause of morbidity and mortality worldwide. Approximately 4.0 million people aged between 20 and 79 years died from DM in 2017, which is equivalent to one death every eight seconds.

The burden of DM is high in developing countries. In 2017 the IDF indicated that almost 79% of diabetics live in low and middle income countries, with two thirds of all cases arising from the Asia Pacific region, mainly China and India (IDF 2017). The high prevalence in these countries could be due to poor health care facilities such as inadequate diabetic management and public diabetes awareness. This is also applicable in African societies including South Africa. Another major factor contributing to the increasing rates of DM in low and middle income countries is urbanization (Levitt et al. 1999). As populations move towards the urban areas particularly in sub-Saharan Africa (SSA), this migration is undoubtedly associated with a shift in lifestyle from a relatively healthy traditional pattern, to urban areas with high rates of obesity due to increased food quantity and reduced quality, low levels of exercise, smoking and increased alcohol availability (Beaglehole & Yach 2003). Although, the majority of diabetics in SSA live in the cities, the population mostly (61.3%) originate from rural areas. In 2017 the IDF reported that the African region had the highest proportion of undiagnosed DM reported to be over 69.2% of adults (IDF 2017). However, the African region had the lowest prevalence of DM with an estimated 15.5 million adults aged 20-79 years having DM compared to other regions worldwide (IDF 2017). Therefore the high rate of death related to DM in Africa could be due to the fact that most of the diabetics are unaware of the disease hence not on treatment.

South Africa (SA) is one of the countries in Africa with the highest number of people with DM. In 2017 the IDF reported that the following countries had the highest prevalence of DM in Africa; Ethiopia (2.6 million), SA (1.8 million), Democratic Republic of Congo (1.7 million), and Nigeria (1.7 million) (IDF 2017). Several studies have reported that there are marked demographic and ethnic variations in the prevalence of DM in SA (Levitt et al. 1993; Motala et al. 2003). Motala et al performed a long term follow up study using 1985 WHO diagnostic criteria for glucose tolerance based on 75 g OGTT and reported that SA Indians have a high crude incidence (9.5%) of type 2 DM which is significantly associated with higher baseline blood glucose, body mass index (BMI) and obesity (Motala et al. 2003). The mixed ancestry population of SA was reported to have the second highest prevalence of type 2 DM (7.1% crude prevalence) after the Indian population but this study was performed 20 years ago using the WHO 1985 criteria (Levitt et al. 1999). However, a more recent cross sectional

study conducted between 2008 - 2009 in the Bellville South community, in the Western Cape province of SA used the updated WHO 1999 criteria and reported an increased prevalence of type 2 DM in individuals of mixed ethnic ancestry aged  $\geq 31$  years with a crude prevalence of 18.1% (Erasmus et al. 2012).

## 1.5. Diabetes diagnostic criteria

The diagnostic criteria for DM have changed several times over the past decades due to increased knowledge and understanding regarding its aetiology and pathogenesis. In the 1960s the criteria were based on OGTT but they were still not sure of the quantity of glucose to be ingested during the test and the diagnostic criteria were not yet standardized (WHO 1965). The National Diabetes Data Group (NDDG) of the United States of America proposed using 75 g OGTT for classification and diagnosis of DM (National Diabetes Data Group 1979) and this was adopted by WHO in 1980 (WHO 1980). Therefore, OGTT is a diagnostic test performed by ingesting a glucose load containing the equivalent of 75 g of anhydrous glucose dissolved in water after an overnight fast of about 10-12 hours. For clinical purposes the blood samples for glucose determination are taken immediately before and 2 hours after the glucose drink. DM is diagnosed when the 2-hour plasma glucose (2h-PG) is greater or equal to 11.1 mmol/L (Helminen et al. 2015). The OGTT is used both in clinical practice and by researchers to assess glucose tolerance (ADA 2017).

The OGTT is considered to be a gold standard test for DM diagnosis (Alberti & Zimmet 1998). The advantage of the OGTT is that it is a minimal risk procedure and it employs commonly available laboratory tests and clinical protocols. It is generally accessible for use in large-scale clinical and epidemiologic studies and has been widely used in evaluation of  $\beta$  cell dysfunction, obesity, prediabetes and DM (Chen et al. 2018). This test is highly sensitive and can be used for screening of DM (Seino et al. 2010). It has been confirmed that compared to FPG and HbA1c, the 2 hour PG diagnose more people with diabetes (ADA 2017). An OGTT is the only means of identifying people with IGT and is frequently needed to confirm or exclude an abnormality of glucose tolerance in asymptomatic people (WHO 2006). However, the OGTT is expensive, less reproducibility and inconvenient since it requires patients to fast and stay in the clinic for at least 2 hours and morning appointments or return visits for confirmatory test (Diabetes Care 2003).

In 1980, the WHO included FPG as one of the diagnostic criteria for DM, but they kept changing the cutoff (WHO 1980; WHO 1985; WHO 1999) as illustrated in Figure 1.1. FPG is a simple blood test performed in the morning after an overnight fast of 10 to 12 hours. DM is diagnosed when FPG is greater or equal to 7.0 mmol/L (WHO 1999). In 1999 the WHO updated their DM diagnostic criteria and recommended the lowering of the diagnostic value of the FPG threshold from 7.8 mmol/l to 7.0 mmol/L because this value was associated with an increased risk of microvascular and macrovascular diabetic complications (WHO 1999). The FPG is highly vulnerable to a number of pre-analytical variables including recent food ingestion, stress, severe illness, and sample storage as well as high within-subject biological variability. Also, a morning appointment is required with return visits for confirmatory tests making it inconvenient (Diabetes Care 2003). The American Diabetes Association (ADA) elected the international Expert Committee (IEC) in 1979 to revise and modify the previously recommended diagnostic criteria for DM by the WHO, 1985 and published its recommendation in 1997 (James et al. 1997).

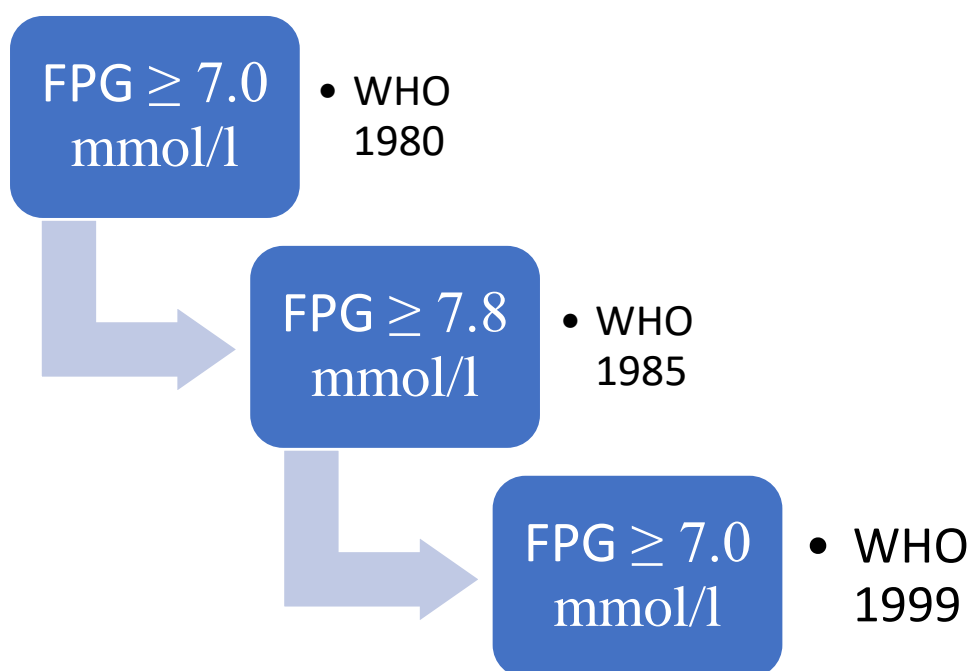


Figure 1.1: Change in diagnostic value for FPG

Since then, the only acceptable tests for the diagnosis of DM were based on a 10-hour FPG  $\geq 7$  mmol/l, a 2-hour OGTT  $\geq 11.1$  mmol/l or random plasma glucose (RPG) of  $\geq 11.1$  mmol/l in a patient with diabetic symptoms. RPG is a blood glucose level taken at any time of the day with no fasting required. It is performed when the person presents with typical diabetic



symptoms such as polyuria, polyphagia, polydipsia and excessive weight loss. This test is convenient but can only be used in symptomatic patients (Helminen et al. 2015). In 2004 the ADA and WHO reached a similar conclusion on the diagnostic criteria of DM (ADA 2004). However, the WHO further proposed a change in the criteria for prediabetes and normoglycaemia which differed from that of the ADA as summarized in table 1.3 (WHO 2006).

The IEC of the ADA, which includes the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) and the National Glycohaemoglobin Standardization Program (NGSP) recently recommended the use of glycated haemoglobin (HbA1c) as one of the diagnostic criteria for DM, with a threshold of  $\geq 6.5\%$  ( $\geq 48$  mmol/mol IFCC) recommended to diagnose DM (IEC 2009). This value was chosen due to the fact that the incidence of retinopathy, which is a common diabetic related complication, was found to be increased after this value (IEC 2009). Subsequently the ADA and WHO modified their diagnostic criteria in 2010 and 2012 respectively in order to incorporate HbA1c as part of the diagnostic criteria as summarized in Table 1.3 ( ADA 2010; John 2012). Individuals having HbA1c levels ranging from 5.7 to 6.4 % (39-46 mmol/mol IFCC) were classified as prediabetic as they are considered to be at risk for developing DM (George & Ja 2017). The value of HbA1c, which is equivalent to the internationally used HbA1c (%) defined by the NGSP, is expressed by adding 0.4% to the HbA1c (JDS) (%) defined by the Japanese Diabetes Society (JDS). It was then recommended that HbA1c testing should be performed using a method that is certified by the NGSP and standardized or traceable to the DCCT reference assay (ADA 2010).

**Table 1.3: Criteria for normoglycaemia, prediabetes and the diagnosis of diabetes  
(Adapted from WHO 2006 and 2011 and ADA 2010)**

	WHO criteria	ADA criteria
<b>Normoglycaemia</b> FPG: 2h-PG: HbA1c:	< 6.1 mmol/L < 7.8 mmol/L Not specified	< 5.6 mmol/L < 7.8 mmol/L < 5.7 %
<b>Prediabetes (IFG/IGT)</b> FPG: 2h-PG: HbA1c:	6.1- 6.9 mmol/L 7.8- 10.9 mmol/L Not specified	5.6 - 6.9 mmol/L 7.8 - 11.0 mmol/L 5.7 - 6.4 %
<b>Diabetes</b> *FPG: **2h-PG: ***RPG: ****HbA1c:	≥ 7.0 mmol/L ≥ 11.1 mmol/L ≥ 11.1 mmol/L ≥6.5 %	≥ 7.0 mmol/L ≥ 11.1 mmol/L ≥ 11.1 mmol/L ≥6.5 %

**Footnotes:**

- According to the WHO either \*/\*\*/\*\*\*/\*\*\*\* can be used to diagnose diabetes at the initial examination of diabetes.
- For confirmation of the diagnosis of diabetes a re-examination is done by repetition of \*/\*\*/\*\*\*/\*\*\*\* on another date but
- But if for initial examination \*\*\*/\*\*\*\* was used, a confirmation should be done with either \*/\*\* since results can't be reliable on repetition of this tests.
- For patients presenting with hyperglycaemia symptoms such as thirst, polydipsia, polyuria, weight loss or the presence of diabetic retinopathy, diabetes can be diagnosed on the first examination with either \*/\*\*/\*\*\*/\*\*\*\*.
- \*\*\*\*Conducted in a laboratory that is NGSP certified and standardized to the DCCT assay
- \*\*\*\* In conditions where HbA1c might be inappropriately low, either \*/\*\* should be used for diagnosis of diabetes
- Neither of the criteria \*/\*\*/\*\*\*/\*\*\*\* is suitable for point of care assays.

## 1.6. Non-enzymatic glycation of proteins

The non-enzymatic glycation of proteins is a spontaneous reaction that occurs between the free aldehyde group of glucose and free amino groups of proteins (Dziedzic et al. 2012). It was first described a century ago by Louis Camille Maillard who named it “Maillard reaction”, defined as the browning reaction between amino acids and simple carbohydrates (Maillard 1912). In the early 1980s this reaction by which glucose chemically bind to amino groups of proteins, without the aid of an enzyme was renamed non enzymatic glycosylation (Monnier & Cerami 1982). However few years later it was renamed non enzymatic glycation reaction in order to differentiate it from enzymatic glycosylation which is posttranslational modification of proteins catalyzed by specific enzymes (Yatscoff et al. 1984).

The non-enzymatic glycation reaction is subdivided into early and advanced steps. The reaction is initiated by the formation of an imine intermediate referred to as Schiff base. This reaction is reversible and occurs over a period of hours. The labile imine will then rearrange itself into stable covalently linked reversible Amadori products, over a period of weeks. In the advanced step, the Amadori product undergoes polymerization reactions including complex rearrangement, cleavage and covalent binding reactions whereby heterogynous structures named advanced glycation end products (AGE) which are nonreversible are formed (Baynes and Thorpe 2000 ; Monnier 2003). A simplified scheme of the non-enzymatic reaction is outlined in Figure 1.2.

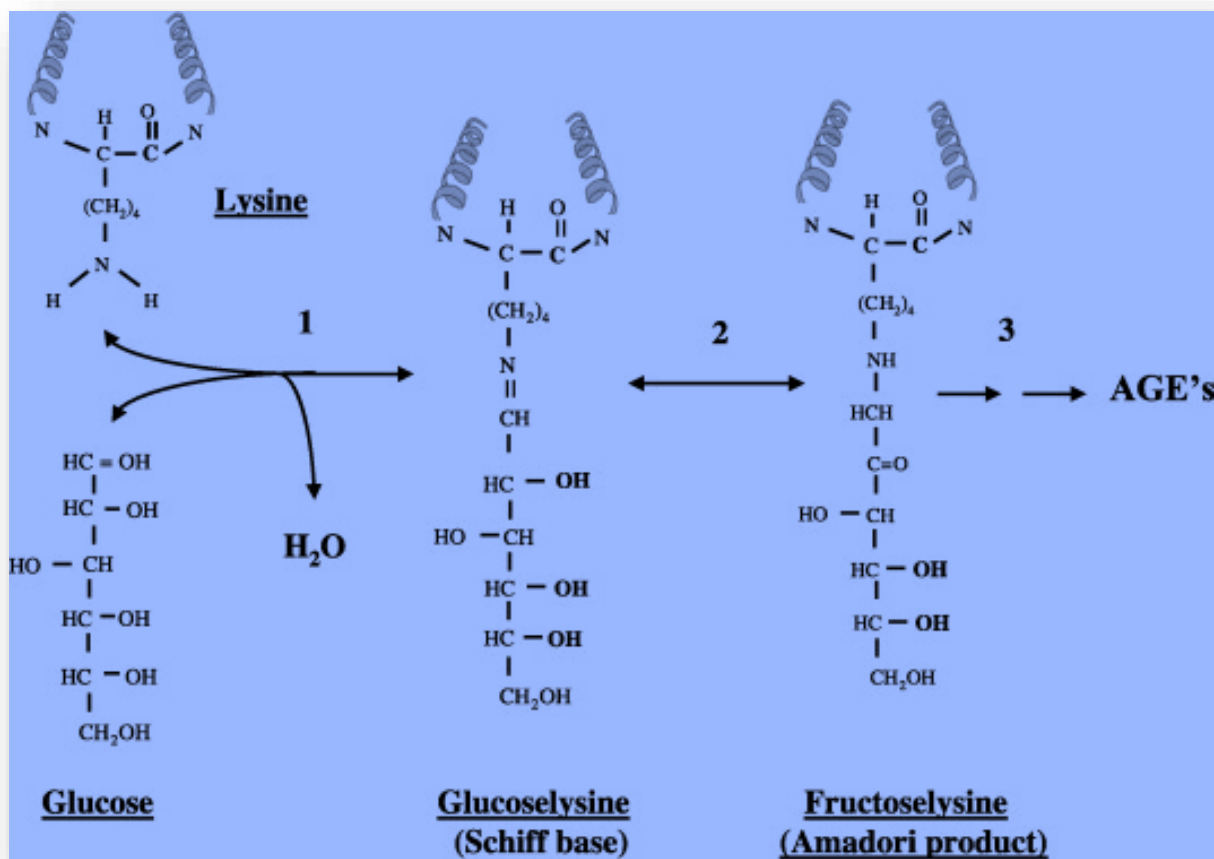


Figure 1.2. Initial steps of the Maillard reaction between lysine and glucose. Adapted from (Szwergold et al. 2002). The formation of Schiff bases, fructoselysines and AGE's are all reversible. Step 1 (formation of Schiff bases) is much faster compared to step 2 (formation of fructoselysines) and step 3 (formation of AGE's).

### 1.6.1. Glycated haemoglobin (HbA1c)

HbA1c as defined by IFCC is a haemoglobin molecule with glucose bound to its N-terminal valine of the  $\beta$  chain ( $\beta$ N-1-deoxyfructosyl-haemoglobin) (Jeppsson et al. 2002). The HbA1c test measures the percent of haemoglobin in circulating erythrocytes that has non-enzymatically reacted with glucose and represents the average plasma glucose concentration for a period of three months, the lifespan of red blood cells (Franco 2012). It was discovered in the late 1960s by Samuel Rahbar when he was scanning blood samples for novel haemoglobin variants and discovered a blurry band "HbA1c" increased in diabetics (Rahbar 1968). However, it was not believed then that HbA1c was a measure of diabetic control until a study performed in diabetic and non-diabetic mice using erythrocytes labelled with radioactive iron to track the cells 'age in mice and results described that HbA1c levels increased over the lifetime of a cell and importantly HbA1c increased 2.8 times faster in

diabetic mice (Koenig & Cerami 1975). HbA1c was then introduced into the clinical laboratories for glycaemic monitoring in 1997 although at that time methods displayed poor precision and there were no calibrators or material with assayed values for quality control purposes (John et al. 2007).

The evidence to use HbA1c for glycaemic control was based upon the results of the two landmark studies, the Diabetes Complications and Control Trial (DCCT) and the UKPDS. The DCCT study was performed in young type 1 diabetics and demonstrated that there is a continuous increase in the risk of complications with increasing HbA1c values (DCCT 1993). The UKPDS study was performed in type 2 diabetics and described that tight glycaemic control by maintaining lower levels of HbA1c using an intensive glucose-control treatment policy substantially reduced the risk of developing diabetes-related microvascular complications (UKPDS 1998). Standardization of HbA1c became an important issue after the publication of the DCCT study. The method used to determine HbA1c in this study was not suitable as a primary reference method and a purified standard for this method could not be prepared. Therefore, the American Association of Clinical Chemistry (AACC) in 1994 established the NGSP in an attempt to harmonize the HbA1c test results to those reported in the DCCT (Little & Goldstein 1995). The NGSP managed to harmonize HbA1c using the results from the DCCT study by a nonspecific Bio- Rex 70 ion exchange HPLC. In 1995 the standardization system of the JDS used a set of calibrators to harmonize results of HbA1c in Japan (Shima 1994) and in 1998 the Swedish Standardization Scheme similarly to NGSP, was based on the MonoS HPLC method as designated comparison method (DCM) for the harmonization of HbA1c measurements (Jeppsson et al. 1986).

HbA1c was then harmonized to the DCCT, but not yet standardized implying that the variation in HbA1c levels were reduced but not yet eradicated. In 1995 the IFCC Working Group (IFCC WG-HbA1c) took it upon themselves to initiate the standardization of HbA1c by developing a standard, consisting of purified HbA1c and HbA0 as calibrators and a primary reference method. They established a laboratory network with two reference methods for HbA1c analysis which included mass spectrometry (HPLC-MS) and capillary electrophoresis (HPLC-CE), both based on enzymatic cleavage of the haemoglobin molecule (Hoelzel and Miedema 1996 ; Finke et al. 1998). However, the results of IFCC HbA1c are reported in mmol/mol and when converted to percentage as reported by NGSP, it becomes approximately 1.5 - 2% lower than DCCT values in non-diabetic subjects causing

confusion for patient and clinicians (Jeppsson et al. 2002). The A1c-derived Average Glucose study group (ADAG) performed a study in 507 subjects (type 1 DM, type 2 DM and non-diabetics) and found a strong relationship between average glucose and HbA1c permitting the use of estimated average glucose (eAG). This made it possible for translation of measured HbA1c so it could be reported in the same units as used for day-to-day monitoring of glycaemia (Nathan et al. 2008). However, even after the IFCC had succeeded in standardizing most of the assays used in the U.S, the use of HbA1c still had disadvantages and it was not suitable as a diagnostic criteria for DM (Genuth et al. 2003) until it was accepted in 2009 (IEC 2009).

The HbA1c test can be performed at any time of the day and does not require a fasting sample which makes it advantageous and a preferred test for assessing glycaemic control. The use of HbA1c can avoid the problem of day-to-day variability of glucose values. Furthermore, less intra-individual variability was observed when compared with FPG and 2-hour OGTT (WHO 2011). However, HbA1c also has limitations including a lower diagnostic performance in specific populations such as pregnant women and non-Hispanic blacks and the risk of over diagnosing DM in the presence of iron deficiency anaemia (Lippi & Targher 2010). In addition, a study performed on our population (mixed ancestry population of Bellville South) by Zemlin et al reported that the recommended cut-off point of HbA1c  $\geq 6.5\%$  to diagnose DM had a low sensitivity for this population and they established that an HbA1c level of  $\geq 6.1\%$  had a higher sensitivity (Zemlin et al. 2011). HbA1c levels were found to be substantially reduced in subjects with increased RBC turnover, end-stage renal disease and haemoglobinopathies (Lippi & Targher 2011). Other disadvantages include large analytical imprecision when not using HPLC, and the higher costs compared to glucose measurement (Rohlfing et al. 2002). However, it has been recommended that the test for HbA1c levels should be performed for glycaemic control at least twice yearly in patients who are meeting treatment goals and who have stable glycaemic control and quarterly in patients whose therapy has changed or who are not meeting glycaemic control (Driskell et al. 2014). In 2009 the IEC of the ADA introduced the use of HbA1c as a diagnostic tool for DM (IEC 2009).

### 1.6.2. Fructosamines

Plasma proteins also undergo non-enzymatic glycation reaction forming fructosamines and / or glycated albumin (GA) (Zafon et al. 2013). Serum fructosamines refers to all serum proteins that undergo glycation whereas serum GA specifically refers to albumin that has

undergone glycation (National Institute of Health 2010). Since the major serum protein is albumin, which has a half-life of 20 days, both fructosamine and GA reflect blood glucose levels for the past 2-3 weeks (Rodriguez-Segade et al. 2011). Therefore both can be used for short term (2-3 weeks) glycaemic control (National Institute of Health 2010). However due to albumin and other plasma protein's greater susceptibility to glycation compared to intracellular proteins such as hemoglobin, the blood levels of fructosamines or GA exhibit a broader fluctuation than those of HbA1c, allowing an earlier detection of rapid changes in blood glucose levels (Rondeau & Bourdon 2011). Hence measurement of fructosamines and GA is regarded as an attractive alternative, especially in patients in whom the measurement of HbA1c may be biased or even unreliable or when quicker detection of changes in glycaemic control is needed, such as in pregnancy (Danese et al. 2015).

Conditions linked to hypoproteinaemia such as pregnancy or malnutrition and also abnormal levels of immunoglobulins (Ig), especially IgA are more likely to affect the concentration of fructosamines (Rodriguez-Segade et al. 1989). In brief, all clinical conditions affecting protein metabolism are more likely to influence the concentration of fructosamines. Furthermore diseases such as liver cirrhosis and hypothyroidism result in a prolonged half-life of albumin leading to increased GA levels, whereas diseases such as nephrotic syndrome decrease the half-life of albumin leading to decreased levels of GA (Okada et al. 2011). Unlike HbA1c, fructosamines are not genetically influenced. This is supported by the findings in non-diabetic monozygotic and dizygotic twins study where it was shown that fructosamine levels were significantly correlated in these twins (Cohen et al. 2006). However, GA is a more preferred intermediate glycaemic marker compared to fructosamines due to reported higher specificity and accuracy (Danese et al. 2015).

### 1.6.3. Advanced glycation end products (AGEs)

The non-enzymatic glycation of proteins forms major intermediates (Schiff bases and Amadori products) which undergo a series of further, slower reactions and rearrangements leading to the formation of AGEs (Szwergold et al. 2002). Whilst AGEs are formed from chemical reactions with sugars, they can also be derived exogenously from tobacco (Nicholl & Bucala 1998) and certain foods, particularly those that are heated and browned (O'Brien et al. 1989).

In the 1980s it was postulated that The Maillard reaction of proteins could be a factor in aging and in the development or worsening of diabetic complications, although the mechanism behind this was not clearly understood (Monnier & Cerami 1982). AGEs are irreversible and it was discovered that they induce aging by accumulating in stable and long lived extracellular proteins such as collagen (Meng et al. 1996), crystallins (Van Boekel & Hoenders 1992) and histones (Gugliucci & Bendayan 1995), consequently having detrimental effects on cell functioning (Hirata et al. 1997) and contributing to diabetic complications and the development of neurodegenerative diseases (Vlassara, Bucala and Striker 1994; Brownlee 2001). It is believed that the interaction of AGEs with receptors for advanced glycation end products (RAGE) on cell surfaces could be the main pathogenic cause of diabetic complications. AGE bind to RAGE forming the AGE-RAGE complexes on plasma proteins, inducing changes in gene expression of cells such as endothelial cells, mesangial cells and macrophages forming modified plasma proteins, they also trigger downstream signaling and transcriptional pathways that results in oxidative stress, inflammation and release of reactive oxygen species (ROS) as illustrated in Figure 1.3 below (Rahbar 2005 ; Oliveira et al. 2013).



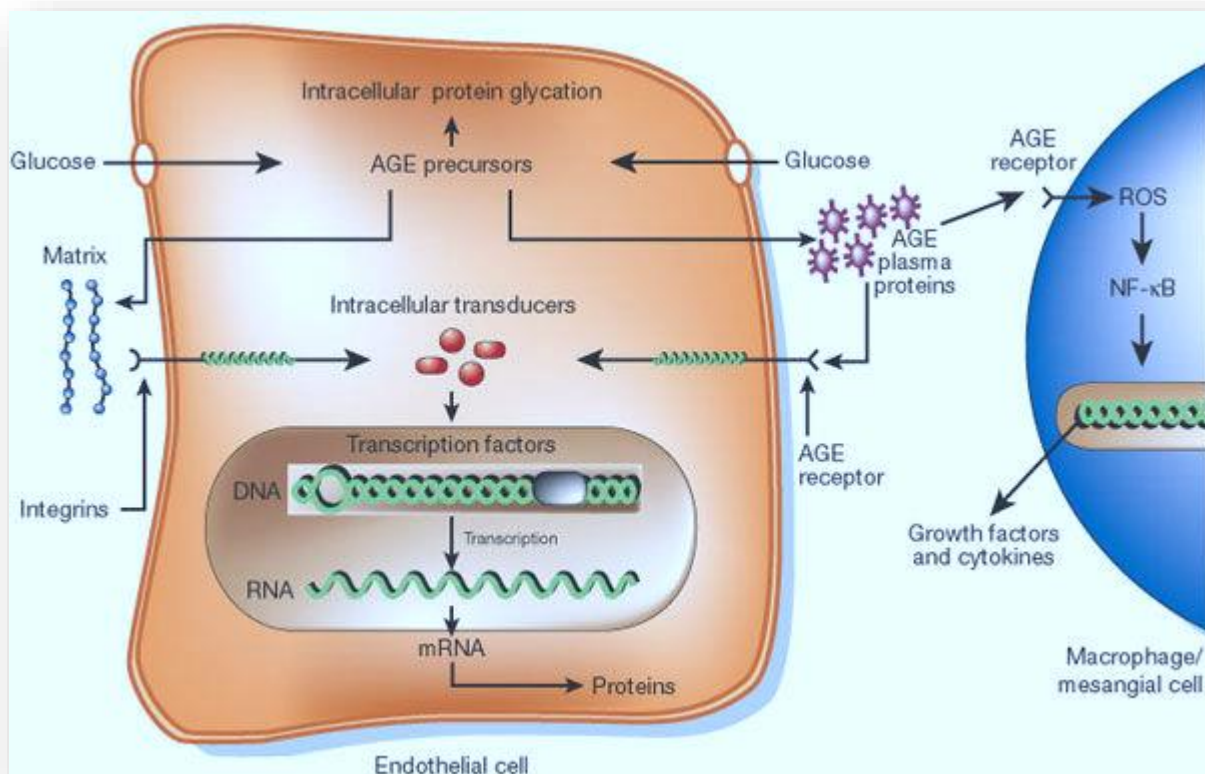


Figure 1.3. Increased production of AGE precursors and its pathologic consequences, adapted from (Brownlee 2001). Mechanisms by which intracellular production of AGE precursors damages vascular cells. Covalent modification of intracellular proteins by dicarbonyl AGE precursors alters several cellular functions. Modification of extracellular matrix proteins causes abnormal interactions with other matrix proteins and with integrins. Modification of plasma proteins by AGE precursors creates ligands that bind to AGE receptors, inducing changes in gene expression in endothelial cells, mesangial cells and macrophages.

## 1.7. Diabetic complications

DM and its complications are a major cause of morbidity and mortality worldwide (Adler et al. 2000). However, hyperglycaemia alone cannot completely explain these complications. The DCCT and UKPDS found that intensive glycaemic control dramatically reduced microvascular complications but did not prevent them (DCCT 1993; UKPDS 1998). In diabetics the formation of AGEs is accelerated by persistent hyperglycaemia resulting in significant adduct accumulation on long-lived macromolecules (Monnier et al. 2005). Diabetic retinopathy is a progressive disorder that affects blood vessels of the retina and

AGEs have been implicated in the initiation and progression of this condition. It has been reported that the expression of RAGE is upregulated in DM and is predominantly localized to glia in the inner retina where they form AGE-RAGE complexes which often occur at higher levels in diabetics (Soulis et al. 1997). Approximately one third of diabetics globally develop some degree of diabetic retinopathy and it is the commonest cause of blindness and loss of vision (Yau et al. 2012). As a result of this vision impairment, poverty in affected families may be exacerbated due to loss of jobs resulting in lack of income.

Ne<sup>-</sup>-(carboxymethyl) lysine (CML) constitutes major AGE accumulated in the renal basement membrane in patients with diabetic nephropathy and associated with upregulation of RAGE on the podocytes cells located on the Bowman's capsule (Tanji et al. 2000). These findings led to conclusions that AGE may also play a role in glomerular injury in acute inflammatory glomerulonephritis. Diabetic nephropathy is damage to kidneys caused by DM and characterized by persistent proteinuria > 300 mg/24 h and increased blood pressure. Diabetic nephropathy can result in end stage renal disease requiring dialysis or transplantation (Andersen et al. 1983).

A study performed in diabetics with a history of foot neuropathic ulceration found that skin auto-fluorescence, which is assumed to reflect tissue AGE accumulation is increased during early stages of diabetic neuropathy and correlates with severity of nerve dysfunction and foot ulceration (Meerwaldt 2005). These findings support the importance of the clinical impact of AGE accumulation in diabetic neuropathy. Diabetic neuropathies present in several ways. The commonest form is a diffuse progressive polyneuropathy affecting mainly the feet and characterized by sensory impairment including burning and numbness which is difficult to treat potentially resulting in amputation of the lower limb (Flemmer & Vinik 2000). This makes foot problems potentially life threatening in diabetics. It has also been reported that foot ulceration leads to prolonged lengths of hospitalization and is a significant cause of morbidity in diabetics (Frykberg 1998).

Macrovascular complications include atherosclerotic diseases such as the coronary artery disease, peripheral arterial disease and stroke (ADA 2006). Atherosclerosis is narrowing of arterial lumen in the peripheral or coronary vascular system resulting from chronic inflammation and injury to the arterial walls in response to accumulation of oxidized lipids. Atherosclerosis results in cardiovascular diseases (CVD) which accounts for approximately 65% of all deaths in diabetics, with the major CVDs such as ischaemic heart disease and

stroke accounting for the greatest proportion of these deaths. In fact diabetics have been described as having a cardiovascular risk equivalent to someone who has already had a myocardial infarction (Whitely et al. 2005). The vascular complications of DM, including kidney diseases, myocardial infarction and stroke are increasing rapidly globally (Borch-Johnsen 2007). AGEs are linked to atherosclerosis in multiple ways including enhancing endothelial dysfunction, elevating vascular low-density lipoprotein (LDL) levels by reducing LDL uptake and promoting plaque destabilization (Goh & Cooper 2008). In addition, experimental studies have demonstrated that AGEs are able to induce vascular calcification, with consequence in the aortic valve (Eupen et al. 2013).

## 1.8. Glycation gap

Among type 1 diabetics (Soros et al. 2010), type 2 diabetics (Rodriguez-Segade et al. 2011) and non-diabetics (Rohlfing et al. 2002) considerable inter-individual differences in HbA1c levels that are not accounted for by corresponding variance in glycaemia levels. This imperfect relationship between HbA1c and glucose levels is due to various non-glycaemic determinants. Other studies have found that HbA1c is influenced by factors such as age, race, BMI, haemoglobinopathies, renal dysfunction, waist circumference, FPG, haematocrit, RBC count, current smoking status and alcohol consumption (Barth et al. 2008; Jansen et al. 2013). Factors that influence the life span of RBC affect HbA1c levels, with an increase in the mean age of RBC resulting in an increase in HbA1c levels and a decrease in mean age of RBC resulting in reduced HbA1c levels. This is supported by a Korean study which demonstrated that older individuals have higher HbA1C levels than younger individuals with similar glucose profiles (Lee et al. 2013). Patients with conditions such as iron deficiency anaemia which is one of the factors increasing RBC lifespan, have increased levels of HbA1c due to the altered lifespan of RBC (Adeoye et al. 2014). Patients with haemolytic anaemia have increased RBC turnover resulting in lower levels of HbA1c (Gram-Hansen et al. 1990). Also, lower HbA1c levels have been observed in patients with chronic liver disease (Schnedl et al. 2005). Haemoglobinopathies such as HbAS (sickle cell trait) and HbC which are more common in SSA have been reported to interfere with measurement of HbA1c resulting in lower HbA1c levels (Bry et al. 2001). Patients with malaria infection have haematological effects such as anaemia due to lower levels of RBCs hence lower levels of HbA1c (Gallager et al., 2009).

HbA1c also exhibits racial and ethnic differences. A study on 4000 individuals participating in the Diabetes Prevention Program, reported that HbA1c levels were significantly higher in Blacks (6.2%), American Indian (6.1%), Asian (6.0%) and Hispanic (5.9%) subjects compared to Whites (5.8%) (Herman et al. 2007). This is in agreement with a study reporting that HbA1c levels are increased in Mexican Americans and Blacks compared to Whites (Cohen et al. 2008). A more recent study performed on 104 Blacks and 104 Whites aged 8 years or older with type 1 DM demonstrated that HbA1c levels overestimate the mean glucose concentration in Blacks with mean HbA1c level being 9.1% in Blacks and 8.3% in Whites (Bergenstal et al. 2017).

Yudkin et al referred to persons with HbA1c levels higher than expected for their plasma glucose levels as “high glycaters” and those with lower than expected levels as “low glycaters” (Yudkin et al. 1990). Discrepancies between HbA1c and fructosamine levels have also been reported in several studies (Macdonald et al. 2008 ; Hempe et al. 2002). Cohen et al then proposed measurement of the glycation gap to address this discrepancy encountered between HbA1c and average plasma glucose levels (Cohen et al. 2003). Glycation gap refers to the difference between measured HbA1c and the value predicted by regression of HbA1c on either fructosamine or GA (Cohen et al. 2003). Similarly McCarter proposed the measurement of haemoglobin glycation index (HGI) which differs from glycation gap in that it measures the difference between measured HbA1c and the value predicted by regression of HbA1c on mean blood glucose measured throughout the day (McCarter et al. 2004).

Several studies have indicated that the glycation gap is consistent within individuals over time, therefore emphasizing the persistence of the underlying mechanism resulting in this variation encountered between HbA1c and other glycaemic measures (Rodriguez-Segade et al. 2015; Kim et al. 2016). These findings have led to assumption that the glycation gap could be a better glycaemic indicator than HbA1c. Furthermore, studies have reported on the association of the glycation gap with diabetic related complications. Cohen et al and Rodriguez-Segade et al reported a positive correlation between the glycation gap and nephropathy (Cohen et al. 2003; Rodriguez-Segade et al. 2012). Additionally, a prospective cohort study performed on 3182 diabetics (Black, White and Asian) found that the glycation gap was significantly associated with retinopathy, nephropathy, macrovascular disease and mortality (Nayak et al. 2013). Hence, it was suggested that the glycation gap could improve

the quality of glycaemic control monitoring, especially in patients whose HbA1c levels do not truly reflect the mean blood glucose (Dziedzic et al. 2012). Similarly, studies on HGI reported that it is reproducible over time within an individual and it is positively correlated with diabetic related complications such as nephropathy and retinopathy (Hempe et al. 2015; McCarter et al. 2004).

However, the glycation gap is also affected by factors such as creatinine levels, mean corpuscular hemoglobin concentration (MCHC) and metformin treatment (Zafon et al. 2013). Higher HbA1c levels relative to fructosamines have been described in patients treated with metformin implying that metformin facilitates glycation of haemoglobin hence overestimation of HbA1c levels (Zafon et al. 2013). It has also been reported that the glycation gap is genetically determined and this is supported by the findings of the study performed in identical and non-identical twins which demonstrated that genetic factors contributed 69% of the glycation gap and only 31% was due to environmental factors (Cohen et al. 2006). This heritability of the glycation gap accounts for one third of the heritability of the HbA1c (Cohen et al. 2006). Since the glycation gap reflects the difference in HbA1c as determined in the intracellular compartments and the extracellular compartment of the RBC and these findings imply that there are genetic factors which influence the glycation reaction in the intracellular compartment but not in the extracellular compartment. Therefore, the glycation gap could be a useful predictor of factors other than glycaemia affecting HbA1c levels. Recently, it was suggested that the glycation gap should be used as an additional tool for glycometabolic monitoring especially for patients with conditions that may affect the reliability of the measurement of fructosamines and HbA1c (Paleari et al. 2016).

## 1.9. Deglycation

### 1.9.1. Discovery of Fructosamine 3 Kinase (FN3K)

With growing evidence suggesting that the accumulation of AGE's is associated with aging and the development of diabetic complications, the discovery of inhibitors of glycation has offered a potential therapeutic target for the prevention of diabetic complications related to non-enzymatic glycation of proteins. Fructosamine 3 kinase (FN3K) is a cellular repair enzyme involved in the deglycation process, a new form of protein repair (Avemaria et al. 2015). FN3K was discovered during a study of cataracts performed in the lens of diabetic rat when a novel sugar phosphate called fructose-3-phosphate (Fru-3P) was identified in the

aging lens (Szwergold et al. 1990). Fru-3P is a potent glycating agent and a potential precursor of an alpha-dicarbonyl sugar, 3-deoxyglucosone (3DG) (Lal et al. 1995) The use of the tool, Phosphorus nuclear magnetic resonance (P-NMR) played a major role in the observation of the formation of Fru-3P first in lenses of diabetic animals then in erythrocytes of both control and diabetic subjects (Petersen et al. 1990). Although there was no known function of Fru-3P then, more investigations were performed on the erythrocytes extracts and it was demonstrated that an adenosine triphosphate (ATP) dependent kinase is involved in the production of Fru-3P. However, due to low affinity ( $K_m \geq 30$  mM) of FN3K for Fru-3P it was argued that the physiological substrate of FN3K was not fructose itself but compounds with closely related structure possibly fructosamine (Petersen et al. 1992).

However, this kinase was poorly characterized until Delpierre and colleagues discovered that this enzyme can be inhibited by a synthetic fructosamine called 1-deoxy-1-morphilino-fructose (DMF) (Delpierre et al. 2000). This discovery led to the identification, purification and cloning of human and mouse fructosamine kinases and alignment of these kinases showed 80% sequence identity. Human FN3K was identified as a 309-amino acid monomeric protein (Delpierre et al. 2000). This enzyme belongs to a family of aminoglycoside kinases and ethanolamine kinases (Delpierre et al. 2000).

### 1.9.2. FN3K specificity, properties and role in deglycation

Cloning of FN3K led to discovery that this kinase phosphorylates a wide variety of fructosamines. FN3K phosphorylates fructoselysine (FL) at their 3-hydroxyl group resulting in formation of fructoselysine 3 phosphate (FL3P) in tissue extracts (Szwergold et al. 1997). The close proximity of ketoamine group and phosphates in the FL3P makes it unstable, therefore it will readily decompose resulting in regeneration of lysine, 3-DG and inorganic phosphate as illustrated in Figure 1.4 below (Szwergold et al. 1997). FN3K is a deglycation and protein repair enzyme. FN3K phosphorylates fructosamine bound proteins leading to regeneration of lysine residues and preventing further reaction to AGEs, Figure 1.4 (Szwergold et al. 1997). However, deglycation is said to occur more rapidly on fructosamines bound to the side chains of lysine and slower on those bound to the side chains of valine due to their lack of accessibility. Therefore, the ability of FN3K to act on fructosamine is decreased as it is bound to  $\alpha$  amino group of amino acids (Delpierre & Schaftingen 2003). Recently, a study performed on 67 subjects (age:  $76 \pm 8$  years) with an aortic valve stenosis, found that the use of ATP-dependent FN3K reduced the concentration

of fructosamine in the aortic valve resulting in increased flexibility of these valves (Cikomola et al. 2016). Hence FN3K potentially plays an important role as a deglycating enzyme especially in the control of diabetic complications in association with non-enzymatic glycation of proteins and supplementation of FN3K may be useful as a repair mechanism.

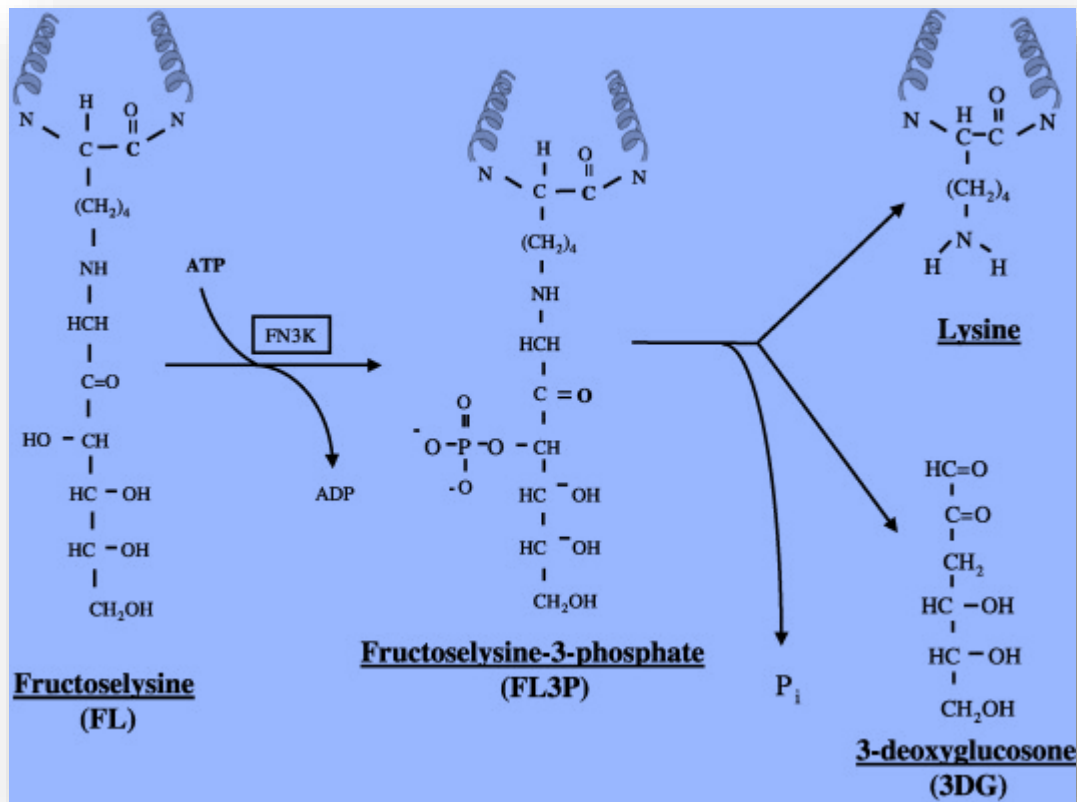


Figure 1.4. Proposed role of FN3K as a catalyst in the decomposition of fructoselysine (FL). Adapted from (Szwergold et al., 2002). Phosphorylation of FL results in the formation of Fructoselysine-3-phosphat (FL3P) which is intrinsically unstable and decomposes spontaneously to lysine, inorganic phosphate and 3-deoxyglucosone, thereby regenerating an unmodified protein.

Inhibition of FN3K using its competitive inhibitor DMF in intact erythrocytes and incubating them in glucose further substantiated its role in deglycation. The subsequent accumulation of HbA1c increased about twofold (Delpierre et al. 2002). Veiga-da-Cunha et al confirmed this and reported that the levels of haemoglobin-bound fructosamines was about 2, 5-fold higher in FN3K  $-/-$  mice than FN3K  $+/+$  or FN3K  $+/-$  mice. Additionally, they found that cytosolic proteins were 1.5 to 1.8 fold more glycated in tissues highly susceptible to glycation such as liver, kidney, brain and skeletal muscle of FN3K $-/-$  mice than those of FN3K  $+/+$  mice (Veiga-da-Cunha et al. 2006). In summary, protein deglycation catalyzed by FN3K is

a process of restoring proteins to their original structure and function prior to the Maillard modification (Delpierre et al. 2002; Beisswenger et al. 2003).

### 1.9.3. FN3K substrate and tissue distribution

FN3K is present in mammals and birds but not in fishes, plants and bacteria. The activity of this enzyme is absent/low in erythrocytes of pigs and chickens due to low intracellular glucose whereas high activities are found in erythrocytes of species such as humans, mice and rats where the intra-erythrocyte glucose concentration is equal to that of the plasma (Delplanque et al. 2004). This enzyme is encoded by the FN3K gene located on chromosome 17q25 and contains six exons encoding 309 amino acids. The FN3K gene is ubiquitous, expressed in all tissues and highly expressed in tissues susceptible to glycation such as kidney, peripheral nerves and heart (Conner et al. 2004). The FN3K gene is also present in low protein turnover tissues such as lenses and erythrocytes (Lal et al. 1993). However it was found that highly basic glycated proteins such as histones and cytochrome-c are the preferred substrates of FN3K because of its high affinity (Szwergold et al. 2001). The presence of this enzyme in many cells and tissues imply that it might be important for the functioning and survival of some cells.

### 1.9.4. Variability of FN3K activity

The activity of FN3K is stable with time in a single individual but varies from subject to subject in a four-fold range (Delpierre et al. 2004). Delpierre et al performed a study in type 1 diabetics and non-diabetics to ascertain whether changes in FN3K activity were associated with distinct alleles of the FN3K gene. They identified six single nucleotide polymorphisms (SNPS) in the FN3K gene and suggested that they might be associated with the enzyme activity (Delpierre et al. 2006). However, of these six SNPs, only two were reported to have a significant association with the FN3K activity. The CC of the G900C (rs1056534) in exon 6 and the GG of the c.-385A/G (rs3859206) in the promoter region were associated with reduced FN3K activity measured in erythrocytes (Delpierre et al. 2006). In addition, Mohas et al further investigated one of the SNPs reported by Delpierre (rs1056534) in type 2 DM (859) and controls (265) and found that the C allele of G900C was associated with lower HbA1c concentration and that this polymorphism was associated with the onset of disease. With the CC allelic variant, type 2 DM was diagnosed at a later age than in case of GC or GG variants ( $p < 0.05$ ) (Mohás et al. 2010).



An Italian study performed on both type 1 and 2 diabetics also confirmed the presence of some FN3K variants previously reported by Delpierre and identified additional genetic variants (c.187 A/C, IVS4-9 delTTG), and two new mutations (c.559 C/T, c.716 A/G). However, only some variants were found to be related to higher HbA1c levels and this was not significant (Mosca et al. 2011). Škrha et al also demonstrated the association of SNP rs1056534 and rs3848403 of FN3K gene with serum levels of RAGE in diabetics (Škrha et al. 2014). Subsequently, Tanhäuserová et al demonstrated that SNP rs1056534 of the FN3K gene was associated with the progression of diabetic nephropathy and cardiovascular morbidity and mortality (Tanhäuserová et al. 2014).

In a White cohort of 70 diabetics (35 with type 1 and 35 with type 2) and 33 controls the promoter of the FN3K gene was analyzed and evaluated for the presence of the two polymorphisms, the c.-385A/G (rs3859206) and the c.-232A/T (rs2256339). Two additional new variants (c.-421C/T; c.-429delATCGGAG) were found in one patient with type 1 DM (Avemaria et al. 2015). A recent study performed in a cohort of African diabetics and controls examining the activity of FN3K also reported a significant correlation with HbA1c values and a unique significant association between FN3K activity and the glycation gap (Cikomola et al. 2016). Therefore, the presence of this polymorphism may affect the protective role of FN3K by either reducing or increasing the activity of the enzyme hence these genetic variants may have a major effect on the progression of DM.

### 1.9.5. Other Repair Enzymes

After the cDNA encoding FN3K was cloned, Collard et al realized that mammalian and bird genomes encode FN3K-related protein (FN3K-RP) that is homologous to FN3K sharing approximately 60% amino acid sequence identity (Collard et al. 2003). The gene encoding for FN3K-RP is located at position 8 Kb upstream of the FN3K gene on chromosome 17q25.3 (Collard et al. 2003). The mammalian and bird FN3K-RP doesn't phosphorylate fructosamines, it catalyzes the phosphorylation of low molecular mass and protein bound ketoamine such as ribulosamines and erythrulosamines and with lower affinity psicosamines, all substrates which FN3K also acts on (Collard et al. 2003; Delplanque et al. 2004). However, these substrates cannot be found in nature hence there are no known substrates of FN3K-RP. Furthermore FN3K-RP phosphorylates proteins on their third carbon (C3) in the D-configuration (D-allose and D-ribose) unlike FN3K, which phosphorylates C3 either in the L- or the D-configuration (Collard et al. 2004; Delplanque et

al.2004).

## Purpose of the study

### Hypothesis:

We hypothesise that SNPs of the FN3K gene influence the levels of the glycation gap.

### Research Question:

What are the effects of the FN3K SNP genotypes on the variability encountered between HbA1c and fructosamines as measured by the glycation gap?

### Aim:

The aim of this study was to examine the role of FN3K genotypes on the variability encountered between HbA1c and fructosamine as measured by the glycation gap.

### Objectives:

- To investigate the allelic variant of FN3K SNP rs1056534 and rs2256339 in normal, pre-diabetic and type 2 diabetics of mixed ancestry descent.
- To assess the variability between HbA1c and fructosamines by determining the glycation gap within normal, pre-diabetic and type 2 diabetics of mixed ancestry descent.

### Problem statement:

Type 2 DM is a growing global problem and a major cause of morbidity and mortality. IDF in 2017 reported that approximately 425 million people globally were diabetic (IDF 2017). The prevalence of DM has been found to be very high in the mixed ancestry population of Bellville South, Western Cape South Africa (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). Non-enzymatic glycation of proteins has been strongly related to the pathogenesis of chronic diabetic complications as well as degenerative changes occurring in the course of aging. The glycation gap which explains the variability between HbA1c and other measures of hyperglycaemia has been associated with the onset of diabetic related microvascular complications such as nephropathy (Nayak et al. 2013). However, evidence has shown that the glycation gap could be genetically determined, and this accounted for one third of the heritability of HbA1c which implies that there are genetic factors which influence the glycation reaction in the intracellular compartment but not in the

extracellular compartment (Cohen et al. 2006). The glycation process may be moderated by an enzymatic deglycation process thought to involve a deglycating enzyme FN3K which forms part of a protein repair system and/or cellular defence. However, subject to subject variations in the activity of FN3K enzyme in erythrocytes has been reported and could potentially affect the levels of HbA1c independently of differences in the blood glucose level. In addition, there are several SNP's in the FN3K gene which are said to influence the activity of the deglycating enzyme (Delpierre et al. 2006).

**Rationale for the study:**

The glycation gap has been suggested to be a better indicator than HbA1C for assessing the risk of death and hospitalization in diabetic dialysis patients. Thus, determining the glycation gap in any individual may be of importance especially in helping with the identification of individuals who are at risk of developing diabetic complications. The role of FN3K in deglycation is considered as a potential intracellular protein repair method and this has given hope for future therapeutic possibilities for diabetic related complications. Furthermore, better understanding of the possible effect of FN3K genetic variants on the progression of the DM and its ability to prevent adverse diabetic outcomes such as microvascular and macrovascular complications, morbidity and mortality in the management of diabetic patients is needed. The increasing prevalence as well as the socioeconomic burden of DM and its complications in developing countries necessitates reliable diagnostic methods, measures of glycaemic monitoring and therapeutic approaches of preventing or inhibiting the development of diabetic related complications which is a major priority in diabetic research.

**Research outputs:**

This study will form the master's research project for the student involved in this study. The abstract of this research article will be presented at national and international conferences and workshops. It will also be published in a peer-reviewed accredited journal. Hence it will be allow further knowledge about the effect of FN3K genotypes of glycation gap. This study will be a valuable contribution to the mixed ancestry community and globally.

## Chapter 2: Methodology

### 2.1. Study design

This was an observational case control study in which mixed-ancestry diabetic and non-diabetic subjects of the Bellville South community, Western Cape, South Africa were compared to identify the frequency of the allelic variants of FN3K. The association between FN3K genotypes and the glycation gap was determined in diabetic, pre-diabetic and normoglycaemic subjects. This study formed part of the large Bellville South study investigating the risk factors for CVDs (Matsha et al. 2012).

### 2.2. Study setting

This study was conducted in Bellville South, which 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 mixed ancestry population (88 %) (Wit et al. 2010). According to the census report of 2011 it was estimated that 24,642 individuals, with about 54 % females and 46 % males are resident in Bellville South (Western Cape Census, 2011). 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. Therefore, this community presents an ideal study setting to investigate the effects of FN3K genotypes on the glycation gap in type 2 DM and non-diabetic people due to the high crude prevalence of type 2 DM (28.2%) (Erasmus et al. 2012).

### 2.3. Sample size

Sample size calculation

The sample size needed for this study was calculated using the DM prevalence of 28.2% reported by Erasmus et al. (2012) for the Bellville South community.

The formulae used was:

$$n = z^2 (pq)/e^2$$

Where:

n = the sample size

z = standard error associated with the chosen level of confidence (1.96)

$p$  = estimated percent in the population

$q = 100 - p$

$e$  = acceptable sample error (5%)

Calculation

$n = z^2 (pq)/e^2$

$n = \{1.96^2(28.2 * (100-28.2))/5^2$

$n = 311$

Therefore, sample size needed was a minimum 311 participants. However; we used more than 311 participants: higher than sample size calculated as we examined different subgroups

## 2.4. Study population

The enrolment of subjects for this case control study was performed between 2014 and 2016. A total of 1988 subjects were recruited and gave informed consent. Anthropometric measurements including body weight, height, waist and hip circumferences as well as blood pressure were taken. Blood samples were taken after an overnight fast as well as after a 2 hour glucose tolerance test (OGTT).

### 2.4.1. Inclusion criteria

All mixed-ancestry participants (diabetic and non-diabetic) enrolled in the study on a voluntary basis and gave informed consent in a language of their choice.

### 2.4.2. Exclusion criteria

Participants who were pregnant, aged less than 20 years, those with acute and chronic conditions and also those who did not volunteer to participate in the study were excluded.

## 2.5. Data collection

### 2.5.1. Clinical data

#### 2.5.1.1. Clinical assessment

Anthropometric measurement, blood pressure measurement and blood collection were obtained. Anthropometric measurements included body weight, body height and waist and

hip circumferences. All anthropometric measurements were performed three times and the average of these measurements used for the final analysis.

#### 2.5.1.1.1. Height

The Omron Body Composition Monitor (BF511: Omron, Japan) was used to measure both height and weight of the participants. The stadiometer was used to measure body height to the nearest 0.1 centimetre (cm). The participant had to stand upright on the flat surface of the stadiometer without shoes (Tolonen et al., 2002). 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.

#### 2.5.1.1.2. Weight

The Omron Body Composition Monitor (BF511: Omron, Japan) was used to measure participants' weight in kilograms (kg). Participants were weighed in light clothing and barefooted. The weight of all participants, except wheelchair bound or those who were posturally impaired was measured. The participant would stand on the centre 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 (Tolonen et al., 2002). 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<sup>2</sup>] (kg/m<sup>2</sup>).

#### 2.5.1.1.3. Waist circumference

A non-elastic tape that had been inspected for calibrations and stretch was used to measure the waist circumference (WaistC) (cm). 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).

#### 2.5.1.1.4. Hip circumference

The hip circumference (HipC) (cm) was measured at the maximal circumference over the buttocks. A non-elastic tape was also used for this measurement. The investigator would place the tape around the buttocks on the widest area over the horizontal 23 plane without pressing tightly against the skin, and then take the measurement (Tolonen et al., 2002).

#### 2.5.1.1.5. 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 and the units are millimetres of mercury (mm Hg) (Pickering et al. 2005).

Blood pressure measurements were performed according to WHO guidelines (WHO, 1999). Blood pressure measurements were taken using an automatic digital blood pressure monitor (Omron M6 Comfort-preformed Cuff Blood Pressure Monitor, Omron). Blood pressure measurements were taken with the participant sitting quietly in a relaxed 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.

#### 2.5.1.1.6. Blood collection

Phlebotomy was conducted by trained nursing sisters. Participants were requested to fast overnight before the morning of blood collection. All blood samples were drawn in a sitting position. Six blood tubes were collected for each participant: three fasting and three 2-hour post OGTT bloods. Self-reported type 2 DM participants (confirmed by either medical records or medications) had only the fasting blood samples taken and no 2-hour bloods were drawn. The following fasting and 2-hour blood samples were collected: 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 centrifuged to obtain serum for serological tests such as serum cotinine, insulin and lipid profile. The purple capped-tubes (whole blood) were used to measure HbA1c levels and for DNA extraction. Blood samples collected were transported daily in an ice-box for processing at an accredited pathology practice, PathCare Reference Laboratory (Cape Town, South Africa) for biochemical analysis of samples.

#### 2.5.1.2. 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 an OGTT. Subjects were asked to fast overnight where after the OGTT was conducted according to WHO guidelines (WHO 1999): 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 second blood sample after 2 hours (2h-PG).

#### 2.5.2. Biochemical data

Biochemical measurements: Plasma glucose concentrations were measured using the hexokinase method (Cobas 6000, Roche Diagnostics; Mannheim, Germany), HbA1c was measured using high performance liquid chromatography (HPLC)(Biorad Variant Turbo), Insulin was measured using a Paramagnetic particle assay (Chemiluminescence), Fructosamine was measured using a Colorimetric test nitro-blue-tetrazolium (Roche Cobas c311), LDL-chol (mmol/L) was measured using an Enzymatic Selective Protection – Endpoint assay (Beckman AU), HDL-chol (mmol/L) using an Enzymatic Immunoinhibition-Endpoint assay (Beckman AU) and the triglycerides were estimated using a glycerol phosphate oxidase in the presence of peroxidase (GPO-POD) Endpoint assay (Beckman AU).

##### 2.5.2.1. Formulas used

**The glycation-gap:** The glycation-gap was determined according to a formula described by Nayak et al. (2013): Glycation Gap = HbA1c - FHbA1c (Fructosamine derived glycated haemoglobin)

$$\text{FHbA1c} = \left[ \left( \frac{\text{fructosamine} - \text{mean fructosamine}}{\text{SD fructosamine}} \right) \times \text{SD HbA1c} \right] + \text{mean HbA1c}$$

**Obesity:** Obesity was classified using the BMI values as proposed by the WHO (WHO 2004, updated 2016). The formulae used is body weight (kg) / (height (m))<sup>2</sup> ((kg/m<sup>2</sup>)).

The classification is then as follows:

- Underweight: < 18.50 kg/m<sup>2</sup>
- Normal range: 18.50 to 24.99 kg/m<sup>2</sup>
- Overweight: ≥ 25.00 to 29.99 kg/m<sup>2</sup>
- Obese: ≥ 30.00 kg/m<sup>2</sup>

**Diabetes:** Participants were classified according to the revised WHO criteria (WHO 2016). Participants were categorized as DM, pre-diabetes (IGT and/or IFG) or normoglycaemia according to their history of DM, as well as their fasting, and 2-hour glucose concentrations.

The glycaemic sub-groups were classified as follow:

- DM: if FBG is ≥7.0 mmol/L or post 2HR BG ≥11.1 mmol/L
- IGT: if FBG is <7.0 mmol/L and post 2HR BG is between ≥7.8 mmol/L and <11.1 mmol/L
- IFG: if FBG is between 6.1 and 6.9 mmol/L and if measured, post 2HR BG is <7.8 mmol/L

### 2.5.3. Genotyping

#### 2.5.3.1. DNA extraction

Human genomic DNA was isolated from whole blood collected in EDTA blood tubes and stored at -20°C. DNA was extracted from 1 - 2 ml of blood using the simple, fast and cost efficient salt extraction method. After thawing the frozen blood samples, it was resuspended into five times volume (~5 - 10 ml) of lysis buffer (155 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM EDTA; pH 7.4) to lyse the blood cells. 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 followed by centrifugation 1,500 rpm for 10 minutes using a Beckman General Purpose centrifuge (Beckman Coulter Inc., CA, USA). The supernatant was discarded and the pellets obtained were washed with 10 ml of phosphate buffered saline (PBS; 2.68 mM KCl, 136 mM NaCl, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, and 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 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 suspension was incubated overnight at 55 °C in the water bath to digest proteins.

The following day, 1 ml of a saturated solution of 6 M NaCl was added and the tubes were vigorously vortexed followed by centrifugation at 2,500 rpm for 10 minutes. The supernatant was carefully transferred to a clean 15 mL polypropylene tube where the DNA was precipitated by adding two volumes (~ 9 ml) of 100 % ice cold ethanol. DNA is insoluble in ethanol and will thus form a string like precipitate which was taken out if sufficiently large or where DNA precipitate was small samples were centrifuged at 8,000 rpm for 30 minutes and the DNA pellet kept.

The DNA pellet was washed with 1 ml of 70 % ethanol, the 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 overnight on a mixer to aid suspension of the pellet. The concentration and purity (A260/A280) of the DNA samples were measured by Nanodrop spectrophotometry (Nanodrop one C, Thermo Fisher Scientific, USA). An A260/A280 ratio between 1.8 and 2.2 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.

#### 2.5.3.2. Real time Polymerase Chain Reaction (RT-PCR) SNP Genotyping.

In the present study genotypes for FN3K SNP G900C (rs1056534) and c. -232A/T (rs2256339) polymorphisms were determined with the Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System 96 well fast from Thermo Fisher Scientific. Taqman SNP Genotyping Assays (primers and reagents) designed by Thermo Fisher Scientifics were used to amplify and detect specific SNPs alleles in the purified genomic DNA samples. The following primers were used: TCGGGCGGGAGTACAGGAGCCCTTC (forward) and TTGGGCACCATGCGAAGGCTGCTCA (reverse) for SNP G900C and primers GGGATACCGGGTCCCTGCAGGGAGC (forward) and CAAGCCCCAGCGCGCCCGCGAAGAG (reverse) for SNP c. -232A/T were used.

The reaction mixture for a 96 well plate was prepared as follows: Firstly, a master mix for 96 samples were prepared in a 2ml Eppendorf tube, consisting of the Taqman SNP genotyping mix, assay primers and distilled water as illustrated in Table 2.1 below. Then using a multichannel pipette, 8  $\mu$ l of the master mix was pipetted into each well of the 96 well plate, then 2  $\mu$ l of the genomic DNA (5ng/mL) samples were added into their specific wells. Three negative controls (NTC's) were included on each plate and 2  $\mu$ l of distilled water were added in control wells instead of genomic DNA samples as illustrated in Table 2.2 below. Upon completion of pipetting all the wells were inspected for uniformity of volume. An optical adhesive film was used to completely cover the wells of the reaction plate.

Table 2.1. The reaction master mix used in PCR amplification

Components	Master Mix 96 well plate	
	Volume per well	Volume for 96 well plate
Master mix		
➤ Taqman SNP genotyping mix	5 $\mu$ l	500 $\mu$ l
➤ Taqman primer mix	0.25 $\mu$ l	25 $\mu$ l
➤ Distilled water	3.75 $\mu$ l	375 $\mu$ l

Table 2.2: Preparation of reaction for 96-well plate for PCR amplification

Components	96 well plate	
	Total Volume per well is 10 $\mu$ l.	
	93 wells for samples	3 wells for controls
Master mix	8 $\mu$ l	8 $\mu$ l
DNA sample	2 $\mu$ l	Not added
Distilled water	Not added	2 $\mu$ l

The run conditions for the Taqman SNP Genotyping assays were optimized on the instrument. The following run conditions were optimal: 30 seconds pre-PCR read at 60.0°C, followed by hold stage at 95.0°C for 10 minutes, 40 cycles of PCR stage at 95.0°C for 15

seconds (Denature) and at 60.0°C for 1 minute (Anneal/extend), then the Post-Read stage at 60.0°C for 30 seconds. The reaction plate was loaded in the QuantStudio™ 7 flex Real time PCR System. After the run the results were obtained from the QuantStudio™ 7 Flex Real-Time PCR System software.

Twelve of our genomic DNA samples were sent for sequencing at Inqaba Biotec (Cape Town) to confirm if the correct target SNPs were amplified. The sequencing protocol used were as follows: the PCR assay was done using the following conditions; EconoTaq PLUS 2X Master Mix Catalog No. 30035 (10µl), gDNA (10-30ng/µl) (1µl), Forward primer (10µM) (1µl), Reverse primer (10µM) (1µl) and Nuclease free water Catalog No. E476 (7µl). After running the PCR, the PCR product was cleaned using ExoSAP protocol as follows; A Exo/SAP master mix was prepared by adding the following to a 0.6ml micro-centrifuge tube: Exonuclease I (NEB M0293) 20U/µl (50.0 µl), Shrimp Alkaline Phosphatase (NEB M0371) 1U/µl (200.0 µl). Then the following reaction mixture was prepared: PCR Mixture (10.0µL) and Exo/SAP Mix (2.5µL). it was then mixed and incubated at 37°C for 30 min after which the reaction was stopped by heating the mixture at 95°C for 5 min. Sequencing was then done with the ABI V3.1 Big dye kit according to manufacturer's instructions and the labelled products were then cleaned with the Zymo Seq clean-up kit. The cleaned products were injected on the ABI3500XL analysers with a 50cm array, using POP7 and the computer software program was used to read the results.

## 2.6. Statistical analysis

Data was analysed using a software program Statistica (Statsoft, <http://www.statsoft.com>). General characteristics of the study group were summarized as count and percentage for dichotomous traits, mean and standard deviation (SD) or median and 25<sup>th</sup>-75<sup>th</sup> percentiles (skewed data) for quantitative traits. SNPs were tested for departure from Hardy-Weinberg Equilibrium (HWE) expectation via a chi square goodness of fit test. One-way ANOVA was used to determine the mean, SD of the glycation gap in the normoglycaemic, pre-diabetic and diabetic sub-groups and the Bonferroni test was used to compare multiple groups of categorized parameters. Spearman Rank Order correlation was used to do correlation studies for non-parametrics (r and p). A P-value of < 0.05 was taken as statistically significant.

## 2.7. Ethical consideration

Ethical approval for the larger study (CPUT/HW-REC 2015/H01) (2<sup>nd</sup> renewal) was granted by the Cape Peninsula University of Technology Faculty of Health and Wellness Sciences Research Ethics Committee and for the sub study (S16/06/100) Stellenbosch University Health Research Ethics Committee. Research was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). Participants were given a written informed consent to sign after fully explaining procedures in the language of their choice.

## Chapter 3: Results

### 3.1. The general characteristics of the study population

The general characteristics of the study population categorized by gender were summarized in Table 3.1. Age (years) was significantly higher in females than in males, mean  $\pm$  SD for females were  $50.3\pm 14.8$  and for males  $47.4\pm 15.8$  ( $p=0.0022$ ). Anthropometric measurements, including BMI ( $\text{kg}/\text{m}^2$ ), WaistC (cm) and the HipC (cm) were significantly higher in females than in males (all  $p<0.0001$ ). Glycaemic measurements, including fasting blood glucose (mmol/L), 2-hour post OGTT glucose (2h-PG) (mmol/L), HbA1c (%), fasting insulin (mIU/L), 2-hours post OGTT insulin (2h- PI) (mIU/L) and the glucose/insulin ratio were all significantly higher in females than in males (All  $p<0.0001$ ). There was no gender difference in fructosamine-S ( $\mu\text{mol}/\text{L}$ ) levels ( $p=0.4114$ ), but similar to the other glycaemic measures, the glycation-gap was also significantly higher in females than in males, mean  $\pm$  SD for females was  $0.18\pm 0.89$  and for males  $-0.24\pm 0.83$  ( $p<0.0001$ ) (Figure 3.1). The lipids, including LDL-Chol (mmol/L), HDL-Chol (mmol/L) and Total-Chol (mmol/L) were significantly (all  $p<0.0001$ ) and the Triglycerides-S (mmol/L) near-significantly ( $p=0.0904$ ) higher in females than in males. The percentage of smokers was significantly higher in males than in females ( $p<0.0001$ ).

Table 3.1. Characteristics of the study population categorized by gender

	Total, N1412	Male, N339	Female, N1073	
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	p-value
Age (years)	49.6 $\pm$ 15.1	47.4 $\pm$ 15.8	50.3 $\pm$ 14.8	0.0022
BMI (kg/m <sup>2</sup> )	29.3 $\pm$ 8.0	24.5 $\pm$ 6.3	30.8 $\pm$ 7.9	<0.0001
WaistC (cm)	92.9 $\pm$ 16.9	86.3 $\pm$ 16.1	94.9 $\pm$ 16.7	<0.0001
HipC (cm)	104.8 $\pm$ 16.3	94.8 $\pm$ 12.2	107.9 $\pm$ 16.1	<0.0001
WHR	0.89 $\pm$ 0.08	0.91 $\pm$ 0.09	0.88 $\pm$ 0.08	<0.0001
SBP (mmHg)	127.8 $\pm$ 24.1	127.2 $\pm$ 24.7	128.0 $\pm$ 23.9	0.6030
DBP (mmHg)	82.1 $\pm$ 14.0	80.9 $\pm$ 15.6	82.5 $\pm$ 13.4	0.0747
Pulse (bpm)	71.4 $\pm$ 12.8	68.6 $\pm$ 13.3	72.3 $\pm$ 12.5	<0.0001
Fasting Blood Glucose (mmol/L)	5.78 $\pm$ 2.91	5.24 $\pm$ 1.94	5.95 $\pm$ 3.13	<0.0001
2 Hours Post Glucose (mmol/L)	6.68 $\pm$ 3.11	5.57 $\pm$ 2.61	7.05 $\pm$ 3.17	<0.0001
HbA1c (%)	6.21 $\pm$ 1.58	5.87 $\pm$ 1.16	6.31 $\pm$ 1.68	<0.0001
Fasting Insulin (mIU/L)*	7.00 (4.40; 10.9)	4.85 (2.80; 8.50)	7.50 (5.00; 11.6)	<0.0001
2 Hours Post Insulin (mIU/L)*	39.6 (21.0; 72.5)	21.6 (9.8; 42.1)	47.7 (25.6; 79.7)	<0.0001
Glucose/Insulin ratio	1.01 $\pm$ 0.93	1.31 $\pm$ 1.10	0.92 $\pm$ 0.85	<0.0001
Fructosamine-S ( $\mu$ mol/L)	258.5 $\pm$ 68.7	262.4 $\pm$ 58.3	257.5 $\pm$ 71.1	0.4114
Glycation gap	0.10 $\pm$ 0.90	-0.24 $\pm$ 0.83	0.18 $\pm$ 0.89	<0.0001
Triglycerides-S (mmol/L)*	1.22 (0.87; 1.71)	1.13 (0.84; 1.66)	1.25 (0.89; 1.73)	0.0904
LDL-Chol (mmol/L)	3.22 $\pm$ 1.03	2.92 $\pm$ 1.01	3.32 $\pm$ 1.02	<0.0001
HDL-Chol (mmol/L)	1.33 $\pm$ 0.36	1.26 $\pm$ 0.38	1.35 $\pm$ 0.35	<0.0001
Total-Chol (mmol/L)	5.18 $\pm$ 1.17	4.82 $\pm$ 1.16	5.30 $\pm$ 1.16	<0.0001
Chol/HDL ratio	4.11 $\pm$ 1.21	4.06 $\pm$ 1.38	4.13 $\pm$ 1.15	0.3861
Serum Cotinine (ng/mL)	135.8 $\pm$ 158.9	155.1 $\pm$ 151.2	129.7 $\pm$ 160.8	0.0113
Smokers, Yes, % (N)	48.6% (668/1374)	60.7% (201/331)	44.8% (467/1043)	<0.0001

Key: \*: median and range (25Q; 75Q)



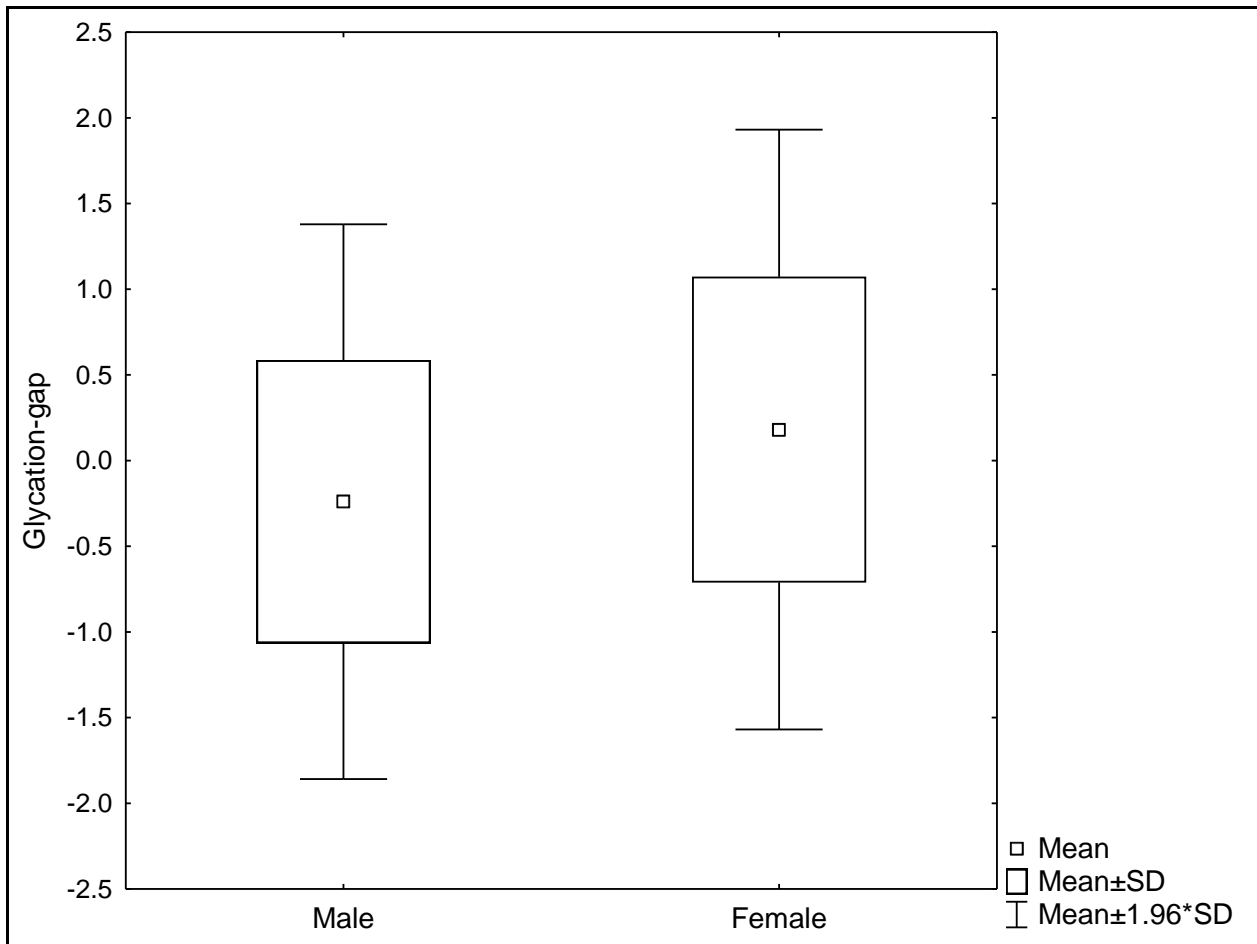


Figure 3.1. The glycation-gap was significantly higher in females than in males, mean  $\pm$  SD respectively  $0.18 \pm 0.89$  and  $-0.24 \pm 0.83$  ( $p < 0.0001$ )

### 3.2. The Hardy-Weinberg Equilibrium (HWE) results

The FN3K SNP G900C (rs1056534) and SNP c. -232A/T (rs2256339) were tested for departure from HWE expectation via a chi square goodness of fit test. Results are summarized in Table 3.2. The table shows the allele and genotype distribution of SNP G900C investigated in the present study. SNP c. -232A/T deviated from Hardy Weinberg Equilibrium (HWE) in the overall sample ( $p=0.0004$ ) and in normal controls ( $p=0.0012$ ), whilst SNP G900C did not deviate in all groups ( $p>0.7281$ ). Therefore, SNP c. -232A/T was left out for the rest of the statistical analysis.

The genotype frequencies for SNP G900C in the glycaemic sub-groups were as follows, GG: 45.9 %, GC: 43.7 %, CC: 10.4 % in normal subjects; GG: 48.6 %, GC: 41.7 %, CC: 9.7% in pre diabetic subjects and GG: 41.7 %, GC: 46.5 %, CC: 11.8 % in the diabetic subjects. There were no significant differences in the SNP G900C genotype frequencies between the glycaemic sub-groups.

Table 3.2. Genotype distributions, minor allele frequencies

	Normoglycaemia	Prediabetes	Diabetes	Overall
N	925	216	271	1412
SNP G900C				
G/G, n (%)	425 (45.9)	105 (48.6)	113 (41.7)	643 (45.5)
G/C, n (%)	404 (43.7)	90 (41.7)	126 (46.5)	620 (43.9)
C/C, n (%)	96 (10.4)	21 (9.7)	32 (11.8)	149 (10.6)
C, n (%)	596 (32.2)	132 (30.6)	190 (35.1)	918 (32.5)
HWE (p-value)	>0.999	0.7893	0.7281	0.9979
SNP c. -232A/T				
T/T, n (%)	373 (40.3)	83 (38.4)	113 (41.7)	569 (40.3)
T/A, n (%)	393 (42.5)	99 (45.8)	113 (41.7)	605 (42.8)
A/A, n (%)	159 (17.2)	34 (15.7)	45 (16.6)	238 (16.9)
A, n (%)	711 (38.4)	167 (38.7)	203 (37.5)	1081 (38.3)
HWE (p-value)	0.0012	0.6214	0.0701	0.0004

### 3.3. The general characteristics of the study population categorized by gender and SNP G900C genotypes.

Clinical and biochemical features of the study population according to gender and genotypes of the G900C polymorphism of the FN3K gene were summarized in Table 3.3. There was a significant difference in the BMI ( $\text{kg/m}^2$ ) and Hip C (cm) in the genotype categories in females,  $p=0.0444$  and  $p=0.0227$  respectively, however this was not observed in males  $p=0.3801$  and  $p=0.7804$ . The fasting blood glucose (mmol/L) was significantly increased across the GG, G/C and C/C genotypes in females, mean  $\pm$  SD were;  $5.74\pm 2.71$ ,  $6.01\pm 3.09$  and  $6.52\pm 4.52$  respectively,  $p= 0.0433$ . The difference was not observed in males. The glycation gap significantly decreased across the GG, G/C and C/C genotype variants in males, mean  $\pm$  SD were  $-0.13\pm 0.86$ ,  $-0.25\pm 0.72$  and  $-0.80\pm 1.04$  respectively,  $p=0.0239$  (Figure 3.2) however the difference was not observed in females. The HbA1c level also decreased across the G/G, G/C and C/C genotype variants in males, however the difference was non-significant ( $p= 0.3009$ ).

Table 3.3. The general characteristics of the study population categorized by gender and SNP G900C genotypes

	SNP G900C genotypes, Females				SNP G900C genotypes, Males			
	G/G N475	G/C N477	C/C N121	p-value	G/G N168	G/C N143	C/C N28	p-value
	Mean ± SD	Mean ± SD	Mean ± SD	p-value	Mean ± SD	Mean ± SD	Mean ± SD	p-value
Age (years)	50.2±14.6	50.3±14.9	50.4±15.2	0.9952	47.6±16.0	46.7±15.8	49.8±15.4	0.6143
BMI (kg/m <sup>2</sup> )	31.1±7.8	30.9±8.1	29.1±7.4	0.0444	25.0±7.2	24.0±5.4	24.2±5.2	0.3801
WaistC (cm)	95.8±17.0	94.7±16.2	92.6±17.0	0.1540	86.6±17.1	85.8±15.3	87.1±14.5	0.8737
HipC (cm)	108.7±16.0	108.1±16.3	104.2±15.6	0.0227	94.3±13.6	95.1±10.5	95.8±11.8	0.7804
WHR	0.88±0.08	0.88±0.08	0.89±0.08	0.4714	0.91±0.09	0.90±0.10	0.91±0.07	0.3239
SPB (mmHg)	127.7±22.9	128.1±24.5	129.1±25.3	0.8423	126.3±24.0	127.6±25.0	131.0±27.9	0.6331
DBP (mmHg)	82.4±13.6	82.6±13.4	82.0±13.3	0.8797	80.4±14.4	80.6±16.4	85.1±18.0	0.3248
Pulse (bpm)	72.7±12.4	72.1±12.9	72.0±11.2	0.6982	68.4±12.7	69.0±14.0	67.5±13.8	0.8313
Fasting Blood Glucose (mmol/L)	5.74±2.71	6.01±3.09	6.52±4.52	0.0433	5.30±2.17	5.16±1.71	5.29±1.59	0.8031
2 HRs Post Glucose (mmol/L)	6.93±3.00	7.21±3.39	6.89±2.88	0.3742	5.45±2.30	5.60±2.76	6.03±3.47	0.5674
HbA1c (%)	6.22±1.50	6.36±1.76	6.46±2.00	0.2568	5.96±1.31	5.79±1.02	5.71±0.73	0.3009
Fasting Insulin (mIU/L)*	5.35 (7.50; 11.70)	7.40 (4.75; 11.85)	7.60 (4.80; 11.20)	0.6232	4.70 (2.80; 8.20)	4.80 (2.80; 9.80)	5.55 (2.90; 9.15)	0.8239
2 HRs Post Insulin (mIU/L)*	46.4 (25.2; 80.3)	47.5 (25.8; 78.2)	52.0 (27.3; 90.5)	0.6392	21.5 (10.6; 41.7)	21.5 (9.8; 43.3)	23.8 (5.9; 53.6)	0.9985
Glucose/Insulin ratio	0.86±0.71	0.94±0.83	1.04±1.29	0.0780	1.33±1.16	1.30±1.08	1.23±0.88	0.8929
Fructosamine-S (µmol/L)	250.9±59.1	263.0±74.6	260.7±94.6	0.1100	263.8±63.1	258.5±50.8	280.2±74.4	0.4537
Glycation gap	0.24±0.81	0.14±0.96	0.15±0.94	0.3735	-0.13±0.86	-0.25±0.72	-0.80±1.04	0.0239
Triglycerides (mmol/L) *	1.24 (0.89; 1.73)	1.26 (0.89; 1.76)	1.22 (0.86; 1.63)	0.6818	1.13 (0.87; 1.64)	1.09 (0.79; 1.65)	1.29 (0.87; 1.99)	0.2936
LDL-Chol (mmol/L)	3.33±1.02	3.34±1.06	3.19±0.85	0.3765	2.97±0.99	2.86±1.04	2.92±0.94	0.6399
HDL-Chol (mmol/L)	1.35±0.38	1.35±0.32	1.35±0.34	0.9744	1.28±0.37	1.22±0.40	1.30±0.37	0.2927
Total-Chol (mmol/L)	5.33±1.18	5.31±1.18	5.16±0.94	0.3687	4.89±1.14	4.72±1.17	4.85±1.16	0.4340
Chol/HDL ratio	4.17±1.18	4.11±1.12	4.04±1.11	0.5202	4.02±1.40	4.13±1.38	3.94±1.23	0.7082
Serum Cotinine (ng/mL)	132.8±159.3	122.4±159.5	145.5±171.5	0.3225	152.8±150.5	151.1±146.7	189.8±178.9	0.4595
Smokers, Yes, % (N)	217/464 (46.8%)	193/460 (42.0%)	57/119 (47.9%)	0.2602	96/161 (59.6%)	88/143 (61.5%)	17/27 (63.0%)	0.9150

Key:

\*: median and range (25Q; 75Q)

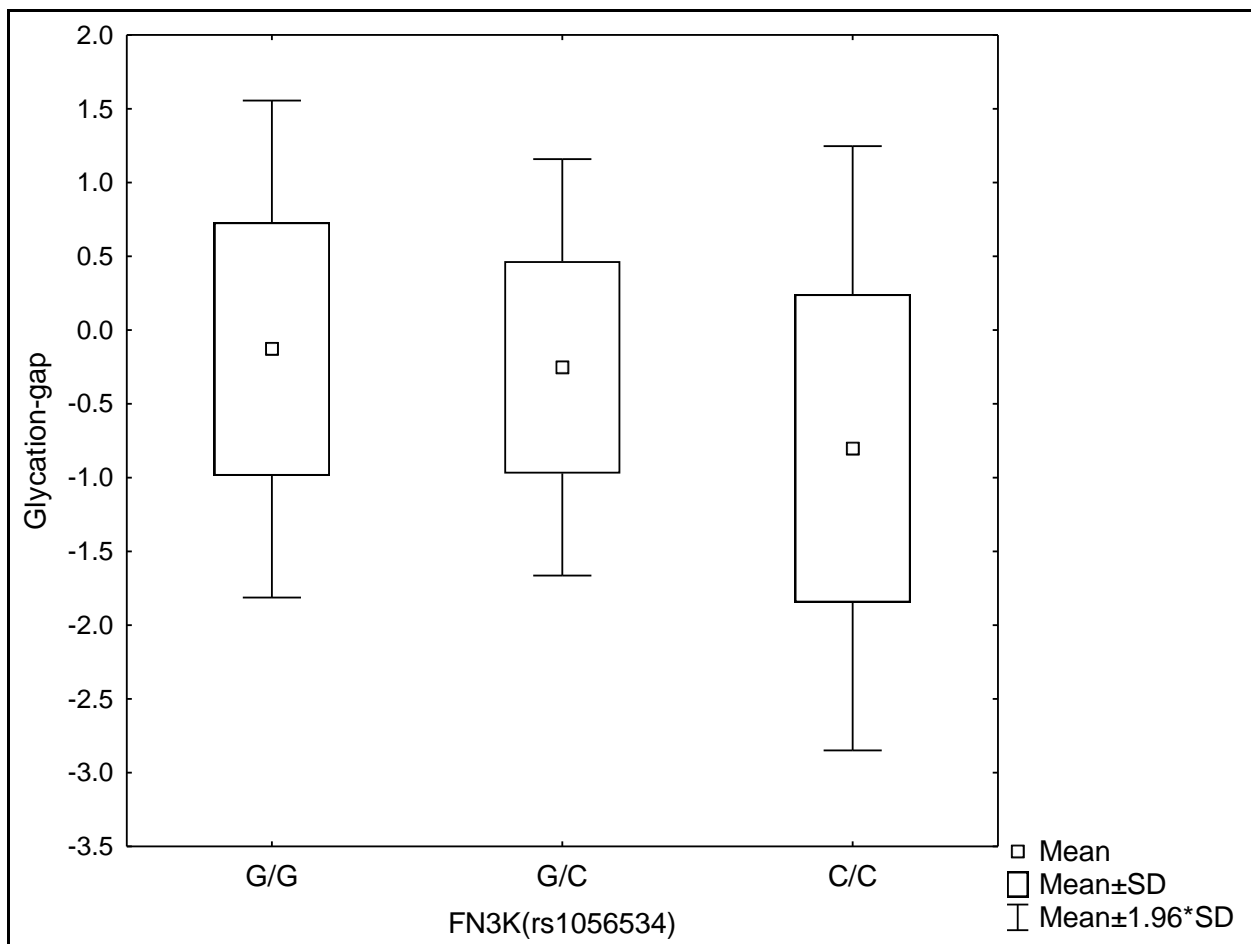


Figure 3.2. The glycation-gap showed a significant decrease through the FN3K SNP G900C (rs1056534) genotypes in males, mean  $\pm$  SD in genotype G/G  $-0.13 \pm 0.86$ , in genotype G/C  $-0.25 \pm 0.72$  and in genotype C/C  $-0.80 \pm 1.04$  ( $p=0.0239$ ). (Results were not significant in females).

### 3.4. Characteristics of the total study population categorized according to the Glycation-gap and glycaemic sub-groups.

Characteristics of the total study population categorized according to the glycation-gap and glycaemic sub-groups were summarized in Table 3.4. There was no significant difference in genotype frequencies (%) observed in the glycation-gap categories (Negative, Neutral and Positive) in normal, pre-diabetic and diabetic subjects ( $p=0.4318$ ,  $p=0.7950$  and  $p=0.1222$  respectively). There was a significant difference in age (years) across the glycation gap categories in diabetic subjects  $p=0.0300$  however no difference was observed in normal and pre-diabetic subjects. There was a significant increase in the BMI ( $\text{kg/m}^2$ ) (all  $p < 0.02$ ) as well as the WaistC (cm) (all  $p < 0.04$ ) across the glycation-gap categories in normal, pre-diabetic and diabetic subjects, while the HipC (cm) increased significantly across the glycation gap categories in normal and pre-diabetic subjects only (all  $p < 0.03$ ).

The glycaemic measures including fasting blood glucose (mmol/L), 2h-PG (mmol/L), HbA1c (%), fasting insulin (mIU/L), 2h-PI (mIU/L) increased significantly across the glycation gap categories in normal subjects (all  $p < 0.02$ ). HbA1c (%) and fasting insulin (mIU/L) increased in the pre-diabetic group from the neutral to the positive glycation gap categories (all  $p < 0.04$ ) (the negative glycation gap category had too few numbers for meaningful statistical analysis). The fasting blood glucose (mmol/L), 2h-PG (mmol/L), HbA1c (%), fasting insulin (mIU/L), 2h-PI (mIU/L) increased significantly from the neutral to the positive glycation gap categories (all  $p < 0.04$ ) (the negative glycation gap category had too few numbers for meaningful statistical analysis). Fructosamine ( $\mu\text{mol/L}$ ) decreased significantly across the glycation-gap categories in normal, pre-diabetic and diabetic subjects (all  $p < 0.0001$ ). Triglycerides (mmol/L) significantly increased across the glycation-gap categories in all glycaemic groups (all  $p < 0.04$ ). The HDL-Chol (mmol/L) significantly decreased across the glycation-gap categories in normal subjects ( $p=0.0004$ ) however no difference was observed in pre-diabetic and diabetic subjects.

Table 3.4. Characteristics of the total study population categorized according to the Glycation-gap and glycaemic sub-groups

G-Gap categorization	Categorized G-gap Normoglycaemic subjects, N501			p-value	Categorized G-gap Pre-DM subjects, N141			p-value	Categorized G-gap DM* subjects, N184			p-value
	Negative	Neutral	Positive		Negative*	Neutral	Positive		Negative**	Neutral	Positive	
	Mean ± SD				Mean ± SD				Mean ± SD			
N (%)	42/501 (8.38%)	429/501 (85.63%)	30/501 (5.99%)		5/141 (3.55%)	119/141 (84.40%)	17/141 (12.06%)		20/184 (10.87%)	115/184 (62.50%)	49/184 (26.63%)	
SNP G900C genotypes												
G/G N (%)	18/42 (42.9%)	184/429 (42.9%)	12/30 (40.0%)		2/5 (40.0%)	61/119 (51.3%)	8/17 (47.1%)		4/20 (20.0%)	50/115 (43.5%)	20/49 (40.8%)	
G/C N (%)	23/42 (54.8%)	196/429 (45.7%)	15/30 (50.0%)		3/5 (60.0%)	46/119 (38.7%)	8/17 (47.1%)		11/20 (55.0%)	56/115 (48.7%)	23/49 (46.9%)	
C/C N (%)	1/42 (2.4%)	49/429 (11.4%)	3/30 (10.0%)		0/5 (0.0%)	12/119 (10.1%)	1/17 (5.9%)		5/20 (25.0%)	9/115 (7.8%)	6/49 (12.2%)	
FN3K(rs1056534) genotypes: Overall P- value				0.4318				0.7950				0.1222
Age (years)	46.8±15.6	48.1±14.5	48.0±11.2	0.8531	60.6±15.1	56.3±14.1	55.0±9.3	0.7220	62.0±13.3	59.7±10.8	55.4±10.3	0.0300
BMI (kg/m <sup>2</sup> )	24.3±5.0	29.2±7.9	33.6±9.4	<0.0001	25.8±5.2	31.8±8.2	36.3±5.3	0.0174	29.2±7.0	32.6±7.0	34.5±6.8	0.0170
WaistC (cm)	82.8±12.1	93.5±16.2	104.5±18.5	<0.0001	91.5±13.0	100.0±15.4	108.6±13.8	0.0373	96.2±12.2	102.5±12.2	108.2±13.6	0.0010
HipC (cm)	99.4±11.1	107.4±15.1	115.1±20.3	0.0001	98.0±10.5	111.1±16.1	118.5±11.2	0.0278	105.3±13.6	111.0±13.8	114.1±13.4	0.0531
WHR	0.83±0.06	0.87±0.08	0.91±0.09	0.0002	0.93±0.07	0.90±0.07	0.92±0.07	0.5047	0.92±0.09	0.93±0.09	0.95±0.07	0.2053
SPB (mmHg)	117.9±21.3	123.6±21.9	125.7±22.4	0.2231	134.4±28.4	129.6±21.6	128.5±18.5	0.8649	143.9±35.5	134.0±19.2	136.0±24.7	0.2054
DBP (mmHg)	80.6±12.3	81.3±13.2	82.1±16.1	0.9046	85.0±7.2	82.0±12.3	81.1±12.0	0.8170	87.9±12.0	83.5±11.5	85.2±12.7	0.2618
Pulse (bpm)	70.6±10.6	69.7±12.1	74.1±13.2	0.1464	64.8±8.5	72.1±13.4	77.5±11.5	0.1152	82.5±17.9	73.8±13.2	81.5±14.7	0.0013



Fasting Blood Glucose (mmol/L)	4.79±0.4 2	4.75±0.5 0	5.02±0.4 9	0.0146	5.28±0.68	5.27±0.62	5.41±0.67	0.6980	12.03±5.16	8.41±3.96	10.31±4.82	0.0006
2 HRs Post Glucose (mmol/L)	5.59±1.2 1	5.55±1.3 2	6.38±1.0 9	0.0032	8.58±0.82	8.88±1.10	8.99±1.38	0.7742	17.76±3.37	12.98±2.89	15.93±3.10	0.0005
HbA1c (%)	5.24±0.3 9	5.61±0.3 9	6.35±0.3 7	<0.0001	5.24±0.55	5.92±0.44	6.61±0.32	<0.0001	8.89±2.21	7.84±2.08	9.63±2.32	<0.0001
Fasting Insulin (mIU/L)***	4.85 (3.60; 7.50)	6.70 (4.30; 9.60)	9.20 (5.90; 16.20)	0.0002	10.10 (6.80; 12.70)	9.00 (5.60; 14.40)	12.60 (8.60; 19.20)	0.0401	7.85 (5.60; 11.15)	9.40 (5.80; 16.70)	13.90 (9.25; 20.30)	0.0026
2 HRs Post Insulin (mIU/L)***	31.8 (13.2; 52.4)	36.1 (20.0; 67.0)	62.8 (39.1; 96.4)	0.0018	105.8 (72.1; 123.4)	78.4 (52.5; 134.8)	84.6 (43.7; 131.9)	0.9935	31.1 (30.6; 40.5)	67.7 (39.4; 95.7)	77.6 (72.4; 99.2)	0.0355
Glucose/Insulin ratio	1.14±0.6 9	0.88±0.6 4	0.65±0.4 8	0.0055	1.00±1.13	0.80±0.67	0.45±0.25	0.881	1.86±1.67	1.14±1.15	1.22±2.01	0.1359
Fructosamine (µmol/L)	275.3±2 2.3	232.6±2 0.0	208.0±1 7.4	<0.0001	269.1±20. 5	240.6±2 0.7	213.9±1 3.6	<0.0001	459.5±126. 2	323.8± 93.3	325.2±8 9.7	<0.0001
Glycation gap	- 1.35±0.3 6	- 0.01±0.5 2	1.30±0.2 6	<0.0001	- 1.21±0.12	0.12±0.50	1.42±0.30	<0.0001	-1.94±1.11	0.13±0.52	1.89±0.90	<0.0001
Triglycerides (mmol/L)***	0.92 (0.68; 1.29)	1.09 (0.81; 1.56)	1.33 (0.83; 1.88)	0.0214	1.12 (0.74; 1.58)	1.35 (1.03; 1.77)	1.99 (1.34; 2.59)	0.0376	1.56 (1.31; 2.07)	1.49 (1.12; 2.17)	1.85 (1.49; 3.00)	0.0077
LDL-Chol (mmol/L)	3.06±1.1 9	3.24±1.0 2	3.08±0.7 6	0.4276	3.04±0.85	3.55±0.95	3.39±0.77	0.4172	3.77±1.00	3.42±1.05	3.34±0.91	0.2717
HDL-Chol (mmol/L)	1.47±0.3 6	1.30±0.3 2	1.17±0.3 3	0.0004	1.24±0.36	1.30±0.31	1.28±0.24	0.8936	1.26±0.29	1.24±0.34	1.17±0.27	0.3553
Total-Chol (mmol/L)	5.09±1.2 9	5.19±1.1 6	4.95±0.8 4	0.4977	4.88±0.94	5.54±1.08	5.39±1.02	0.3657	5.84±1.25	5.35±1.18	5.33±1.05	0.1952
Chol/HDL ratio	3.61±1.0 7	4.16±1.1 2	4.61±1.5 3	0.0009	4.22±1.67	4.45±1.13	4.31±0.95	0.8061	4.79±1.16	4.52±1.30	4.70±1.08	0.5513
Serum Cotinine (ng/mL)	98.3±14 5.1	143.1±1 61.4	157.7±1 79.9	0.1898	79.4±108. 0	117.7±1 62.5	157.2±1 38.4	0.5281	45.0±89.0	92.1±1 54.7	82.7±14 8.5	0.4194
Smokers, Yes, % (N)	14/42 (33.3%)	208/427 (48.7%)	16/30 (53.3%)	0.1332	2/5 (40.0%)	45/119 (37.8%)	10/17 (58.8%)	0.2559	4/20 (20.0%)	28/115 (24.3%)	13/49 (26.5%)	0.8480

Key:

DM\*: Screen-detected and known DM combined

\*\*: Numbers too low for meaningful statistical analysis

\*\*\*: Median and range (25Q; 75Q)

### 3.5. Correlation analysis.

Correlation between the general characteristics of the study population and the Glycation-gap, categorized by gender are summarized in Table 3.5. The glycation-gap is positively correlated with the BMI in the total group, males and females;  $r=0.3694$ ,  $r=0.1973$  and  $r=0.3428$  respectively (all  $p < 0.01$ ) (Total group, Figure 3.3). Furthermore, the glycation-gap was positively correlated with Waist (cm), Hip (cm) and WHR in all groups, with the strongest correlations in the total and female groups. The glycation-gap is further positively correlated with fasting blood glucose (mmol/L), 2h-PG (mmol/L), fasting insulin (mIU/L) and 2h- PI (mIU/L) in the total group as well as in females (All  $< 0.0001$ ). In males the positive correlation was with fasting insulin (mIU/L) only ( $p < 0.0001$ ).

The glycation-gap was positively correlated with triglycerides (mmol/L) in the total group and females  $r=0.2540$  and  $r=0.3063$  respectively (all  $p < 0.0001$ ). In contrast, the glycation-gap was negatively correlated with HDL-Chol (mmol/L) in the total group, males and females;  $r=-0.2031$ ,  $p < 0.0001$ ;  $r=-0.1696$ ,  $p=0.0294$  and  $r=-0.2670$ ,  $p < 0.0001$  respectively.

Table 3.5. Correlation between the general characteristics of the study population and the Glycation-gap, categorized by gender

Glycation-gap	Total, N826		Males, N167		Females, N659	
	r	p-value	r	p-value	r	p-value
Age (years)	0.0280	0.4223	-0.0419	0.5913	0.0566	0.1466
BMI (kg/m <sup>2</sup> )	0.3694	<0.0001	0.1973	0.0111	0.3428	<0.0001
WaistC (cm)	0.3749	<0.0001	0.2016	0.0092	0.3765	<0.0001
HipC (cm)	0.3151	<0.0001	0.1748	0.0243	0.2696	<0.0001
WHR	0.2423	<0.0001	0.1978	0.0106	0.3072	<0.0001
SPB (mmHg)	0.0914	0.0086	-0.0578	0.4580	0.1449	0.0002
DBP (mmHg)	0.0322	0.3555	-0.1403	0.0706	0.0720	0.0647
Pulse	0.1747	<0.0001	0.0762	0.3276	0.1715	<0.0001
Fasting Blood Glucose (mmol/L)	0.1981	<0.0001	0.0535	0.4923	0.2159	<0.0001
2 Hours Post Glucose (mmol/L)	0.1948	<0.0001	-0.0444	0.6053	0.2147	<0.0001
HbA1c (%)	0.5128	<0.0001	0.4453	<0.0001	0.5307	<0.0001
Fasting Insulin (mIU/L)	0.3368	<0.0001	0.2093	0.0070	0.3241	<0.0001
2 Hours Post Insulin (mIU/L)	0.2240	<0.0001	0.0560	0.5126	0.2045	<0.0001
Glucose/Insulin ratio	-0.2753	<0.0001	-0.2315	0.0031	-0.2420	<0.0001
Fructosamine-S (µmol/L)	-0.4156	<0.0001	-0.4699	<0.0001	-0.3858	<0.0001
Triglycerides (mmol/L)	0.2540	<0.0001	0.0245	0.7529	0.3063	<0.0001
LDL-Chol (mmol/L)	0.0502	0.1505	-0.0227	0.7717	0.0470	0.2291
HDL-Chol (mmol/L)	-0.2031	<0.0001	-0.1696	0.0294	-0.2670	<0.0001
Total-Chol (mmol/L)	0.0229	0.5119	-0.0369	0.6355	0.0089	0.8191
Chol/HDL ratio	0.2011	<0.0001	0.1103	0.1584	0.2403	<0.0001
Serum Cotinine (ng/mL)	0.0556	0.1111	0.0934	0.2297	0.0914	0.0191

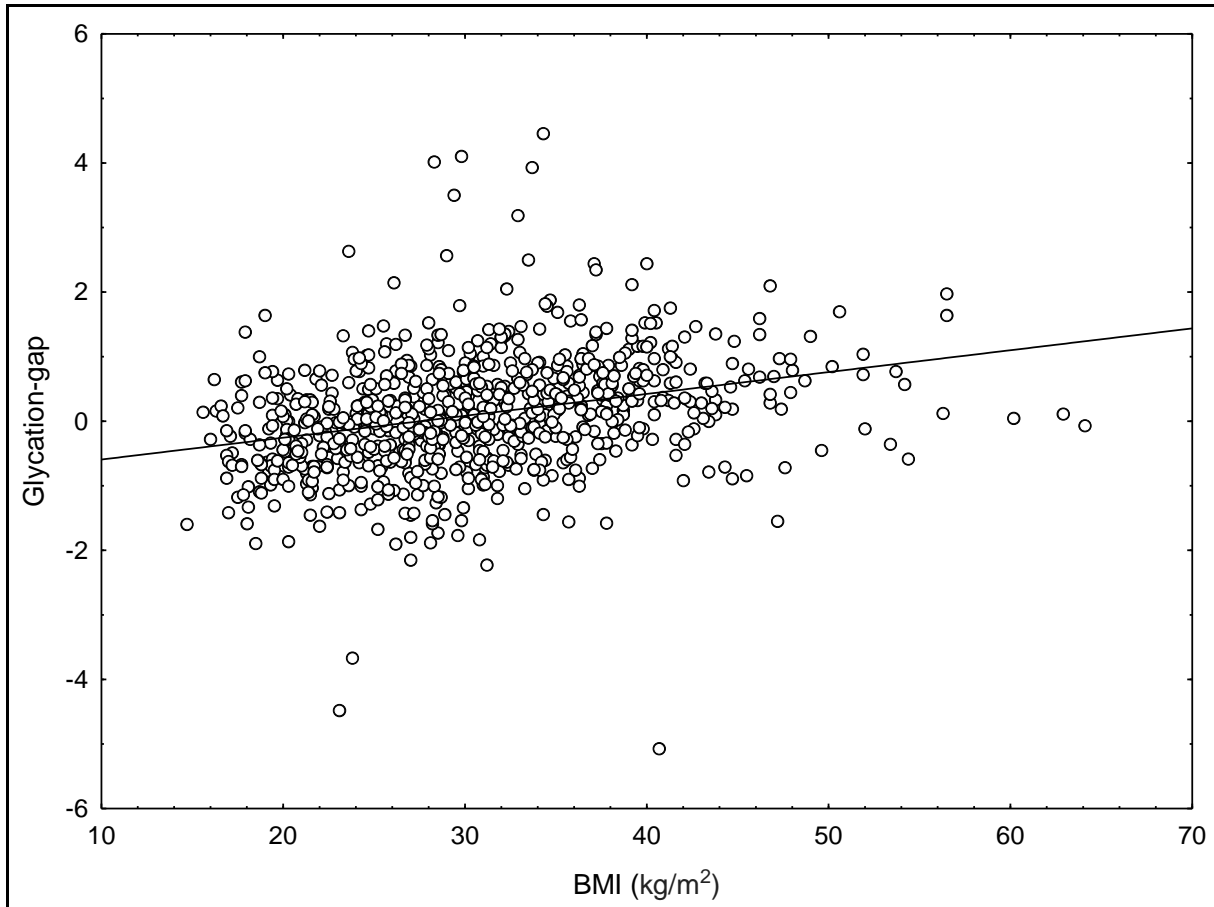


Figure 3.3. The glycation-gap showed a significant positive correlation with the BMI (kg/m<sup>2</sup>) in the total group of subjects,  $r=0.3694$ ;  $p<0.0001$ . (Similar results were shown in the male and female groups)

## Chapter 4: Discussion

### 4.1. Introduction

In this study, we investigated whether the genotypes of the FN3K gene had an effect on the variability encountered between HbA1c and fructosamine as measured by the glycation gap. Our results found that the glycation gap differed significantly across the genotypes of the FN3K SNP G900C.

The IDF estimated that 425 million people (20-79 years) around the world had DM in 2017 and this number is projected to increase to 629 million by 2045. Furthermore, it was reported that 4.0 million people aged between 20 and 79 years died from DM in 2017 with the highest proportion (0.23 million) of death occurring in the African region (IDF 2017). Non-enzymatic glycation of proteins has been implicated in the development of diabetic complications (Stitt et al. 2010). These proteins, such as HbA1c, fructosamine and glycated albumin may be useful in monitoring DM control and treatment, and recently HbA1c was also recommended for the diagnosis of DM (WHO 2011). HbA1c is a clinically relevant glycoprotein as it reflects the average plasma glucose for the past 2 - 3 months (Jeppsson et al. 2002). However, despite the reliability and standardization of HbA1c assays, several studies have reported inter-individual variations in the glycation of haemoglobin which may be due to factors other than glycaemia in type 1 DM (Cohen et al. 2003), type 2 DM (Rodriguez-Segade et al. 2011) and in non-diabetic subjects (Yudkin et al. 1990). Fructosamine reflects the average plasma glucose for a shorter period than HbA1c and may be more readily influenced by short-term changes in blood glucose but its concentration in plasma is much more stable than that of glucose itself. Fructosamine level can also be influenced by non-glycaemic factors such as clinical conditions that affect protein metabolism (Schleicher et al. 1993) and pathologic conditions linked to hypoproteinemia (Rodriguez-Segade et al. 1989).

Therefore, Cohen et al described the glycation gap which was used to study non-glycaemic factors which influence the glycation of haemoglobin (Cohen et al. 2003). Recent studies have found that the glycation gap is consistent over time within individuals (Nayak et al. 2011; Rodriguez-Segade et al. 2011) and is associated with diabetic complications as well as morbidity and mortality (Cohen et al. 2003; Rodriguez-Segade et al. 2011; Nayak et al. 2013). Therefore, determination of the glycation gap may be important to identify those who are at risk of developing diabetic complications

and also for therapeutic decisions. Recent identification of an enzyme FN3K as a part of a protein repair system opposing to the consequences of hyperglycaemia was of great interest (Delpierre et al. 2000). This enzyme catalyses the deglycation reaction which reverses the glycation of proteins thereby alleviating cellular damage due to accumulation of glycated proteins and protecting from diabetic complications (Szwergold et al. 2002). However, animal model studies have demonstrated that there is variation in the activity of FN3K enzyme between subjects (Veiga-da-cunha et al. 2006). Delpierre et al found that this variability in enzyme activity was associated with genetic polymorphisms in the FN3K gene (Delpierre et al. 2006). Recently Dunmore et al found that the variation in the glycation of proteins as measured by the glycation gap may be associated with differences in FN3K activity (Dunmore et al. 2018).

Therefore, in the present study we investigated the effects of the FN3K SNP genotypes on the glycation gap. We analysed two FN3K SNPs G900C (rs1056534) and c. -232A/T (rs2256534) located on exon 6 and the promoter region of the FN3K respectively in a total of 1412 subjects (925 normal, 216 pre-diabetic and 271 type 2 DM) aged  $\geq 20$  years. Out of the six SNPs of the FN3K described for the first time by Delpierre et al (Delpierre et al. 2006), we chose these two SNPs because they were previously reported to be associated with FN3K activity and levels of HbA1c (Mohás et al. 2010; Mosca et al. 2011). The genotypes TT of SNP c. -232A/T and CC of SNP G900C have been associated with increased activity of FN3K enzyme (Delpierre et al. 2006). However, in our study the polymorphism c.-232A/T didn't follow the Hardy-Weinberg Equilibrium and was therefore excluded from further analysis. The polymorphism G900C followed the Hardy-Weinberg Equilibrium and was therefore further evaluated. We found no significance difference in the genotype frequencies of G900C between the glycaemic groups (normal, pre-diabetic and type 2 DM), this is similar to the findings of Mohas et al who observed no significant differences in the genotype frequencies of SNP G900C between the type 2 diabetics and controls (Mohás et al. 2010). Delpierre et al also reported that the allele frequency was not significantly different between diabetics and controls (Delpierre et al. 2006). However, in our study the glycation gap levels were significantly higher in females compared to males ( $p < 0.0001$ ), the large number of females as compared to males and some confounders may partially have caused this difference.

## 4.2. FN3K genotypes and the glycation gap

According to our knowledge this is the first study to establish the role of FN3K genotypes on the glycation gap globally. Our research question was whether the glycation gap is indeed affected by the genotypes of the FN3K and this was supported by our results. We found a significant decrease in the glycation gap across the G900C genotypes (GG, GC and CC), with the lowest level in the CC genotype ( $P=0.0239$ ). Similarly, the concentration of HbA1c decreased across the G900C genotypes but this difference was not statistically significant ( $P=0.3009$ ). However, these findings were only observed in males. We postulate that this might be due to the fact that in our population the females were obese (BMI  $30.8\pm 7.9$ ) whereas the males were normal weight (BMI  $24.5\pm 6.3$ ). In our study the glycation gap was significantly positively correlated with BMI, waist and hip circumference. The positive glycation gap subgroup had significantly higher BMI, waist and hip circumference compared to the neutral and negative glycation gap groups across all glycaemic groups.

This is in agreement with the results of a study performed by Dunmore et al who analysed 148 White type 2 diabetics with a mean age of 61.3 years and identified two glycation gap groups that were distinctly dichotomised with a consistently positive (67) or negative glycation gap (81) ( $> +0.5\%$  or  $> -0.5\%$  HbA1c) and the glycation gap was calculated using the same formula as the one used in our study. They found that the negative glycation gap group had lower BMI levels compared to the positive glycation group in type 2 diabetics, mean  $\pm$  SD ( $30.2\pm 5.2$ ) and ( $35.4\pm 6.7$ )  $p<0.001$  respectively (Dunmore et al. 2018). Similarly, Nayak et al analysed 3182 White, Asian and Black type 2 diabetics ( $\geq 18$  years of age) and found that the glycation gap significantly positively correlated with BMI (Nayak et al. 2013).

A recent retrospective cohort study was performed in 105 Korean subjects (mean age of 54.5) with type 2 DM. They calculated the glycation gap using the method described by Cohen et al (2003) where FHbA1c was derived from HbA1c-Fructosamine regression equation, which is different from the formula used in our study which was described by Nayak et al (2013). In this formula FHbA1c was derived from simultaneously measured fructosamine standardized to the HbA1c distribution. However, instead of fructosamine they determined glycated albumin and the glycation gap was defined as the difference between the measured HbA1c level and that predicted from the glycated

albumin level, as calculated using the HbA1c-glycated albumin regression equation. They demonstrated that BMI, visceral fat area and abdominal subcutaneous fat were all significantly positively associated with the glycation gap (Kim et al. 2016). All these formulae found similar results to what was obtained in our study. However, to our knowledge, our study is the first to examine FN3K SNPs. Therefore, since the negative glycation gap implies that there is less glycation of haemoglobin than expected and positive glycation gap implies that there is more glycation of haemoglobin than expected, it may be postulated from these findings that individuals with higher BMI have higher rates of glycation and this may be expected as obesity is considered to be a risk factor for the development of type 2 DM (Motala et al. 2003). However, since in our study the males were normal weight we can postulate that the effect of the SNP G900C genotypes on the glycation gap was independent of obesity. Therefore it can be postulated that the glycation gap is influenced by genetic factors and this supports the findings of the monozygotic and dizygotic female twins study (40 and 46 pairs respectively) which demonstrated a strong correlation between monozygotic ( $r= 0.65$ ) than dizygotic ( $r= 0.48$ ) twins implying that the glycation gap is strongly inheritable (Cohen et al. 2006)

However, our study was limited by the lack of the measurement of the FN3K activity. We attempted to determine the FN3K activity using the recently developed colometric assay during the training provided by the developers in Belgium, UZ Gent Hospital (Cikomola et al. 2016). However, we couldn't find any enzyme activity using this method even after several troubleshooting efforts. It would have been interesting to study the FN3K genotypes, enzymatic activity and the glycation gap together. However, previous studies have reported on the activity of FN3K and expression in human and animal models (Veiga Da Cunha et al. 2006; Delpierre et al. 2006). The first study to report on the reasons behind the varying activity of FN3K enzyme is the study by Delpierre and associates. They reported on an association between FN3K enzyme activity and polymorphisms in the FN3K gene in a Belgian cohort of 31 type 1 diabetics and 26 controls without any selection in terms of age, sex, duration of disease and presence of chronic complications. They described that the CC genotype of the G900C was associated with reduced enzymatic activity measured in erythrocytes (Delpierre et al. 2006). However this study was limited due to the smaller sample size.

A second study by a Hungarian research group reported on the correlation of FN3K polymorphisms with HbA1c and the onset of DM. They enrolled a cohort of type 2 diabetics (859) and controls (265)



and genotyped those with a PCR restriction fragment length polymorphism (RFLP) method and determined that the CC genotype of the G900C was associated with later onset of type 2 DM than in the case of GC or GG genotypes. They further found that the CC genotype was associated with lower levels of HbA1c (Mohás et al. 2010). However, Delpierre et al reported that HbA1c was a poor substrate of FN3K due to its N-terminal valine of the  $\beta$ -chains which is glycated during its formation (Delpierre et al. 2006). Evidence for the association between the CC genotype and HbA1c was obtained from the analysis of a Caucasian cohort (70 diabetics, 35 type 1 DM and 35 type 2 DM) and 33 controls which were evaluated using PCR and direct sequencing of the FN3K gene. They demonstrated that the CC genotype of the G900C was associated with lower concentration of HbA1c (Mosca et al. 2011).

A further study performed in 314 subjects aged between 57 and 77 years with type 2 DM to investigate whether genetic variability in some genes might stimulate the progression of diabetic nephropathy and the morbidity and mortality associated with DM found that SNP G900C was associated with the progression of diabetic complications including nephropathy and cardiovascular morbidity and mortality (Tanhäuserová et al. 2014). Škrha et al conducted a research in 595 White (311 men and 284 women) aged between 18 to 93 years. These consisted of 126 healthy controls, 129 type 1 diabetics and 340 type 2 diabetics. They determined that a decreased concentration of soluble receptor for advanced glycation end products (sRAGE) associated with the GG genotype of G900C (Škrha et al. 2014). A recent study by Dunmore et al described the association of FN3K activity with the glycation gap. They analysed 148 White type 2 diabetics with a mean age of 61.3 years and reported that the activity and levels of FN3K were significantly higher in the negative glycation gap group compared to the positive glycation gap group (Dunmore et al. 2018).

Taking into consideration that HbA1c is a principal measurement in the calculation of the glycation gap, it can be assumed that when the HbA1c levels are high, the value of the glycation gap would be high and vice versa (Nayak et al. 2013). Hence, considering our results and findings of the studies described above, it can be postulated that the CC genotype of G900C is associated with increased FN3K activity in erythrocytes thereby resulting in lower levels of HbA1c and lower glycation gap level, whereas the GG genotype of G900C is associated with decreased activity of FN3K and higher levels of HbA1c and higher glycation gap. This is demonstrated in Figure 4.1. We postulate that the difference in the glycation of proteins as measured by the glycation gap may be

due to the variation in FN3K genotypes affecting the activity of the FN3K enzyme thus the levels of HbA1c. Therefore, since the glycation gap is an important predictor of risk of DM complications, we postulate that the presence of the CC genotype of G900C SNP serves as a defender against the risk of developing DM related complications. However, our study was limited as when performing real time PCR for SNP genotyping only negative controls were included without positive controls. However, our real time data is considered valid since some of our samples were repeated and others had been sent for sequencing which served as positive controls for the SNP of interest.

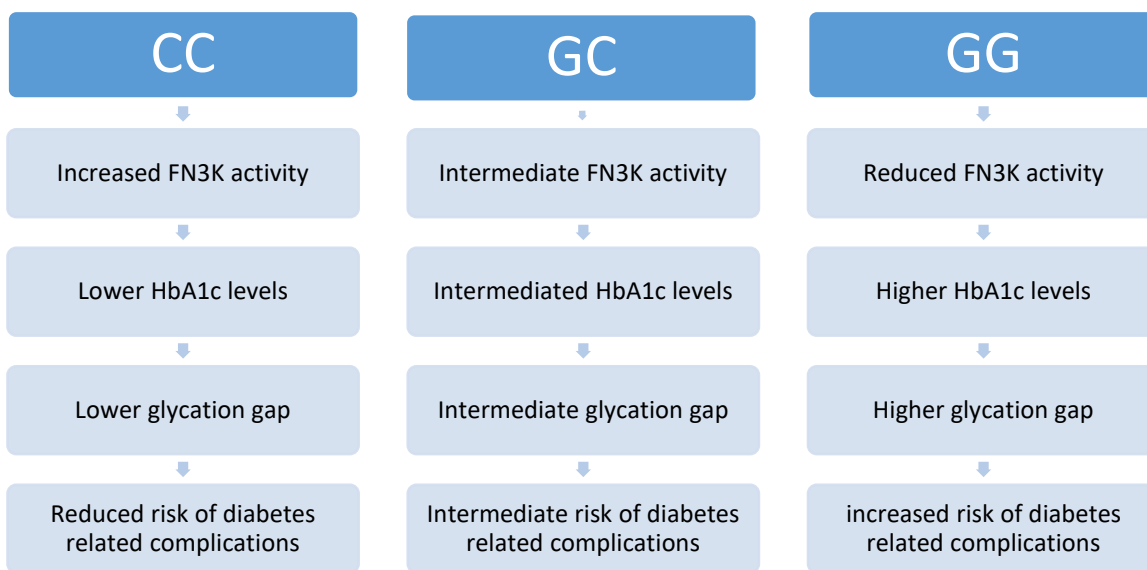


Figure 4.1: Summarized role of FN3K SNP G900C genotypes (CC, GC and GG)

### 4.3. Glycation gap correlation analysis

Furthermore, in our study we found a moderate positive correlation ( $r=0.51$ ) between the glycation gap and HbA1c in the total subjects. This is similar to Cohen who performed a study in 153 type 1 diabetics aged 15 years and older and calculated the glycation gap as the difference between measured HbA1c and HbA1c predicted from the fructosamine based on the HbA1c-Fructosamine regression equation. They reported a moderate positive correlation  $r=0.48$  between the glycation gap and HbA1c (Cohen et al. 2003). However, Chalew et al performed a study in 62 type 1 diabetics and calculated the glycation gap according to Cohen et al (2003) and found a stronger correlation

between the glycation gap and HbA1c ( $r=0.7$ ) (Chalew et al. 2005). This is contrary to our findings and could have been due to the fact that their glycation gap was determined in type 1 diabetics whereas ours was determined in type 2 diabetics as well as prediabetic and normal subjects. Also their study population was much smaller than ours.

Paleari et al explored the glycation gap in a selected group of nondiabetic and diabetic subjects, a total of 157 subjects (68 males and 89 females) aged between 18 and 75 years and they observed a strong positive correlation between the glycation gap and HbA1c in nondiabetic subjects ( $r = 0.9468$ ), and a weaker correlation in subjects with HbA1c above the upper limit for non-diabetic subjects ( $r=0.5091$ ) (Paleari et al. 2016). This study supports the findings of our study and provides evidence that the weak correlation found between the glycation gap and HbA1c in our study could be due to the fact that our nondiabetics and diabetics were analysed together. Accordingly, the findings of the present study imply that HbA1c only explains 51% of the variation in the glycation gap while 49% is explained by other confounding factors.

In our study we found a negative correlation ( $r= -0.4156$ ,  $p<0.0001$ ) between the glycation gap and fructosamines as expected according to the concept of the glycation gap. Our findings are similar to the results of Dziedzic et al who studied 93 healthy subjects (63 women and 30 men), between the ages of 18-79 and found a significant negative correlation between fructosamine and the glycation gap ( $r = -0.3$ ,  $p = 0.003$ ) (Dziedzic et al. 2012). However, our results contradict Rodriguez-Segade et al who studied 2314 type 2 diabetics (1118 males and 1196 females) with a mean age of 59.9 years and determined the glycation gap according to Cohen et al (2003). They found that mean fructosamine levels did not differ significantly among the glycation gap groups (Rodriguez-Segade et al. 2011). Their subjects had diabetic nephropathy which may have affected the levels of fructosamines hence no association was found (Rodriguez-Segade et al. 2011). Our results are also contrary to what Cosson et al found. They investigated the association of the glycation gap and DM complications in 925 type 2 diabetics with a mean age of 57.8 years and found an unexpected positive association between the glycation gap and fructosamine (Cosson et al. 2013). This may be due hypoproteinaemia resulting from nephropathy and macroproteinuria thereby affecting the levels of fructosamines (Cosson et al. 2013).

In our study we also found no correlation between the glycation gap and age in the total group. Our results contradict those of Dziedzic et al who found a significant positive correlation between age and the glycation gap ( $r = 0.34$ ,  $p = 0.0006$ ) in 93 healthy subjects (63 women and 30 men) between the ages of 18-79 (Dziedzic et al. 2012). This may have been because their glycation gap was only determined in healthy subjects in this study, whereas in our study it was determined in diabetic, prediabetic and normal subjects. Evidence for this postulation come from the study by Paleari et al who explored the glycation gap on a sub-group of 111 healthy subjects (49 females and 62 males) and found that the correlation between the glycation gap and age was slightly weaker if non-healthy individuals are added into the analysis ( $r = 0.4486$  vs  $0.4217$ , respectively) (Paleari et al. 2016). However, these findings contradict the findings of a retrospective cohort study performed in 105 Korean subjects (mean age of 54.5) with type 2 DM which found a weak negative correlation between the glycation gap and age ( $r = -0.203$ ,  $P = 0.008$ ) (Kim et al. 2016).

Further analysis of the glycation gap in our study found that there were several other confounding factors independent of glycaemia which need to be taken into consideration. We found that the glycation gap correlated significantly with triglycerides and HDL cholesterol. The positive glycation gap group had increased levels of triglycerides and decreased levels of HDL cholesterol, whereas the negative glycation group had decreased levels of triglycerides and increased levels of HDL cholesterol. Low levels of HDL cholesterol and high levels of triglycerides are part of the diabetic dyslipidaemia and are a major risk factor for cardiovascular disease in DM (Chehade et al. 2013). Low levels of HDL cholesterol and high levels of triglycerides in type 2 DM are associated with the risk of macroangiopathy which is linked to cardiovascular diseases (Hermans & Valensi 2018). It can be postulated from these findings that the positive glycation gap might be associated with diabetic dyslipidaemia. Our findings agree with the findings of the study performed by Cosson et al in a cohort of 925 adults with type 2 DM who described that increasing glycation gap tertiles were positively associated with dyslipidaemia (Cosson et al. 2013).

Others have also reported on the association of the glycation gap and DM complications showing that type 2 diabetics with high glycation gap are at a high risk of developing microvascular or macrovascular complications (Cohen et al. 2003; Rodriguez-Segade et al. 2011; Nayak et al. 2013). However, due to the cross-sectional nature of our study, we could not determine whether any association with diabetic complications.

## Chapter 5: Conclusion

In conclusion, our study provided evidence that there are active genetic mechanisms in the intracellular RBC compartment resulting in varying activity of the enzyme FN3K, thus affecting the levels of HbA1c through the deglycation process. The males in our study had normal BMI and as the varying genotypes of SNP G900C was only observed in males, it can be postulated that the glycation gap variations observed are independent of BMI. These findings support the protective role of the CC genotype of the polymorphism G900C against the effects of non-enzymatic protein glycation reactions in DM. Therefore, it can be concluded that identification of the G900C polymorphism in an early stage of the disease could be useful, especially in therapeutic decisions and prediction of a better prognosis. Furthermore, the present study provided evidence that the glycation gap varies with demographic factors and is heritable, clearly showing that the concept of the glycation gap needs to be treated with caution and more understanding of it is required. Hence, the findings of the present study still need to be confirmed and followed up.

### 5.1. Limitations of the study

Even though our study population was not small, we had a limited number of diabetics (271) compared to the normal subjects. When these were then further subdivided according to various genotypes of G900C SNP of FN3K, the groups became smaller hence decreasing the statistical power of our results. Another limitation was the disproportionate number of females compared to males (1073 versus 339). Additionally, the Bellville South population may have high prevalence of smokers and smoking may be unseen confounder and we didn't control for it. Therefore, for the glycation gap analysis, the two groups were analysed together which might have influenced our results since we found a significant difference in the glycation gap between males and females ( $p < 0.0001$ ) which is similar to what other studies have reported (Cohen et al. 2006; Nayak et al. 2011). A further limitation of our study was that we used fructosamine which is a marker for the extracellular glycation and short term glycaemic control for the determination of the glycation gap. Fructosamine is known to be affected by proteinuria and protein turn over which may have influenced the glycation gap. The high glycation gap observed may reflect the presence of proteinuria. Instead we should have used glycated albumin because the percentage of glycated albumin is not influenced by the albumin concentration (Yeungb et al. 1992).

Furthermore, for the glycation gap analysis the normal, pre-diabetic and diabetic subjects were analysed together because further subdivision of these glycaemic groups into glycation gap categories could have resulted in smaller numbers making our results unreliable. However, we found no significant difference observed in the glycation gap among the glycaemic groups (normal, pre-diabetic and diabetic). Another major limitation of the present study was we didn't control for the glycation gap confounding factors including BMI, waist and hip circumference, HDL cholesterol and triglycerides which might have influenced our results. Hence, no conclusion could be made regarding the glycation gap in the present study as it was not known if our findings were independent of these factors as they significantly correlated with the glycation gap.

## 5.2. Clinical implications

There is considerably less evidence about various genotypes of FN3K SNPs that might confer protection against DM and its related complications. Our findings are very useful in research because they show evidence that FN3K has a protective role in type 2 DM. The association of the CC genotype of G900C with low levels of the glycation gap confirm that FN3K can significantly reduce the risk of developing diabetic related complications. Furthermore, we provided evidence that the glycation gap can be an important research tool that can be used to evaluate underlying factors beyond glycaemic control which might be influencing the development of diabetic complications. Therefore since the glycation gap is easily accessible it may be useful in DM management and could help with developing new therapeutic approaches and allow consideration of targeted interventions.

## 5.3. Recommendation for future studies

We recommend that larger prospective studies are necessary to investigate the effects of FN3K genotypes on the glycation gap. It would be interesting to correlate FN3K genotypes with enzyme activity and the glycation gap in diabetics and those with various diabetic microvascular and macrovascular complications. This is necessary for better understanding of the role of this various FN3K genotypes in DM and its related complications as well as its possible role in the management of the disease. Furthermore, future studies can investigate if the presence of specific genotypes in FN3K SNPs is associated with the development of diabetic complications and if they can help in early diagnosis of complications resulting in a better prognosis of the disease. Although several

studies have shown that the glycation gap is consistent over time within individuals, it is associated with DM microvascular and macrovascular complications as well as DM related mortality. It has also been shown that the glycation gap is associated with several demographic factors. In the present study we confirmed that it is affected by the genotypes of G900C SNP of FN3K. Therefore, there is a huge “gap” that still needs to be filled and more studies should be performed in order to understand the mechanisms behind this phenomenon independent of the confounding factors. Clinical trials could be necessary to help determine how best to use the glycation gap in clinical practice. Animal model studies could be necessary to explore the therapeutic possibilities of FN3K enzyme for DM and its complications.

## Reference

- American Diabetes Association. (1997). Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes care*, 20, 1183-1197.
- ADA, 2017. Classification and Diagnosis of Diabetes. , 40(January), pp.11–24.
- ADA, 2006. Diagnosis and Classification of Diabetes Mellitus.
- ADA, 2010. Diagnosis and classification of diabetes mellitus. *Diabetes care*, 33 Suppl 1, pp.S62-9.
- Adeoye, S. et al., 2014. Anaemia and Haemoglobin A1c level: Is there a case for redefining reference ranges and therapeutic goals? *BJMP*, 7(1), p.6.
- Adler, A. I., Stratton, I. M., Neil, H. A. W., Yudkin, J. S., Matthews, D. R., Cull, C. A., ... & Holman, R. R. (2000). Association of systolic blood pressure with macrovascular and microvascular complications of type 2 diabetes (UKPDS 36): prospective observational study. *Bmj*, 321(7258), 412-419.
- Alberti, K.G.M.M. & Zimmet, P.Z., 1998. Definition , Diagnosis and Classification of Diabetes Mellitus and its Complications Part 1 : Diagnosis and Classification of Diabetes Mellitus Provisional Report of a WHO Consultation. , pp.539–553.
- Andersen, A. et al., 1983. *Diabetologia*. , 1, pp.496–501.
- Anon, 1999. WHO\_NCD\_NCS\_99.2.pdf. , p.66.
- Avemaria, F. et al., 2015. Possible role of fructosamine 3-kinase genotyping for the management of diabetic patients. , 53(9), pp.1315–1320.
- Babenko, A.P. et al., 2006. Activating mutations in the ABCC8 gene in neonatal diabetes mellitus. *New England Journal of Medicine*, 355(5), pp.456–466.
- Barth, J.H., Marshall, S.M. & Watson, I.D., 2008. Consensus meeting on reporting glycosylated haemoglobin (HbA1c) and estimated average glucose (eAG) in the UK: report to the National Director for Diabetes, Department of Health. *Diabetic Medicine*, 25(4), pp.381–382.
- Baynes, J.W. & Thorpe, S.R., 2000. Glycooxidation and lipoxidation in atherogenesis. *Free Radical Biology and Medicine*, 28(12), pp.1708–1716.
- Beaglehole, R. & Yach, D., 2003. Globalisation and the prevention and control of non-communicable disease: the neglected chronic diseases of adults. *The Lancet*, 362(9387), pp.903–908.
- Beisswenger, P.J. et al., 2003. Alpha-oxoaldehyde metabolism and diabetic complications.



- Biochemical Society transactions*, 31(Pt 6), pp.1358–1363.
- Bergenstal, R.M. et al., 2017. Racial Differences in the Relationship of Glucose Concentrations and Hemoglobin A1c Levels. *Annals of Internal Medicine*.
- Borch-Johnsen, K. (2007). The metabolic syndrome in a global perspective. The public health impact--secondary publication. *Dan Med Bull*, 54(2), 157-159.
- Brownlee, M., 2001. Biochemistry and molecular cell biology of diabetic complications. *Nature*, 414(6865), pp.813–820.
- Bry, L., Chen, P.C. & Sacks, D.B., 2001. Effects of hemoglobin variants and chemically modified derivatives on assays for glycohemoglobin. *Clinical chemistry*, 47(2), pp.153–163.
- Cartee, G.D., 2015. Mechanisms for greater insulin-stimulated glucose uptake in normal and insulin-resistant skeletal muscle after acute exercise. *American journal of physiology. Endocrinology and metabolism*, 309(12), pp.E949-59.
- Census, W. cape, 2011. *Census 2011 Municipal report Western Cape*,
- Cervin, C. et al., 2008. Genetic similarities between latent autoimmune diabetes in adults, Diabetes in Adults , type 1 diabetes , and type 2 diabetes. *Diabetes*. 2008; 57 (5): GENETICS OF LADA. , pp.1433–1437.
- Chalew, S.A. et al., 2005. A comparison of the Glycosylation Gap and Hemoglobin Glycation Index in patients with diabetes. , 19, pp.218–222.
- Chehade, J.M., Gladysz, M. & Mooradian, A.D., 2013. Dyslipidemia in Type 2 Diabetes : Prevalence , Pathophysiology , and Management. , (October 2012), pp.327–339.
- Chen, M. E., Aguirre, R. S., & Hannon, T. S. (2018). Methods for Measuring Risk for Type 2 Diabetes in Youth: the Oral Glucose Tolerance Test (OGTT). *Current diabetes reports*, 18(8), 51.
- Cikomola, J.C. et al., 2016. A simple colorimetric assay for measuring fructosamine 3 kinase activity. *Clinical Chemistry and Laboratory Medicine (CCLM)*.
- Cohen, R.M. et al., 2003. Discordance between HbA1c and fructosamine: evidence for a glycosylation gap and its relation to diabetic nephropathy. *Diabetes care*, 26(1), pp.163–167.
- Cohen, R.M. et al., 2006. Evidence for independent heritability of the glycation gap (glycosylation gap) fraction of HbA1c in nondiabetic twins. *Diabetes care*, 29(8), pp.1739–1743.
- Cohen, R.M. et al., 2008. Relationship of prospective GHb to glycated serum proteins in incident

- diabetic retinopathy: implications of the glycation gap for mechanism of risk prediction. *Diabetes care*, 31(1), pp.151–153.
- Collard, F. et al., 2003. A mammalian protein homologous to fructosamine-3-kinase is a ketosamine-3-kinase acting on psicosamines and ribulosamines but not on fructosamines. *Diabetes*, 52(12), pp.2888–2895.
- Collard, F. et al., 2004. Fructosamine 3-kinase-related protein and deglycation in human erythrocytes. *The Biochemical journal*, 382(Pt 1), pp.137–143.
- Conner, J.R., Beisswenger, P.J. & Szwegold, B.S., 2004. The expression of the genes for fructosamine-3-kinase and fructosamine-3-kinase-related protein appears to be constitutive and unaffected by environmental signals. , 323, pp.932–936.
- Costa, B. et al., 2007. Relevance of casual undetected hyperglycemia among high-risk individuals for developing diabetes. *Diabetes research and clinical practice*, 78(2), pp.289–292.
- Danese, E. et al., 2015. Advantages and pitfalls of fructosamine and glycated albumin in the diagnosis and treatment of diabetes. *Journal of diabetes science and technology*, 9(2), pp.169–176.
- Delpierre, G. et al., 2000. Conversion of a synthetic fructosamine into its 3-phospho derivative in human erythrocytes. *The Biochemical journal*, 352 Pt 3, pp.835–839.
- Delpierre, G. et al., 2002. Fructosamine 3-kinase is involved in an intracellular deglycation pathway in human erythrocytes. *The Biochemical journal*, 365(Pt 3), pp.801–808.
- Delpierre, G. et al., 2000. Identification, cloning, and heterologous expression of a mammalian fructosamine-3-kinase. *Diabetes*, 49(10), pp.1627–1634.
- Delpierre, G. et al., 2006. Variability in erythrocyte fructosamine 3-kinase activity in humans correlates with polymorphisms in the FN3K gene and impacts on haemoglobin glycation at specific sites. *Diabetes & metabolism*, 32(1), pp.31–39.
- Delpierre, G. & Schaftingen, E. Van, 2003. Fructosamine 3-kinase , an enzyme involved in protein deglycation. , 31, pp.1354–1357.
- Delpierre, G. et al., 2004. Identification of fructosamine residues deglycated by fructosamine-3-kinase in human hemoglobin. *The Journal of biological chemistry*, 279(26), pp.27613–27620.
- Delplanque, J. et al., 2004. Tissue distribution and evolution of fructosamine 3-kinase and fructosamine 3-kinase-related protein. *The Journal of biological chemistry*, 279(45), pp.46606–46613.

- Diabetes Care, D.O.F., 2010. Diagnosis and classification of diabetes mellitus. *Diabetes Care*, 33(SUPPL. 1).
- Diabetes Care, R.D., 2003. Follow-up report on the diagnosis of diabetes mellitus : The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus
- Diabetes Care., 2004. Diabetes mellitus and other categories of description of diabetes. , 27.
- Diabetes Care. 1998. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Report of the expert committee on the diagnosis and classification of diabetes mellitus.;21(Suppl 1):S3-519
- Diabetes Control and Complications Trial Research Group. (1993). The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *New England journal of medicine*, 329(14), 977-986.
- Dillard-cannon, E., 2014. Management of adult diabetic ketoacidosis. , pp.255–264.
- Doria, A. et al., 1999. Phenotypic characteristics of early-onset autosomal-dominant type 2 diabetes unlinked to known maturity-onset diabetes of the young (MODY) genes. *Diabetes care*, 22(2), pp.253–261.
- D.P.P.R, Group., 2009. 10-year follow-up of diabetes incidence and weight loss in the Diabetes Prevention Program Outcomes Study. *The Lancet*, 374(9702), pp.1677–1686.
- Driskell, O.J. et al., 2014. Reduced Testing Frequency for Glycated Hemoglobin , HbA 1c , Is Associated With Deteriorating Diabetes Control. , 37(January), pp.2731–2737Dunmore, S.J. et al., Evidence that differences in fructosamine-3-kinase activity may be associated with the glycation gap in human diabetes. , 44(0).
- Dziedzic, M. et al., 2012. Level of glycation gap in a healthy subject. , 19(4), pp.842–845.
- Cosson, E. et al., 2013. Glycation Gap Is Associated With Macroproteinuria but Not With Other. , 36(September 2012).
- Erasmus, R.T. et al., 2012. High prevalence of diabetes mellitus and metabolic syndrome in a South African coloured population: Baseline data of a study in Bellville, Cape Town. *SAMJ: South African Medical Journal*, 102(11), pp.841–844.
- Eupen, M.G.A. Van et al., 2013. Plasma levels of advanced glycation endproducts are associated with type 1 diabetes and coronary artery calcification. , pp.1–9.
- Fetita, L., 2006. REVIEW : Consequences of Fetal Exposure to Maternal. , 91(10), pp.3718–3724.
- Finke, A. et al., 1998. Preparation of a candidate primary reference material for the international

- standardisation of HbA1c determinations. *Clinical chemistry and laboratory medicine*, 36(5), pp.299–308.
- Flemmer, M. C., & Vinik, A. I. (2000). Evidence-based therapy for type 2 diabetes: The best and worst of times. *Postgraduate medicine*, 107(5), 27-47.
- Franco, R.S., 2012. Measurement of red cell lifespan and aging. *Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie*, 39(5), pp.302–307.
- Frykberg, R., 1998. Pressures in Diabetic Foot Ulceration. , (August 2017).
- Gallagher, E. J., Le Roith, D., & Bloomgarden, Z. (2009). Review of hemoglobin A1c in the management of diabetes. *Journal of diabetes*, 1(1), 9-17.
- Gavin III, J.R. et al., 1997. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes care*, 20(7), p.1183.
- Genuth, S. et al., 2003. Implications of the United kingdom prospective diabetes study. *Diabetes care*, 26 Suppl 1, pp.S28-32.
- George, J.A. & Ja, G., 2017. Should haemoglobin A 1c be used for the diagnosis of diabetes mellitus in South Africa ? Should haemoglobin A 1c be used for the diagnosis of diabetes mellitus in South Africa ? , 9677(September), pp.1–7.
- Goh, S. & Cooper, M.E., 2008. The Role of Advanced Glycation End Products in Progression and Complications of Diabetes. , 93(April), pp.1143–1152.
- Goldstein, B.J. & Müller-Wieland, D., 2016. *Type 2 diabetes: principles and practice*, CRC Press.
- Gottsä, A., Bakhtadze, E. & Berger, B., 2005. Definition, Prevalence,  $\beta_2$ -Cell Function, and Treatment. , 54(December), pp.1–5.
- Gram-Hansen, P., et al. "Glycosylated haemoglobin (HbA1c) as an index of the age of the erythrocyte population in non-diabetic patients." *European journal of haematology* 44.3 (1990): 201-203.
- Group, D.R., 1993. The New England Journal of Medicine Downloaded from [nejm.org](http://nejm.org) on August 5, 2017. For personal use only. No other uses without permission. Copyright © 1993 Massachusetts Medical Society. All rights reserved.
- Gugliucci, A. & Bendayan, M., 1995. Histones from diabetic rats contain increased levels of advanced glycation end products. *Biochemical and biophysical research communications*, 212(1), pp.56–62.

- Hancock, C.R. et al., 2008. High-fat diets cause insulin resistance despite an increase in muscle mitochondria. , 105(22).
- Hawa, M.I. et al., 2013. Adult-onset autoimmune diabetes in Europe is prevalent with a broad clinical phenotype: Action LADA 7. *Diabetes Care*, 36(4), pp.908–913.
- Helminen, O. et al., 2015. OGTT and random plasma glucose in the prediction of type 1 diabetes and time to diagnosis. , pp.1787–1796.
- Hempe, J.M. et al., 2002. High and low hemoglobin glycation phenotypes in type 1 diabetes: a challenge for interpretation of glycemic control. *Journal of diabetes and its complications*, 16(5), pp.313–320.
- Hempe, J.M. et al., 2015. The Hemoglobin Glycation Index Identifies Subpopulations With Harms or Benefits From Intensive Treatment in the ACCORD Trial. , 38(June), pp.1067–1074.
- Herman, W.H. et al., 2007. Differences in A1C by race and ethnicity among patients with impaired glucose tolerance in the Diabetes Prevention Program. *Diabetes care*, 30(10), pp.2453–2457.
- Hermans, M.P. & Valensi, P., 2018. Elevated triglycerides and low high-density lipoprotein cholesterol level as marker of very high risk in type 2 diabetes.
- Hirata, C. et al., 1997. Advanced glycation end products induce expression of vascular endothelial growth factor by retinal Müller cells. *Biochemical and biophysical research communications*, 236(3), pp.712–715.
- Hoelzel, W. & Miedema, K., 1996. Development of a reference system for the international standardization of HbA1c/glycohemoglobin determinations. *Journal of the International Federation of Clinical Chemistry / IFCC*, 8(2), pp.62-64,66-67.
- Imagawa, A., 2004. Discovery and establishment of fulminant type 1 diabetes mellitus and new classification of type 1 diabetes mellitus based on it. *J Jpn Diabetes Soc.*, 47, pp.796–797.
- Imagawa, A. et al., 2003. Fulminant type 1 diabetes: a nationwide survey in Japan. *Diabetes care*, 26(8), pp.2345–2352.
- International Diabetes Federation, I.D.F., 2017. IDF\_DA\_8e-EN-final.pdf.
- International Diabetes Federation, I.D.F., 2015. *DIABETES*,
- International Diabetes Federation, I.D.F., 2014. Global guideline for type 2 diabetes. *Diabetes research and clinical practice*, 104(1), pp.1–52.
- International Diabetes Federation, I.D.F., 2013. IDF diabetes atlas. *Brussels: International Diabetes Federation*.

- International Expert Committee, I.C.E., 2009. International Expert Committee report on the role of the A1C assay in the diagnosis of diabetes. *Diabetes care*, 32(7), pp.1327–1334.
- James, R. et al., 1997. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus.
- Jansen, H. et al., 2013. Determinants of HbA1c in nondiabetic Dutch adults: genetic loci and clinical and lifestyle parameters, and their interactions in the Lifelines Cohort Study. *Journal of internal medicine*, 273(3), pp.283–293.
- Jeppsson, J.-O. et al., 2002. Approved IFCC reference method for the measurement of HbA1c in human blood. *Clinical Chemistry and Laboratory Medicine*, 40(1), pp.78–89.
- Jeppsson, J.O. et al., 1986. Measurement of hemoglobin A1c by a new liquid-chromatographic assay: methodology, clinical utility, and relation to glucose tolerance evaluated. *Clinical chemistry*, 32(10), pp.1867–1872.
- John, W.G. et al., 2007. HbA1c standardisation: history, science and politics. *The Clinical biochemist.Reviews / Australian Association of Clinical Biochemists*, 28(4), pp.163–168.
- John, W. G., Hillson, R., & Alberti, S. G. (2012). Use of haemoglobin A1c (HbA1c) in the diagnosis of diabetes mellitus. The implementation of World Health Organisation (WHO) guidance 2011. *Practical Diabetes*, 29(1), 12-12a.
- Juneja, R. et al., 2001. Islet Cell Antibodies and Glutamic Acid Decarboxylase Antibodies, But Not the Clinical Phenotype, Help to Identify Type 1 1/2 Diabetes in Patients Presenting With Type 2 Diabetes. , 50(9), pp.1008–1013.
- Kahn, S. E., Hull, R. L., & Utzschneider, K. M. (2006). Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature*, 444(7121), 840.
- Kim, M.K. et al., 2016. Journal of Diabetes and Its Complications Discordance in the levels of hemoglobin A1C and glycated albumin : Calculation of the glycation gap based on glycated albumin level. *Journal of Diabetes and Its Complications*, 30(3), pp.477–481. Available at: <http://dx.doi.org/10.1016/j.jdiacomp.2015.12.022>.
- Knip, M., Veijola, R., Virtanen, S. M., Hyöty, H., Vaarala, O., & Åkerblom, H. K. (2005). Environmental triggers and determinants of type 1 diabetes. *Diabetes*, 54(suppl 2), S125-S136.
- Knip, M. & Siljander, H., 2008. Autoimmune mechanisms in type 1 diabetes. *Autoimmunity reviews*, 7(7), pp.550–557.

- Kobayashi, T. et al., 1993. Immunogenetic and Clinical. , 16(5), pp.780–788.
- Koenig, R.J. & Cerami, A., 1975. Synthesis of hemoglobin A1c in normal and diabetic mice: potential model of basement membrane thickening. *Proceedings of the National Academy of Sciences of the United States of America*, 72(9), pp.3687–3691.
- Lal, S. et al., 1993. Detection of fructose-3-phosphokinase activity in intact mammalian lenses by <sup>31</sup>P NMR spectroscopy. *The Journal of biological chemistry*, 268(11), pp.7763–7767.
- Lal, S. et al., 1995. Metabolism of fructose-3-phosphate in the diabetic rat lens. *Archives of Biochemistry and Biophysics*, 318(1), pp.191–199.
- Lee, H. et al., 2013. Optimal hemoglobin A1 C Cutoff Value for Diagnosing type 2 diabetes mellitus in Korean adults. *Diabetes research and clinical practice*, 99(2), pp.231–236.
- Levitt, N.S. et al., 1999. Modifiable risk factors for Type 2 diabetes mellitus in a peri-urban community in South Africa. *Diabetic Medicine*, 16(11), pp.946–950.
- Levitt, N. S., Katzenellenbogen, J. M., Bradshaw, D., Hoffman, M. N., & Bonnici, F. (1993). The prevalence and identification of risk factors for NIDDM in urban Africans in Cape Town, South Africa. *Diabetes care*, 16(4), 601-607.
- Lippi, G. & Targher, G., 2011. A laboratory standpoint on the role of hemoglobin A1c for the diagnosis of diabetes in childhood: more doubts than certainties? *Pediatric diabetes*, 12(3pt1), pp.183–186.
- Lippi, G. & Targher, G., 2010. Glycated hemoglobin (HbA1c): old dogmas, a new perspective? *Clinical chemistry and laboratory medicine*, 48(5), pp.609–614.
- Little, R.R. & Goldstein, D.E., 1995. Endocrine (standardization of glycohemoglobin measurement). *Analytical Chemistry*, 67(12), p.393R–397R.
- Macdonald, D.R. et al., 2008. Clinical impact of variability in HbA1c as assessed by simultaneously measuring fructosamine and use of error grid analysis. *Annals of Clinical Biochemistry*, 45(Pt 4), pp.421–425.
- Maillard, L.C., 1912. Réaction de Maillard. *Action des acides aminés sur les sucres: formation des mélanoidines par voie mé thodique.Compte-rendu de l'Académie des sciences* tome, 154, pp.66–68.
- Matsha, T.E. et al., 2012. Cardiovascular Topics The 30-year cardiovascular risk profile of South Africans with diagnosed diabetes , undiagnosed diabetes , pre-diabetes or normoglycaemia : the Bellville , South Africa pilot study. , 23(1), pp.5–11.

- McCarter, R.J. et al., 2004. Biological variation in HbA1c predicts risk of retinopathy and nephropathy in type 1 diabetes. *Diabetes care*, 27(6), pp.1259–1264.
- Meerwaldt, A.R., 2005. Increased accumulation of skin advanced glycation end-products precedes and correlates with clinical manifestation of diabetic neuropathy. , pp.1637–1644.
- Meng, J. et al., 1996. Advanced glycation end products of the Maillard reaction in aortic pepsin-insoluble and pepsin-soluble collagen from diabetic rats. *Diabetes*, 45(8), pp.1037–1043.
- Mohás, M. et al., 2010. A polymorphism within the fructosamine-3-kinase gene is associated with HbA1c levels and the onset of type 2 diabetes mellitus. *Experimental and clinical endocrinology & diabetes*, 118(03), pp.209–212.
- Monnier, V.M., 2003. Intervention against the Maillard reaction in vivo. *Archives of Biochemistry and Biophysics*, 419(1), pp.1–15.
- Monnier, V.M. & Cerami, A., 1982. Nonenzymatic glycosylation and browning in diabetes and aging: studies on lens proteins. *Diabetes*, 31(Supplement 3), pp.57–63.
- Monnier, V.M., Sell, D.R. & Genuth, S., 2005. Glycation products as markers and predictors of the progression of diabetic complications. *Annals of the New York Academy of Sciences*, 1043(1), pp.567–581.
- Mosca, L. et al., 2011. Genetic variability of the fructosamine 3-kinase gene in diabetic patients. , 49(5), pp.803–808.
- Motala, A.A. et al., 2003. High incidence of Type 2 diabetes mellitus in South African Indians : a 10-year follow-up study. , pp.23–30.
- Nathan, D.M. et al., 2007. Impaired fasting glucose and impaired glucose tolerance: implications for care. *Diabetes care*, 30(3), pp.753–759.
- Nathan, D.M. et al., 2008. Translating the A1C assay into estimated average glucose values. *Diabetes care*, 31(8), pp.1473–1478.
- National Diabetes Data Group, N.D.D.G., 1979. No Title.
- National Institute of Health, N.I.H. of, 2010. US Renal Data System, USRDS 2010 Annual data report: atlas of chronic kidney disease and end-stage renal disease in the United States. *National Institute of Diabetes and Digestive and Kidney Diseases*.
- Nayak, A.U. et al., 2013. Association of glycation gap with mortality and vascular complications in diabetes. *Diabetes care*, 36(10), pp.3247–3253.
- Nayak, A.U. et al., 2011. Evidence for consistency of the glycation gap in diabetes. *Diabetes care*,



34(8), pp.1712–1716.

- Nicholl, I.D. & Bucala, R., 1998. Advanced glycation endproducts and cigarette smoking. *Cellular and molecular biology (Noisy-le-Grand, France)*, 44(7), pp.1025–1033.
- Nordwall, M. et al., 2015. Impact of HbA1c, followed from onset of type 1 diabetes, on the development of severe retinopathy and nephropathy: the VISS Study (Vascular Diabetic Complications in Southeast Sweden). *Diabetes care*, 38(2), pp.308–315.
- O'Brien, J., Morrissey, P.A. & Ames, J.M., 1989. Nutritional and toxicological aspects of the Maillard browning reaction in foods. *Critical Reviews in Food Science & Nutrition*, 28(3), pp.211–248.
- Ogurtsova, K. et al., 2017. IDF Diabetes Atlas : Global estimates for the prevalence of diabetes for 2015 and 2040. *Diabetes Research and Clinical Practice*, 128, pp.40–50. Available at: <http://dx.doi.org/10.1016/j.diabres.2017.03.024>.
- Okada, T. et al., 2011. Influence of proteinuria on glycated albumin values in diabetic patients with chronic kidney disease. *Internal medicine*, 50(1), pp.23–29.
- Oliveira, M.I.A. et al., 2013. RAGE receptor and its soluble isoforms in diabetes mellitus complications. *Jornal Brasileiro de Patologia e Medicina Laboratorial*, 49(2), pp.97–108.
- Paleari, R. et al., 2016. Clinica Chimica Acta Glycation gap : An additional tool for glycometabolic monitoring. *Clinica Chimica Acta*, 463, pp.27–31. Available at: <http://dx.doi.org/10.1016/j.cca.2016.10.004>.
- Palmer, 2003. What ' s in a Name. , 26(2), pp.8–10.
- Perry, I.J., 1999. Impaired fasting glucose and cardiovascular disease. *Diabetes care*, 22(11), pp.1919–1920.
- Petersen, A. et al., 1992. Fructose metabolism in the human erythrocyte. Phosphorylation to fructose 3-phosphate. *The Biochemical journal*, 284 ( Pt 2)(Pt 2), pp.363–366.
- Petersen, A. et al., 1990. Identification of sorbitol 3-phosphate and fructose 3-phosphate in normal and diabetic human erythrocytes. *The Journal of biological chemistry*, 265(29), pp.17424–17427.
- Pickering, T.G. et al., 2005. Recommendations for Blood Pressure Measurement in Humans and Experimental Animals Part 1 : Blood Pressure Measurement in Humans A Statement for Professionals From the Subcommittee of Professional and Public Education of the American Heart Association Council on. , pp.142–161.

- Rahbar, S., 1968. An abnormal hemoglobin in red cells of diabetics. *Clinica Chimica Acta*, 22(2), pp.296–298.
- Rahbar, S., 2005. The discovery of glycated hemoglobin: a major event in the study of nonenzymatic chemistry in biological systems. *Annals of the New York Academy of Sciences*, 1043(1), pp.9–19.
- Ramachandran, A. et al., 2006. The Indian Diabetes Prevention Programme shows that lifestyle modification and metformin prevent type 2 diabetes in Asian Indian subjects with impaired glucose tolerance (IDPP-1). *Diabetologia*, 49(2), pp.289–297.
- Rodriguez-Segade, S. et al., 1989. Effects of various serum proteins on quantification of fructosamine. *Clinical chemistry*, 35(1), pp.134–138.
- Rodriguez-Segade, S. et al., 2012. Estimation of the glycation gap in diabetic patients with stable glycemic control. *Diabetes care*, 35(12), pp.2447–2450.
- Rodriguez-Segade, S. et al., 2015. Influence of the glycation gap on the diagnosis of type 2 diabetes. *Acta Diabetologica*, 52(3), pp.453–459.
- Rodriguez-Segade, S. et al., 2011. Progression of nephropathy in type 2 diabetes: the glycation gap is a significant predictor after adjustment for glycohemoglobin (Hb A1c). *Clinical chemistry*, 57(2), pp.264–271.
- Rohlfing, C. et al., 2002. Biological variation of glycohemoglobin. *Clinical chemistry*, 48(7), pp.1116–1118.
- Rohlfing, C.L. et al., 2002. Defining the relationship between plasma glucose and HbA(1c): analysis of glucose profiles and HbA(1c) in the Diabetes Control and Complications Trial. *Diabetes care*, 25(2), pp.275–278.
- Rondeau, P. & Bourdon, E., 2011. The glycation of albumin: structural and functional impacts. *Biochimie*, 93(4), pp.645–658.
- Schleicher ED, Olgemöller B, Wiedenmann E, Gerbitz KD. Specific glycation of albumin depends on its half-life. *Clin Chem*. 1993;39:625-628.
- Schnedl, W.J. et al., 2005. Glycated hemoglobin and liver disease in diabetes mellitus. *WMW Wiener Medizinische Wochenschrift*, 155(17), pp.411–415.
- Seino, Y. et al., 2010. Report of the Committee on the classification and diagnostic criteria of diabetes mellitus. , pp.2–20.
- Shaw, J.E. et al., 1999. Impaired fasting glucose or impaired glucose tolerance. What best

- predicts future diabetes in Mauritius? *Diabetes care*, 22(3), pp.399–402.
- Shima, K., 1994. Interlaboratory difference in HbA1c measurement in Japan-the interim report of the committee on an interlaboratory standardization of HbA1c determination. *J Jpn Diabetes Soc.*, 37, pp.855–864.
- Škrah, J. et al., 2014. Fructosamine 3-Kinase and Glyoxalase I Polymorphisms and Their Association With Soluble RAGE and Adhesion Molecules in Diabetes. , 63.
- Soita, D., 2013. DAVID JONAH SOITA STUDENT NUMBER : 206155654 CARDIOVASCULAR DISEASE RISK PROFILE OF THE SOUTH-AFRICAN MIXED ANCESTRY POPULATION WITH HIGH INCIDENCE OF DIABETES MELLITUS : BASELINE AND THREE YEAR FOLLOW-UP THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENT F. , (December).
- Soros, A.A. et al., 2010. Hemoglobin glycation index: a robust measure of hemoglobin A1c bias in pediatric type 1 diabetes patients. *Pediatric diabetes*, 11(7), pp.455–461.
- Soulis, T. et al., 1997. Advanced glycation end products and their receptors co-localise in rat organs susceptible to diabetic microvascular injury. , pp.619–628.
- Stitt, A.W., Jenkins, A.J. & Cooper, M.E., 2010. Advanced glycation end products and diabetic complications. , pp.1205–1223.
- Szwergold, B.S. et al., 1997. Identification of a novel protein kinase activity specific for Amadori adducts on glycated proteins. In *Diabetes*. AMER DIABETES ASSOC 1660 DUKE ST, ALEXANDRIA, VA 22314, p. 419.
- Szwergold, B.S., Howell, S. & Beisswenger, P.J., 2001. Human fructosamine-3-kinase: purification, sequencing, substrate specificity, and evidence of activity in vivo. *Diabetes*, 50(9), pp.2139–2147.
- Szwergold, B.S., Howell, S.K. & Beisswenger, P.J., 2002. Nonenzymatic glycation/enzymatic deglycation: a novel hypothesis on the etiology of diabetic complications. In *International Congress Series*. Elsevier, pp. 143–152.
- Szwergold, B.S., Kappler, F. & Brown, T.R., 1990. Identification of fructose 3-phosphate in the lens of diabetic rats. *Science*, 247(4941), p.451.
- Tanhäuserová, V. et al., 2014. Genetic variability in enzymes of metabolic pathways conferring protection against non-enzymatic glycation versus diabetes-related morbidity and mortality. *Clinical chemistry and laboratory medicine*, 52(1), pp.77–83.

- Tanji, N. et al., 2000. Expression of Advanced Glycation End Products and Their Cellular Receptor RAGE in Diabetic Nephropathy and Nondiabetic Renal Disease. , pp.1656–1666.
- Tolonen, H. et al., 2002. Recommendation for indicators, international collaboration, protocol and manual of operations for chronic disease risk factor surveys. *Project EHRM, European Health Risk Monitoring Project www-publications from the EHRM Project Helsinki, Finland: National Public Health Institute.*
- Turner, R. et al., 1997. Early report UKPDS 25 : autoantibodies to islet-cell cytoplasm and glutamic acid decarboxylase for prediction of insulin requirement in type 2 diabetes. , 350, pp.1288–1293.
- UKPDS Group, U.K.P.D.S., 1998. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *The Lancet*, 352(9131), pp.837–853.
- Van Boekel, M.A.M. & Hoenders, H.J., 1992. In vivo glycation of bovine lens crystallins. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, 1159(1), pp.99–102.
- Veiga-Da-Cunha, M.V. et al., 2006. Increased protein glycation in fructosamine 3-kinase-deficient mice. *Biochemical journal*, 399(2), pp.257–264.
- Vlassara, H., Bucala, R. & Striker, L., 1994. Pathogenic effects of advanced glycosylation: biochemical, biologic, and clinical implications for diabetes and aging. *Laboratory investigation; a journal of technical methods and pathology*, 70(2), pp.138–151.
- Wenzlau, J.M. et al., 2008. SIC30A8 is a major target of humoral autoimmunity in type 1 diabetes and a predictive marker in prediabetes. *Annals of the New York Academy of Sciences*, 1150, pp.256–259.
- Whiteley Lucinda & Sandosh Padmanabhan, David Hole, C.I., 2005. Should Diabetes Be Considered a Coronary Heart Disease Risk Equivalent ? Results from 25 years of follow-up in the Renfrew and Paisley Survey.
- Whiting, David R., et al. "IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030." *Diabetes research and clinical practice* 94.3 (2011): 311-321.
- Wit, E. De et al., 2010. Genome-wide analysis of the structure of the South African Coloured Population in the Western Cape. , pp.145–153.
- World Health Organization, W.H.O., 2006. Definition and diagnosis of diabetes mellitus and

- intermediate hyperglycemia: report of a WHO/IDF consultation. *World Health Org.*
- World Health Organization, W.H.O., 1965. No Title. *Expert Committee on Diabetes Mellitus: First Report.*
- World Health Organization, W.H.O., 1980. WHO Expert Committee on Diabetes Mellitus Second report. *World Health Organization Tech Rep Ser.*, 646, pp.1–80.
- World Health Organization. (1994). Concern for Europe's tomorrow: health and environment in the WHO European Region, summary. World Health Organization. Regional Office for Europe.
- World Health Organization, W.H.O., 2011. Use of glycated haemoglobin (HbA1c) in diagnosis of diabetes mellitus: abbreviated report of a WHO consultation.
- World Health Organization. (2013). Diagnostic criteria and classification of hyperglycaemia first detected in pregnancy. World Health Organization. WHO Essential Medicines and Health Products Annual Report 2015. World Health Organization, 2016.
- World Health Organization. Guideline: Sugars intake for adults and children. World Health Organization, 2015.
- Yatscoff, R.W., Tevaarwerk, G.J. & MacDonald, J.C., 1984. Quantification of nonenzymically glycated albumin and total serum protein by affinity chromatography. *Clinical chemistry*, 30(3), pp.446–449.
- World Health Organization. (1999). Definition, diagnosis and classification of diabetes mellitus and its complications: report of a WHO consultation. Part 1, Diagnosis and classification of diabetes mellitus (No. WHO/NCD/NCS/99.2). Geneva: World health organization.
- Yau, J.W. et al., 2012. Global prevalence and major risk factors of diabetic retinopathy. *Diabetes care*, 35(3), pp.556–564.
- Yeungb, V.T.F. et al., 1992. The inter-relationships between albuminuria, plasma albumin concentration and indices of glycaemic control in non-insulin-dependent diabetes mellitus. , 210, pp.179–185.
- Yorifuji, T. et al., 2004. Neonatal diabetes mellitus and neonatal polycystic, dysplastic kidneys: phenotypically discordant recurrence of a mutation in the hepatocyte nuclear factor-1 $\beta$  gene due to germline mosaicism. *The Journal of Clinical Endocrinology & Metabolism*, 89(6), pp.2905–2908.
- Yudkin, J.S. et al., 1990. Unexplained variability of glycated haemoglobin in non-diabetic subjects not related to glycaemia. *Diabetologia*, 33(4), pp.208–215.
- Zafon, C. et al., 2013. Variables Involved in the Discordance between HbA1c and Fructosamine :

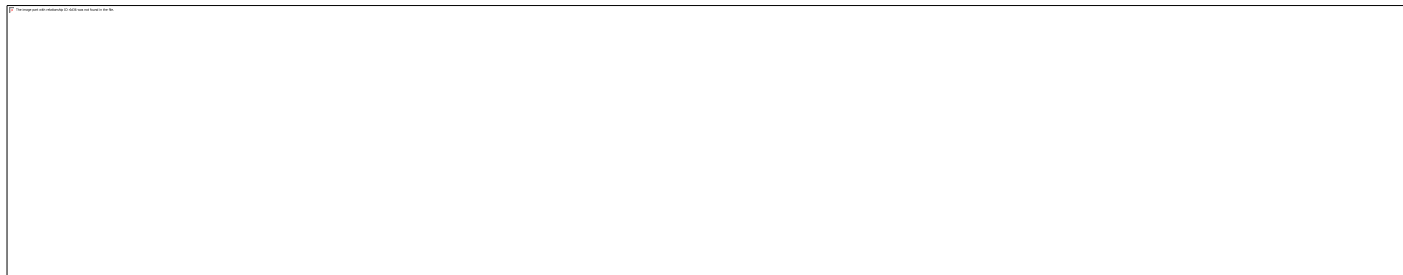
The Glycation Gap Revisited. , 8(6), pp.1–5.

Zemlin, A.E. et al., 2011. HbA1c of 6.5% to diagnose diabetes mellitus—does it work for us?—  
The Bellville South Africa study. *PloS one*, 6(8), p.e22558.

Zimmet, P. (1995). The pathogenesis and prevention of diabetes in adults: Genes, autoimmunity  
and demography. Based on the Eli Lilly Lecture at the 15th International Diabetes Federation  
Congress, Kobe, Japan—November 1994. *Practical Diabetes International*, 12(1), 7-8.

# Appendices

## Appendix A: Ethics Approval



### Approval Notice

#### Response to Modifications- (New Application)

25-Oct-2016  
MOTSHWARI,  
DIPUO DD

Ethics Reference #: S16/06/100

**The effect of fructosamine 3 kinase (FN3K) genotypes on glycation gap in type 2 diabetic and non-diabetic mixedite:**

**ancestry population in South Africa.**

Dear Ms DIPUO MOTSHWARI,

The **Response to Modifications - (New Application)** received on **09-Sep-2016**, was reviewed by members of **Health Research Ethics Committee 1**

via Expedited review procedures on **04-Oct-2016** and was approved.

Please note the following information about your approved

research protocol: Protocol Approval Period: **25-Oct-2016 -24-**

**Oct-2017**

Please remember to use your **protocol number (S16/06/100)** on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

### After Ethical Review:

Please note a template of the progress report is obtainable on [www.sun.ac.za/rds](http://www.sun.ac.za/rds) and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372

Institutional Review Board (IRB) Number:

IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

## Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health ([healthres@pgwc.gov.za](mailto:healthres@pgwc.gov.za) Tel: +27 21 483 9907) and Dr Helene Visser at City Health ([Helene.Visser@capetown.gov.za](mailto:Helene.Visser@capetown.gov.za) Tel:

+27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and documents please visit: [www.sun.ac.za/rds](http://www.sun.ac.za/rds)

If you have any questions or need further assistance, please contact the HREC office at .

### Included Documents:

20160922 MOD Cover letter  
20160922 MOD Budget  
SC28316051813280.pdf  
20160922 MOD HREC mods letter  
2016 Investigators declaration  
(1).pdf HREC Application Form.pdf  
General Checklist(Eng)\_V2 June  
2014\_final.pdf ID zemlin.pdf  
SU\_Consent\_to\_Participate\_in\_Research.doc  
c CURRICULUM VITAE OF Motshwari  
DD.pdf ID dephney.pdf  
PROTOCOL.docx  
final proposal.docx  
20160922 MOD Consent  
form PROTOCOL  
SYNOPSIS.docx  
Abridged TANDI MATSHA CV\_2016.pdf  
20160922 MOD HREC Application  
Form SPECIFIED BUDGET.pdf  
Short CV A Zemlin 2015.pdf  
declaration form Matsha.pdf  
CV\_Andre Pascal Kengne biosketch\_2016 (for Ambros).pdf  
INFORMATION LEAFLET.doc  
CURRICULUM VITAE OF RAJIV T ERASMUS.pdf

Sincerely,

Franklin Weber  
HREC Coordinator  
Health Research Ethics Committee



## Appendix B: Salt Extraction method (Laboratory Protocol 2014)

(Adapted from <http://www.liv.ac.uk/~kempsj/IsolationofDNA.pdf>).

Day 1:	Day 2:
<p>Work on ice Add to 50 ml tubes:</p> <ul style="list-style-type: none"> <li>• 1 - 5 ml blood</li> <li>• 10 ml lysis buffer at 4 °C</li> <li>• [OR 10 ml blood + 30 ml lysis buffer]</li> </ul> <p>Vortex, put on ice for 5 min, repeat 3x Spin 1,500 rpm, 10 min at 4 °C Discard supernatant KEEP PELLETT Add to pellet:</p> <ul style="list-style-type: none"> <li>• 10 ml PBS (to rinse)</li> </ul> <p>Vortex Spin 1,500 rpm, 10 min at 4 °C Discard supernatant <i>If pellet is still red, repeat:</i> Add 10 ml PBS Vortex Spin 1,500 rpm, 10 min at 4 °C Discard supernatant KEEP PELLETT Dissolve pellet in:</p> <ul style="list-style-type: none"> <li>• 3 ml nucleic lysis buffer</li> <li>• 30 µl proteinase K (10 mg/ml)</li> <li>• 300 µl 10 % SDS</li> </ul> <p>Vortex Incubate overnight at 55°C (waterbath full)</p>	<p>Don't work on ice Add to volume in tube:</p> <ul style="list-style-type: none"> <li>• 1 ml 6 M NaCl</li> </ul> <p>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:</p> <ul style="list-style-type: none"> <li>• two volumes 100 % ethanol at -20 °C</li> </ul> <p>(~ 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 PELLETT Tip tube dry on paper towel Dissolve DNA pellet in::</p> <ul style="list-style-type: none"> <li>• 100 - 200 µl distilled water</li> </ul> <p>(depending on size of pellet) Put overnight on turning apparatus to dissolve</p>
	Day 3:
	<p>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</p>

## 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 <sub>2</sub> PO <sub>4</sub> (1.47 mM) 1.15 g Na <sub>2</sub> HPO <sub>4</sub> (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 <sub>4</sub> Cl 1 M stock KHCO <sub>3</sub> 100 mM stock EDTA	70.13 g/200 ml/saturated	10 mg/ml proteinase K
Lysis buffer	10 % SDS	
155 mM NH <sub>4</sub> Cl (15.5 ml from 1 M stock) 10 mM KHCO <sub>3</sub> (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 C: Consent Form

### PARTICIPANT INFORMATION AND INFORMED CONSENT FORM FOR RESEARCH INVOLVING GENETIC STUDIES

TITLE OF RESEARCH PROJECT: PROGRESSIVE RESEARCH ON RISK FACTORS OF TYPE 2 DIABETES AND CARDIOVASCULAR DISEASES IN SOUTH AFRICA

REFERENCE NUMBER:

PRINCIPAL INVESTIGATORS: Professor Tandi Matsha (Cape Peninsula University of Technology)  
Professor Rajiv Erasmus (Stellenbosch University) Professor Andre Kengne (SA Medical Research Council)

Project manager: Dr Gloudina Maria Hon (Cape Peninsula University of Technology)

ADDRESS: Obesity and chronic diseases of lifestyle Department of Biomedical Sciences Faculty of Health & Wellness Sciences  
Cape Peninsula University of Technology, Bellville

CONTACT NUMBER: Prof T Matsha 021 959 6366 or email: matshat@cput.ac.za

Ethics approval: Cape Peninsula University of Technology Ethics  
Reference number: CPUT/SW-REC 2015/H01  
University of Stellenbosch Ethics Reference number: N14/01/003

We would like to invite you to participate in a research study that involves genetic analysis and possible long-term storage of blood or tissue specimens. 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 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 agree to take part initially.

This research study has been approved by the ethics Faculty of Health & Wellness Sciences of the Cape Peninsula University of Technology and it will be conducted according to international and locally accepted ethical guidelines for research, namely the Declaration of Helsinki, and the SA Department of Health's 2004 Guidelines: Ethics in Health Research: Principles, Structures and Processes.

Genetic material, also called DNA or RNA, is usually obtained from a small blood sample. Occasionally genetic material is obtained from other sources such as saliva or biopsy specimens. (A biopsy is a tiny piece of tissue that is cut out e.g. from the skin or from a lump, to help your doctor make a diagnosis.) Genes are found in every cell in the human body. Our genes determine what we look like and sometimes what kind of diseases we may be susceptible to. Worldwide, researchers in the field of genetics are continuously discovering new information that may be of great benefit to future generations and also that may benefit people today, who suffer from particular diseases or conditions.

This research study seeks to address the increasing problem of diabetes and cardiovascular diseases such as heart attack and stroke amongst the mixed ancestry or coloured population of South Africa. In this study we shall identify people with diabetes and those at high risk of diabetes as well as investigate the environmental and genetic risk factors that predispose some individuals to the development of diabetes and cardiovascular diseases. Examples of environmental factors include body weight, diet, and physical activity. Additionally, this project aims to investigate whether oral health is a risk factor for diabetes and cardiovascular diseases. In this study we shall investigate whether some individuals have early cardiovascular diseases by using an ultrasound machine. This project also aims to collect genetic material (blood) to analyze for certain variants and to store excess material for future research. When a large group of patients with similar diseases has been collected, meaningful research into the disease processes may become possible.

Our research team has previously conducted a similar research study involving the coloured community and found out that more than 18 out of 100 individuals had diabetes but did not know. We also found that some of the risk factors associated with diabetes in other populations were not necessarily the same as those affecting the coloured population of South Africa. You have therefore been invited to take part in this research study to assist in establishing the risk factors for diabetes and cardiovascular diseases affecting the coloured people of South Africa.

- A. You will be requested to provide information about your medical history, family history and information on eating, drinking and smoking habits. Completion of the questionnaire will take no longer than 30 minutes.
- B. You shall be requested to provide a record of the medication you are currently taking, therefore if you are taking chronic medication, you shall be requested to provide this to the research team to record the medication.
- C. Measurement such as weight, height, waist and hip will be done.

D. Fasting Venous Blood (20ml) will be collected thereafter you will be asked to drink a glucose solution (glucose content 75g). After two hours another venous blood (10ml) will be collected. The blood will be used to determine whether you have diabetes or you are at high risk for developing diabetes.

E. The other tests that will be determined from your blood sample are: Cholesterol, triglycerides, creatine levels to assess your kidney function, liver enzymes to assess your liver, and biochemical markers for inflammation.

F. A finger prick blood sample (a drop of blood), to be taken at the same time of the first venous blood sample, may also be required from you. The finger prick blood sample will be used to test for diabetes or the risk of developing diabetes on a point-of-care test instrument. Researchers will compare the finger prick point-of-care diabetes test with that of the send away venous blood laboratory test and would be able to establish whether the point-of-care test provides the same accurate results as that of the laboratory. Point-of-care testing may in the future be used to provide fast and accurate results without the need to send blood away to a laboratory for processing. This may be of benefit to people undergoing testing for diabetes as results would be available within a few minutes.

G. The remainder of the blood sample will be used for genetic and future research studies. The serum and DNA may be stored for several years until the technology for meaningful analysis becomes available. No pharmaceutical agent (medication) will be tested in the study.

H. For oral health, research study personnel will extract wooden toothpick, flocked brush, and mouthwash saliva samples from you to test for the presence of *Porphyromonas gingivalis* as an indicator for periodontal disease. Flocked brush and wood toothpick sampling will involve inserting devices in the subgingival crevice between the last upper premolar and the first upper molar. The device will sweep down the anterior surface of the first upper molar with the direction of motion away from the gum to minimize any potential discomfort. Mouthwash sampling will involve rinsing with 10 ml sterile saline solution for 20 seconds.

I. Early cardiovascular diseases will be performed by means of an ultrasound machine.

J. The research team will follow up on you on a yearly basis and some of these test may be repeated. The investigators wish to follow you up for your entire life. In the unfortunate event that you are deceased during the study period. The study team will review stats SA data and/or medical records to ascertain whether the cause of death was due to diabetes or cardiovascular diseases. . If you do not wish to be followed up on a yearly basis and your Statistics SA and/or medical records not to be accessed in the unfortunate event that you are deceased whilst being a participant in the study, you will have an opportunity to request that it be not accessed when you sign the consent form.

K. Radio imaging techniques will be done on consenting subjects. These include (i) ultra sound to assess whether you have signs of early cardiovascular diseases, (ii) computed tomography scan (CT-scan) to accurately assess the fat content that is dangerous for cardiovascular diseases (iii) Dual-energy X-ray absorptiometry (DXA) devices will be used to study the morphology of the liver. These radio imaging techniques involve

radiation which can be harmful if one is exposed excessively. For this study a low dose radiation will be used for acquisition of the images thereby minimizing radiation exposure to the participant. . If you do not wish to undergo any of these radio imaging techniques, you will have an opportunity to decline when you sign the consent form.

L. An eye examination will be done to test your eye vision and any other abnormalities in the eye. For this examination, drops placed in your eyes widen (dilate) your pupils to allow the doctor to better view inside your eyes. The drops may cause your close vision to blur for a short while.

A slight bruising might occur after blood has been drawn from the arm but this will heal quickly. After the administration of the glucose solution, you may feel nauseous and dizzy in which case you must notify the medical personnel. A medical nurse or doctor will be present on all occasions. You may also learn that you have diabetes, in which case you will be referred to your health care giver with the results for further treatment and management. If during the study it is discovered that you have changes in your genes that may lead to a serious disease, a genetic counsellor at the expense of the principal investigators will counsel you. Radio imaging techniques such as the CT-scan involves radiation which can be harmful if one is exposed excessively. For this study a low dose radiation will be used for acquisition of the images thereby minimizing radiation exposure to the participant.

Your personal results will be made known to you only if they indicate that you may:

- Have diabetes, thereafter, you will be referred to your local health centre or general practitioner for further investigations and treatment.
  - Have a condition or predisposition to developing diabetes that is treatable or avoidable e.g. by a lifestyle modification.
  - Need genetic counselling.
- However, participants with normal results who wish to know their results are free to contact the research team and their results will be given upon written request.

The blood samples may be stored indefinitely to accommodate new technologies that may develop. In the event that a technology is not available in South Africa to analyse your blood sample, your blood specimen may be sent to another country with the technology either now or at a later date. However, if your specimen is to be sent to another country, permission to do so will be sought from relevant bodies. Your blood specimen will be stored at the Cape Peninsula University of Technology.

Your blood will only be used for genetic research that is directly related to Diabetes and cardiovascular diseases. Also if the researchers wish to use your stored blood for additional research in this field they will be required to apply for permission to do so from the ethics Faculty of Health & Wellness Sciences of the Cape Peninsula University of Technology. If you do not wish your blood specimen to be stored after this research study is completed you will have an opportunity to request that it be discarded when you sign the consent form.

Your identity will be recorded once and kept confidential throughout. This is to allow the principal investigators to convey information that may be beneficial to you. Access will be limited to the principal investigators by assigning a special study code to all your data and blood samples. This means that your sample will be identified with a special study code that will remain linked to your name and contact details. However, during the entire research study, your blood specimens will be anonymised and the research staff won't be able to associate it with your name and contact details. You shall also be supplied this code so that if at anytime the investigators need to contact you, you may only identify yourself using your special code. Any scientific publications, lectures or reports resulting from the study will not identify you.

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.

You will not be paid to take part in this study although your out-of-pocket expenses may be reimbursed. The expenses that will be covered by the research team are those that include transportation to a hospital radiography department should you consent to radio imaging.

You should inform your family practitioner or usual doctor that you are taking part in a research study. You can contact

Prof T Matsha at 021 959 6366 or [matshat@cput.ac.za](mailto:matshat@cput.ac.za),  
If you have any further queries or encounter any problems, you can also contact the Cape Peninsula University of Technology Health and Wellness Sciences Research Ethics Committee,  
Chairperson: Prof Engel-hills at 0219596570 or [EngelhillsP@cput.ac.za](mailto:EngelhillsP@cput.ac.za) or

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

By signing below, I ..... agree to take part in a research project that includes genetic research study entitled (PROGRESSIVE RESEARCH ON RISK FACTORS OF TYPE 2 DIABETES AND CARDIOVASCULAR DISEASES IN SOUTH AFRICA).

I declare that:

- I have read or had read to me this information and consent form and 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 pressurised to take part.
- I have received a signed duplicate copy of this consent form for my records.

I agree that my blood or tissue sample can be stored indefinitely after the project is completed but that it is anonymised with all possible links to my identity removed, and that the researchers may then use it for additional research in this or a related field. Once my sample is anonymised, my rights to that sample are waived. My sample may be shipped to another laboratory in SA or abroad to be used in other research projects in this or a related field

OR

I agree that my blood or tissue sample can be stored indefinitely, but I can choose to request at any time that my stored sample be destroyed. My sample will be identified with a special study code that will remain linked to my name and contact details. I have the right to receive confirmation that my request has been carried out.

OR

Please destroy my blood sample as soon as the current research project has been completed.

I consent that the research team may follow me up for yearly check-up AND in the unfortunate event that I am deceased whilst still part of the study, I consent that the team may access Statistics SA and/or my medical records to ascertain whether the cause of my death was due to diabetes or cardiovascular diseases.

OR



I do not consent to follow me up for yearly check-up BUT in the unfortunate event that I am deceased whilst still part of the study, I consent that the team may access Statistics SA and/or my medical records to ascertain whether the cause of my death was due to diabetes or cardiovascular diseases.

OR

I do not consent to follow me up for yearly check-up AND in the unfortunate event that I am deceased whilst still part of the study, I do not consent that the team accessing Statistics SA and/or my medical records to ascertain whether the cause of my death was due to diabetes or cardiovascular diseases.

- I consent to ultra sound techniques to assess if I have early cardiovascular diseases
- I do not consent to ultra sound techniques that assess if I have early cardiovascular diseases

AND

- I consent computed tomography scan (CT-scan) to accurately assess the fat content that is dangerous for cardiovascular diseases
- I do not consent to computed tomography scan (CT-scan) that accurately assess the fat content that is dangerous for cardiovascular diseases

AND

- I consent to Dual-energy X-ray absorptiometry (DXA) used to study body composition.
- I do not consent Dual-energy X-ray absorptiometry (DXA) used to study body composition

Signed at (place) ..... on (date)

.....

Finger print

.....

.....  
Signature of participant      Signature of witness

I (name) ..... declare that:

- I explained the information in this document to .....
- 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.
- I did/did not use a interpreter. (If a interpreter is used then the interpreter must sign the declaration below.

Signed at (place) ..... on (date)  
..... 2016.

.....  
.....  
Signature of investigator    Signature of witness

I (name) ..... declare that:

- I assisted the investigator (name) ..... to explain the information in this document to (name of participant) ..... Using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (place) ..... on (date)  
..... 2016.

.....  
.....  
Signature of interpreter    Signature of witness

