# THE USE OF GLYCATED HAEMOGLOBIN IN DETERMINING GLYCAEMIC CONTROL AND ASSESSING RISK OF RENAL DAMAGE IN DIABETIC PATIENTS IN ABUTH, ZARIA

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**MARCH, 2021**

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**A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY IN HUMAN PHYSIOLOGY**

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**ZARIA, NIGERIA**

**MARCH, 2021**

## Declaration

I declare that the research work in this dissertation entitled **"The Use of Glycated Haemoglobin in Determining Glycemic Control and Assessing Risk of Renal Damage in Diabetic Patients in ABUTH, Zaria"** has been carried out by me in the Department of Human Physiology, Faculty of Basic Medical Sciences, College of Medical Sciences, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree at this or any other Institution.

| ______________________ | ______________________ | ______________________ |
|---|---|---|
| Ibrahim Oladayo MUSTAFA | Signature | Date |

## Certification

This dissertation entitled **"The Use of Glycated Haemoglobin ($HbA1_C$) in Determining Glycemic Control and Assessing the Risk of Renal Damage in Diabetic Patients in ABUTH, Zaria"** by Ibrahim Oladayo MUSTAFA meets the regulations governing the award of the degree of Doctor of Philosophy (PhD) in Human Physiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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## Dedication

I dedicate this treatise to my family and to diabetic patients as we continue to seek better treatment, better management and indeed cure for the condition.

## Abstract

Glycated haemoglobin ($HbA1_C$) is being reported to vary in level for a given blood glucose range from individual to individual especially along racial line. This study was therefore carried out to investigate the local $HbA1_C$ normal level and determine extent of (if any) variation from the World Health Organization(WHO) recommended threshold for the onset of diabetes mellitus(DM) and pre-diabetes using blood glucose as a benchmark. And also look into the correlation between glycated hemoglobin A1c (HbA1c) and the risk of developing diabetic nephropathy among diabetic patients attending Ahmadu Bello University Teaching Hospital (ABUTH) Zaria, Nigeria and determine the level or range of glycated hemoglobin at which the risk of nephropathy becomes pronounced. One hundred and fifty two (152) volunteer subjects were used for the study comprising of 101 diabetic subjects and 51 non-diabetic control subjects. Blood sample, 5ml, was collected from each of the subjects after about 8 to 10 hours of overnight fasting. About 3ml of the sample was centrifuged and the serum analysed for fasting blood glucose (FBG) and serum creatinine (SCr). The Glomerular Filtration Rate (GFR) was then calculated from the serum creatinine values using Cockroft-Gault equation. The remaining volume of blood, about 2ml, was transferred into EDTA bottles and analysed immediately for glycated haemoglobin ($HbA1_C$).Thirty-seven (37) of the diabetic subjects and twenty-three (23) of the non-diabetic subjects had mean $HbA1_C$ levels of 6.96% and 6.29% respectively that correspond to mean FBG levels of 91.37mg/dL and 79.16mg/dL respectively. No significant correlation was found between HbA1c and GFR but on selecting subjects with HbA1c level $\geq$ 9% and testing the correlation between their HbA1c and GFR, the correlation became significant and negatively ($r = -0.35$). The glycated haemoglobin (HbA1c) levels of diabetic patients attending Ahmadu Bello University Teaching Hospital and non-diabetic control subjects was distinctly higher for a

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## List of Abbreviations

ABUTH- Ahmadu Bello University Teaching Hospital

ANOVA- Analysis of Variance

BG- Blood Glucose

BMI- Body Mass Index

$Cc_r$- Creatinine Clearance

DAN- Diabetes Association of Nigeria

DCCT- Diabetes Complications and Control Trial

DM- Diabetes Mellitus

EDTA- Ethylene Diamine Tetraacetic Acid

eGFR- Estimated Glomerular Filtration Rate

FBG- Fasting Blood Glucose

FIA- Fluorescence Immunoassay Meter

FPG- Fasting Plasma Glucose

GDM- Gestational Diabetes Mellitus

GFR- Glomerular Filtration Rate

GIT- Gastro Intestinal Tract

Hb- Haemoglobin

HbA1c- Glycated Haemoglobin

IFCC- International Federation of Clinical Chemistry and Laboratory Medicine

OGTT- Oral Glucose Tolerance Test

SCr- Serum Creatinine

SD- Standard Deviation

SPSS- Statistical Package for Social Sciences

WHO- World Health Organization

# CHAPTER ONE

## 1.0 INTRODUCTION

### 1.1 Backgroundto theStudy

Diabetes mellitus (DM) is a disease that can be a serious threat to public health as the number of adults that will be suffering from the disease by the year 2030 is estimated to be 439 million (Shaw *et al*., 2010). It was estimated that as of 2014 about 387 million people have DM worldwide, representing 3.8% of the adult population (Shi and Hu, 2014). Also, it is predicted that Africa will witness a 110% increase in prevalence of DM between 2013 and 2035, with most of the affected people suffering from type 2 DM; And among countries in Africa, Nigeria has the highest number of cases of this disease with 5 million people, about 5% of the population affected (Oghagbon, 2014).

As our understanding of DM has evolved over the past 25–50 years, the options for diagnostic criteria have changed too. The days of tasting urine to know if it is sweet have evolved into portable devices and laboratory machines running multiple tests per hour to diagnose and monitor DM (Capula *et al*., 2013).

In the 1960s, identification of type 2 (previously referred to as adult onset or non-insulin-dependent) DM using the oral glucose tolerance test (OGTT) had become established. Unfortunately, there were inconsistencies concerning how the test should be performed, the quantity of glucose that should be ingested and the diagnostic blood glucose cut-off points. By 1980, the World Health Organization (WHO) had standardized these parameters, and since then fasting plasma glucose (FPG) values have become more commonly used for diagnosis, especially in the United States (Leslie *et al*., 2017).

Glycated haemoglobin (haemoglobin $A1_C$ or $HbA1_C$) is a type of haemoglobin primarily used to identify the average plasma glucose concentration over extended periods of time (at least 6-8 weeks) (Kilpatrick *et al*., 2009). It is formed in a non-enzymatic pathway (irreversibly by the condensation of glucose to the N-terminal lysine and valine of the beta-chain of haemoglobin A (HbA)) due to the haemoglobin's normal exposure to high plasma levels of glucose (Miedema, 2005). Glycation of haemoglobin has been implicated (as a biomarker) in nephropathy and retinopathy in DM (Saleh, 2015).

In the normal 120-day life span of the red blood cell, glucose molecules join haemoglobin, forming glycated haemoglobin (Paterson *et al*., 1998). Once a haemoglobin molecule is glycated, it remains in that form (Sacks *et al*., 2002). A buildup of glycated haemoglobin in the red cell reflects the average level of glucose to which the cell has been exposed during its life cycle (Chalew and Hempe, 2008). The HbA1c level is proportional to average blood glucose concentration over the previous four weeks to three months (Makris *et al*., 2008).

The results of haemoglobin $A1_C$ ($HbA1_C$) measurements are used to evaluate quality of glycaemic control in individuals with DM (Cerami, 2012).

Microvascular complications such as retinopathy, nephropathy and neuropathy occur in diabetics (Beisswenger, 2010). Largely due to these complications, globally, DM is said to be the fifth leading cause of death (Roglic *et al*., 2005).

Prevention of DM and its complications, early detection of disease stages, and therapeutics that would act in the presence of hyperglycaemia to prevent, delay or reverse the complications are the major concerns (Marshall and Flyvbjerg, 2006). Biomarkers such as glycated haemoglobin, glycated serum proteins (fructosamine), 1,5-anhydroglucitol (1,5-AG), urea and electrolytes, and serum creatinine are studied for understanding the

mechanisms of hyperglycaemia-induced metabolic abnormalities (Marshall and Flyvbjerg, 2006).

Diabetic nephropathy, a leading cause of kidney failure and one of the key complications of diabetics, is defined by either microalbuminuria or by an increase in serum creatinine level, which is in turn used in the calculation of estimated GFR (eGFR) in diabetic patients (Amos *et al*., 1997).

While microalbuminuria is a very sensitive test in people with Type 1 DM, testing for microalbuminuria alone may miss many cases of diabetic kidney disease in those with Type 2 (Koroshi, 2007). Therefore, it is very important to test the kidney function by measuring the serum creatinine level (Lewis and Maxwell, 2014).

Using the serum concentration of creatinine in an equation that takes into account the person's weight, age, sex, and race, one can estimate the GFR to evaluate kidney function. The higher the blood creatinine level, the lower the GFR and the worse shape the kidneys are in (Waad-Allah *et al*., 2012). Normal eGFR ranges from 90 to 120 ml/min/1.73m$^2$ (Waad-Allah *et al*., 2012).

In patients with DM, $HbA1_C$ is used to assess and monitor effectiveness of treatment (glycaemic control) given over a past period, usually the past two to three months. The results from $HbA1_C$ testing can then be used to determine the course of future treatment for the patient in order to guard against hyperglycaemic-induced complications such as nephropathy, which is reported to complicate approximately 30% of type 2 diabetic patients (Khan *et al*., 2004).

It has been shown that HbA1c whatever its limitations remains the strongest predictor available for at least the microvascular complications of DM such as neuropathy,

nephropathy and retinopathy (Hirsch and Brownlee, 2010). Unfortunately, clinicians caring for people with DM encounter individuals in whom HbA1c and blood glucose simply do not match (Robert and Christopher, 2012). This discordant information leaves clinicians with the question of how to care for these patients especially when it occurs in people with reliable blood glucose records and ostensibly normal peripheral blood and reticulocyte counts, without evidence of haemoglobinopathy, haemolytic disorder, blood loss/transfusion or nutritional deficiency such as iron, folate or vitamin $B_{12}$ (Robert and Christopher, 2012).

There is also the challenge of an imperfect relationship between HbA1c and blood glucose in populations. For instance, at an HbA1c level of 6.0%, the mean blood glucose (at 95% CI) ranges from 100 to 152 mg/dL overlapping with the mean blood glucose (95% CI) at an HbA1c level of 7.0% which is 123–185 mg/dL (Nathan *et al*., 2008). That is, for a level of 6.0% HbA1c, there are statistically significant number of subjects that had their blood glucose levels between 100mg/dL and 152mg/dL. Also, for a level of 7.0% HbA1c, there are statistically significant number of subjects that had their blood glucose levels between 123mg/dL and 185mg/dL. Indicating that at both levels of 6.0% and 7.0% HbA1c, which is a wide range, there are subjects with blood glucose levels between 123mg/dL and 152mg/dL. This is what made Herman and Cohen (2012) suggest that reliance only on HbA1c could miss persons with DM and falsely diagnose those without the disease.

These differences between glucose and HbA1c appear to be at least partly real and result from other intervening physiologic factors and mechanisms rather than from fluctuations in blood glucose or measurement errors (Robert and Christopher, 2012). These factors/mechanisms include erythrocyte life span, iron handling and glucose distribution across the erythrocyte membrane and perhaps other yet-to-be-discovered mechanisms (Florez, 2010). And sufficient evidence is emerging on the varying erythrocyte life span

among people, large enough to result in different HbA1c in two individuals that have the same blood glucose level (Lindsell *et al*., 2008).

A good example of evidence supporting an explanation based on physiology — which forms the basis of our research — is the number of reports of a consistent difference in the relationship between HbA1c and glucose between persons of different races, most notably African-Americans and Caucasians (Selvin *et al*., 2011).

## 1.2 Statement ofResearch Problem

The results of haemoglobin A1c (HbA1c) measurements are used to evaluate quality of glycaemic control (Cerami, 2012) and to measure the risk of diabetic complications such as renal damage (Aslan, 2011) in individuals with DM. However, the accuracy of $HbA1_C$ test results is being reported to be doubtful and highly varied amongst individuals especially along racial lines; with reports of significantly higher levels in non-Caucasian diabetic patients for a given blood glucose (BG) range (Herman *et al*., 2009). As pointed out in a book by Diabetes Association of Nigeria (DAN) in 2013 titled "Clinical Practice Guidelines for Diabetes Management in Nigeria", the $HbA1_C$ test is subject to certain limitations such as conditions that affect erythrocyte turnover (haemolysis, blood loss) and haemoglobin variants in individual patient, making it possible for $HbA1_C$ result not to correlate with the patient's clinical situation (Chinenye *et al*., 2013). Similarly, in a review by Oghagbon (2014), it is suggested that despite the usefulness of HbA1c in monitoring diabetic patients in Nigeria and despite that its relationship to prevalence of diabetic complications has been proven in various studies, caution must be taken in the application of HbA1c in our environment due to its variability with race/ethnicity. And if possible, individualization of HbA1c target goals is advised, so as not to over-treat and tip patients into hypoglycaemia, especially those who have hypoglycaemia unawareness or history of severe hypoglycaemia. For instance, in a

multi-center study involving seven teaching hospitals in Nigeria, 68% of diabetic patients have their mean HbA1c level >7.0% (supposedly high enough for onset of complication) but only about half of these patients (35.5%) developed complications (Chinenye *et al*., 2012), suggesting a difference in the HbA1c "at risk of complication" level in Nigerian patients. If these variabilities and limitations are not investigated and determined, patients might remain (mysteriously) sick or become far worse than what they presented with.

## 1.3 Justification of the Study

Many of the findings about the racial differences in $HbA1_C$level were based on the research done abroad and using African-Americans as representative subjects for Blacks with little done on African subjects. Consequent to the conclusion of an earlier study based on Zaria residents recommending studiesto be carried out on assessment of HbA1c threshold for the diagnosis of diabetes and pre-diabetes in specific populations (Lawal *et al*., 2018), this studyinvestigates the local peculiaritiesin $HbA1_C$ levels and thresholdfor the diagnosis of diabetes and pre-diabetes and determines theextent of these peculiarities.

The opinion that black people have higher values of glycated haemoglobin $A1_C$ for corresponding levels of glycaemia either due to genes, erythrocyte turnover or erythrocyte permeability to glucose is supported by a research published in the journal of American Diabetes Association by Bruce*et al*. (2013), where they stated that for a given degree of glycaemia, $HbA1_C$ levels vary among different ethnic groups and that ethnicity needs to be taken into consideration when using $HbA1_C$ to assess glycaemic control or to set glycaemic targets.

## 1.4 Aim and Objectives of the Study

### 1.4.1 Aim

The aim of this studywas to investigate the correlation between $HbA1_C$, blood glucose and tendency for development of renal complication in diabetic patients attending ABUTH, Zaria.

### 1.4.2 Objectives

The objectives of this study were to:

i. determine the levels of $HbA1_C$, fasting blood glucose (FBG), serum creatinine and GFR in diabetic patients and control subjects
ii. correlate $HbA1_C$ with FBG, serum creatinine and estimated glomerular filtration rate(eGFR)in diabetic patients and control subjects
iii. correlate FBG with serum creatinine and eGFR in patients and control subjects
iv. determine the proportion of study participants with "abnormal"$HbA1_C$ levels

## 1.5 NullHypothesis

Glycated haemoglobin ($HbA1_C$) level does not have any local peculiarity in its use in determining glycaemic control and assessing risk of renal damage in diabetic patients attending Ahmadu Bello University Teaching Hospital Zaria, Nigeria.

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

## 2.1 Diabetes Mellitus

DM is a metabolic disease or condition characterized by hyperglycaemia (abnormally high blood glucose) (American Diabetes Association, 2011). The hyperglycaemia could be caused by defects in insulin secretion, defects in insulin action or usually a combination of both (Tirosh *et al*., 2005). The diagnosis of DM can be challenging, as it is usually not made on a single blood test; albeit an elevated fasting blood glucose will raise suspicion that a patient may have DM, and this often times leads to additional workup and testing.

### 2.1.1 Diagnosing DM

Criteria for the diagnosis of DM according to World Health Organization, 2000:

i. Symptoms of DM, plus a casual plasma glucose concentration of 11.1 mmol/L (200 mg/dL), where *casual* is defined as *any time of day without regard to the time since the patient's last meal*. Classic symptoms of DM include polyuria, polydipsia and unexplained weight loss.
ii. Fasting blood glucose level of 7.0 mmol/L (126 mg/dL), where *fasting* is defined as *no caloric intake for at least eight hours*.
iii. Two-hour post-load glucose of 11.1 mmol/L (200 mg/dL) during an Oral Glucose Tolerance Test (OGTT). The test should be performed as described by WHO, using a glucose load containing the equivalent of 75 grams of anhydrous glucose dissolved in water.

In the absence of certainhyperglycaemia, these criteria should be confirmed by repeating the test on a different day. The OGTT (Oral Glucose Tolerance Test) test is not recommended for routine clinical use.

Glycated haemoglobin, or haemoglobin A1c (HbA1c), is more robust both analytically and functionally, since neither fasting nor a glucose load are required. HbA1c also has a high positive predictive value for DM at a cutoff above 6.5% (or 48 mmol/mol, as recommended by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), 2007.

People may have DM by other criteria and at lower HbA1c levels. A level of HbA1c of 6.0% (IFCC 42 mmol/mol) or higher is usually considered abnormal and warrants further investigation (Leslie *et al*., 2017).

2.1.2 Types of DM

There are many different forms of DM albeit there are two major forms that account for 98% of cases; type 1 and type 2 DM (The Expert Committee on the Diagnosis and Classification of DM, 1997).

Type 1 DM (previously known as insulin-dependent DM or juvenile DM) and type 2 DM (previously known as non-insulin-dependent DM or adult onset DM) represent two distinct disease processes with about 90% of all cases being type 2, primarily presenting during adulthood(Kahn *et al*., 2010).

By definition, type 2 diabetic patients do not depend on insulin for survival. In contrast, type 1 DM, which represents about 5–10% of cases (American Diabetes Association, 2017), is an immune-mediated DM that results from cellular-mediated autoimmune destruction of the beta cells of the pancreas (Skyler *et al*., 2016).

Type 1 DM usually presents in children and requires insulin that, by the previous definition, means they depend on insulin for survival (Gale, 2005). This distinction can be ambiguous, especially when type 1 DM is diagnosed in adulthood, or where type 2 DM is diagnosed in childhood. Thus neither insulin dependence nor age at diagnosis is categorical features of type 1 DM (Insel *et al*., 2015).

Physiologically, increased insulin secretion compensates for reduction in insulin sensitivity. In type 2 DM, individuals have insulin resistance and the insulin deficiency is usually relative, in contrast to the absolute insulin deficiency that is seen in type 1 DM(Skyler *et al*., 2016).

Many patients with type 2 DM are obese, and obesity itself contributes to some degree of insulin resistance. But insulin secretion is also defective in these patients and unable to compensate for the insulin resistance (Torgerson *et al*., 2004).

In type 1 DM however, the autoimmune destruction of the beta cells of the pancreas results in decreased or (in later stages) no insulin secretion. The rate of beta cell destruction may be varied, and there are multiple genetic predispositions related to this variation (Orban *et al*., 2009).

*2.1.2.1 Type 1 DM*

This is caused by beta cell destruction, usually leading to absolute insulin deficiency. According to American Diabetes Association (2014)it could either be:

i. Immune-mediated or
ii. Idiopathic (Unknown cause)

Type 1 autoimmune (Immune-mediated) DM is a result of insulin deficiency of variable severity, especially in children, leading to insulin-dependent DM. In Western countries,

almost all patients have the immune-mediated form of DM, which actually can occur at any age, but is the second most common chronic disease of childhood after asthma (Dabelea *et al*., 2014).

Lack of insulin, caused by the autoimmune destruction of islet cells, is typical of type 1 DM. The interaction of the environment with an underlying genetic susceptibility leads to an autoimmune response, which damages or destroys insulin-secreting cells (Bluestone*et al*., 2010).

The risk of developing childhood-onset autoimmune DM is about 1 in 400 in the general population; 1 in 100 (risk) in the adult population; about 6 in 100 for a sibling of a diabetic patient and about 50 in 100 (50%) in an identical twin of a diabetic patient (Soltesz, 2007).

Despite the increasing incidence of type 1 DM in children, especially very young children, by about 2020, it is predicted that the majority of children with DM will have type 2 DM (Atkinson *et al*., 2014). The disease incidence of type 1 DM is increasing, especially in very young children, but that incidence remains far below that of adult-onset type 2 DM (Leslie *et al*., 2017).

Idiopathic DM occurs in about 20% of Type 1 patients where specific pathogenesis of the condition remains unclear (Gianani *et al*., 2010).

Slow progression to insulin deficiency in patients with autoimmune DM can occur, with about 10% of adult patients presenting initially with a non-insulin-requiring form of type 1 DM called latent autoimmune DM of adults (LADA) (Naik *et al*., 2009).

LADA is characterized by the presence of DM-associated antibodies to glutamic acid decarboxylase (GADA). This is likely a form of type 1 autoimmune DM, which also encompasses juvenile-onset insulin-dependent DM and some cases with ketosis-prone DM (KPD). Autoimmune type 1 DM is associated with other autoimmune diseases (notably

autoimmune thyroid disease and celiac disease), which also show genetic susceptibility, largely mediated by the human leukocyte antigen (HLA) genes of chromosome 6 (Gale, 2005).

Other immune response genes and a variant of the insulin gene are also involved in type 1 autoimmune DM. The nature of the environmental factor, however, remains unclear (Bach and Chatenoud, 2012).

*2.1.2.2 Type 2 DM*

This may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance. It is a common chronic disease predominantly responsible for the global epidemic of DM. The disease involves reduced insulin sensitivity (insulin resistance) at the supposed insulin sensitive tissues (Ramachandran, 2006).

The rise in cases of obesity allied to sedentary lifestyle, in the context of industrialization, and the increased consumption of energy-rich foods, likely contribute to the dramatic rise in the occurrence of type 2 DM (Torgerson, 2004).

Because of the slow and subtle development of hyperglycaemia in type 2 DM, it frequently is not diagnosed for many years until it becomes severe enough for patients to develop vivid symptoms and/or complications (Lalla *et al*., 2013).

This is said to be concerning, as diabetic patients are at risk for both macrovascular and microvascular complications. Type 2 DM therefore, is due to the interaction of the environment with an underlying genetic susceptibility leading to loss of glucose homeostasis (fig 2.1) (Melendez-Ramirez *et al*., 2010).The heritability of type 2 DM is high, and genes associated with that risk include genes involved in the development of the pancreas and genes associated with the risk of obesity.

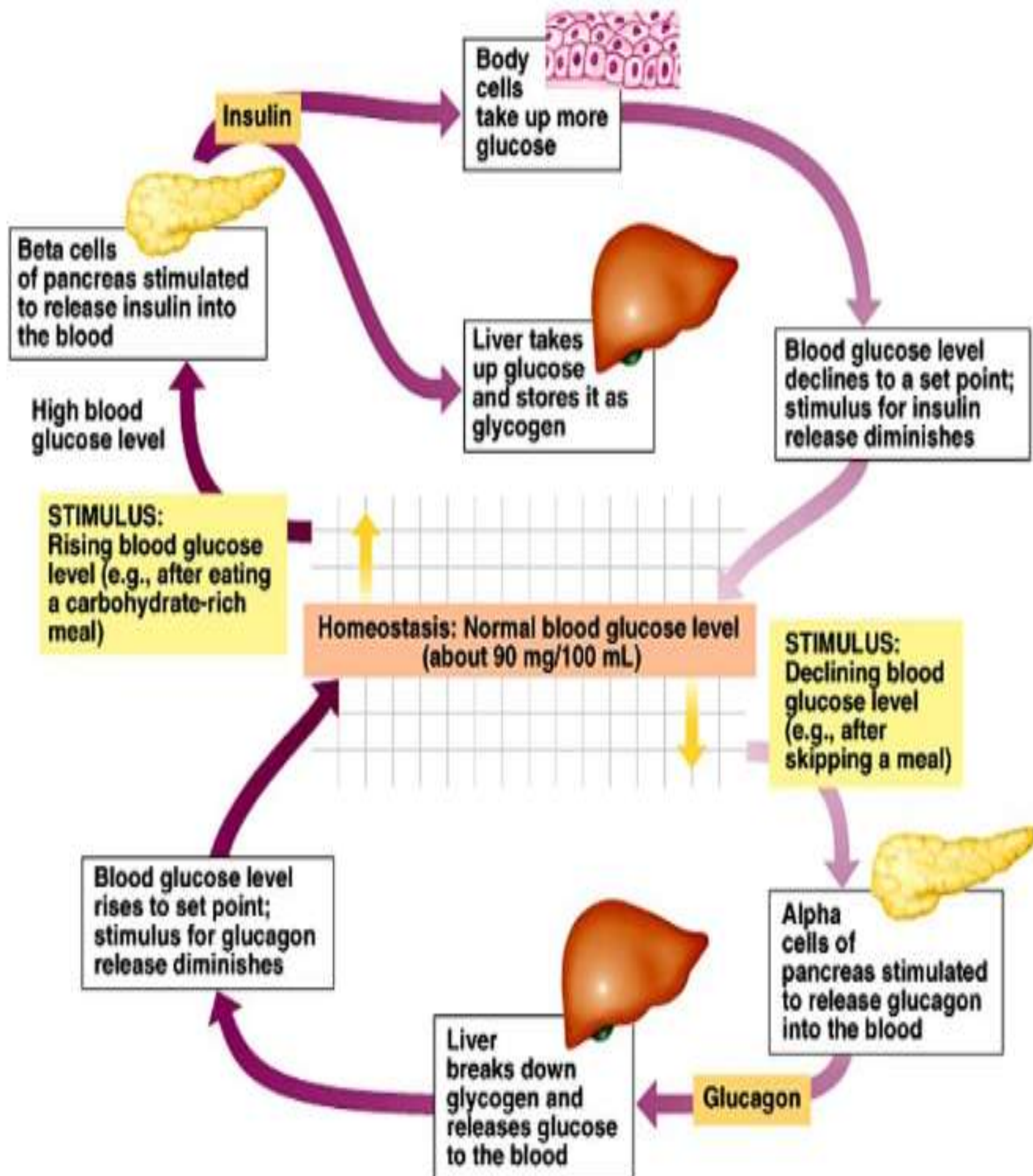


Fig 2.1**Glucose Homeostasis Maintained by Insulin and Glucagon** (Pearson Education Inc., 2003)

A typical patient with type 2 DM is overweight (average body mass index [BMI] at presentation, >27 kg/m$^2$) (Wilkin, 2009).

Other independent risk factors for DM include being born to a mother with gestational DM, high birth weight or exceptionally low birth weight. Low birth weight predisposes both to DM and to obesity, as intrauterine malnutrition may preprogram the baby to respond inappropriately in a calorie-rich environment (Thunander *et al*., 2008; Hattersley *et al*., 2009).

Rates of progression to manifest type 2 DM are variable, but the disease usually presents in adult life. It is predicted that childhood-onset type 2 DM will become the prevalent form of the disease by about 2020 (Whiting *et al*., 2011).

About 85% of type 2 DM patients have the metabolic syndrome: a cluster of hyperglycaemia, obesity, hypertension, low HDL cholesterol and raised triglycerides. This syndrome is no more than the sum of its parts, and the term is now used cautiously. It does, however, represent the multiple nature of the disease process reflecting the dominant effect of insulin insensitivity (Leslie *et al*., 2017).

*2.1.2.3 Other Specific Types of DM according to WHO, 2011 include:*

i. Genetic defects of beta cell function
ii. Genetic defects in insulin action
iii. Diseases of the exocrine pancreas (Secondary DM)
iv. Endocrinopathies (Secondary DM)
v. Drug- or chemical-induced (Secondary DM)
vi. Infections (Secondary DM)
vii. Uncommon forms of immune-mediated DM (Secondary DM)

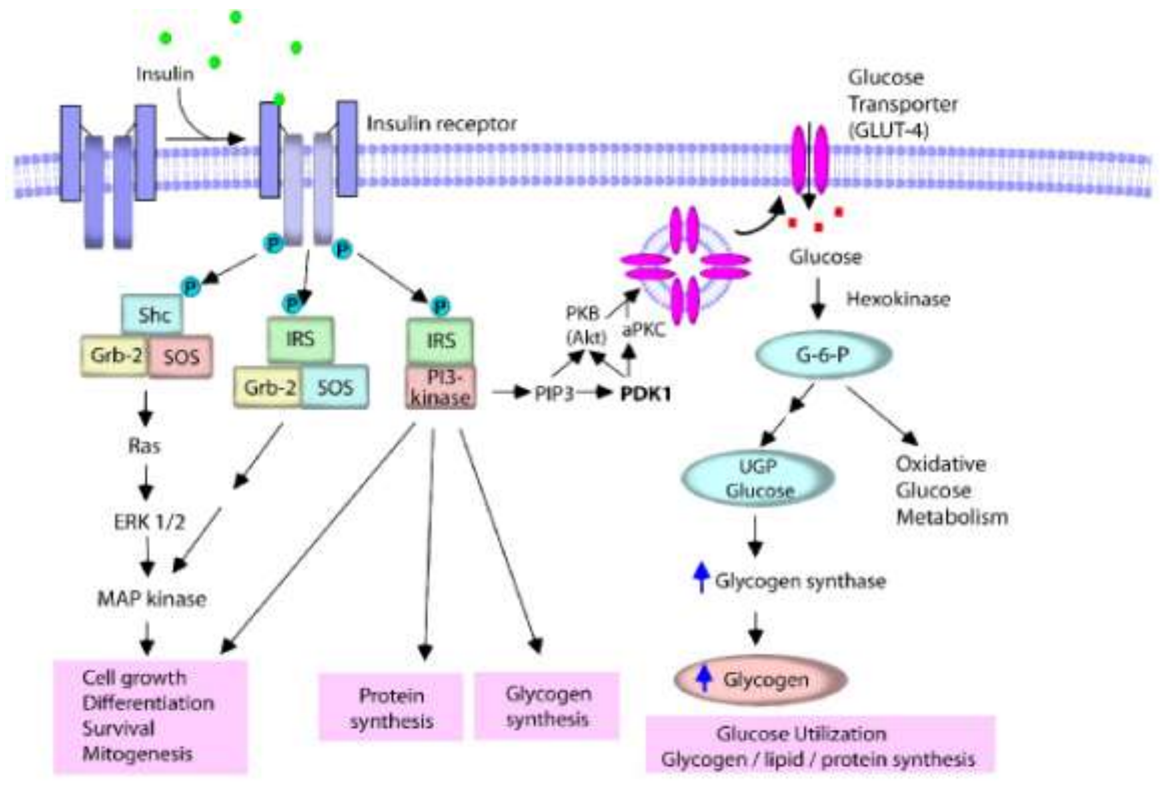


Fig 2.2**Insulin Signalling** (Mangmool *et al*., 2017).

viii. Other genetic syndromes sometimes associated with DM
ix. Gestational Diabetes Mellitus (GDM)

While DM is defined by an increase in blood glucose, the cause of the hyperglycaemia is due to inadequate insulin secretion or reduction in insulin sensitivity. Insulin is the key hormone in the metabolism of glucose. Glucose enters in the blood from these three main sources:

i. The gastrointestinal tract (GIT), from ingested carbohydrates, which are hydrolysed or converted in the liver
ii. Release from the liver glycogen stores and other glycogen stores (a process known as glycogenolysis)
iii. By new glucose synthesis from precursors such as lipids and proteins (a process called gluconeogenesis)

Since insulin plays a key role in liver glucose metabolism and in the use of glucose by muscles and adipose (fat) cells through its signalling pathway (fig 2.2), it follows that inadequate insulin levels will tend to cause increased blood glucose. The metabolic disturbances of DM therefore reflect the broad action of insulin in the metabolism of glucose (DeFronzo*et al*., 2013).

### 2.1.3 Clinical presentations of DM

Patients with DM can present either with symptoms due to the high glucose or with thecomplications of DM. According to Unger and Parkin(2010) the classic triad of symptoms associated with DM that are directly due to high blood glucose include:

i. Polyuria
ii. Thirst
iii. Weight loss

These symptoms are associated with DM, irrespective of its cause, but are most often found in children with type 1 DM and, at an extreme, can be associated with diabetic ketoacidosis. Clinical indications of type 2 DM may be minimal until significant clinical complications present. Early indicators include one or more of the following symptoms, (Garber *et al.*, 2016):

i. Increased thirst/urination
ii. Infections slow to heal
iii. Blurred vision

Diagnosis is usually suggested from routine diagnostic tests.

2.1.4 DMdiagnostic tests

*2.1.4.1 Urine glucose*

Glycosuria (appearance of glucose in urine) is not diagnostic of DM, but should be an alert for the need for further investigation. About 1% of the population has renal glycosuria, inherited as an autosomal dominant or autosomal recessive trait associated with a low renal threshold for glucose (Khunti *et al.*, 2013).

*2.1.4.2 Blood glucose*

Blood glucose has been the gold standard for the diagnosis of DM. Fasting blood glucose remains a valuable diagnostic aid in Nigeria. In disease management, blood glucose, estimated either by the patient using capillary blood or by laboratories using whole blood (venous or capillary), is valuable as it gives immediate information regarding the quality of blood glucose control. HbA1c differs in that it represents an average over the past three months, influenced mostly by the immediate past 30 days (Kester *et al.*, 2012).

*2.1.4.3 HbA1c*

HbA1c has the advantage of being accurate, simple and, now, reproducible with worldwide standardization and harmonization of the assays. One advantage HbA1c has over glucose measurement is the lack of a fasting requirement and the difficulties with OGTT (Oral Glucose Tolerant Test), but the precise cut-off to diagnoseDM remains controversial (Sherwani *et al.*, 2016).

A level of 6.5% (IFCC (International Federation of Clinical Chemistry) 48 mmol/mol) is specific for the diagnosis of DM in most studies, but lacks sensitivity and may miss many cases. The accuracy of the test is further complicated by many factors, which modify levels of HbA1c due to biological variability, genetic factors (such as red cell life span, ethnicity and haemoglobinopathies), environmental factors (for example, iron deficiency) and interferences (for example, vitamin C) (Ziemer *et al.*, 2010).

## 2.2 **Blood Glucose**

In people that are healthy, blood glucose concentration is maintained within a very narrow limit, with some fluctuations after meal. Glucose concentration increases after meals, but typical meals will not raise blood glucose above 144 mg/dL, and normoglycaemia should be restored within four hours in healthy people (Duckworth *et al.*, 2009).Glucose-containing non-esterified fatty acids complexes are stored as glycogen. For a 70-kg man, 700–1000g of (hydrated) glycogen in total is stored majorly in the liver (60–125g) and skeletal muscles (400–600g). Glycogen is synthesized from glucose and gluconeogenic substrates such as lactate, pyruvate, glycerol and some amino acids (Moore *et al.*, 2012).The liver is central in the homeostasis of glucose because it absorbs and stores glucose (as glycogen) after eatingand releases glucose into the circulation between meals (Fig 2.4).Because the kidneys are

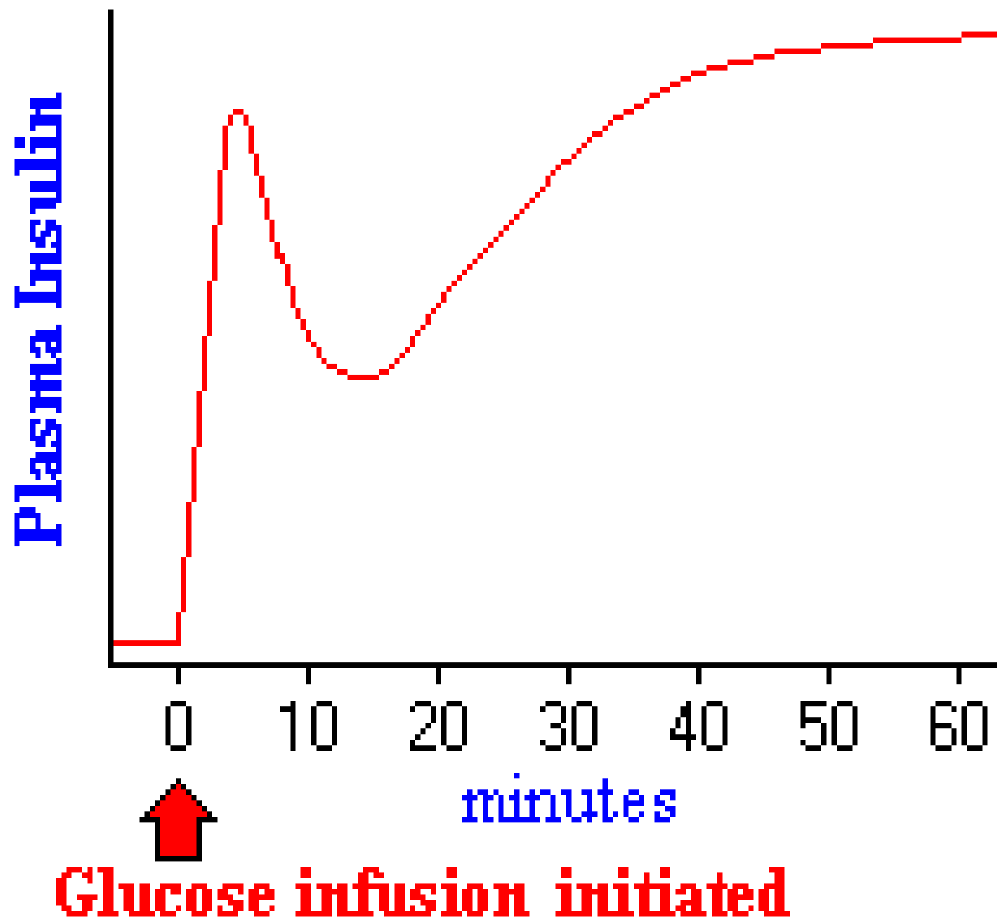


Fig 2.3**Insulin Action** (vivo.colostate.edu).

also important for glucose homeostasis, hypoglycaemia may also occur during renal failure (Rizza, 2010).

Glucose is produced by gluconeogenesis (fig 2.4) in the liver, where a two 3-carbon molecule such as glycerol (derived from the breakdown of fat), is combined with lactate or pyruvate (derived from anaerobic glycolysis), or other amino acids, to create 6-carbon glucose (Kowalski and Bruce, 2014).

Glucose provides approximately 40–60% –on a regular diet – of total energy expenditure during the day and is the predominant fuel post-absorption or during exercise. However, cells may also use ketone bodies or fatty acids for their energy supply and switch between these metabolic fuels. Glucose, is trapped within a cell (since all glucose transporters, the GLUTs, are potentially bidirectional) by being phosphorylated on entry by a family of hexokinases (e.g., glucokinase) (Augustin, 2010).

Glucokinase (or phosphorylation by glucokinase) is a rate-limiting step in themetabolism of glucose, so this enzyme is a crucial determinant of insulin secretion from beta cells. Loss-of-function mutations of glucokinase cause one form of maturity onset DM of youth (MODY) (Perry *et al*., 2017).

### 2.2.1 Insulin

Insulin is the major hormone that regulates energy storage and release. The protein (insulin) is encoded by genes located on chromosome No. 11 and expressed in beta cells (fig 2.5) in the islets of Langerhans in the pancreas that synthesize and release the hormone (Bosco *et al*., 2010).

Before the release of insulin as an active hormone, it exists as a prohormone known as proinsulin, in which a connecting chain of amino acids, C-peptide, maintains the structure.

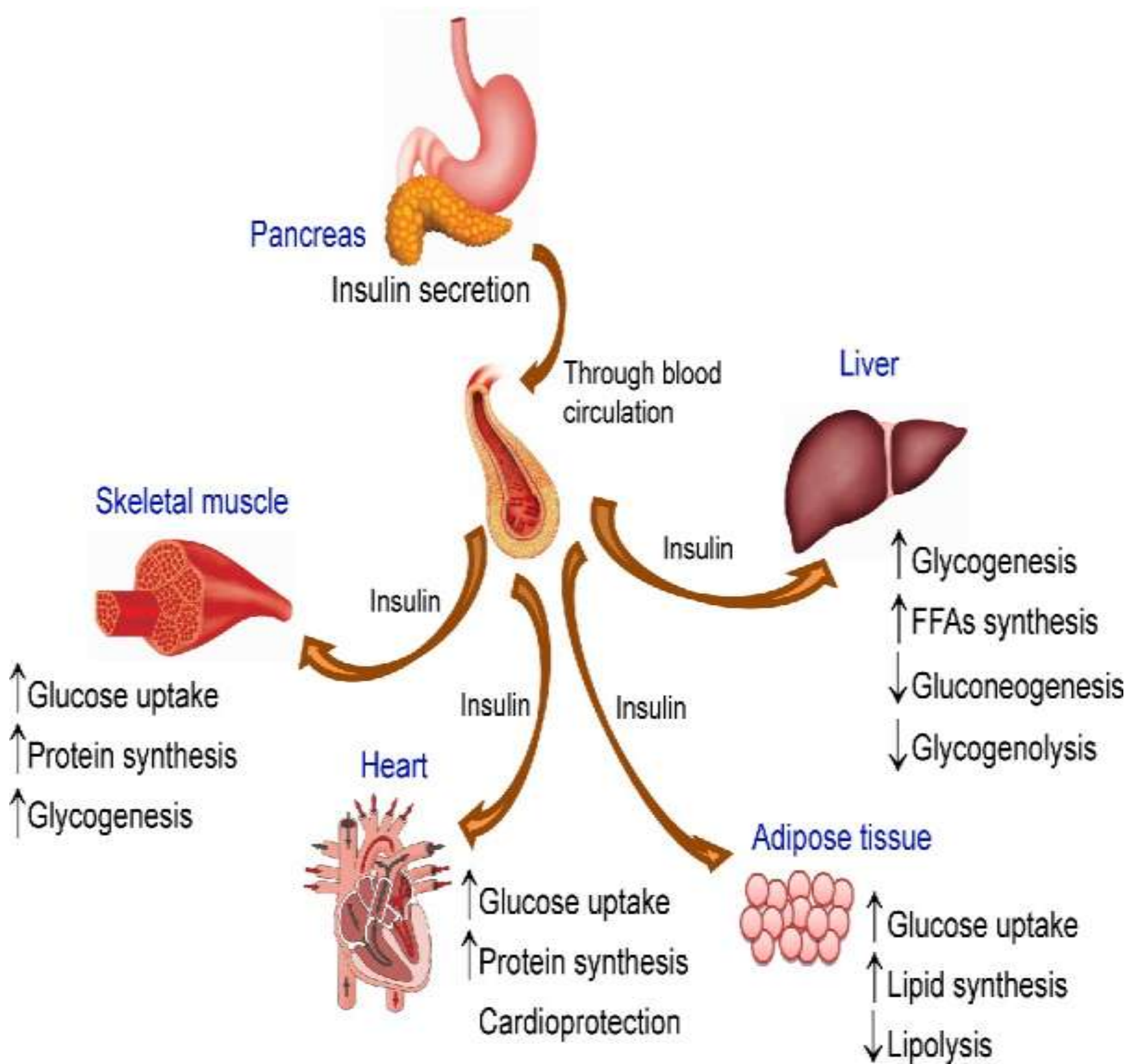


Fig 2.4**Glucose Homeostasis** (Mangmool *et al*., 2017).

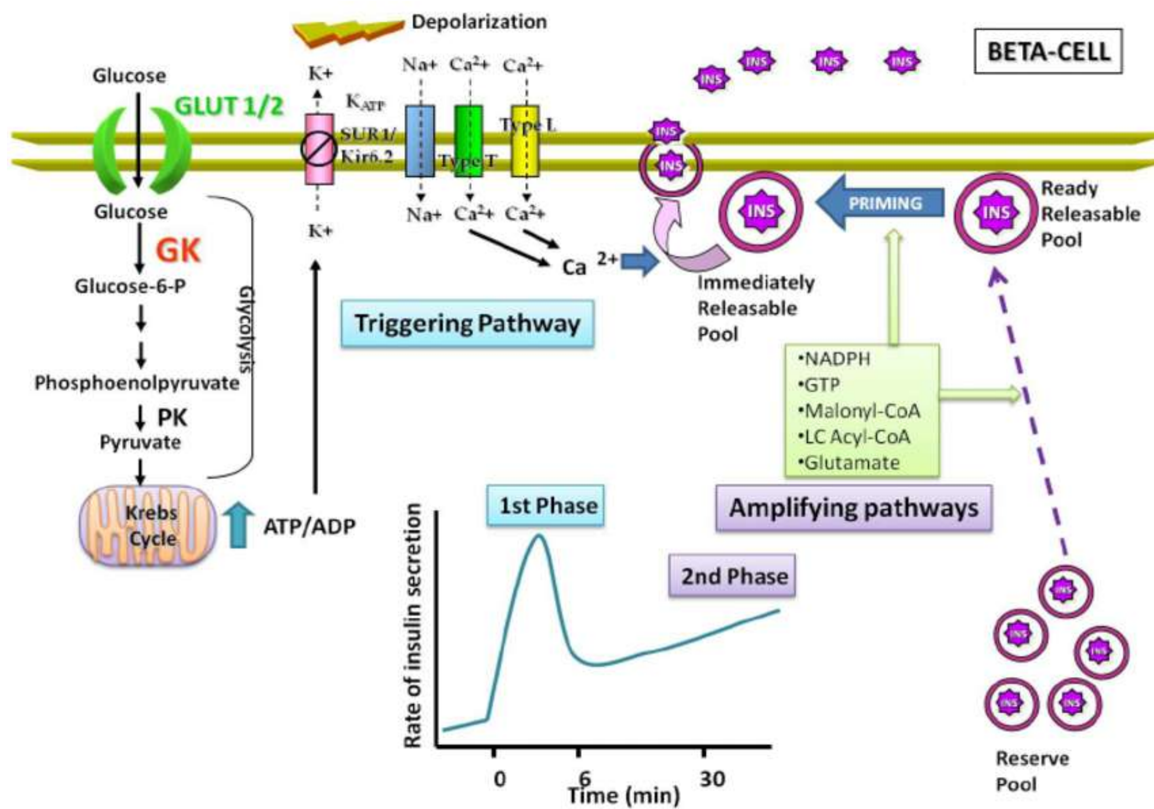


Fig 2.5**Mechanism of Insulin Release** (Luisa Lazo-de-la-Vega-Monroy and Fernandez-Mejia, 2011).

When the relatively biochemically-inactive C-peptide is cleaved from proinsulin, the active hormone – insulin – is produced and is ready for secretion (Barreto *et al*., 2010).

Insulin, upon secretion enters the portal circulation of the liver, a prime target of insulin action. The liver then extracts and degrades about 50% of secreted insulin. While insulin is the major regulator of intermediary metabolism, its actions can be modified by other hormones like glucagon, adrenaline and steroids (Henquin, 2011).

Insulin is a peptide hormone with a molecular weight of 5807 Daltons and comprising of 51 amino acids which are organized in two chains linked by two disulfide bonds (fig 2.6) (Leto and Saltiel, 2012).

Inadequate insulin secretion and/or resistance to insulin are the causes of all forms of DM. An ATP-dependent and sulfonylurea-sensitive potassium ($K^+$) channel on the membrane of the beta cell relays the signal that leads to $K^+$ channel closure, influx of calcium and secretion (exocytosis) of insulin (Fred and Welsh, 2009).

The most important stimulant of this channel is hyperglycaemia, while sulfonylureas, which stimulate the channel, are a group of drugs used in therapy (fig 2.7). Insulin secretion is directly related to food intake and sugar content within the food that is consumed (Komatsu *et al*., 2013).

## 2.3 GlycaemicControl

DM is associated with damage to blood vessels, nerves, kidneys and the eyes. These changes impact large blood vessels (macrovascular disease) and small blood vessels (microvascular disease), andlevel of blood glucose is a major determinant of these risks (Rask-Madsen and Khan, 2012).

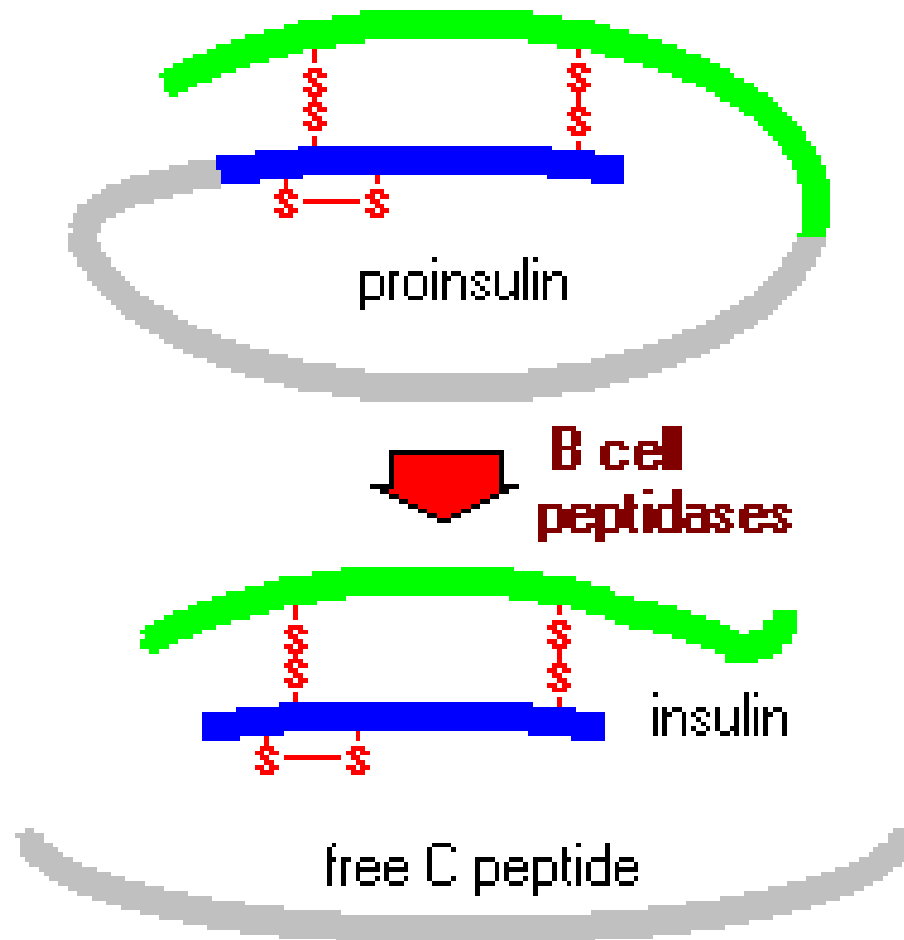


Fig 2.6**Insulin Structure Including the Disulphide Bridges** (S-S) (vivo.colostate.edu).

The macrovascular diseases associated with DM include cardiovascular, cerebrovascular and peripheral vascular disease. And these conditions, clinically, are associated with angina, stroke and claudication, respectively (Holman *et al*., 2008).

The risk of developing clinically significant macrovascular disease is five times higher in a diabetic patient than in a non-diabetic individual. The major modifiable risk factors associated with this disease complication include smoking, obesity, hypertension and dyslipidemia, and, to a degree, hyperglycaemia (Chakera *et al*., 2015). The configuration of these risk factors aside from smoking, make up the metabolic syndrome which is the aggregate of its parts and, therefore, a valuable guide to clinicians to remind them of the width of management schemes.

Microvascular disease on the other hand, is associated with retinopathy, neuropathy and nephropathy, all typical resultants of damage to smaller capillaries. Clinically, these conditions are associated with visual impairments, numbness of the feet, and protein in the urine, respectively (Brownlee, 2005).

In worst cases, these same microvascular complications can lead to blindness, foot ulcers/amputations and kidney failure in respective order. Major modifiable risk factors associated with microvascular disease are the same as for macrovascular disease: smoking, obesity, hypertension, dyslipidemia and hyperglycaemia, but with hyperglycaemia being a more dominant factor in this case. Given the differential effect of hyperglycaemia on microvascular disease compared with macrovascular disease, it is said that DM is a condition encompassing two diseases: one disease associated with macrovascular disease (and its associated risk factors) and the other associated with microvascular disease (predominantly due to hyperglycaemia) (De Block and Van Gaal, 2009).

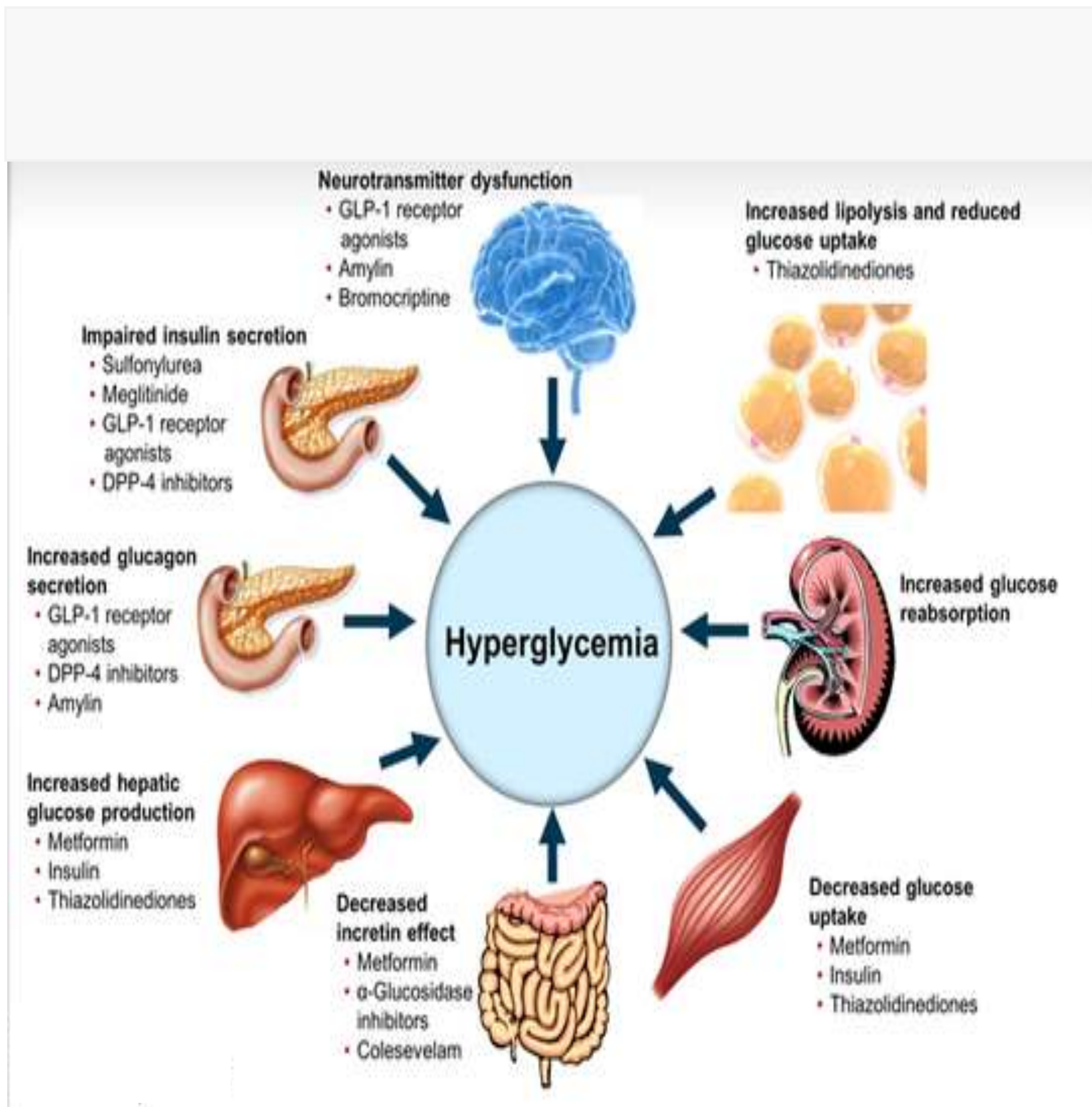


Fig 2.**7 Type II DM** (Mangmool *et al*., 2017).

### 2.3.1 Management ofDM

DM is a disease that carries a substantial cost due to the prevalence of the disease (type 2 especially), the chronicity of the disease, the severity of the complications, and the fact that both the disease and it complications can be treated. Direct costs due to treatment, diagnosis and medical care approximately equal indirect costs due to loss of financial output through illness or death – at least in industrialized countries – with the yearly mean cost of DM-related healthcare in Nigeria equalling about 50 million dollars (International Diabetes Federation, 2014).

Management of DM is aimed at the risk factors, which predispose to complications, and the identification and treatment of these complications. Key approaches include education (sensitisation about the disease), diet, exercise, oral hypoglycaemic-drug therapy, injectables such as exenatide or liraglutide, insulin and bariatric surgery (Blonde and Russell-Jones, 2009).

#### *2.3.1.1 Management of type 1 DM*

Children with type 1 DM require insulin treatment from the time they are diagnosed. However, the majority of adult patients with autoimmune DM do not require insulin, at least initially, and the majority of them remain insulin-independent for many years. Insulin regimens include either multiple insulin injections, insulin injections with a mix of fast-acting and slow-acting insulin, or continuous subcutaneous insulin infusion pumps (Pickup, 2012).

#### *2.3.1.2 Management of type 2 DM*

Patients with type 2 DM are usually managed with oral medications or injections other than insulin – at least for a start. The typical treatment is cumulative, with diet and exercise

initially; oral medication later on; then progressively more tablets or injectable drugs (such as a GLP-1 agonist or insulin) (Butler *et al*., 2013).

Insulin regimens often start with slow-acting insulin at bedtime, but may then progress to similar regimens as for type 1 DM, though usually not including subcutaneous insulin infusion pumps. The role of bariatric surgery is still uncertain, but surgery is offered as an option to patients that have gross obesity and hyperglycaemia refractory to conventional treatment (Schauer*et al*., 2012).

The number of therapies, the variable responses to them and the range of side effects has led to a more personalized approach, as set out in the many recent guidelines. Oral therapies currently used include metformin, sulfonylureas, glinides, dipeptidyl peptidase IV (DPPIV) inhibitors, sodium-glucose transporter (SGLT2) inhibitors, glitazones and acarbose. Injectables for therapy include GLP-1 agonists and insulin (Berthe *et al*., 2007).

*2.3.1.3 Diet and exercise*

Since surplus caloric intake and insufficient exercise are fundamental to the epidemic of type 2 DM, it adds up that diet and exercise are key to the management of type 2 DM and indeed to all forms of DM, as well as efforts to prevent progression of impaired glucose tolerance to DM (Fatourechi *et al*., 2009).

Long-term adherence to any dietary plan is notoriously difficult; and dietary advice is largely empirical. A sensible approach is to suggest that the diet should not be different from that proposed for the healthy population, perhaps with an emphasis on the avoidance of refined sugar. Overweight patients (BMI 25–30 kg/m2) should be started on a weight-reducing diet of about 4–6 MJ (megajoules) or 1000–1600 kcal daily. While low-fat diets have only a small

impact on the serum cholesterol, they can limit increases in serum triglycerides (Franz *et al*., 2007).

Then alcohol, if not forbidden, its intake should be regulated and energy content taken into consideration. Patients on insulin should avoid alcoholic binges as they may precipitate severe hypoglycaemia. A daily salt intake of no more than 2.3 g per day is recommended to limit hypertension (Volaco, 2018).

*2.3.1.4 Managing complications*

The management of diabetic complications is focussed on the prevention of these complications in the first place. Much of the time spent on DM care is based on the premise that prevention is not just possible, but also cost-effective (Faech *et al*., 2015).

Managing macrovascular disease in DM is the same as managing cardiovascular, cerebrovascular and peripheral vascular disease in general. On the contrary, microvascular complications are unique to DM. So the treatment of diabetic retinopathy for instance, includes laser photocoagulation for proliferative retinopathy or anticytokine therapy for macular oedema, as well as vitrectomy for an unresolved vitreous haemorrhage. And angiotensin receptor inhibitors are employed early to limit progression to diabetic nephropathy (Leslie, 2010).

## 2.4 **Red Blood Cells**

### 2.4.1 Haemoglobin

Red blood cells (also referred to as erythrocytes) are the most common type of blood cells and they deliver oxygen to body tissues via a cardiovascular system. They take up oxygen in the alveoli and exchange it for carbon dioxide and the exchange of gases occurs by the process of simple diffusion: higher pressure oxygen diffuses from the alveoli to a lower

pressure of oxygen in the blood, whereas carbon dioxide diffuses in the opposite direction according to the concentration gradient (Piperno *et al*., 2011).

This is done, of course, continuously to bring fresh air into the lungs and the alveoli and to exhale carbon dioxide through an active process of respiration that requires contraction of skeletal muscles (Seifter *et al*., 2005). The mature red blood cell is produced by a process called "normoblastic erythropoiesis" (Fig 2.8).

This occurs entirely in the red bone marrow that can be found in vertebrae, ribs, skull, sternum, scapula, and proximal ends of the limb bones (Macleod, 1977). Erythrocytes are formed in the stroma of the bone marrow (Macleod, 1977; Pallis, 1998). The formation of erythroblast is controlled by a glycoprotein hormone, erythropoietin which is produced in the kidneys from a plasma substrate (erythrogenin) in response to oxygen deficiency (alkalosis)(Baciu and Ivanof, 1984).

Erythropoiesis is inhibited by a rise in the level of circulating red blood cells to subnormal values and stimulated by anaemia and by hypoxaemia (Macleod, 1977). The normal period for norrmoblastic erythropoiesis is 4 days (Pallis, 1998).

The formation of red blood cell itself requires the usual nutrients and structural materials, in addition to iron, vitamin B12 and folic acid. The developing red blood cell is organized for the biosynthesis of some $4 \times 10^8$ molecules of haemoglobin that ultimately make up 95% of its dry weight (Harris and Kellermeyer, 1974).

The haemoglobin of the adult erythrocyte affects the transfer of oxygen and carbon dioxide throughout the body. If haemoglobin was not maintained within the corpuscle units, the resulting haemoglobin solution of 15 g 100 $ml^{-1}$ would be too viscous for efficient circulation (Roughton, 1963).

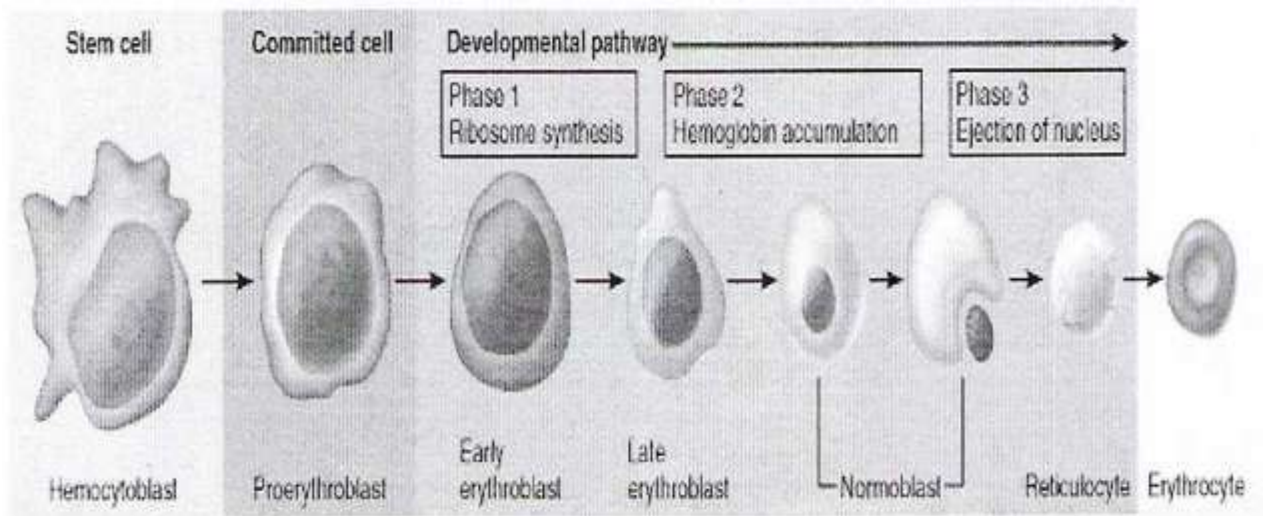


Fig 2.8:**Normoblastic Erythropoiesis** (Pearson Education Inc., 2003).

It has been demonstrated that haemoglobin in lysates, at an intracorpuscular concentration of 5 mM Hb, has a viscosity less than that of the erythrocyte suspended in the same diluting medium (Cokelet and Meiselman, 1968).

The human red blood cell has proved to be a useful tool for many studies, as it has the particular advantage that the plasma membrane is the only membrane protein. Plasma membrane preparations (ghosts) can be produced from intact cells by simple hypotonic shock and then subsequent washings to remove the resealed haemoglobin (Dodge *et al*., 1963; Steck and Kant, 1974; Seymour *et al*., 1982).

Lifewould not have been easy if we hadto rely only on our lungs and the water (plasma) in our blood to transport oxygenthrough our bodies. Fortunately, we have some chemical assistance in theform of a protein called haemoglobin (Fig 2.9).

Haemoglobin (Hb) possesses a molecular weight of 64,450 dalton. Hb is a tetramer molecule made up of four subunits, in which each contains a haeme moiety conjugated to a polypeptide whereby the Haeme is an iron-containing porphyrin derivative (Omar, 2013).

There are two pairs of polypeptides in each Hb molecule. In normal adult HbA1, the two types are α2β2, in which α chain contain 141 amino acid residues and the β chain contain 146 amino acid residues. In foetal Hbf, the β polypeptide chains are replaced by γ chains of 146 amino acid residues each, but only 10 individual residues differ from those in β chains (Omar, 2013). When Hb binds to oxygen the distance between the iron (II) ions of the β1 and β2 polypeptide chains is markedly reduced from 40 Å to 33 Å. The changes in contacts between α and β chains are of special interests.

In the transition from oxy- to deoxyhaemoglobin, large structural changes take place at two of the four contact regions, namely α1 β2 and the identical α2 β1 contacts, but not at the

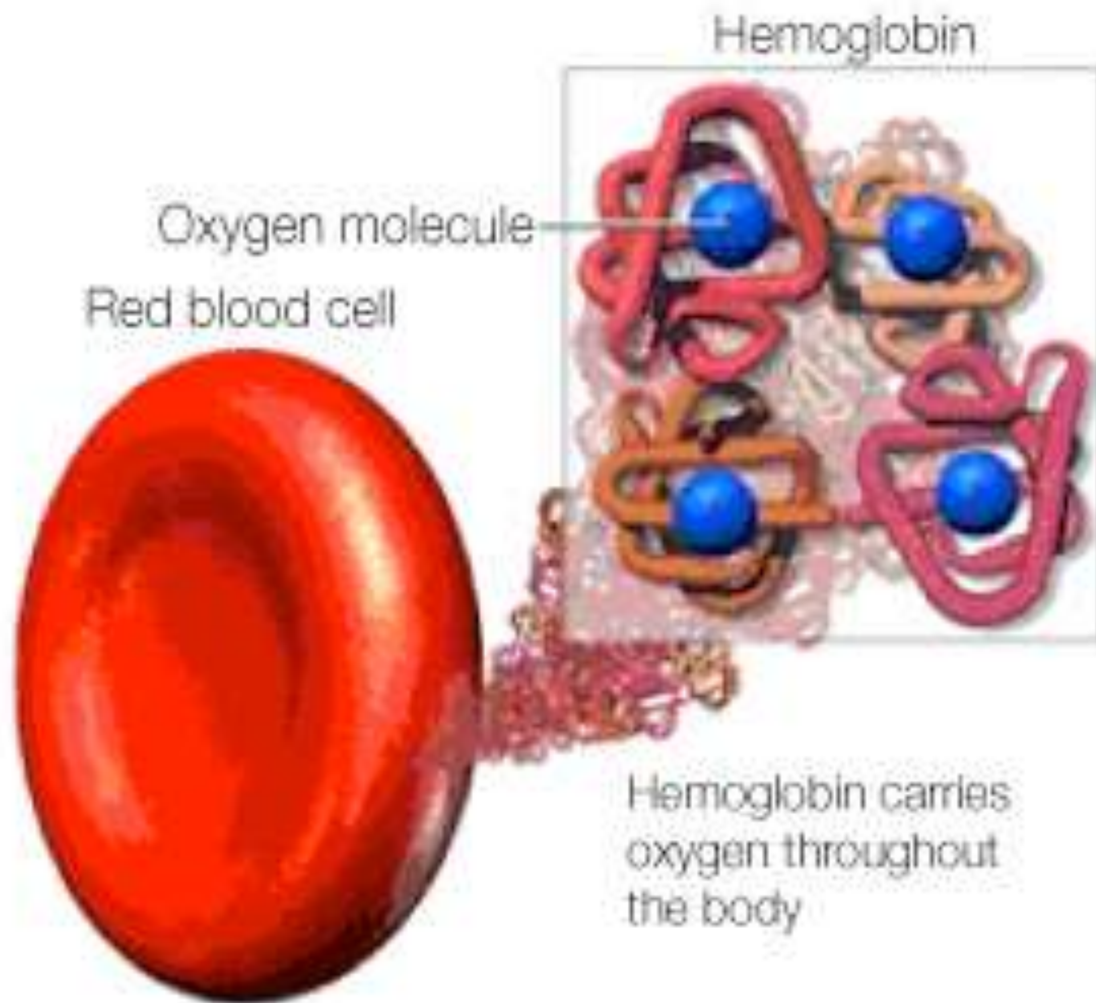


Fig 2.9:**Summary of Haemoglobin Structure**(Pearson Education Inc., 2003).

other α1 β1 and α2 β2 contacts. The α1 β1 pair rotates 15 degrees with respect to the other pair and atoms at the interface between these pairs shift by as much as 6 Å. In fact, α1 β2 contact region acts as a switch between two alternative structures. The two forms of interface are stabilized by different sets of hydrogen bonds. This interface is closely connected to the haeme groups, and so the structural changes in it affect the haemes and vice versa. The quaternary structure of deoxyhaemoglobin is termed the T (taut or tense) form, whereas R (relaxed) form is termed for oxyhaemoglobin (Omar, 2013).

## 2.5 **Glycated Haemoglobin($HbA1_C$)**

HbA1c is the chromatographic fraction of haemoglobin that has glucose attached to the N-terminal valine (or lysine) of the β-chain (Karami and Baradaran, 2014).

Effective monitoring of diabetic control (and diagnosis) requires a good marker for estimation of the average blood glucose over a longer period and glycated haemoglobin (HbA1c) fulfils this requirement for such a reliable compass to guide therapy (fig 2.10). The glycation reaction depends on how long red blood cells have been in circulation and the ambient (blood) glucose levels. As red cells have a lifespan of 3–4 months, HbA1c then logically should reflect the average blood glucose levels in the preceding three months (Loh*et al.*, 2015).

Thus, in patients with DM, $HbA1_C$ is used to assess and monitor effectiveness of treatment (glycaemic control) (fig 2.11) given over a past period, usually the past two to three months. The results from $HbA1_C$ testing can then be used to determine the course of future treatment for the patient in order to guard against hyperglycaemic-induced complications (Khan *et al.*, 2004).There are aspects of the measurement of HbA1c that are problematic. Although in some laboratories the precision of HbA1c measurement is similar to that of plasma glucose, global consistency with both assays remains a problem (International Expert Committee,

2009). Whether it is the glucose or HbA1c assay that is used, consistent and comparable data that meet international standards are required. This is beginning to happen in many countries but evidently is still not standard across the world. Within any country, it is axiomatic that results for glucose and HbA1c ought to be consistent between laboratories.

The National Glycohaemoglobin Standardization Program (NGSP) (Little *et al.*, 2001) was established following the completion of the Diabetes Complications and Control Trial (DCCT). For many years it was the sole basis for improved harmonization of HbA1c assays. More recently the International Federation of Clinical Chemists (IFCC) established a working group on HbA1c in an attempt to introduce an international standardization program (Hoezel *et al.*, 2004).

An important part of this effort was establishment of reference method procedures for HbA1c. Currently, both the NGSP and the IFCC base their evaluations on reference method procedures that have further enhanced the harmonization of HbA1c assays across manufacturers. Finally in the USA, the College of American Pathologists (CAP) has mandated more stringent criteria for individual assays to match assigned values for materials provided in the CAP proficiency programme (Goldstein *et al.*, 2004).

A further major factor concerns costs and availability of HbA1c assays in many countries. Also, the situation in several of these countries will be exacerbated by high prevalence of conditions such as haemoglobinopathies, which affect HbA1c measurement. A report published in 2009 by an International Expert Committee on the role of HbA1c in the diagnosis of DM recommended that HbA1c can be used to diagnose DM and that the diagnosis can be made if the HbA1c level is 6.5% (International Expert Committee, 2009).

Diagnosis should be confirmed with a repeat HbA1c test, unless clinical symptoms and plasma glucose levels >11.1mmol/l (200 mg/dl) are present in which case further testing is

not required. Levels of HbA1c just below 6.5% may indicate the presence of intermediate hyperglycaemia. The precise lower cut-off point for this has yet to be defined, although the ADA (American Diabetes Association) has suggested 5.7 – 6.4% as the high risk range (Diagnosis and classification of Diabetes, 2010).

While recognizing the continuum of risk that may be captured by the HbA1c assay, the International Expert Committee recommended that persons with a HbA1c level between 6.0 and 6.5% were at particularly high risk and might be considered for DM prevention interventions. In view of this and of the advances in technology over recent years, members of the consultation agreed that HbA1c may be used to diagnose DM providing that appropriate conditions apply, i.e. standardized assay, low coefficient of variability, and calibration against IFCC standards (Hanas and John, 2010).

Furthermore, each country should decide whether it is appropriate for its own circumstances. The choice of diagnostic method will depend on local considerations such as cost, availability of equipment, population characteristics, presence of a national quality assurance system etc. Policy-makers are advised to ensure that accurate blood glucose measurement be generally available at the primary health care level, before introducing HbA1c measurement as a diagnostic test (American Diabetes Association (ADA), European Association for the Study of Diabetes (EASD), International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), and the International Diabetes Federation (IFD), 2007).

The consultation concluded that there is insufficient evidence to make any formal recommendation on the interpretation of HbA1c levels below 6.5%. Long term prospective studies are required in all major ethnic groups to establish more precisely the glucose and HbA1c levels predictive of microvascular and macrovascular complications.A workinggroup

should be established to examine all aspects of HbA1c and glucose measurement methodology (Ucar *et al*., 2013).

The diagnosis of DM in an asymptomatic person should not be made on the basis of a single abnormal plasma glucose or HbA1c value. At least one additional HbA1c or plasma glucose test result with a value in the diabetic range is required, either fasting, from a random (casual) sample, or from the oral glucose tolerance test (OGTT) (Sacks, 2011).

The diagnosis should be made by the best technology available, avoiding blood glucose monitoring meters and single-use HbA1c test kits (except where this is the only option available or where there is a stringent quality assurance programme in place). It is advisable to use one test or the other but if both glucose and HbA1c are measured and both are "diagnostic" then the diagnosis is made. If one only is abnormal then a further abnormal test result, using the same method, is required to confirm the diagnosis (WHO, 2011).

More and more asymptomatic subjects are being detected as a result of screening programmes so that diagnostic certainty is paramount. If such tests fail to confirm the diagnosis of DM, it will usually be advisable to maintain surveillance with periodic re-testing until the glycaemic status becomes clear (Little *et al*., 1986).

An HbA1c of 48 mmol/mol (6.5%) is recommended as the cutoff point for the diagnosis of DM (World Health Organization, 2011).

Mathematical models and clinical data show that mean glycaemia in the 30 days immediately preceding blood sampling contributes approximately 50% to the final result, whereas days 90–120 preceding contribute only about 10% (Goldstein *et al*., 1994), as visualized in Fig 2.12.

**A1c and Blood Sugar**

| A1c (%) | Average Blood Sugar (mg/dL) |
|---|---|
| 4 | 68 |
| 5 | 97 |
| 6 | 126 |
| 7 | 152 |
| 8 | 183 |
| 9 | 212 |
| 10 | 240 |
| 11 | 269 |
| 12 | 298 |
| 13 | 326 |
| 14 | 355 |

Fig 2.10:**$HbA1_C$and Glucose Equivalence**(*WebMD* Medical Reference, 2016)

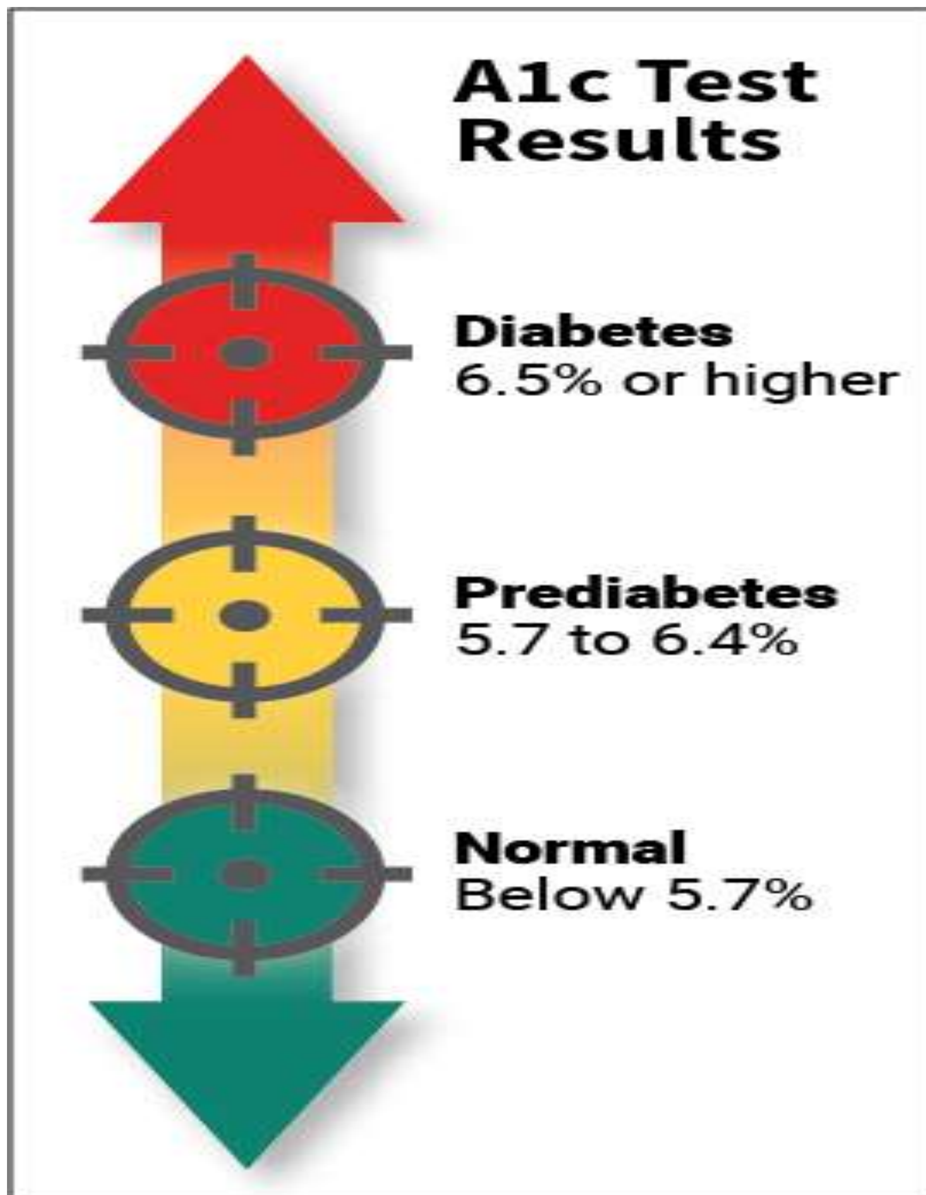


Fig 2.11: **$HbA1_C$ Chart**(*WebMD* Medical Reference, 2016).

Glycated haemoglobin though a widely accepted, clinically useful, diagnostic, and prophylactic glycaemic marker, there are still limitations much of which borders on and the techniques used in measuring or reporting (referencing) the glycated haemoglobin tests and haemoglobin variants (Ucar *et al*., 2013).

### 2.5.1 Diagnostic potentials of HbA1c

The ADA (American Diabetes Association) has recently recommended HbA1c with a cut-point ≥6.5% for diagnosing DM as an alternative to fasting blood glucose (FBG ≥7.0 mmol/L)-based criteria (Gillett, 2009).

The levels of HbA1c are strongly correlated with FBG (Khan *et al*., 2007). FBG, 2-hour post-glucose load plasma glucose, and oral glucose tolerance tests are recommended for the diagnosis of DM only if HbA1c testing is not possible due to unavailability of the assay, patient factors that preclude its interpretation, and during pregnancy (Herman and Fajans, 2010). HbA1c provides a reliable measure of chronic glycaemia and correlates well with the risk of long-term DM complications, so that it is currently considered the test of choice for monitoring and chronic management of DM. However, the cut-point of HbA1c from the diagnostic point of view is still controversial. Among diabetics, the blood glucose levels increase in the blood and the glucose attaches to the haemoglobin molecule in a concentrationdependent manner. The glucose-bound (glycated) haemoglobin or $HbA1_C$ provides the average glucose levels in an individual’s blood as it becomes glycated with the haemoglobin. It is important to note that the $HbA1_C$ levels are directly proportional to the blood glucose levels.

A simple blood glucose test such as a fasting glucose test (FGT) is a measure of glucose concentration present in an individual’s blood at a given point of time (Nathan *et al*., 2007).

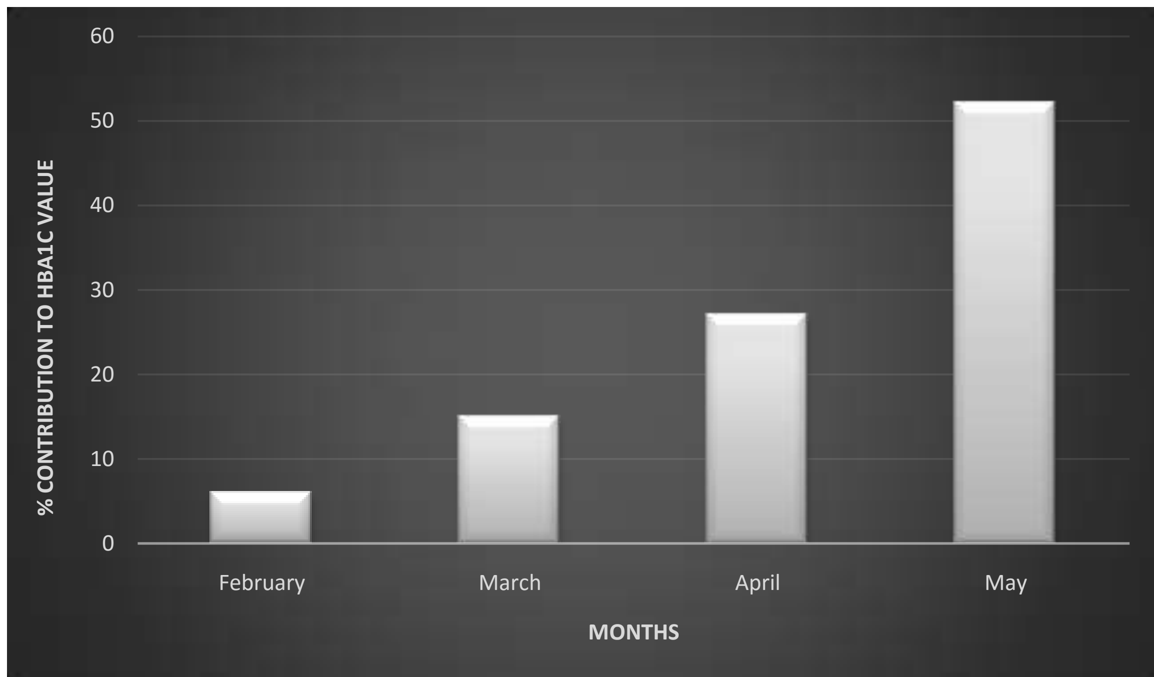


Fig 2.12:**Cumulative Influence of RBC and Plasma Glucose Level on $HbA1_C$ Test** (Goldstein *et al*., 1994).

The blood used for the FGT may be obtained through a needlestick of a finger or directly from the arm. A new techno logy, continuous glucose monitoring, has arrived in the market, which allows for non-prick readings (Pandit, 2012).

A small chip is implanted under the skin, which provides continuous glucose monitoring readings to the sensor kept outside, and if the glucose levels are higher or lower, it sends a special signal to the sensor, thus alerting the patient and/or the health-care provider for intervention (Tansey *et al*., 2011).

The FGT is an excellent test for "in the moment" glucose levels, but it does not provide detailed information about the time course trend of the glucose levels. The $HbA1_C$ test, however, is a marker of the average glucose levels spread over a two- to three-month period. Contrary to popular belief, along with the type 2 DM, the $HbA1_C$ is also used to diagnose, manage, and monitor the type 1 DM as well (Ehehalt *et al*., 2010).

In a series of 12,785 male diabetic patients, Khan *et al*. 2015 have shown that the $HbA1_C$ cut-point of 6.5% was associated with 3.78% false-negative predictions while majority of the falsenegative patients had borderline FPG (7.0–8.0 mmol/L) and $HbA1_C$ (6.0%–6.5%), and therefore belonged to at-risk category on the basis of $HbA1_C$ alone criteria. These findings suggest that the status of individuals with HbA1c between 6.0% and 6.5% should be verified by combined FPG and $HbA1_C$ criteria.

Recently, Khan *et al*. 2015 have provided regression equations for interconversions between the levels of FGT and $HbA1_C$ for predicting their expected values in diabetic patients.

Not requiring fasting and also not being bound by the time of the day on the part of the patient, the $HbA1_C$ is a very convenient test to administer and evaluate (Juarez *et al*., 2014).DM, the silent killer, can be detected earlier, and an appropriate treatment regimen can

be implemented sooner than later among people. The blood glucose data available from HbA1c are used in prescribing and monitoring the medicines for DM and pre-diabetes, along with exercise and diet (Bozkaya *et al*., 2010).

The accuracy of this test has continued to evolve over the last several years and is becoming the go-to option for SOC (Society of Clinicians) for detecting blood glucose values among patients in clinics. According to the National Glycohaemoglobin Standardization Program (NGSP), which developed the $A1_C$ tests, the accuracy has continued to evolve and got more precise over time (Little, 2003).

The HbA1c is recommended to be performed at least twice a year in DM patients with stable blood glucose levels (American Diabetes Association, 2011).

Still, there is no substitute for the daily (several times a day) monitoring of the blood glucose, particularly those on insulin regimen as the readings dictate the amount of insulin that a patient must take before each meal. The estimated average glucose can also be calculated from the actual HbA1c levels to help individuals with DM to correlate these levels with the daily monitoring of glucose levels (Bozkaya *et al*., 2010; Kim *et al*., 2013).

The HbA1c levels differ for different DM patients, depending on their history of DM and whether they are on tablets or long-term and/or short-term insulin dosage (Bozkaya *et al*., 2010).

Type 2 DMmanifests itself in terms of hyperglycaemia due to compromised insulin production (no production or nonavailability) (Buse *et al*., 2011).

The significance of the $HbA1_C$ test lies in the diagnosis and the prognosis of the DM patients, which lends it to a detailed understanding of insulin and insulin resistance. There is a direct correlation between $HbA1_C$ and insulin resistance, where $HbA1_C$ has been shown to be more

strongly associated with the insulin sensitivity in healthy subjects with normal glucose tolerance (Lin *et al*., 2014).

The $HbA1_C$ test has revealed minimal overlap in values between normal glucose tolerance in subjects with type 2 DM while comparing the glycaemic spectrum for insulin resistance. As a result, $HbA1_C$ is a reliable biomarker and an excellent indicator of insulin resistance for testing individuals for DM and pre-diabetes (Borai *et al*., 2011).

Kwon *et al*. 2015 evaluated the clinical usefulness of $HbA1_C$ in diagnosing gestational DM (GDM) and predicting the risk of future type 2 DM development among GDM patients. $HbA1_C$ showed high sensitivity with relatively low specificity for diagnosis of GDM in pregnant women but was a potential predictor of postpartum DM. The prognostic value of $HbA1_C$ for postpartum DM was evaluated by receiver-operating characteristic curve analysis, with a sensitivity of 78.6% and a specificity of 72.5% at a cut-off value of 5.55%.

A retrospective cohort study on women who delivered and had an early screening $HbA1_C$ test performed at □20 weeks of gestation showed that nearly one-third of those patients in the $HbA1_C$ 5.7%–6.4% group (27.3%) experienced the development of GDM compared with only 8.7% in the $HbA1_C<5.7\%$ group (Fong *et al*., 2014).

Thus, women with $HbA1_C$ 5.7%–6.4% have a significantly higher risk of progression to GDM compared with women with normal $HbA1_C$ values and should be considered for closer GDM surveillance and possible intervention. Although paired values of blood glucose and serum fructosamine were also reported for the screening of GDM, there were significant fluctuations during several antenatal visits (Khan *et al*., 2006, 2007).

### 2.5.2 Prognostic potentials of $HbA1_C$

$HbA1_C$ is not only a useful biomarker of long-term glycaemic control but also a good predictor of lipid profile; thus, monitoring of glycaemic control using $HbA1_C$ could have additional benefits of identifying DM patients who are at a greater risk of cardiovascular complications (Khan *et al*., 2007).

Thus, a single $HbA1_C$ test provides valuable information that can be used for the management of chronic diseases. In a series of 1,011 type 2 diabetic patients, HbA1c exhibited direct correlations with cholesterol, triglycerides, and low density lipoprotein cholesterol and inverse correlation with high-density lipoprotein cholesterol. There was a linear relationship between HbA1c and dyslipidemia as the levels of serum cholesterol and triglycerides were significantly higher and that of high-density lipoprotein cholesterol were significantly lower in patients with worse glycaemic control as compared to patients with good glycaemic control(Martin-Timon *et al*., 2014).

Elevated level of HbA1c has been identified as a significant risk factor for cardiovascular diseases and stroke in subjects who may have DM (Martin-Timon *et al*., 2014). A community-based population study on 11,092 non-diabetic patients found that elevated HbA1c level was strongly associated with the risk of cardiovascular disease and mortality (Selvin *et al*., 2010).

High levels of HbA1c were associated with an increased risk of recurrence of atrial tachyarrhythmia in patients with type 2 DM and paroxysmal atrial fibrillation undergoing catheter ablation (Lu *et al*., 2015).

Even an increase of 1% in HbA1c concentration was associated with about 30% increase in all-cause mortality and 40% increase in cardiovascular or ischemic heart disease mortality, among individuals with DM (Khaw *et al*., 2001).

Whereas reducing the HbA1c level by 0.2% could lower the mortality by 10% (Khaw *et al*., 2001).

Vaag, 2006 has suggested that improving glycaemic control in patients with type 2 DM may be more important than treating dyslipidemia for the prevention of both microvascular and macrovascular complications.

Cicek *et al*. 2011 determined the effect of HbA1c on the outcomes of primary percutaneous coronary intervention (PCI) for ST-segment elevation myocardial infarction (STEMI). They observed that in-hospital mortality and major adverse cardiac events were significantly higher in patients with HbA1c >6.5% (11%) compared with the group of patients with HbA1c between 5.7% and 6.4% (2.8%) or HbA1c >5.6% (0.9%). Out of the total 374 patients, 196 (63.6%) patients without a history of DM had elevated HbA1c >5.7%, with 31 (10.1%) of them having HbA1c >6.5%.

On the basis of 12-month follow-up of 1,433 patients with stable angina who underwent coronary angiography, it was concluded that high level of baseline HbA1c appeared to be an independent predictor for the severity of coronary artery disease and poor outcome in patients with stable coronary artery disease (Hong *et al*., 2014).

As the levels of HbA1c increased, patients were more likely to have prior cardiovascular disease and a more unfavourable baseline cardiovascular risk profile in a cohort of AMI patients. Although the admission glucose levels may represent a marker for increased risk in the acute and subacute setting after AMI, HbA1c, being a surrogate for more chronic

dysglycaemia, is clearly a more useful marker of patients with greater long-term risk of death (Timmer *et al*., 2011).However, in an observational multi-center study on 608 patients with STEMI who underwent primary PCI, the admission level of HbA1c was not found to be an independent prognostic marker for short-term outcomes in STEMI patients treated with primary PCI (Tian *et al*., 2013).

A prospective cohort of 2,519 non-diabetic patients undergoing elective coronary angiography for suspected stable angina pectoris did not show any association between HbA1c levels and prognosis, questioning an independent role of glycaemia in the pathogenesis of atherosclerotic complications in non-diabetic patients (Rebnord *et al*., 2015).

Recently, Wang *et al*.2015 have shown that HbA1c level is not a significant and independent marker for the severity of angiography (stenosis) in ACS patients.

Kompoti *et al*.2015 investigated the clinical significance of HbA1c levels on admission in the intensive care unit as a prognostic marker for morbidity and mortality in critically ill patients. The findings showed that HbA1c is a useful tool for the diagnosis of a previously undiagnosed DM in critically ill patients, and HbA1c at admission is significantly associated with intensive care unit mortality.

Pimentel *et al*. 2015 have shown that HbA1c ≥6.5% is not enough to be used alone in the diagnosis of post-transplantation DM in renal transplant patients. However, the combined use of HbA1c cut-off points of >5.8% and ≥6.2% would reduce the number of oral glucose tolerance tests by 85% and the use of an algorithm with HbA1c in combination with FPG proved to be the most efficient strategy to diagnose or rule out post-transplantation DM (Pimentel *et al*., 2015).

Poor glycaemic control (HbA1c >8%) has been associated with decreased survival in the general population of diabetic patients on maintenance haemodialysis, suggesting that moderate hyperglycaemia increases the risk for all-cause mortality of diabetic maintenance haemodialysis patients in Han Chinese population (Li *et al*., 2014).

Helminen *et al*. 2015 assessed the utility of HbA1c levels in predicting the clinical disease in genetically predisposed children with multiple autoantibodies. They observed that a 10% increase in HbA1c levels in samples obtained 3–12 months apart predicted the diagnosis of clinical disease, suggesting the usefulness of HbA1c as a marker for predicting the time to diagnosis of type 1 DM in children with multiple autoantibodies.

2.5.3 HbA1c test units

As is usually the case with most of the units, the United States and European Union and other countries do not agree with the units of HbA1c measurements (Karami and Baradaran, 2014).

In the US, the HbA1c levels are expressed in terms of percentage of the Diabetes Control and Complications Trial units (Little and Sacks, 2009; Loh *et al*., 2015).

The United Kingdom, New Zealand, and Australia, along with many other European and Asian countries, however, express the HbA1c levels as millimoles per mole, keeping in reference with the recommendations of the International Federation of Clinical Chemistry (IFCC) (Hanas and John, 2010; Consensus Committee, 2007).

The International HbA1c Consensus Committee has recommended that the HbA1c levels must be reported in terms of System International (SI) units (millimoles per mole, with no decimal places), which relate better scientifically to a valid measure of HbA1c. The NGSP still recommends using the units in terms of the percentage with one decimal place, for example, an HbA1c level below 5.7% is considered as normal (Loh *et al*., 2015).

The SI units allow for avoiding any confusion between the reported HbA1c levels and the traditional fasting glucose levels expressed as millimoles per liter. All of these units can be easily converted using one of the online calculators and the values are interchangeable including those expressed as mg/dLand also allow for calculating the estimated average glucose results. It is important to note that the HbA1c levels, expressed in millimoles per mole, must not be confused with blood glucose levels, which are expressed in millimoles per litre, and provide an average long-term trend. The following equation will help to obtain the SI units from the HbA1c expressed in terms of the percentage: HbA1c SI unit (mmol/mol) (HbA1c NGSP unit in % × 10.93) − 23.50. For example, if the HbA1c is 5.7% (Diabetes Control and Complications Trial), then the HbA1c SI unit (mmol/mol) (IFCC) can be calculated as HbA1c SI unit (mmol/mol) (5.7 × 10.93) − 23.50 = 38.8 mmol/mol (IFCC) (Hanas and John, 2010).

2.5.4 $HbA1_C$range

Non-DM usually falls within the 4.0%–5.6% HbA1c range. The pre-diabetes usually has the HbA1c levels as 5.7%–6.4%, while those with 6.4% or higher HbA1c levels have DM (Xu *et al*., 2014).

Since DM is associated with several comorbidities, the recommendations for individuals with DM include a healthy lifestyle (diet and exercise) and maintaining the HbA1c levels below 7.0% (Lin *et al*., 2015).

DMrelated complications are directly proportional to the levels of HbA1c – the increase in the HbA1c levels also increases the risk of such complications. Using HbA1c as a SOC (Society of Clinicians') test also provides some complications for the health-care providers and the patients alike. For example, in anaemic (low haemoglobin) patients or those with shorter RBC lifespan (glucose-6-phosphate dehydrogenase deficiency, sickle-cell disease,

etc.), the HbA1c levels may be compromised indicating a false "good" result (Forbes *et al*., 2005).The excessive use of vitamin C, B, and E supplements and increased levels of cholesterol, liver, and kidney diseases can also present abnormally high levels of HbA1c (Luk *et al*., 2013; Xu *et al*., 2014).

Dyslipidaemia, which is an imbalance of lipids and fats circulating in the blood stream, is another debilitating disease associated with DM (Khan, 2007; Mooradian, 2009).

However, maintaining healthy glucose levels for type 2 diabetics is of paramount importance and may help in preventing micro- and macrovascular complications (Fowler, 2008).

The HbA1c is also used routinely for testing gestational DM among pregnant women (Capula *et al*., 2013).

Other researchers have utilized the serum fructosamine and blood glucose for the screening of GDM. Both these tests allow the healthcare providers to establish whether the pregnant women, with associated risk factors, had developed DM before the pregnancy, which may have gone undiagnosed. If the HbA1c levels are not monitored closely to establish acceptable glycaemic control, the higher levels of HbA1c may cause the long-axis cardiac dysfunction in the developing foetus (Rizzo *et al*., 1994; Hornberger, 2006).

There is a direct correlation between reduced HbA1c levels and reduced percentage of mortality. Maintaining healthy levels of the HbA1c significantly ameliorates the risk of cardiovascular diseases among individuals with DM (Pradhan *et al*., 2007).

### 2.5.5 Methods for HbA1c analysis

The HbA1c analysis methods can be divided into two categories: methods based on the charge differences and methods based on the structural differences. Ion-exchange chromatography and capillary electrophoresis belong to the first category, while

immunoassay, enzymatic assay, and affinity chromatography belong to the second category. Thus, the routine determination of HbA1c can be achieved by methods based on different principles such as immunoturbidimetry, boronate affinity chromatography, ion-exchange high-performance liquid chromatography (HPLC), and enzymatic assay (Weykamp *et al*., 2009; Lee *et al*., 2013; Ucar *et al*., 2013).

Özçelik *et al*. 2010 measured HbA1c in blood from 120 patients with pre-diabetes and DM using three different methods including turbidimetric inhibition immunoassay (TINIA), particle-enhanced immunoturbidimetric assay (PEITT), and HPLC. Although the average HbA1c measured by HPLC (7.52% ± 1.40%) was significantly higher than the other methods, including TINIA (7.12% ± 1.66%) and PEITT (7.26% ± 1.39%), there was good concordance between results of PEITT and HPLC methods ($r = 0.9401$).

The measured total time spent on 120 samples was 45 minutes for TINIA, 39 minutes for PEITT, and 384 minutes for HPLC (Özçelik *et al*., 2010). Recently, capillary 2-FP analyzer has been found to be suitable for HbA1c measurement, and sometimes, it showed some advantages with respect to the HPLC analyzers tested, especially when Hb variants are present (Dessi *et al*., 2015).

Since the HbA1c test is now recommended for diagnosing DM and minimal variation of the concentration affects the clinical therapy, it is very important that the results are reliable and interference free. One must become more stringent that any unacceptable results are detected, not reported and each method is evaluated for Hb variant interference (Lin *et al*., 2012).

There are at least 30 different laboratory methods commercially available to measure the proportion of HbA1c in blood (International Federation of Clinical Chemistry and Laboratory Medicine, 2007). Studies have also reported significant bias among analytical methods to measure HbA1c levels (Marinova *et al*., 2013).

Therefore, standardization and comparability of HbA1c results with different methods appear to be an important issue. In 1995, the IFCC established a Working Group (IFCC WG-HbA1c) to achieve international standardization of HbA1c measurement (Hoelzel and Miedema, 1996).

A reference measurement procedure for HbA1c was developed based on the proteolytic digestion of red cell haemoglobins followed by quantitative peptide mapping by HPLC-mass spectrometry or HPLC-capillary electrophoresis (Kobold *et al*., 1997).

The reliability of HbA1c measurement depends on bias (related to proper calibration) and precision (related to the reproducibility of the method). Quality goals can be derived from biological variation, clinical needs, or the state of the art. For HbA1c, a generally accepted rule of thumb is that clinicians interpret a difference of 5 mmol/mol (0.5%) between successive patient samples as a significant change in glycaemic control (Little *et al*., 2011).

2.5.6 Accessibility to HbA1c testing for diagnosis

Although most laboratories in tertiary care hospitals are well equipped with modern instrumentation including HPLC, many of the primary care centres in low- and middle-income countries do not have access to HPLC, some are still struggling with outdated methods or doubtful point-of-care devices that may not be reliable to monitor DM. For accurate results, small clinics and health centres have to be dependent on accredited clinical laboratories for HbA1c analysis (Lee *et al*., 2013).However, this strategy becomes more expensive due to the additional cost of sample transportation.

Recently, Fokkema *et al*. 2009 evaluated the feasibility of HbA1c measurements from dried blood spots collected on filter paper and compared the HbA1c from filter paper (capillary blood) with HbA1c measured in venous blood. HbA1c on filter paper was

extremelycorrelated with routine HbA1c (r = 0.987) while the evaluation of samples collected at home showed comparable HbA1c values by filter paper and routine sampling methods. Most of the participants (83%) said that they would like the filter method to be brought into practice, suggesting that HbA1c sampling on filter paper is an acceptable sampling alternative for analysis of HbA1c.

It is anticipated that a finger prick sample collection on a filter paper would be more convenient for remote and rural health-care centres to send the samples of HbA1c analysis to dedicated laboratories. Moreover, the good relationship and concordance between the immunoturbidimetric and HPLC methods may support the reliability of properly standardized immunoturbidimetric methods for preliminary screening of DM in remote areas (Hanwi *et al*., 1995; Metus *et al*., 1999; Groche *et al*., 2003).

2.5.7 Physiological changes due to haemoglobin glycation

An increase in HbA1c as observed in conditions of poor diabetic control has been associated with increased blood viscosity (Leiper *et al*., 1984).

Glycosylation of haemoglobin and increased glucose levels tends to affect RBC properties, lowering the RBC flexibility and increasing their aggregation tendency, leading to increased blood viscosity (Buhler *et al*., 2001).

Glycosylation of haemoglobin may also affect membrane lipid protein interactions in RBCs, altering their internal viscosity, modifying viscoelastic properties of erythrocyte membranes, and impairing RBC deformability (Watala *et al*., 1992).

There is also evidence that glycosylation of haemoglobin impairs nitric oxide (NO)-related relaxation of human mesenteric vessels (Valejo *et al*., 2000).

Haemoglobin glycosylation is also reported to alter NO (Nitric Oxide) binding with thiols resulting in lowered NO bioavailability and impaired vasodilatation in rabbit aortic rings (James *et al*., 2004).

Another mechanism by which glycosylation of haemoglobin is proposed to be vasoactive is via the formation of reactive oxygen species (Angulo *et al*., 1996).

Glycosylation of haemoglobin also lowers oxygen-carrying capacity, thereby promoting hypoxia and its related systemic vascular vasodilatory adaptations and responses (Paffett and Walker, 2007).

Glycosylation of haemoglobin appeared to lead to blood pressure reduction in type 2 diabetic patients untreated for hypertension (Cabrales *et al*., 2008). Since 8%–10% HbA1c is considered to be a threshold beyond which the effects of haemoglobin glycosylation become significant, these investigators determined mean arterial blood pressure for patients not treated for hypertension below and above 9% HbA1c and found significant reduction in mean arterial blood pressure below the threshold (86.2 ± 3.9 mmHg) as compared to above the threshold (93.1 ± 12.5 mmHg) (Cabrales *et al*., 2008).

### 2.5.8HbA1c measuring and referencing techniques

The importance of HbA1c as a major diagnostic tool is well recognized and it is not surprising that many commercial assays have been developed. Specificities of the methodsare different and, with them, potentially, the HbA1c values. Thus, in order to enable optimal clinical use, results among different methods should be equivalent. The IFCC Reference System for HbA1c serves as the analytical anchor for the standardization of all commercial HbA1c methods (Hanas and John, 2010).

During the first years after the discovery of HbA1c, each method, and perhaps each laboratory had its own reference values. For optimal clinical use (e.g., uniform clinical guidelines, comparison of scientific studies) equivalence of results is desirable. Equivalence can be achieved with harmonization or standardization (Miller *et al.*, 2011).

With harmonization, commercial methods are calibrated against a designated comparison method and material so all methods align to each other. With standardization, calibration is against a scientifically sound reference measurement procedure. Harmonization could thus be said to lead to a relative truth and standardization to the absolute truth. The need for equivalent results was well recognized and inspired several national harmonization initiatives (Little and Sacks, 2009).

In the United States, the designated method was the same method used in the Diabetes Control and Complications Trial (DCCT), which has been shown to be stable over several years and was directly linked to clinical data. This led to a nationwide program with international affiliations organized by the National Glycohaemoglobin Standardization Program (NGSP) (Little *et al.*, 2001).

Similar initiatives achieved harmonization in Japan (JDS/JSCC) and Sweden (Mono-S). Unfortunately, all were based on designated comparison methods, and it is not surprising that results of these chosen methods were different. This situation caused confusion, and therefore the IFCC developed a reference method to achieve worldwide standardization (Consensus Committee, 2007).

2.5.9 IFCC referencing method

The IFCC Reference Method (IFCC-RM)is based on the concept of metrological traceability. Pure HbA1c and $HbA_0$ are mixed to prepare primary calibrators that are used to calibrate the IFCC-RM (Jeppsson *et al*., 2002).

Erythrocytes are washed and lysed, followed by enzymatic cleavage. The resulting hexapeptides are quantified with either HPLC-mass spectrometry or HPLC-capillary electrophoresis (Figure 2.13).

With the IFCC-RM, values are assigned to whole blood panels that serve as secondary calibrators for manufacturers. The IFCC-RM is embedded in a global network of reference laboratories where IFCC-RM values are assigned to the IFCC secondary reference materials. These are then used by the manufacturers to assign values to their kit calibrators and subsequently used by the clinical laboratories to calibrate their instruments. This material and method traceability chain warrants that worldwide HbA1c results reported to diabetologists and patients are traceable to the IFCC Reference Method, allowing global guidelines with uniform decision limits for diagnosis and therapy. Independent control is achieved by EQA/PT organizers using samples to which values have also been assigned with the IFCC-RM (Hoelzel *et al*., 2004).

With the use of a master equation based on many years of NGSP/IFCC network-to-network comparisons, **NGSP = (0.09148 x IFCC) + 2.152**. An HbA1c value of 48 mmol/mol in IFCC units translates into 6.5% HbA1c in NGSP units (Table 2.1).

2.5.10 Haemoglobinvariants (haemoglobinopathy and thalassaemia)

Haemoglobinopathy is a hematologic disorder induced by an alteration in the genetically determined primary structure of haemoglobin (α, β, γ and/or δ chain), usually causing

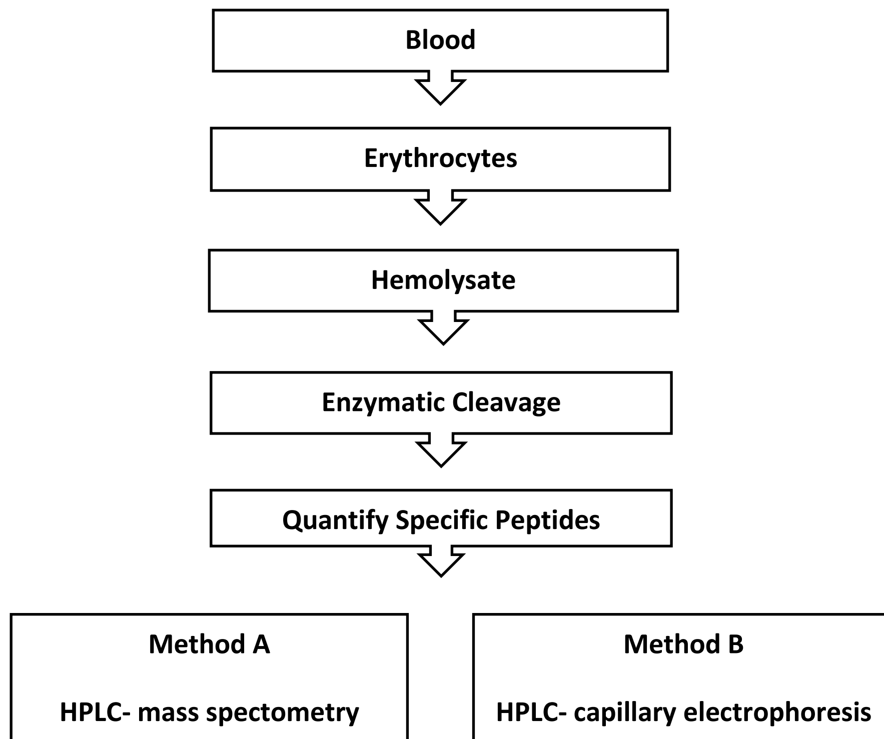


Fig 2.13:**Steps in the IFCC Reference Method** (Ucar *et al*., 2013).

anaemia. Haemoglobinopathies are generally autosomally inherited. The most common and best-known haemoglobinopathy is HbS (sickle cell anaemia). In addition, thalassemias are abnormalities of globin chain production, the most common ones being α- and β-thalassemia. Haemoglobinopathies are found in all parts of the world while thalassemias are mostly seen in populations in Southeast Asia and the Mediterranean (Williams and Weatherall, 2012).Certainly, HbS is the most common haemoglobin variant in Africa, North America and Central Europe (Piel *et al*., 2013), while HbG and HbE are the most common Hb variantsfound in Southeast Asia (Hardison *et al*., 2002). Table 2.2 summarizes the various forms of haemoglobinopathies and their characteristics.

As a general consideration, the interference due to the presence of a haemoglobin variant may be due to the potential effect the variant may have on normal red cell physiology, the true analytical interference of the haemoglobin variants as evaluated by the specific analytical method used to measure HbA1c, or the postanalytical issue related to reporting the result of HbA1c.

2.5.11 Effect of variant on red cell physiology

It should always be taken into account that in the case of homozygosis or double heterozygosis for a haemoglobin variant – such as in subjects with sickle cell anemia homozygous for HbS, or with HbSC – the determination of HbA1c is not feasible because no HbA is present, and other indicators of glycaemic control such as the determination of glycated plasma proteins or fasting blood glucose have to be used (Lin*et al*., 2012).

When haemolytic anaemia is suspected or known, the red cell life span may be reduced and, therefore, the interpretation of the HbA1c value should be considered suspect, so alternative methods should be used to confirm clinical interpretations.

Table 2.1: **$HbA1_C$ Result Referencing Methods and Equivalences**(American Diabetes Association, 2012).

| CONDITION | IFCC $HbA1_C$ | NGSP/DCCT $HbA1_C$ |
|---|---|---|
| DM | >48 mmol/mol | ≥ 6.5% |
| Risk of DM | 39 – 46 mmol/mol | 5.7 – 6.4% |
| Low-risk/normal | <39 mmol/mol | < 5.7% |

Table 2.2: **Main Characteristics of the Most Common Haemoglobin Variants** (Leslie *et al.,* 2017).

| Name | Amino Acid | DNA | Electrophoresis | HPLC (Cation Exchange) | Stability | Occurrence | Other Information |
|---|---|---|---|---|---|---|---|
| HbS | ß6(A3)Glu→Val | GAG→GTG | Can be separated from HbA at both alkaline and acidic pH | Can be separated from HbA | Stable | Heterozygotes and homozygotes found in many ethnic groups, but predominantly among black persons and in some Indian tribes | Quantity in heterozygotes comprises 35–40%; the percentage is lower with coexting α-thal; hemolytic anemia in homozygosis or in HbSC, HbSD, HbS-β-thal (sickle cell disease, SCD) |
| HbC | ß6(A3)Glu→Lys | GAG→AAG | Can be separated from HbA at both alkaline (position of HbA2) and acidic pH | Can be separated from HbA | Stable | Predominantly in black persons; also reported in many other racial and/or ethnic groups | Quantity in heterozygotes: 25–45%; homozygosity is a mild hemolytic condition; SCD is clinically significant |
| HbD-Punjab | ß121(GH4)Glu →Gln | GAA→CAA | Can be separated from HbA at alkaline pH, position similar to HbS | Can be separated from HbA | Stable | Primarily found in Indus River Valley (Punjab, Pakistan) and northwestern India; widespread in China, UK, the Netherlands, Australia, Greece, Balkan countries and Turkey | Quantity in heterozygotes: ~40%; found in combination with HbS, HbC, HbE, β-thal, α-thal and in the homozygous state; severe sickle cell disease when co-inherited with HbS; HbD-Punjab |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | | is also known as HbD-Los Angeles |
| HbE | ß26(B8)Glu→Lys | GAG→AAG | Can be separated from HbA at alkaline pH (position of HbA2; separated from HbA2 by capillary electrophoresis) | Can be separated from HbA, eluting with HbA2 | Mildly Unstable | Widespread in the Far East, also in combination with various Hb variants and with different β-thal alleles (thal major) | Quantity in heterozygotes (with 4 α genes): ~30%; microcytosis in approximately 90% of heterozygotes; microcytosis is more marked in homozygotes, together with mild to moderate anemia; clinical features of thalassemia intermedia in compound heterozygotes HbE/β-thal |
| HbG-Philadelphia | α68(E17)Asn→Lys | AAC→AAA | Can be separated from HbA at alkaline pH, position similar to HbS | Can be separated from HbA | Stable | The most common α-chain variant in African-Americans and Afro-Caribbeans; also present in Northern Italy and Sardinia and in a few Chinese families | Quantity in heterozygotes may vary from 20–25% to 40–45% if α-thal 2 (3.7 kb del) is associated; the occurrence with HbS and/or HbC is rather common |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| HbO-Arab | β121(GH4)Glu →Lys | GAA→AAA | Can be separated from HbA at alkaline pH, position similar to HbA2 | Can be separated from HbA | Stable | Found mainly in Balkan countries, Arabian penninsula, Egypt and throughout the Western Hemisphere | Quantity in heterozygotes: 30–40%; found in combination with HbS, HbC, α-thal, β-thal and in the homozygous state; severe sickle cell anemia in combination with HbS |
| Hb Lepore-Boston | δβ hybrid(δ through87;β116) | -- | Can be separated from HbA at alkaline pH, position similar to HbS | Can be separated from HbA, position partially overlapped to HbA2 | Stable | Italy, Romania, Balkan countries, Greece, Turkey, Cyprus, Jamaica, Cuba, UK, Australia and Mexico | Quantity in heterozygotes: 7–13%, found in combination with HbS, HbC, β-thal; also in homozygous; relatively mild sickling disorder in combination with HbS |

In carriers of HbS and HbD-Punjab, the red cell life span is generally normal, while in the heterozygous HbAc, some cases with a reduction in red cell life span have been reported (Prindle and McCurdy, 1970).

2.5.12 Analytical interference of variant on the method of measurement

In immunochemical methods, antibodies directed against the last 4–10 amino acids of the β chains are employed. For enzymatic methods, fructosyl peptide cleavage products are evaluated. The eventual haemoglobin variants having mutations within the primary structure that is analyzed (such as HbS and HbC) can therefore potentially influence the HbA1c result. On the contrary, if the amino acid substitution is far away from this β-terminal region (HbD-Punjab, HbE), it is very unlikely that the haemoglobin variant will interfere with the determination of HbA1c. Whereas in net charge separation methods, ion-exchange (mostly cation-exchange) HPLC methods are included, as well as the various methods based on electrophoresis — capillary electrophoresis (CE) being the most recent one. All haemoglobin variants with an isoelectric point different from that of HbA (7.20) can be, in theory, separated, using a technique based on the net charge difference (Lee*et al.*, 2013). Generally:

(HbA1c)% = (HbA1c) x 100/(total Hb) (Kobold*et al.*, 1997)

If the haemoglobin variant (HbX) and its glycated adduct (HbX1c) migrate separately from HbA and HbA1c, respectively, then the presence of a definite amount of HbX in the sample has negligible effects on the quantification of HbA1c. Indeed, the majority of the actual HPLC and CE systems calculate the relative abundance of HbA1c according to the formula:

(HbA1c) adjusted, % = (HbA1c) x 100/([total Hb] – [HbX])* (Miller*et al.*, 2011)

(*This correction is effective if HbX is eluting after HbA and generally is not performed if HbX is migrating/eluting before HbA.)

### 2.5.13 Other limitations affecting HbA1c measurement

#### *2.5.13.1Chemical modification of haemoglobin*

Essentially, three haemoglobin adducts may be formed as posttranslational modifications of human haemoglobin – consequently affecting the results of HbA1c tests. Particularly, haemoglobin adducts may be formed from the reaction of human haemoglobin with isocyanic acid (carbamylated Hb), the adduct with acetylated compounds (acetylated Hb), and that formed by reaction of haemoglobin with glutathione (glutathionyl Hb) (Marinova *et al.*, 2013).

#### *2.5.13.2Carbamylation (by uremia in renal failure)*

Human haemoglobin-free amino groups of the β chains can react with isocyanic acid, a product formed by spontaneous decomposition of urea or by the oxidation of thiocyanate by myeloperoxidase and the product of such a reaction is carbamylated haemoglobin (cHb) (Jaisson *et al.*, 2011).

Despite some previous reports, a 2013 report showed that the elevated cHb had no clinically significant differences on the methods evaluated (Little *et al.*, 2013).

Instead, reduction in red cell life span in patients with renal failure was shown to be a consideration for HbA1c results for these patients (Szymezak *et al.*, 2009).

#### *2.5.13.3 Acetylation (by aspirin)*

The same N-terminal amino groups reacting with glucose may react with acetylating agents to form an acetyl-aminoderivative. Aspirin (acetyl salicylic acid) is a very common drug, and low-dose aspirin is recommended for both primary and secondary cardiovascular disease prevention in diabetic patients (American Diabetes Association, 2007).

Some studies have pointed out a potential interference of aspirin in HbA1c assays, especially in the HPLC and electrophoretic methods (Camargo *et al*., 2006).

This is because acetylated Hb is essentially very similar to HbA1c with regard to its electric charge and mobility with these techniques. However, a quite recent randomized, double-blind, placebo-controlled study on 12 patients, assuming an aspirin dose of 300 mg/day or identical placebo for eight weeks, was not able to confirm these findings. Considering that the recommended preventive dose of aspirin is equal to 75–162 mg/day, we may conclude that aspirin in vivo normally does not interfere with the determination of HbA1c (Camargo *et al*., 2008).

*2.5.13.4 Glutathionyl Hb*

It is well known that the storage of red blood cells for a prolonged period of time under blood banking conditions may accumulate irreversible damage to the red cells that ultimately enhances hemolysis and oxidative stress after transfusion (Kanias and Acker, 2010).

In most of the ion-exchange HPLC systems, glutathionyl Hb elutes before HbA, forming another minor derivative (HbA1d or HbA3), which is usually well-resolved from HbA1c. And generally, interference is unlikely, as the binding site of glutathione to human haemoglobin β chains is far away from the β N-terminal residues where glucose is bound (Jeppsson *et al*., 1996).

*2.5.13.5 Altered lifespan of the red cell*

Indeed, the presence of iron deficiency anemia, which is associated with increased red cell survival, may produce HbA1c values higher than those expected from the average blood glucose control (Coban *et al*., 2004).

On the contrary, the presence of hemolytic anemias may result in lower HbA1c values due to a decreased red cell survival (Gallo *et al*., 1975).

*2.5.13.6 Thalassemia*

In thalassemia syndromes, severe anaemias may be present in various forms (thalassemia major), and a milder anaemia may be present in the minor forms. In carriers of β-thalassemia, significant reduction of red cell survival has been proven (Gallo *et al*., 1975).

Despite that, Polage *et al*. 2004 were unable to prove, in subjects without DM or with a mild form of hyperglycaemia, any effect of the β-thalassemia carrier condition on the glycated haemoglobin as measured by various separation and immunochemical techniques. With just one technique (Synchron methods), there was a significant effect, probably related to a nonlinearity of the HbA1c measurement in the low total haemoglobin range. Unfortunately, there are no data to prove that, in diabetic patients, carriers of β-thalassemia may alter their glycated haemoglobin results (Little *et al*., 2011).

*2.5.13.7 Pregnancy*

It is undoubtedly of great importance in the prenatal care of women with DM, but it is less clear as to its value during pregnancy in general. In the UK, the NICE (National Collaborating Centre) guideline on DM in pregnancy recommends that HbA1c should not be used routinely for assessing glycaemic control in the second and third trimesters of pregnancy. The IDF global guideline on pregnancy and DM recommends not using routine measurement of HbA1c for management of gestational DM (GDM) (Capula*et al*., 2013).

*2.5.13.8 Ethnicity*

This is of particular interest to, and forms the basis of the present study. The accuracy of $HbA1_C$ test results is being reported to be doubtful and highly varied amongst individuals

especially along racial lines; with reports of significantly higher levels in non-Caucasian diabetic patients for a given blood glucose (BG) range (Herman *et al*., 2009). For instance, in a study carried out by Herman *et al*. (2016), it is concluded that the clinical implications of racial differences in $HbA1_C$, if not taken into consideration, could do great harm to the patient. Also, as pointed out in "Clinical Practice Guidelines for Diabetes Management in Nigeria", a book by Diabetes Association of Nigeria (DAN), the $HbA1_C$ test is subject to certain limitations such as conditions that affect erythrocyte turnover (haemolysis, blood loss) and haemoglobin variants in individual patient, making it possible for $HbA1_C$ result not to correlate with the patient's clinical situation (Chinenye *et al*., 2013). Similarly, and more applicably in a review by Oghagbon 2014, it is suggested that despite the usefulness of HbA1c in monitoring diabetic patients in Nigeria; and despite that its relationship to prevalence of diabetic complications has been proven in various studies, caution must be taken in the application of HbA1c in our environment due to its variability with race/ethnicity. And if possible, individualization of HbA1c target goals is advised, so as not to over-treat and tip patients into hypoglycaemia, especially those who have hypoglycaemia unawareness or history of severe hypoglycaemia. The opinion that black people have higher values of glycated haemoglobin $A1_C$ for corresponding levels of glycaemia either due to genes, erythrocyte turnover or erythrocyte permeability to glucose is supported by a research by Bruce*et al*. (2013), where they stated that for a given degree of glycaemia, $HbA1_C$ levels vary among different racial groups and that race needs to be taken into consideration when using $HbA1_C$ to assess glycaemic control or to set glycaemic targets.

Iron handling, glucose distribution across erythrocyte membrane and rates of uptake into the membrane, rates of glucose attachment to or release from haemoglobin and rates of intra-erythrocytic glucose metabolism are all physiologic factors/mechanisms that influence HbA1c levels (Florez, 2010).

## 2.6 Diabetic Nephropathy

Diabetic nephropathy develops in patients with several years of medical history of DMand could lead to renal failure (Nazar, 2014).

Cases of diabetic nephropathy is increasing randomly every year. It is recognised as the most common complication in DM patients. Governments are now spending enormous sums on kidney patients every year due to increasing prevalence of diabetic nephropathy. According to the UK prospective study, it is expected to further rise about 25% in the next 10 years (Adler *et al*., 2003).

It is a clinical syndrome characterised by the insistent increase in serum creatinine that should be confirmed by continuous decline in the glomerular filtration rate (GFR).

Diabetic nephropathy is characterised by different events. The characteristic occurrence is thickening of the glomerular basement membrane (GBM). After renal damage, the thickening of the basement membrane starts, which leads to pathologic modifications in mesangial and vascular cells. It includes formation of AGEs, accumulation of polyols, and activation of protein kinase C (Cooper, 1998).

It leads to activation of the inflammatory pathway playing a significant role in the damage of the GBM. Secondly, the renal hemodynamic anomaly is similar in both type I and type II DM (Ritz *et al*., 1997).

An initial physiologic abnormality is glomerular hyper-filtration related to intraglomerular hypertension. The exact pattern observed in the pathophysiology of diabetic nephropathy(Cooper, 1998), is:

i. Hyperglycaemia
ii. Thickening of GBM

Table 2.3: **Advantages and disadvantages of assays for glucose and HbA1c** (WHO, 2011)

| | Glucose | HbA1c |
|---|---|---|
| Patient preparation prior to collection of blood | Stringent requirements if measured for diagnostic purposes. | None. |
| Processing of blood | Stringent requirements for rapid processing, separation and storage of plasma or serum minimally at 4°C. | Avoid conditions for more than 12hr at temperatures >23C. Otherwise keep at 4C (stability minimally 1 week). |
| Measurement | Widely available | Not readily available worldwide |
| Standardization | Standardized to reference method procedures. | Standardized to reference method procedures. |
| Routine calibration | Adequate. | Adequate. |
| Interferences: illness | Severe illness may increase glucose concentration. | Severe illness may shorten red-cell life and artifactually reduce HbA1c values. |
| Haemoglobinopathies | Little problem unless the patient is ill. | May interfere with measurement in some assays. |
| Haemoglobinopathy traits | No problems. | Most assays are not affected. |
| Affordability | Affordable in most low and middle income country settings. | Unaffordable in most low and middle-income country settings. |

iii. Glomerular hyper-filtration

iv. Impaired endothelial integrity

v. Onset of microalbuminuria

vi. Impairment of nitric oxide transport

vii. Loss of afferent/efferent auto-regulatory control

viii. Continued loss of glomerular filtration capabilities

A clinically asymptomatic point of failure follows with development of microalbuminuria (30 mg albumin per day) to macroalbuminuria (>300 mg albumin per day). Once overt nephropathy (macroalbuminuria) has been established, renal function falls at a significant but alterable rate (decline in GFR of 220 ml/min/year). The rate of decline depends on type of DM, genetic predisposition, glycaemic control and blood pressure. Later stages may also be supplemented by clinically significant albuminuria, oedema, and nephrotic syndrome. Ultimately, the distinguishing clinical picture of renal failure develops (Cooper, 1998).

It is thought that the development of nephropathy occurs in similar fashion in both types of DM (Cooper, 1998).

The natural history of diabetic nephropathy is divided into five stages (Adler *et al*., 2003):

*Stage 1*: Renal pathology develops at the onset of DM. The growth of the kidney increases by several centimetres. By the time of diagnosis, the GFR and urinary albumin excretion (UAE) have been increased. It can be controlled at this level by onset of insulin.

*Stage 2*: The second phase typically lasts for 5-15 years after diagnosis of DM. The characteristics of the second phase include:

GFR remains elevated due to hyperfiltration

Kidneys remain hypertrophied and UAE rate stays normal

Glomerular filtration rate (GFR) is still higher than normal during this stage. Some diabetic patients continue in this stage throughout their lives (Usama, 2017).

*Stage 3*:

- Microalbuminuria is present. It occurs in 30-50% of patients after DM onset, 80% of whom go on to develop overt nephropathy over 10-15 years.
- GFR remains elevated or returns to normal range
- Blood pressure starts to rise in 60% of patients

Histological changes-progression is as seen in stage 2.

*Stage 4*: This stage is also known as clinical nephropathy or overt nephropathy. The characteristic histological features of stage four are formation of the Kimmelstiel-Wilson nodule (focal glomerular sclerosis) and macroproteinuria. It can progress to nephrotic in 30% of patients or may decline in 80% depending on deterioration of GFR.

*Stage 5*: As the GFR continues to decline, ESRD may develop. Diabetic nephropathy is considered the most common cause of ESRD because of associated autoimmune neuropathy and cardiac disease. The stages of chronic kidney disease (CKD) are mainly based on measured or estimated GFR. There are five stages but kidney function is normal in stage 1 and minimally reduced in stage 2.

Microvascular complications such as retinopathy, nephropathy and neuropathy occur in DM (Beisswenger, 2010).

And largely due to these complications, globally, DM is said to be the fifth leading cause of death (Roglic *et al*., 2005).

Prevention of DM and its complications, early detection of disease stages, and therapeutics that would act in the presence of hyperglycaemia to prevent, delay or reverse the complications are the major concerns (Marshall and Flyvbjerg, 2006).

Biomarkers such as glycated haemoglobin, glycated albumin, fructosamine, 1,5-anhydroglucitol (1,5-AG), urea and electrolytes, and serum creatinine are studied for understanding the mechanisms of hyperglycaemia-induced metabolic abnormalities (Marshall and Flyvbjerg, 2006).

Diabetic nephropathy, a leading cause of kidney failure and one of the key complications of diabetics, is defined by either microalbuminuria or by an increase in serum creatinine level, which is in turn used in the calculation of estimated GFR (eGFR) in diabetic patients (Amos *et al*., 1997).

### 2.6.1 Pathogenesis of diabetic nephropathy

The excessive production of reactive oxygen species (ROS) is one of the hallmarks of diabetic kidney. ROS overproduction is the main cause of diabetic nephropathy (Giacco and Brownlee, 2010).

Hyperglycaemia induces nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme activity and is responsible for ROS overproduction (Gill and Wilcox, 2006).

Up-regulation of sodium glucose transporter 2 (SGLT2) in the brush border of proximal convoluted tubules (PCT) is another pathway of ROS overproduction. SGLT2 up-regulation causes uric acid (UA) overproduction with consequent NADPH oxidase-ROS induction (Bjornstad *et al*., 2015).

Excess ROS mediates podocyte apoptosis and alteration in the slit diaphragm podocin protein (Figure 2.12) (Eid *et al*., 2016), increases intracellular oxidative stress, mitochondrial injury,

adenosine triphosphate (ATP) depletion (Sanchez-Lozada *et al*., 2012; Cristobal-Garcia *et al*., 2015;), endothelial injury, RAS activation and increased epithelial-mesenchyme transition (EMT) with consequent fibrosis (Ryu *et al*., 2013).

ROS overproduction activates the nuclear factor-κB (NF-κB) within the kidney (Wada and Makino, 2013).

NF-κB translocates to the nucleus to trigger several genes like those encoding transforming growth factor-β (TGF-β), chemokine ligand 2 (CCL2) also known as monocyte chemoattractant protein-1 (MCP-1) and intercellular Adhesion Molecule 1 (ICAM1) (Yang *et al*., 2008; Kashihara *et al*., 2010).

This leads to macrophage recruitment and excess collagen deposition within the diabetic kidney (Figures2.14, 2.15 and 2.16). Beside activation of NF-κB, ROS activates protein kinase C (PKC) and mitogen-activated protein (MAP) kinase within mesangial cells (MCs). All these factors stimulate overproduction of extracellular matrix proteins (Figure 2.14) (Kashihara *et al*., 2010).

Activation of mammalian target of Rapamycin (mTOR) is another feature of diabetic nephropathy. Hyperglycaemia stimulates phosphatidylinosiol-3 kinase (PI3K) and protein kinase B (AKT) pathways, with subsequent activation of mTOR. Activated mTOR is responsible for basement membrane thickening, mesangial matrix expansion (Estacio and Schrier, 2001), and renal fibrosis. The mTOR induced renal fibrosis is a consequence of fibroblast proliferation, EMT and the expression of TGF-β and connective tissue growth factor (CTGF, CCN2) (Lieberthal and Levine, 2009; Kume *et al*., 2014).

Stimulation of MCP1 by mTOR leads to increased macrophage recruitment within the interstitium of the kidney (Kume *et al*., 2014).

In addition, increased mTOR activity can aggravate tubular epithelial damage and apoptosis (Figure 2.17) (Velagapudi *et al*., 2011).

CCN2 is the newer alternative name of CTGF. It has an eminent role in diabetic nephropathy (Wang *et al*., 2015). Within the diabetic kidney, CCN2 is detected in almost all cell types (Ito *et al*., 1998). When exposed to high glucose, the glomeruli of diabetic rats and human MCs express a high activity of CCN2 (Murphy *et al*., 1999).

In the diabetic kidney, CCN2 expression is stimulated by TGF-β1, AGE, and angiotensin II (AII). The CCN2 stimulates EMT, fibroblast proliferation, and extracellular matrix accumulation (Figure 2.18) (Wang *et al*., 2015).

In addition, SGLT2 contributes to hyperglycaemia-induced PCT cell senescence. Knocking down of SGLT2 can abort in vitro induction of P21 in PCT when exposed to hyperglycaemia. P21 inhibits cyclindependent kinase (CDK). CDK is an inhibitor of cell senescence (Figure 2.19) (Hayflick, 2003; Kitada *et al*., 2014).

While microalbuminuria is a very sensitive test in people with Type 1 DM, testing for microalbuminuria alone may miss many cases of diabetic kidney disease in those with Type 2 (Koroshi, 2007).

Therefore, it is very important to test the kidney function by measuring the serum creatinine level (Lewis and Maxwell, 2014).

Using the serum concentration of creatinine in an equation that takes into account the person's weight, age, sex, and race, one can estimate the GFR to evaluate kidney function.

The higher the blood creatinine level, the lower the GFR and the worse shape the kidneys are in (Waad-Allah *et al*., 2012).

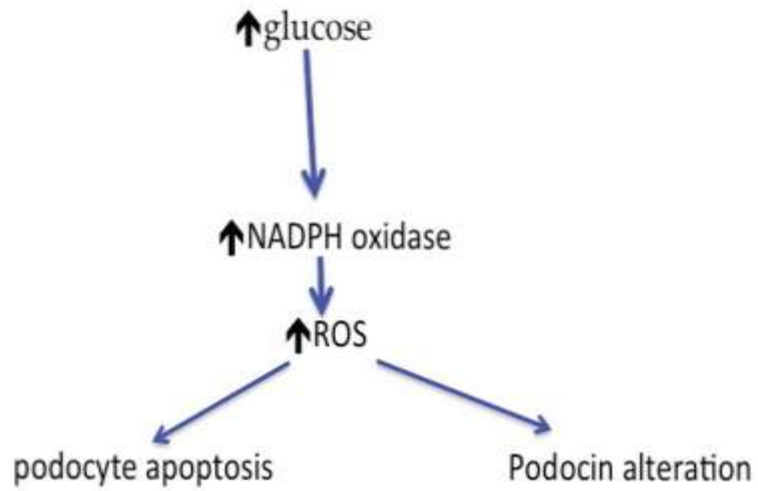


Fig 2.14: **Reactive oxygen species mediated podocyte injury and podocin protein alteration. NADP = Nicotinamide Adenine Dinucleotide Phosphate; ROS = Reactive Oxygen Species** (Usama *et al*., 2017).

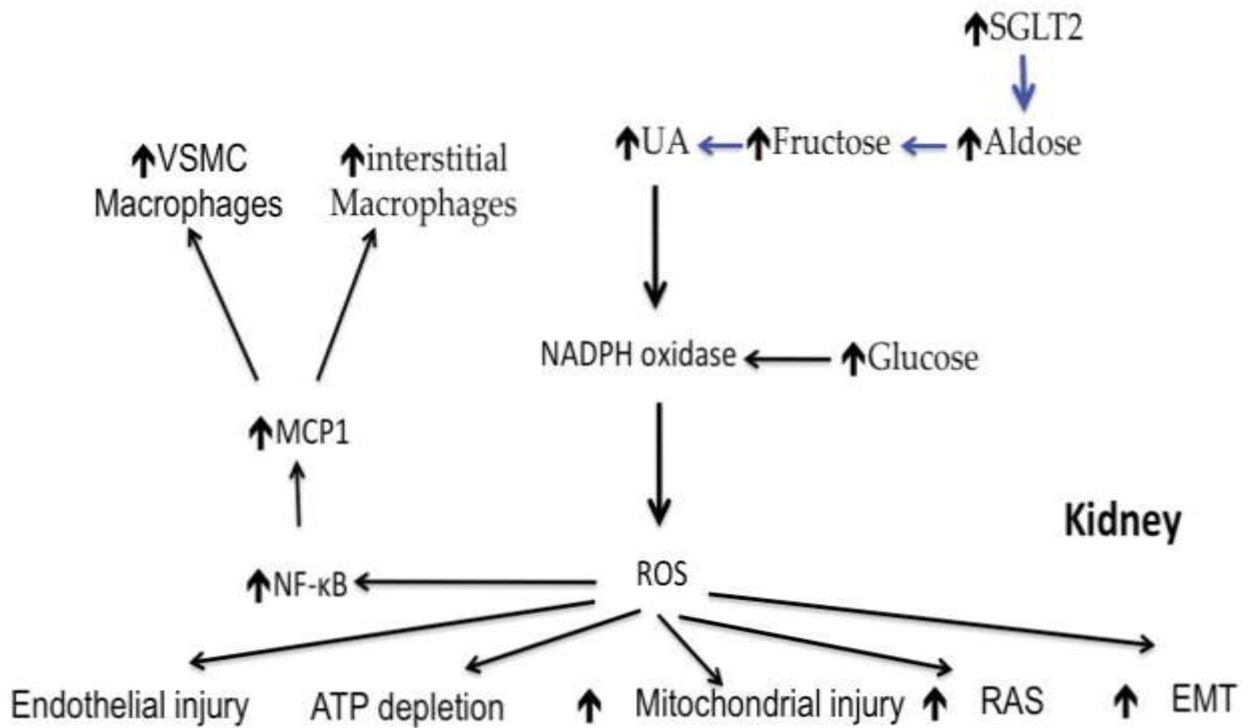


Fig 2.15: **Different pathogenic mechanisms of kidney injury possibly induced by uric acid. UA= uric acid; ROS= reactive oxygen species; NF-κB= Nuclear Factor kappa B; MCP1= Macrophage Chemoattractant protein-1; RAS= Renin angiotensin system; EMT= Epithelium mesenchyme transition; VSMC= Vascular smooth muscle cells** (Usama *et al*., 2017).

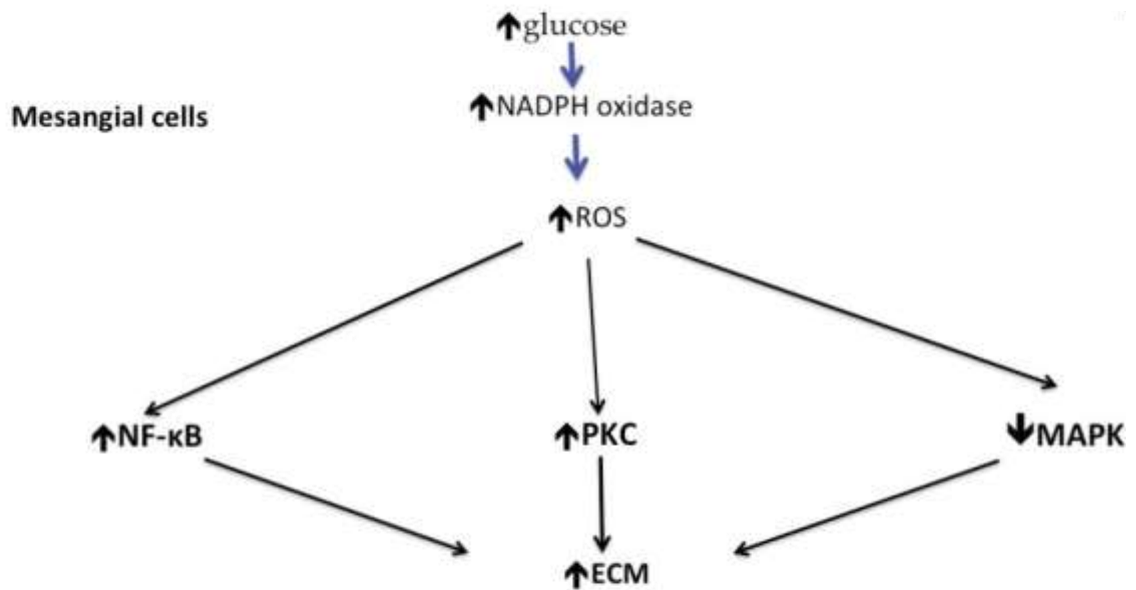


Fig 2.16: **Hyperglycemia induced mesangial expansion. NADP= Nicotinamide adenine dinucleotide phosphate; ROS= Reactive oxygen species; NF-κB= Nuclear Factor kappa B; PKC= Protein kinase C; MAPK= Mitogen activated protein kinase; ECM= Extracellular matrix** (Usama *et al*., 2017).

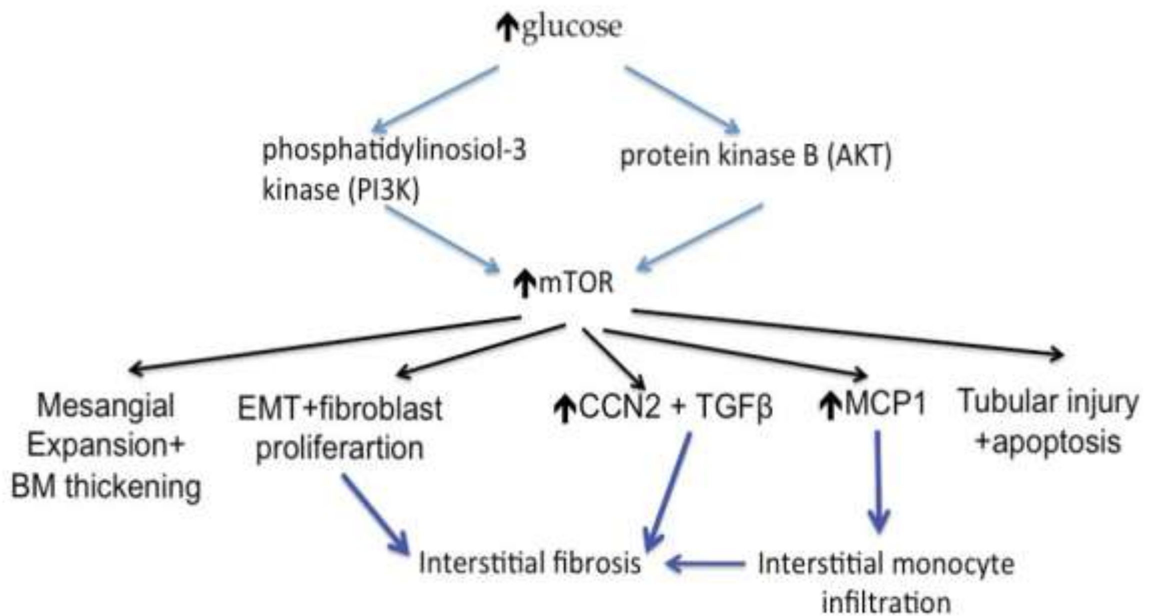


Fig 2.17: **Consequences of mTOR activation induced by hyperglycemia. mTOR= mammalian target of rapamycin; BM= basement membrane; EMT= Epithelium mesenchyme transition; CCN2= Connective tissue growth factor; TGFβ= Transforming Growth Factor β; MCP1= Macrophage chemoattractant protein** (Usama *et al*., 2017).

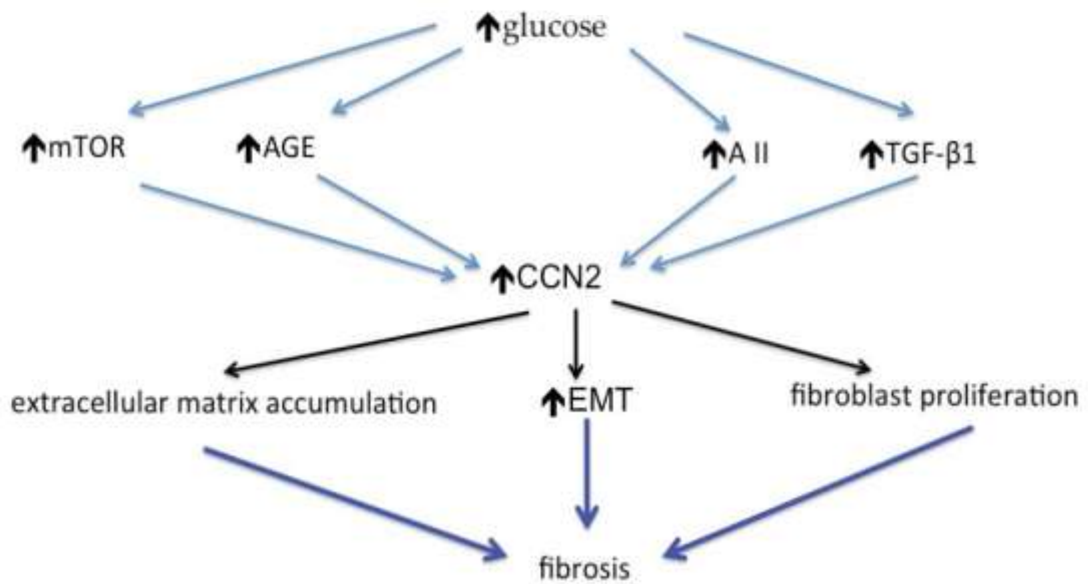


Fig 2.18: **CCN2 mediated glomerular and interstitial fibrosis. mTOR= Mammalian target of rapamycin; AGE = Advanced glycation endproducts; A II= Angiutensin II; TGFβ = Transforming Growth Factor β; CCN2 = Connective tissue growth factor; EMT= Epithelium mesenchyme transition** (Usama *et al*., 2017).

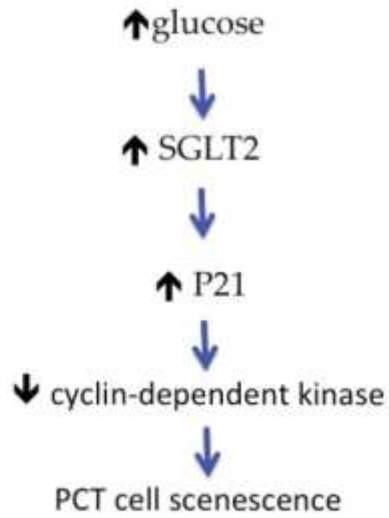


Fig 2.19: **SGLT2 mediated PCT cell senescence. SGLT = Sodium glucose transporter; PCT = Proximal convoluted tubules** (Usama *et al*., 2017).

Normal eGFR ranges from 90 to 120 ml/min/1.73m$^2$ (Waad-Allah *et al*., 2012). One such equation is the Cockroft-Gault equation (Cockroft and Gault, 1976).

Cockroft-Gault Equation:

$$C_{Cr}\ (\text{ml/min}) = \frac{(140 - \text{Age}) \times \text{Weight} \times (0.85 \text{ if female})}{72 \times S_{Cr}}$$

Where $C_{Cr}$ is the rate of creatinine clearance and it equals the GFR, glomerular filtration rate.

It is suggested by Nazar, 2014 that the number one goal in the management (prevention and treatment) of diabetic nephropathy is optimal blood glucose control.

The risk of diabetic nephropathy is strongly linked to poor glycaemic control in both Type 1 and Type 2 DM (Mazze *et al*., 1995; Stratton*et al*., 2000).In addition, there is strong evidence that tight blood sugar control has a significant impact on primary prevention of diabetic nephropathy (Diabetes Control and Complications Trial (DCCT), 1993;United Kingdom Prospective Diabetes Study(UKPDS), 1998).

However, tight glycaemic control is not always an easy task. The studies favouring the early control of glucose level were mostly performed on patients with normal albuminuria or in the early stage of diabetic nephropathy. Other studies addressing intensive blood glucose control in advanced kidney disease produced unreliable results as they are confounded by the presence of other comorbid conditions, for example cardiovascular disease and hypertension. At the very least, glucose is a significant and clinically relevant marker for the metabolic abnormality that leads to nephropathy, as shown in the Diabetes control and complications trial (DCCT) and other treatment trials that exhibit decreased nephropathy with lowered serum glucose (The DCCT Research Group, 1993).

In type 2 diabetic patients associated with diabetic nephropathy, strict glycaemic control may increase renal histology but does not provide protection against macrovascular complications (Scichri *et al*., 2000).

The most appropriate target for glycated haemoglobin for patients with diabetic nephropathy is 7.0%, especially for high-risk patients with established cardiovascular disease. Recently, the ADVANCE study inveterate the expected reductions in new onset microalbuminuria and nephropathy in patients with nearly normal glycaemic control (HbA1c of 6.5% versus 7.3%). However, tense glycaemic control in patients with advanced kidney disease has failed to lower the rate of cardiovascular events (The Action to Control Cardiovascular Risk in Diabetes Study Group, 2008).

The American Diabetes Association and the European Association for the Study of Diabetes Guidelines recommend lifestyle modification first and then propose the addition of basal insulin, sulfonylurea and thiazolidinediones if HbA1c still exceeds in the initial stages of diabetic nephropathy (American Diabetes Association, 1999).

CHAPTER THREE

# 3.0 MATERIALS AND METHODS

## 3.1 Materials

The materials used were syringes (10ml) and Needles (21G), EDTA Bottles, 5ml and 10ml (Chemistry)Plain (Serum) Bottles, Weighing Scale (Vins Medical, England), Stadiometer (Vins Medical, England), $HbA1_C$ Analyzer (Finecare Corporation, China) and Full-Auto Chemistry Analyzer(Erba Diagnostics Mannheim, Germany)

## 3.2 Methodology

### 3.2.1 Study site

Zaria is a major city in Kaduna State in North-western Nigeria. It has savannah vegetation and a population of about 975,153 people (National Population Commission, 2017). The inhabitants of Zaria are of diverse ethnicity and livelihood. Zaria houses Nigeria's largest University, Ahmadu Bello University. Ahmadu Bello University Teaching Hospital (ABUTH) is located in Shika, a distance of about 5 km from the university's main campus. The hospital is modern and serves patients with myriad forms of ailments including DM.

### 3.2.2 Study design

The diabetic volunteer-subjects were those receiving treatment in Ahmadu Bello University Teaching Hospital (Shika) Zaria, Nigeria, and for at least duration of one year. The control non diabetic subjects were volunteers gotten from Zaria residents that meet the criteria for selection. They were motivated to participate partly because they did the tests (check-up) for free and got their results for any attending personal use.They also got little gifts for participating.

Fig 3.1 **Maps of the location of ABUTH Shika, Zaria, Nigeria**(Google).

3.2.3 Sample size determination

$$n = \frac{Z^2 P(1-P)}{d^2}$$

(Daniel, 1999)

Where:

n = Sample size, Z = Z statistic for a level of confidence (for the level of confidence of 95%, Z's value is 1.96), P = Expected prevalence or proportion (expressed in proportion of 1 instead of percentage), d = Precision (expressed in proportion of 1 instead of percentage).

Choosing a prevalence of 10% (Edo and Akhuemokhan, 2012) at 95% confidence interval, the expected prevalence P = 10% (or 0.1) and Precision = 5% (or 0.05), (Naing *et al*., 2006).

Substituting the values in the equation:

$$n = \frac{1.96^2 \; 0.1 \; (0.9)}{0.05^2} = 138$$

This indicates that the sample size should at least be 138 (for both experimental and control subjects), but 152 subjects were used for the study, with the attrition rate at 10%.

3.2.4 Study subjects

Participants consisted of 152 subjects (both genders)of whom 101 were diabetic and 51 were non diabetic. Convenience sampling method was used.

3.2.5 Inclusion criteria

Diabetic patients that have had the disease for at least a year were selected for the study. TheirDMwas Type II DM with average age of patients being 50 years. Also the control (non-diabetic) subjects were of a similar average age.

3.2.6 Exclusion criteria

Subjects with conditions that affect haemoglobin synthesis and erythrocyte production; or with evidence of chronic medical conditions like hypertension, renal failure, liver disease and urinary tract infection were excluded from the study. Patients with DM for less than a yearsince diagnosis were also excluded from the study. For the control group, the subjects with fasting blood glucose levels> 120 mg/dl wereremoved from participating as control subjects because fasting blood glucose level greater than 120 mg/dl was considered hyperglycaemic.

## 3.3 Data Collection

3.3.1 Ethical approval

Ethical approval for the study was obtained from the Ethical Committee on Human Research of Ahmadu Bello University with the approval number: ABUCUHSR/2017/002 (Appendix I) in accordance with the Helsinki declaration.

3.3.2 Informed consent

After filling the Consent Form to Participate in the Study (Appendix II), any willing participant then voluntarily filled the Informed Written Consent form (Appendix III).

3.3.3Questionnaire

Information on DM history andsocio-economic lifestyle was collected viaquestionnaire(Appendix IV). All subjects were non-smokers and did not take alcohol.

3.3.4Anthropometric measurements

Weight of subjects(in kilogram)wasobtained using a weighing scale (by Vins Medical England) and their heights(in metres) by a stadiometer (also manufactured by Vins Medical England). The Body Mass Index(BMI) expressed as $kg/m^2$(weight divided by the height squared) was then calculated from the weight and height of each of the subjects.

3.3.5Laboratory methods

Blood sample,5ml, was collected from each subject in the morning after about 7 to 10 hours of overnight fasting via venepuncture. About 3 to 4ml of the blood was then transferred from syringe into a 10ml plain (chemistry) bottle while the remaining 1 to 2ml was transferred into a 5ml EDTA sample bottle. The blood samples in the plain bottles were then taken to the laboratory and centrifuged at 4000 rpm for 10 minutes in order to separate the serum. The clear,separated fluid which is the serum (at the top of the mixture) was then carefully pipetted out and transferred into 5ml plain bottles and stored in the freezer (at about $-18^0$C) for later analysis. The blood samples in the EDTA bottles were analyzed immediately for glycated haemoglobin A1c (HbA1c). The EDTA bottles were used specifically in order to prevent clotting.

3.3.6 Sample analysis

*3.3.6.1 The HbA1c test principle*

The Finecare$^{TM}$uses the sandwich immunodetection method to measure percentage of HbA1c in human blood (Meng *et al.*, 2012).After the blood sample was mixed with the buffer – which induces haemolysis of the red blood cells to release the haemoglobin – and added to

the sample well of the test cartridge, the fluorescence-labelled detector HbA1c antibody binds to HbA1c in blood specimen. As the sample mixture migrates on the nitrocellulose matrix of test strip by capillary action, the complexes of detector antibody and HbA1c are captured to HbA1c antibody that has been immobilized on test strip. The fluorescence-labelled detector Hb antibody binds to Hb in blood specimen; the complexes are captured to Hb antibody that has been immobilized on test strip. Signal intensity of fluorescence is proportional to concentrations of HbA1c and Hb in blood specimen. The ratio between inflorescent signals of HbA1c and Hb is the ratio between HbA1c and Hb.

*3.3.6.1.1 HbA1c test procedure*

The glycated haemoglobin (HbA1c) level in the blood samples was measured using the Finecare™ fluorescence immunoassay (FIA)meter and cartridges.Firstly, the ID Chip(that comes with the Finecare™ test kit) was inserted into the meter to get it ready. 10 µL of whole blood was then transferred by a micropipette into the buffer tube containing thebuffer, and comes with the Finecare™test kit. The mixture of buffer and blood specimen was mixed well by inverting the tube back and forth for a minute after which 75 µL of the sample mixture was pipetted out and loaded onto the sample well of the test cartridge. The test cartridge was then inserted into the test cartridge holder in the meter and “Test” was clicked by a touch pen on the display touch screen present on the meter. After 5 minutes the result of the HbA1c test was shown on the display screen and also printed out on a pre-installed paper roll after “Print” was clicked.

*3.3.6.2. The chemistry analyzer test principle*

The Analyzer employs the colorimetric analysis method where the amount of resultant colour change produced by mixing the serum sample (containing the measured analyte) with the measured analyte’s specific reagent is proportional to the amount of the analyte present in a

quantified amount of the serum sample (Zhang and Yuan, 2018). For instance, a quantified amount of serum is mixed with glucose reagent. The reaction process resulting from themixture produces a colour change. The intensity of the colour change is proportional to the amount of glucose present in the quantified amount of serum sample that was mixed with glucose reagent. A photometer can then measure the colour change and determine the amount of glucose per quantified amount of serum sample. The same procedure is done for determining amount of creatinine using creatinine's reagent. The procedure in more complex and fine-tuned steps has been automated within the Erba® Mannheim XL-200 Full-Auto Chemistry Analyzer.

*3.3.6.2.1 Serumcreatinine and glucose test procedure*

The levels of creatinine and glucose present in the serum samples were measured using the automated Erba® MannheimXL-200 Full-Auto Chemistry Analyzer by Erba Diagnostics Mannheim, Germany. First the reagents for the specific analytes to be measured (in this case creatinine and glucose) were loaded into the reagent turntable after whichcontrol tests were run with the machine to ensure proper and effective working condition. The serum samples were then brought out of the freezer and defrosted for about 30 minutes. The samples were then poured into the sample cups – 30 cups at a time – that fit into the sampletray of the Analyzer. The machine was then started and monitored by an adjoiningcomputer monitor as it analyses the loaded samples batch by batch at 30 samples per batch. Each sample label was carefully tagged with its corresponding cup position in the machine. After analysing each batch, the (results)levels of creatinine and glucose present in each sample was then displayed on the computer screen and carefully copied out. The procedure was repeated until all the samples were run and results obtained.The creatinine clearance or GFR was estimated for each sample from its serum concentration of creatinine using the Cockroft-Gault equation (Cockroft and Gault, 1976) where creatinine clearance ($C_{Cr}$) = GFR:

$$C_{Cr}\ (ml/min) = \left((140 - Age) \times Weight / (72 \times S_{Cr})\right) \times (0.85\ if\ female)$$

Plate I: **The Finecare™ fluorescence immunoassay (FIA) meter, cartridges and ID chip**

Plate II: **The Automated Erba® Mannheim XL-200 Full-Auto Chemistry Analyzer and Reagents for different Analytes**

## 3.4 **Statistical Analysis**

Results were presented as mean ± SD and data were analysed using independent student's t-test, while relationships between variables were determined using Pearson's correlation test. A linear regression analysis was also conducted to generate a regression equation. A *p*-value of <0.05 was considered significant. All analyses were done using IBM SPSS (Statistical Package for Social Sciences) 24.

CHAPTER FOUR

# 4.0 RESULTS

## 4.1 Comparing the Mean Values of Parameters of Diabetic Subjects with Non-Diabetic Subjects

The mean values of the measured parameters of diabetic patients were compared with those of the healthy (non-diabetic) control subjects (Table 4.1). The diabetic patients had significantly ($p$<0.001) higher levels of HbA1c (9.31% ± 3.24%), FBG (160mg/dL ± 87.61mg/dL) and SCr (0.96mg/dL ± 0.91mg/dL) than the control subjects with 5.67%± 0.71%, 76.31mg/dL ± 8.89mg/dL and 0.85mg/dL ± 0.18mg/dL respectively. The GFR in the patients is significantly ($p$<0.001) lower than that of the control subjects. However, no significantBMI difference observed between the two categories.

## 4.2 The Correlation between Parameters for the Diabetic and Non-Diabetic Control Subjects

The diabetic patients' parameters and the parameters for the non-diabetic control were analysed separately (*Tables 4.2* and *4.3* respectively). With itself, the variables expectedly gave a perfect correlation of 1. For the diabetics, FBG and HbA1c showed a strong positive correlation with an r value of 0.84. The relationship was also significant at a $p$ level of 0.01. Also the GFR and SCr showed a significant correlation ($P$=0.01) with an inverse correlation coefficient r of -0.49. The FBG and HbA1c though showed negative correlations with the GFR, the relationships did not show any significance. Similarly, FBG and HbA1c did not show significant relationship with SCr.For the control, only GFR and SCr showed a significant ($P$= 0.05) correlation with each other with an r value of -0.32 (Table 4.3).

Table 4.1: **Mean Values of Measured Parameters of Diabetic Subjects and Non-Diabetic Control Subjects**.

| Parameters | Diabetic Mean ± SD (n= 101) | Control Mean ± SD (n = 51) | *p*-value | *t*-value |
|---|---|---|---|---|
| HbA1c(%) | 9.31 ± 3.24 | 5.67* ± 0.71 | 0.001 | 10.78 |
| FBG(mg/dL) | 160.62 ± 87.61 | 76.31* ± 8.89 | 0.001 | 9.57 |
| GFR(mL/min) | 83.26 ± 26.93 | 100.66* ± 27.29 | 0.001 | -3.75 |
| SCr(mg/dL) | 0.96 ± 0.91 | 0.85* ± 0.18 | 0.001 | 3.56 |
| BMI(kg/m$^2$) | 25.75 ± 5.21 | 25.09 ± 4.93 | 0.449 | 0.76 |

The asterisk (*) indicates significant difference at the level of $P \leq 0.001$. HbA1c = Glycated Haemoglobin, FBG = Fasting Blood Glucose, GFR = Glomerular Filtration Rate, SCr = Serum Creatinine and BMI = Body Mass Index.

Table 4.2: **The Relationship (Correlation) between the Parameters ($HbA1_C$, FBG, GFR And SCr) for the Diabetic Subjects.**

| | HbA1c(%) | FBG(mg/dL) | GFR(mL/min) | SCr(mg/dL) |
|---|---|---|---|---|
| HbA1c(%) | 1 | | | |
| FBG(mg/dL) | 0.84* | 1 | | |
| GFR (mL/min) | -0.02 | -0.05 | 1 | |
| SCr(mg/dL) | 0.19 | 0.11 | -0.49* | 1 |

Asterisked (*) R-values indicates significant correlation at the level of P = 0.01.HbA1c = Glycated Haemoglobin, FBG = Fasting Blood Glucose, GFR = Glomerular Filtration Rate and SCr = Serum Creatinine

Table 4.3: **The Relationship (Correlation) between the Parameters (HbA1c, FBG, GFR and SCr) for the Non-Diabetic Control Subjects.**

| | HbA1c (%) | FBG (mg/dL) | GFR (mL/min) | SCr (mg/dL) |
|---|---|---|---|---|
| HbA1c (%) | 1 | | | |
| FBG (mg/dL) | 0.10 | 1 | | |
| GFR (mL/min) | -0.15 | -0.20 | 1 | |
| SCr (mg/dL) | 0.16 | 0.26 | -0.32* | 1 |

Asterisked (*) R-value indicates significant correlation at the level of P = 0.05. HbA1c = Glycated Haemoglobin, FBG = Fasting Blood Glucose, GFR = Glomerular Filtration Rate and SCr = Serum Creatinine

### 4.2.1 Correlation between HbA1c and GFR of Patients with HbA1c ≥ 9%

In table 4.4, the patients with HbA1c of 9% and above were selected and a correlation analysis was carried out to test the relationship between their HbA1c and GFR. A positive correlation was seen, significant at the level of $P < 0.05$. 43 out of the 101 patients had HbA1c greater than 9%. Table 4.5 shows the values of their measured parameters.

### 4.2.2 Correlation Graphs that Show the Relationships between HbA1c and FBG for the Diabetic Patients and between HbA1c and GFR for Diabetic Patients that Have Their HbA1c ≥ 9.0%.

Figures 4.1 and 4.2 below are graphsthat show the relationships between HbA1c and FBG for the diabetic patients and between HbA1c and GFR for diabetic patients that have their HbA1c ≥ 9.0%.

### 4.2.3 Correlation (Regression) Equation for $HbA1_C$ and FBG among the Diabetic Patients

After establishing a strong positive correlation between HbA1c and FBG among the diabetic patients a linear regression analysis was conducted and a regression equation ($y = mx + c$) generated:

$$y = 0.031x + 4.29$$

Where y is the dependent variable HbA1c, and x is the independent variable FBG. “m” is the slope of the graph and it equals 0.031 while c is a constant and it equals 4.29. This was then used to generate a chart of varied hypothetical FBG values in order to see their respective equivalent HbA1c values (Table 4.6). See also Fig 4.4.

Table 4.4: **Correlation between HbA1c and GFR of Patients with HbA1c ≥ 9%**

| | HbA1c (%) | GFR (mL/min) |
|---|---|---|
| HbA1c (%) | 1 | -0.35* |
| GFR (mL/min) | -0.35* | 1 |

Asterisked (*) R-values indicates significant correlation at the level of $P< 0.05$.HbA1c = Glycated Haemoglobin and GFR = Glomerular Filtration Rate

Table 4.5: **Mean Values of Measured Parameters of the Patients with HbA1c ≥ 9%**

| Parameters | Mean ± SD (n= 43) |
|---|---|
| HbA1c (%) | 12.58 ± 2.02 |
| FBG (mg/dL) | 234.06 ± 82.44 |
| GFR (mL/min) | 85.05± 21.06 |
| SCr (mg/dL) | 0.91 ± 0.12 |

HbA1c = Glycated Haemoglobin, FBG = Fasting Blood Glucose, GFR = Glomerular Filtration Rate and SCr = Serum Creatinine

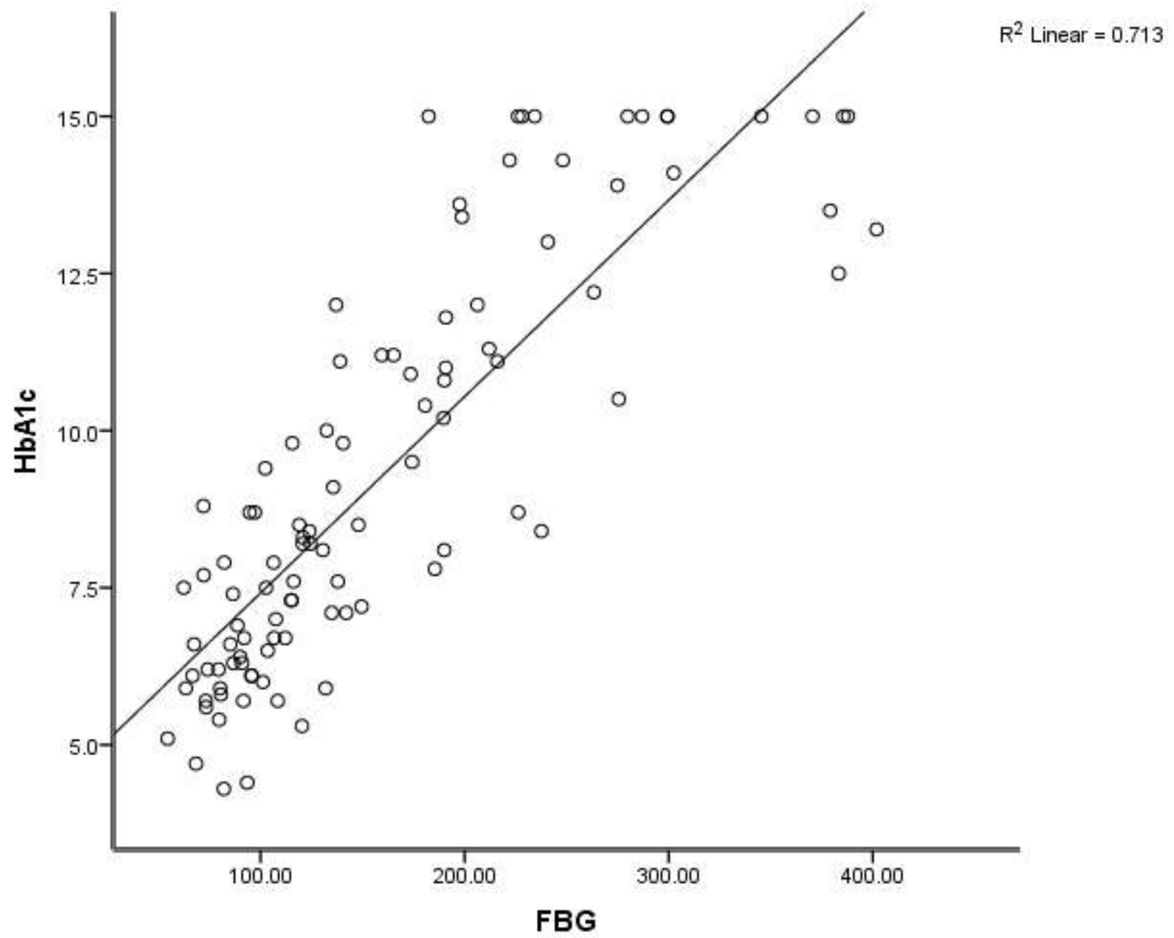


Fig 4.1: **Relationship (with line of best fit) between HbA1c and FBG in the Diabetic Subjects. The Correlation was Significant at a Level of P < 0.001.**

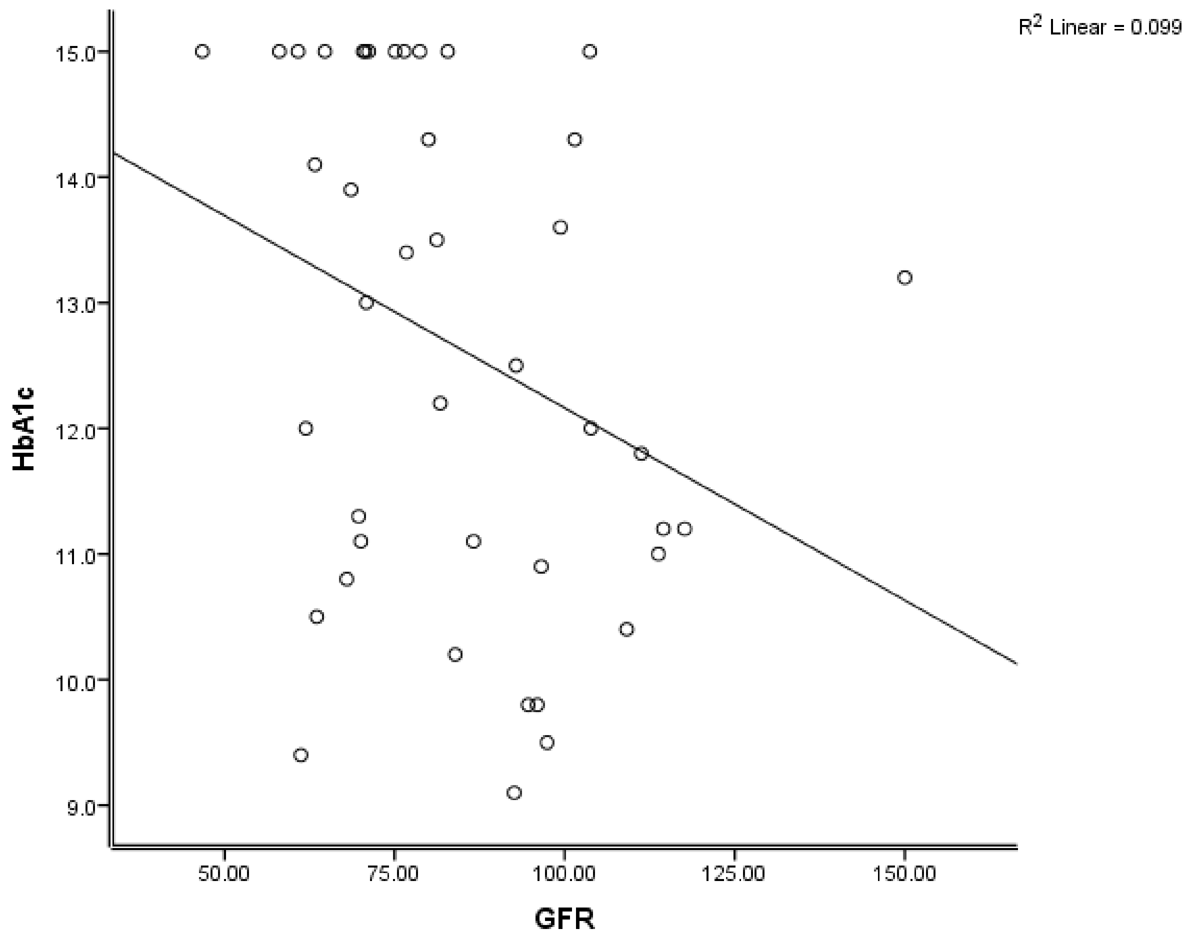


Fig 4.2: **Relationship (with line of best fit) between HbA1c and GFR in Diabetic Subjects with HbA1c Level ≥ 9%. The Correlation was Significant at a Level of P < 0.05**.

Table 4.6: **Glucose Values and Equivalent Glycated Haemoglobin Levels Based on the RegressionEquation: y = 0.031x + 4.29 obtainedfrom this Study Compared with the Supposed Standard Equivalents from WebMD.**

| FBG (mg/dL) | HbA1c (%) | |
|---|---|---|
| | WebMD | This Study |
| 68 | 4 | 6.40 |
| 97 | 5 | 7.30 |
| 126 | 6 | 8.20 |
| 152 | 7 | 9.00 |
| 183 | 8 | 9.96 |
| 212 | 9 | 10.86 |
| 240 | 10 | 11.73 |
| 269 | 11 | 12.63 |
| 298 | 12 | 13.53 |
| 326 | 13 | 14.40 |
| 355 | 14 | 15.30 |

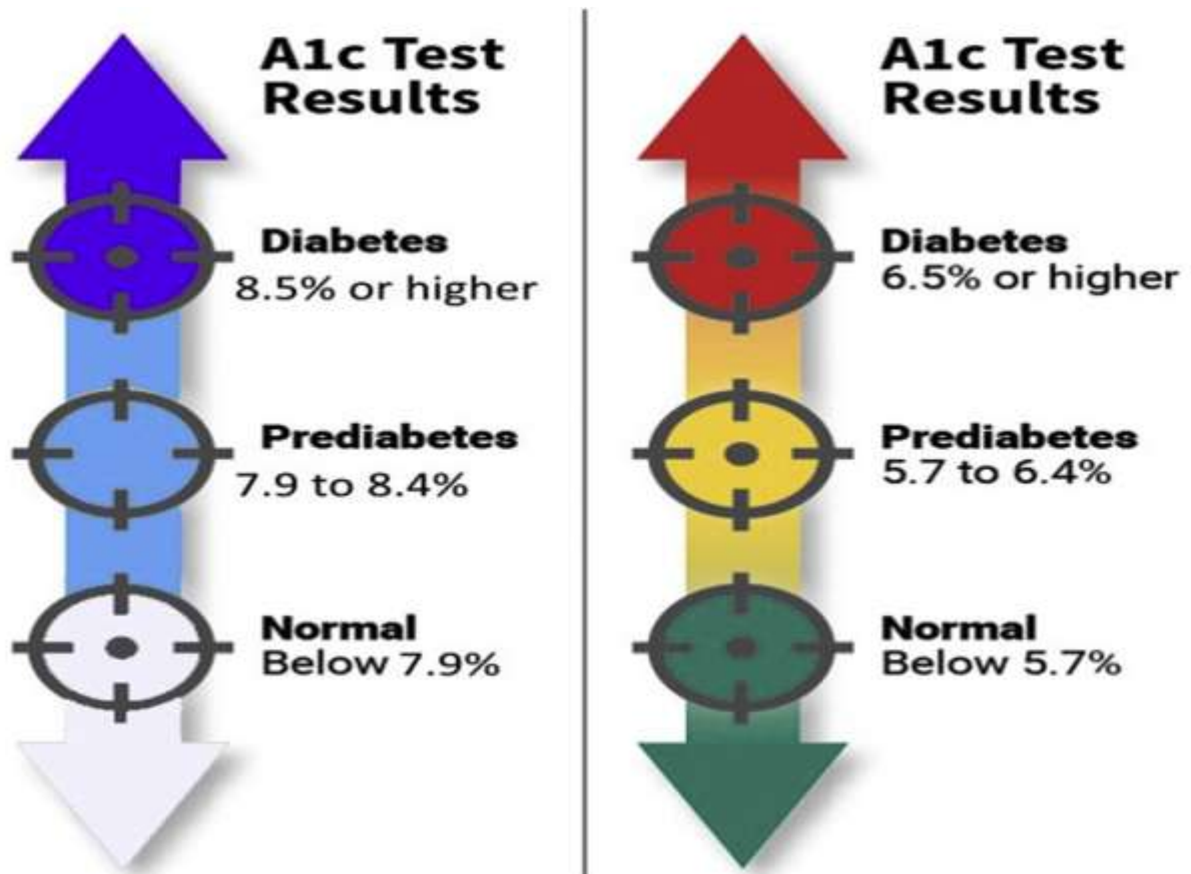


Fig 4.3: **The Glycaemic Chart obtained from this Study (left) and the one from WebMD (right)**.

## 4.3 **Proportion of Study Participants with "Abnormal" or Deviating HbA1$_C$Levels**

Subjects that had HbA1c at 5.7% – the purported point of onset of pre-diabetes – orgreater were selected and their FBG compared with their HbA1c. Those whose FBG is correspondingly high (at the prediabetic or diabetic level) are classified as "conforming" while those whose FBG is within the normal range are classified as "deviating".

Table 4.7: **Proportion of Diabetic Subjects with Deviating HbA1c Levels (5.7% and above) Not Conforming to their Corresponding FBG Levels**.

| | n = 64<br>Conforming HbA1c | n = 37<br>Deviating HbA1c | t | *p*-value |
|---|---|---|---|---|
| % of total N | 63.37 | 36.63 | | |
| HbA1c (%) | 10.67 ± 3.31 | 6.96* ± 1.08 | 8.25 | < 0.001 |
| FBG (mg/dL) | 200.65 ± 87.15 | 91.37* ± 15.89 | 9.75 | < 0.001 |
| SCr (mg/dL) | 0.95 ± 0.18 | 0.99* ± 0.20 | -0.92 | < 0.001 |
| GFR (mL/min) | 82.99 ± 22.19 | 83.72* ± 33.95 | -0.12 | < 0.001 |

The asterisk (*) indicates significant proportion at the level of $p < 0.001$. **Conforming HbA1c** = HbA1c level that matches its supposed glucose range. **Deviating HbA1c** = HbA1c level that does not match (higher than) its supposed glucose range.HbA1c = Glycated Haemoglobin, FBG = Fasting Blood Glucose, GFR = Glomerular Filtration Rate and SCr = Serum Creatinine

Table 4.8: **Proportion of Non-Diabetic Control Subjects with Deviating HbA1c Levels (5.7% and above) not Conforming to their Corresponding (Normal Range) FBG Levels.**

| | n = 28<br>Conforming HbA1c | n = 23<br>Deviating HbA1c | t | *p*-value |
|---|---|---|---|---|
| % of total N | 54.90 | 45.10 | | |
| HbA1c (%) | 5.16 ± 0.35 | 6.29* ± 0.51 | -9.36 | < 0.001 |
| FBG (mg/dL) | 73.97 ± 8.94 | 79.16* ± 8.13 | -2.15 | < 0.001 |
| SCr (mg/dL) | 0.81 ± 0.15 | 0.90* ± 0.20 | -1.71 | < 0.001 |
| GFR (mL/min) | 101.90 ± 24.91 | 99.16* ± 30.45 | 0.35 | < 0.001 |

The asterisk (*) indicates significant proportion at the level of $P < 0.001$. **Conforming HbA1c =** HbA1c level that matches its supposed glucose range. **Deviating HbA1c =** HbA1c level that does not match (higher than) its supposed glucose range.HbA1c = Glycated Haemoglobin, FBG = Fasting Blood Glucose, GFR = Glomerular Filtration Rate and SCr = Serum Creatinine.

CHAPTER FIVE

# 5.0 DISCUSSION

Glycated haemoglobin ($HbA1_C$) is being reported to vary in level for a given blood glucose range from individual to individual especially along racial line. In accordance to the recommendation of a study by Lawal *et al*., 2018 that used volunteer-subjects recruited from Zaria population,this study wascarried out to investigate the local $HbA1_C$threshold amongst diabetic patients attending ABUTH Zaria and non-diabetic Zaria residents, that can be used in the management of diabetes and in the diagnosis of diabetes and pre-diabetes respectively, using blood glucose as a benchmark.

The rate of glycation of haemoglobin – the level of HbA1c – is generally proportional to the level of blood glucose (American Diabetes Association, 1999; Little *et al*., 2001; Khan *et al*., 2004; Leslie *et al*., 2017). In conformity, the results obtained from this study showed that as the glycated haemoglobin increased the level of blood glucose also increased proportionally. This trend is observed more clearly among the diabetic subjects where there is a wide range of increasing blood glucose to be compared with correspondingly increasing HbA1c.

This indicates that glycated haemoglobin (HbA1c) is still useful as glycaemic marker among the study subjectsso far any local variations or peculiarities are considered as suggested by Hanas and John, 2010.

Though not out of the normal range, the average serum creatinine level among the diabetic subjects used for this study was significantly higher than the average serum creatinine level among the non-diabetic control subjects used for the study.

Serum creatinine, the by-product of creatine metabolism is used as a marker for kidneyfunction(Lewis and Maxwell, 2014) whereby increasing level of serum creatinine

indicates decreasing kidney function, a less healthy kidney or renal damage (Marshall and Flyvbjerg, 2006).

DM is a disease that often causes compromised kidney function especially when not managed properly and eventually leads to kidney damage a condition known as diabetic nephropathy (Nazar, 2014).

As the diabetic subjects used for the study had been diagnosed of the condition for one year or more, relative increase in their serum creatinine may point to a depreciating function of their kidneys. Measuring the level of serum creatinine of diabetic patients at intervals helps in monitoring the health of their kidney in effort to guard against diabetic nephropathy which is reported to be the third most common cause of chronic renal failure in Nigeria (Sanusi *et al*., 1997).

Since the patients in this studywere receiving treatment in the hospital, their average serum creatinine level that is found to be within the normal range might be due to this treatment as intervention is said to curtail the progression of loss of kidney function (Holman *et al*., 2008). Perhaps, a more accurate renal-function index such as the glomerular filtration rate (GFR) (Perkovic *et al*., 2013) that considers the age, weight, gender among other parametric data of the individual will reveal a truer state of theirkidneys.

Though still proportional to the level of fasting blood glucose, the results obtained from this study indicate that the HbA1c range that should be generally considered normal in the locality of the subjectsused – Zaria and indeed Nigeria – is significantly higher than what was presupposed.

The results from HbA1c testing are used even in Nigeria here to monitor blood glucose level (Onyemelukwe *et al*., 2020) thereby determining the course of future treatment for the patient in order to guard against hyperglycaemic-induced complications (Robert and Cristopher,

2012). However, the accuracy of these results is being reported to be doubtful and highly varied amongst individuals especially along racial lines; with reports of significantly higher levels in non-Caucasian diabetic patients for a given blood glucose range (Cohen *et al*., 2006; Herman *et al*., 2009).These higher HbA1c levels observed in the study subjects for a normal blood glucose range suggests “raising the bar of normality” or the target point (of HbA1c) in the treatment of diabetic patients to guard against overtreatment and hypoglycaemia.

Earlier, a multi-centre study involving seven teaching hospitals in Nigeria revealed that 68% of diabetic patients had their mean HbA1c levels greater than 7% but only about half of these showed sign of complications development (Chinenye *et al*., 2012) suggesting that the “at risk of complication” level of HbA1c is different among Nigerian patients.

In the end, we are able to identify a trend in the HbA1c levels of diabetic patients that attend Ahmadu Bello University Teaching Hospital Zaria and indeed Zaria's very diverse residents in general even though only instant samples were collected and not continual monitoring. It is still accurate though, since the within individual day-to-day variability of HbA1c level is even said to be less than 0.02 (Sacks *et al*., 2002).

This study has thus come up with a chart with which diabetic patients can be managed. In contrast to a study by Sakpa and Idemudia, (2014) carried out in the University of Benin Teaching Hospital, Benin City, Edo state, Nigeria, the HbA1c and fasting blood glucose of the non-diabetic control subjects in this study though showed some correlation with each other, the correlation is weak and insignificant. This is simply due to values of $HbA1_C$ and fasting blood glucose that are all within the narrow normal range among the individuals.

Glomerular filtration rate (GFR), an index for renal function can be estimated from the level of serum creatinine using an equation that takes into account the individual's age, weight and gender (Cockroft and Gault, 1976) such that the more the serum creatinine concentration the

lower the GFR relative to the person's age, weight and gender. In DM, the GFR is known to drop proportionally to increasing blood glucose level (Waad-Allah *et al*., 2012). In other words, the renal function reduces in proportion to how badly the condition (DM) is being managed.

A similar trend is confirmed by this study whereby significant correlation between HbA1c and GFR is seen only at levels of HbA1c higher than 9%. The GFR decreases as the HbA1c level increases from 9% onward. There was no significant decrease in GFR with increasing HbA1c below the level of 9% HbA1c amongst the diabetic patients and of course among the non-diabetic control subjects.

This occurred because below the level of 9% haemoglobin glycation, there is not the expected correspondingly high FBG level among the patients which would actually compromise the renal function and cause the GFR to drop – HbA1c being just a marker of glycaemic level. Thus, it is only in the patients with HbA1c above 9% that depreciating kidney function with increasing HbA1c was seen.

This further lends credence to the evidence that the HbA1c level is normally high in this part of the world for a given blood glucose range. Also, among the two groups (diabetic and non-diabetic control) the serum creatinine though positively correlated with HbA1c, the relationship was not significant. It should be noted though that the level of serum creatinine is influenced by age, weight and gender which the estimation of GFR takes into consideration (Cockroft and Gault, 1976).

Amongst the control subjects, the serum creatinine level increased with increasing fasting blood glucose (positive correlation), while the GFR dropped with increasing fasting blood glucose (negative correlation) but these relationships were not significant (weak correlation). It is noted though that these are normal, non-diabetic control subjects with normal FBG

(fasting blood glucose) and normally functioning kidney. Thus all values are within the narrow range of normality.

Similar relationships between FBG, serum creatinine and GFR were observed among the diabetic subjects when tested generally. But at higher values of FBG (> 136 mg/dL) synonymous to >8.5% HbA1c value, the GFR dropped significantly with increasing FBG. It is interesting and worthy to note that it is around the same level (□ 9%) that significant correlation between the rising HbA1c and the dropping GFR was observed among the patients. This conjointly points at that HbA1c level (of □ 9%) as an important point where diagnosis of DM and prognosis of complications – especially nephropathy – can be made.

The findings of the present study which point to a local peculiarity in what should be regarded as normal HbA1c is buttressed by earlier studies that indicate a high hereditability of native HbA1c estimated to be approximately 50% (Delpierre *et al*., 2006; Kera *et al*., 2008).

From this study it is seen that about 37% of diabetic subjects used and 45% of non-diabetic control subjects had HbA1c levels that do not match their glucose level – they had higher HbA1c (at the supposed diabetic or prediabetic level) for their (normal) blood glucose range. This indicates that 37 of the patients and 23 of the control subjects were capable of being over-treated or misdiagnosed respectively.

The contributory genetic factors are similar to those indicated by Florez (2010) which are involved in physiologic factors such as glycaemic control (for example, b-cell function, insulin sensitivity and incretin physiology), and non-glycaemic factors (for example,RBC glucose transport, deglycation pathways and altered RBC turnover) (Delpierre *et al*., 2006; Kera *et al*., 2008). Other factors include iron handling, glucose distribution across, and rates

of uptake into the erythrocyte membrane, rates of glucose attachment to or release from haemoglobin and rates of intra-erythrocytic glucose metabolism (Florez, 2010).

This implies that the genetic factors involved in the manifestation of these physiologic factors having been inherited by generations will then determine the HbA1c normal-level (uniqueness) of such locality.

CHAPTER SIX

# 6.0 CONCLUSION AND RECOMMENDATIONS

## 6.1 Conclusion

It was seen from this study that $HbA1_C$ is useful in determining glycaemic control in diabetic patients in the effort to guard against renal damage in these patientsif the bar of normal level of $HbA1_C$ or target point of HbA1c to be achieved with treatment is raised slightly higher. This study therefore concludes that:

i. the levels of glycated haemoglobin (HbA1c), fasting blood glucose (FBG) and serum creatinine (SCr) were significantly higher in the diabetic patients than in the non-diabetic control subjects, while the estimated glomerular filtration rate (eGFR) was significantly lower in the diabetic patients than in the non-diabetic control subjects.

ii. the level of glycated haemoglobin (HbA1c) increases proportionally with increase in the fasting blood glucose (FBG) among the diabetic patients and the correlation between the two parameters was positive and very strong. Also there was a positive correlation between glycated haemoglobin (HbA1c) and fasting blood glucose (FBG) among the non-diabetic control subjects.

iii. there was no significant correlation between glycated haemoglobin (HbA1c) and eGFR at lower levels of HbA1c but their correlation became significant at higher HbA1c levels. So also, there was a significant correlation between the fasting blood glucose (FBG) and estimated glomerular filtration rate (eGFR) at higher levels of FBG.

iv. there were significant number of diabetic patients and non-diabetic control subjects with “abnormal” or deviating levels of glycated haemoglobin (HbA1c) that tend to be significantly higher than expected for a given blood glucose range.

## 6.2 Recommendations

It was recommended from the findings of the present study that:

i. the possibility of a patient having an “abnormal” or deviating glycated haemoglobin ($HbA1_C$) – higher than conventional – level should be considered while caring for diabetic patients;

ii. the possibility of an individual having an “abnormal” or deviating glycated haemoglobin ($HbA1_C$)– higher than conventional – level should be considered while testing for the presence or absence of DM;

iii. further study is needed to investigate the underlying physiologicfactors responsible for the “abnormal” glycated haemoglobin ($HbA1_C$) level observed among some individuals. Further investigation on the relationship between glycaemia, $HbA1_C$ and risk of renal damage is also needed;

iv. the study used instant (one-time) values obtained once from each subject. A more intense study that uses continual data obtained at intervals from subjects will be useful.

## 6.3 Contributionto Knowledge

i. The study reveals a significant number of diabetic subjects (36.63% of them with an average HbA1c level of $6.96 \pm 1.08$ and average FBG of $91.37 \pm 15.89$) and control non-diabetic subjects (45% of them with an average HbA1c level of $6.29\pm 0.51$ and average FBG of $79.16\pm 8.13$) that tend to have higher glycated haemoglobin ($HbA1_C$) levels for a normal blood glucose range, and could either be over treated or misdiagnosed of DM respectively.

ii. A regression equation, $y = 0.031x + 4.29$ that can be used for the interconversion between glycated haemoglobin ($HbA1_C$) and fasting blood glucose level in this environment was generated by this study.

iii. A new reference chart of glycated haemoglobin levels and their glucose equivalences by which care can be provided for diabetic patients was built by this study.

iv. Glycated haemoglobin (HbA1c) of $< 8.5\%$ is set as a new target in the management and prevention of risk of renal damage among diabetic patients.

v. This study has provided a gateway for the underlying physiologic factors responsible for such a relatively wide range in normal HbA1c level to be investigated in the nearest future.

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# APPENDICES

## Appendix I

Ethical Approval for the Study

**AHMADU BELLO UNIVERSITY, ZARIA**

**DIRECTORATE OF ACADEMIC PLANNING & MONITORING**

Vice Chancellor

Director:

Tel. Zaria – 234-069-551912 Fax. 234 (069) 550022 Telegram UNIBELLO Zaria e-mail dapm@abu.edu.ng

**Appl No.:** ABUCUHSR/2017/Human Physiology/002 27th June, 2018

**Approval No:** ABUCUHSR/2017/002

Dr. Yusuf Tanko
Department of Human Physiology,
College of Health Sciences,
Ahmadu Bello University,
Zaria.

Sir,

**PROVISIONAL APPROVAL OF RESEARCH TITLED "THE USE OF GLYCATED HEMOGLOBIN (HbA1c) IN DETERMINING GLYCEMIC CONTROL AND ASSESSING THE RISK OF RENAL DAMAGE IN DIABETIC IN ABUTH, ZARIA"**

This is to convey the provisional approval of the ABUCUHSR to you for the aforestated study domiciled in Department of Human Physiology of the Ahmadu Bello University. The approval is predicated on the assumption that you shall maintain and cater for the study subjects as indicated in your application.

Monitoring of the Research by spot checks, invitations, interactions with the subjects any other means the Committee deems fit shall be undertaken at the convenience of the Committee.

This approval can and shall be revoked should a significant breach in the terms and condition of the approval occur. It is hence your responsibility to ensure that the agreed terms are maintained to the end of the Study.

The said approval shall be posted on the ABUCUHSR Page on the University's website.

Note upon completion of the research, ethical clearance certificate will be issued.

**Prof. I.H. Nock**
ABUCUHSR Chairman

Cc. Director, DAPM
Director, IC &ICT
Provost, College of Health Sciences
HOD, Human Physiology
Prof. J.A. Randawa

**Appendix II**

Consent Form to Participate in the Study

Phone No…………………….

Serial No……………………..

Hospital No…………………..

Age…………………………..

The research is designed to evaluate **THE USE OF GLYCATED HAEMOGLOBIN IN DETERMINING GLYCAEMIC CONTROL AND ASSESSING RISK OF RENAL DAMAGE IN DIABETIC PATIENTS IN ABUTH, ZARIA.** Blood will be required for the study. The results obtained will be treated with confidentiality. You are free to participate or decline in this study without any consequence. This study will hopefully improve your present health status and that of others in the future. If you desired to participate in the study kindly sign the attached form below.

Thank You.

## Appendix III

Informed Written Consent

I…………………………………………...........of……………………………………...agr eed to participate in this study of **"THE USE OF GLYCATED HAEMOGLOBIN IN DETERMINING GLYCAEMIC CONTROL AND ASSESSING RISK OF RENAL DAMAGE IN DIABETIC PATIENTS IN ABUTH, ZARIA".** The full procedure and the probable benefits of the tests were fully explained to me.

I was informed that a sample of my blood will be collected for the test. If I so wish, the test results will be communicated to me in confidence.

I made this consent willingly without being subjected to any pressure.

Participant Name…………………………....... Signature……………………

Witness Name………………………………… Signature…………………...

Researchers Name……………………………. Signature……………………

## Appendix IV

Questionnaire for Evaluating The Use of Glycated Haemoglobin in Determining Glycaemic Control and Assessing Risk of Renal Damage in Diabetic Patients in ABUTH, Zaria

S/No. ………………. Date: ………………

Name: ……………………………………………

Age: ………………….

Gender: ………………………

Height (meters): ……………………….

Weight (kilogram): ………………………

Calculated BMI (Body Mass Index): ……………….

Number of Years Since Diagnosed with Diabetes: ………….

Smoker: Yes ……… No ………

Do You Take Alcohol: Yes ………. No ……….

Source of Livelihood: ………………………………

$HbA1_C$: ………………

FBG: …...............

SCr: …………..

Calculated GFR: ………………………