Sudan University of Science and Technology

Collage of Graduate Studies

Evaluation of Serum Cystatin C Level among Type 2 Diabetes Mellitus Patients in Khartoum State

تقويم مستوي السيستاتين سي في مصل مرضي السكري النوع الثاني في ولاية الخرطوم

A dissertation submitted in partial fulfillment for requirements of MSc Degree in Medical Laboratory Science (Clinical Chemistry)

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الآية

بسم الله الرحمن الرحيم

قال تعالي:

وفِي الأَرْضِ آيَاتٌ لِّلْمُوقِنِينَ* وفِي أَنفُسِكُمْ أَفَلا تُبْصِرُون""

[سورة الذاريات: الآيات 20– 21]

DEDICATION

I dedicate this research to my parents, brothers,family,friends and teachers, who taught us to think, understand and express.

Acknowledgement

*First I wish to thank **Allah** for granting me the Confidence and Success to complete this study.*

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List of Abbreviations

ADA	American Diabetes Association
BMI	Body Mass Index
BUN	Blood Urea Nitrogen
DM	Diabetes Mellitus
DN	Diabetic Nephropathy
eGFR	estimated GFR
eGFRcr	Glomerular filtration rate estimated using serum creatinine
GFR	Glomerular Filtration Rate
HDL	High Density Lipoprotein
KDa	Kilo Dalton
NGSP	National Glycated hemoglobin Standardization Program
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OGTT	Oral Glucose Tolerance Test
SD	Stander Deviation
UKPDS	United kingdom prospective diabetes study
WHO	World Health Organization

Abstract

Diabetes Mellitus (DM) is a systemic metabolic disorder, Diabetic nephropathy (DN), is a common cause of end stage renal disease worldwide. Cystatin C has been identified as a new, promising, and easily measurable marker for detecting mild decrease in GFR with highly specificity and sensitivity. Study aims to evaluate serum cystatin C among type 2 DM during a period of March-August 2015 in Khartoum state.

A hundred and twenty subjects classified as 60 healthy apparently as control group and 60 type 2 DM as case, blood samples were collected to measure serum cystatin C, HbA1c by ichroma™ immunofluorescence reader and BMI was calculated using Quetelet index formula. The results of frequency showed type 2 DM is common in female 37(61.67%) than male 23(38.33%) with ratio of 1:1.6. also results found that, mean cystatin C level was higher in type 2 DM patients versus control group with P-value = 0.000, and significant increase in mean concentration of cystatin C in uncontrolled DM compared with control DM patients P-value < 0.004. Personal correlation results showed positive correlation between cystatin C and age, duration, BMI, and HbA1c (R-value 0.417 and P-value 0.000), (R-value 0.272 and P-value 0.0.036), (R-value 0.260 and P-value 0.004), (R-value 0.340 and P-value 0.008) respectively, and no correlation with gender with R-value 0.051 and P-value 0.580.

The study conclude that, serum cystatin C level is higher in type 2 DM specially uncontrolled DM patients, and positive correlation between serum cystatin C and age, duration, BMI and HbA1Care noticed.

مستخلص البحث

مرض السكري هو اضطراب في نظام التمثيل الغذائي،الاعتلال النفروني لدي مرضي السكري هو المسبب الشائع للمستوي الثالث من امراض الكلي في العالم.

السيستاتين سي اصبح معروفا علي انه مؤشر جديد ، واعد وسهل القياس لاكتشاف اقل نقصان في معدل التصفية الكلوية ، باعلي درجة تخصصية وحساسية.

هذه الدراسة تهدف الي تقييم مستوي السيستاتين سي في مصل المرضي المصابين بمرض السكري النوع الثاني في الفترة من مارس الي اغسطس 2015 م في ولاية الخرطوم.

مائة وعشرون شخص مصنفين الي 60 شخص صحي ظاهريا كمجموعة ضابطة و 60 شخص يعاني من مرض السكري النوع الثاني، تم جمع عينات الدم لقياس مستوي السيستاتين سي في المصل وقياس المستوي التراكمي للهموقلوبين المرتبط مع الجلكوز باستخدام اجهزة تم قياس معامل كتلة الجسم حسابيا باستخدام المعادلة المحسوبة من ناتج قسمة الوزن بالكيلوجرام علي مربع الطول بالمتر.

اظهرت نتائج التردد ان مرضي السكري النوع الثاني شائع لدي الاناث وعددهم 37 بنسبة 61.67% من الرجال وعددهم 23 بنسبة 38.33% بمعدل 1.6 الي 1 .

ايضا الدراسة اوجدت ان متوسط مستوي السيستاتين سي اعلي في مرضي السكري النوع الثاني مقارنة بالمجموعة الضابطة بنسبة ثقة 0.000، وذيادة ذات دلالة احصائية في متوسط مستوي تركيز السيستاتين سي في مرضي السكري غير المتحكم فيه مقارنة بمرضي السكري المتحكم فيه بنسبة ثقة 0.004.

واظهرت نتائج الارتباط الشخصي علاقة ايجابية بين مستوي السيستاتين سي والعمر، مدة المرض، معامل كتلة الجسم والمستوي التراكمي للهموقلوبين المرتبط مع الجلكوز بنسبة مقاربة وثقة (0.417 ، 0.000)، (0.272، 0.036)، (0.260، 0.004)، (0.340، 0.008) علي التوالي، ولا توجد علاقة ذات دلالة احصائية بين مستوي السيستاتين سي المصلي والجنس بنسبة مقاربة 0.051 ثقة 0.580.

تتلخص هذه الدراسة في ان مستوي السيستاتين سي في مصل مرضي السكري النوع الثاني اعلي، وخاصة مرضي السكري غير المتحكم فيه، كما لوحظوجود علاقة ايجابية بين مستوي السيستاتين سي في مصل المرضي المصابين بمرض السكري النوع الثاني والعمر، مدة المرض، معامل كتلة الجسم والمستوي التراكمي للهموقلوبين المرتبط مع الجلكوز.

Chapter one

Introduction and Literature review

1.1 Introduction

Diabetes Mellitus(DM) is a systemic metabolic disorder characterized by a tendency to chronic hyperglycemia with disturbances in carbohydrate, fat and protein metabolism that arise from a defect in insulin secretion or action or both.It is a common condition, with a prevalence of approximately 4% in the western world. There are two distinct types: In type 1 DM there is destruction of pancreatic cells leading to a decrease in, and eventually cessation of insulin secretion. Approximately 10% of all patients with diabetes have type 1. They have an absolute requirement for insulin. In type 2 DM, insulin secretion is defective and delayed, and there is resistance to its action. The prevalence of both types of diabetes is increasing(Marshall*et al*., 2012).

The long-standing elevation of blood glucose causes the chronic complicationsof diabetes premature atherosclerosis, retinopathy, nephropathy, and neuropathy. How hyperglycemia causes the chronic complications diabetes is unclear. In cells where entry of glucose is not dependent insulin, elevated blood glucose leads to increased intracellular glucose and its metabolites. For example, increased intracellular sorbitol contributes to the formation of cataracts. Further, hyperglycemia promotes the condensation of glucose with cellular proteins in a reaction analogous to the formation of HbA1c. These glycated proteins mediate some of the early micro vascular changes of diabetes(Richard and Denise. 2011).

Diabetic nephropathy is the leadingcause of renal failure in the United States. It is defined by proteinuria > 500mg in 24 hours in the setting of diabetes, but this is preceded by lower degreesof proteinuria, or "microalbuminuria."Microalbuminuria is defined as albuminexcretion of 30–299 mg/24 hours. Without intervention, diabetic patients withmicroalbuminuria typically progress to proteinuria and overt diabetic nephropathy.As many as 7% of patients with type2 diabetes may already have

microalbuminuriaat the time they are diagnosedwith diabetes (Gross *et al.,* 2005).

Inthe UKPDS, the incidence of microalbuminuriawas 2% per year in patients withdiabetes type 2, and the 10-year prevalenceafter diagnosis was 25%(Gross *et al.,*2005; Adler*et al.,*2003).

Assessment of diabetic nephropathy: The most tests used to assess renal function are those that assess either the GFR or the integrity of the glomerular filtration barrier, and estimate of the GFR can be made by measuring the urinary excretion of substance that is completely filtered from the blood by the glomeruli and is not secreted, reabsorbed or metabolized by the renal tubule (clearance) **(**Marshall and Bangert.2008**)**.

Cystatin C has been identified as a new, promising, and easily measurable marker for prompt detection of early kidney failure(Levin. 2005; Dharnidharka *et al.,* 2002).Cystatin C is produced at a constant rate by nucleated cells and released into the bloodstream with a half-life of 2 h(Filler *et al.,* 2005).

Cystatin C is freely filtered and almost completely taken up and degraded, but not secreted, by proximal tubular cells. Several studies have used direct measures of GFR as the gold standard to compare cystatin C with creatinine and creatinine- derived estimates of GFR (Mussap and Plebani. 2004).

1.2 Diabetes Mellitus

Diabetes Mellitus (DM) is a systemic metabolic disorder characterized by a tendency to chronic hyperglycemia with disturbances in carbohydrate, fat and protein metabolism that arise from a defect in insulin secretion or action or both. It is a common condition, with a prevalence of approximately 4% in the western world. There are two distinct types: In type 1 DM there is destruction of pancreatic cells leading to a decrease in, and eventually cessation of insulin secretion. Approximately 10% of all patients with diabetes have type 1. They have an absolute requirement for insulin. In type 2 DM, insulin secretion is defective and delayed, and there is resistance to its action. The prevalence of both types of diabetes is increasing (Marshall *et al*., 2012).

1.2.1Types of diabetes mellitus

The current criteria for diagnosis of diabetes rely on the etiology of disease. Four forms of diabetes have been classified. These four forms are type 1, type 2, gestational diabetes, and other specific causes of diabetes (Arneson and Brickell.2007).

1.2.1.1 Type 1 diabetes

Type 1 diabetes is characterized by lack of insulin production and secretion by the beta cells of the pancreas. One cause of the hyperglycemia of type 1 diabetes mellitus is an autoimmune destruction of the beta cells of the pancreas. The cell mediated response causes infiltrationof the pancreas and reduction in the volume of beta cells. As a protein hormone, insulin acts through chemical responses to receptors on the cells of target tissues. In the muscle, insulin stimulates glucose uptake into cells and enhances glycogenesis. In adipose tissue, insulin stimulates glucose uptake into cells and enhances lipogenesis. In the liver, insulin has a negative effect, inhibiting gluconeogenesis and glycogenolysis. Auto antibodies are present in the circulation of many individuals with type 1 diabetes. There appears to be a

genetic susceptibility to development of auto antibodies, with certain histocompatibility antigens predominant in the type 1 diabetes population. However, the development of disease is complex; triggering factors, such as rubella, mumps, and other viral infection, and chemical contact may be necessary for progression of disease (Arneson and Brickell.2007).

1.2.1.2 Type II diabetes

Type two characterized by decline in insulin action due to the resistance of tissue cells to the action of insulin. The problem is intensified by the inability of the beta cells of the pancreas to produce enough insulin to counteract the resistance. Thus, type 2 diabetes is a disorder of both insulin resistance and relative deficiency of insulin .Insulin resistance syndrome, also known as metabolic syndrome and syndrome X, affects the metabolism of many nutrients, including glucose, triglycerides, and high-density lipoprotein (HDL) cholesterol. Individuals who are diagnosed with metabolic syndrome may show abdominal obesityand high blood pressure. Such individuals are at increased risk for cardiovascular disease. The etiology of type 2 diabetes is complex and multifaceted. There is evidence to show that there is an association of obesity with the development of type 2 diabetes. Other factors, such as family history of type 2 diabetes and lack of physical activity, have also been associated with the disorder. Previous diagnosis of gestational diabetes is a risk factor for type2 diabetes, as are increasing age, hypertension, and dyslipidemia. Increased risk for developing the disease is also associated with membership in certain racial and ethnic groups, such as African-Americans, Hispanic-Americans, Native Americans, Asian Americans, and Pacific Islanders (Arneson and Brickell.2007).

1.2.1.3 Gestational diabetes

Is similar in etiology to type 2 diabetes; however, it is defined as diabetes that is diagnosed in pregnancy. Pregnancy is associated with increased tissue cell resistance to insulin. Most pregnant women will compensate with increased secretion of insulin; those individuals who are unable to compensate may develop gestational diabetes. The hyperglycemia of gestational diabetes diminishes after delivery; however, the individual who has developed gestational diabetes is at higher risk for the development of type 2 diabetes thereafter (Arneson and Brickell.2007).

1.2.1.4 Other types of diabetes

The fourth form of diabetes is termed other specific causes of diabetes. This form of hyperglycemia may be the secondary result of non–insulin-related events. Blood glucose levels are increased in endocrine disorders, such as Cushing's syndrome; in exocrine disorders, such as cystic fibrosis; and as a response to specific drugs, such as protease inhibitors and glucocorticoids. Other causes of this form of diabetes are the result of genetic defects that affect pancreatic beta cells or the action of insulin. The disorders of diabetes differ in their presentation as well as their etiology. Approximately 10% of diabetics are of the type 1 variety. The type 1 disease state usually occurs as acute illness, while type 2 diabetes progresses slowly over time (Arneson and Brickell.2007).

Type 1 glucose blood levels are usually more severe than type 2. Type 1 diabetics are more likely to develop ketoacidosisthan are type 2 diabetics. Due to the etiology of disease, type 1 diabetics are insulin dependent, while most type 2 diabetics are not. Type 1 diabetics are younger (18 years old when diagnosed) and thinner; type 2 diabetics are usually older (40 years old when diagnosed) and more likely to be obese. However, these characteristics of presentation are not uniform to all type 1 and type 2 diabetics. Type 1

diabetes may be diagnosed after the age of 18 years. Type 2 diabetes may develop in obese children. Type 2 diabetics may need insulin if glycaemia cannot be controlled by other measures (Arneson and Brickell.2007).

1.2.2Diagnosis of diabetes mellitus (WHO criteria)

Symptoms of hyperglycemia (e.g. polyuria, polydipsia, unexplained weight loss, visual blurring, genital thrush, lethargy) and raised venous glucose detected once - fasting ≥ 126mg/dl (7 mmol/L) or random ≥ 200mg/dl (11.1 mmol/L) or raised venous glucose on 2 separate occasions - fasting ≥ 126mg/dl (7 mmol/L), random ≥ 200mg/dl (11.1 mmol/L) or oral glucose tolerance test – 2h value ≥ 200mg/dl (11.1 mmol/L) (Bishop*et al.,* 2005).

The diagnostic criteria for diabetes mellitus were modified by the Expert Committee to allow for earlier detection of the disease. According to ADA recommendations, all adults older than age 45 years should have a measurement of fasting blood glucose every 3 years unless the individual is otherwise diagnosed with diabetes. The criteria suggested three methods of diagnosis, each of which must be confirmed on a subsequent day by any one of the three methods. These methods are 1) symptoms of diabetes plus a random plasma glucose level of ≥ 200 mg/dl (11.1mmol/L), 2) a fasting plasma glucose of ≥ 126 mg/dl (7mmol/L), or 3) an oral glucose tolerance test (OGTT) with a 2-hour post-load (75-g glucose load) level ≥ 200 mg/dl (11.1mmol/L). The preferred test for diagnosing diabetes is the measurement of the fasting plasma glucose level(Longmore*et al.,*2007).

1.2.3Complications of diabetes mellitus

Diabetes is a group of chronic diseases characterized by hyperglycemia. Modern medical care uses a vast array of lifestyle and pharmaceutical interventions aimed at preventing and controlling hyperglycemia. In addition to ensuring the adequate delivery of glucose to the tissues of the body,

treatment of diabetes attempts to decrease the likelihood that the tissues of the body are harmed by hyperglycemia (Fowler. 2008).

The importance of protecting the body from hyperglycemia cannot be overstated; the direct and indirect effects on the human vascular tree are the major source of morbidity and mortality in both type 1 and 2 diabetes. Generally, the injurious effects of hyperglycemia are separated into macro vascular complications (coronary artery disease, peripheral arterial disease, and stroke) and micro vascular complications (diabetic nephropathy, neuropathy, and retinopathy). It is important for physicians to understand the relationship between diabetes and vascular disease because the prevalence of diabetes continues to increase in the United States, and the clinical armamentarium for primary and secondary prevention of these complications is also expanding (Fowler. 2008).

1.2.3.1 Acute complication of diabetes mellitus

Acute complication of diabetes mellitus includes:

1.2.3.1.1Hypoglycemia: Probably most common cause of coma seen in diabetes patients

1.2.3.1.2Diabetic ketoacidosis: Diabetic ketoacidosis may be precipitated by infection, acute myocardial infarction or vomiting. The clinical consequences of diabetic ketoacidosis are due to hyperglycemia causing plasma hyperosmolarity.

1.2.3.1.3Hyperosmolal non-ketotic coma: The term hyperosmolar or 'precoma' is usually confined to condition in which there is marked hyperglycemia but not detectable ketoacidosis**(**Marshall and Bangert. 2008**)**.

1.2.3.1.4Lactic acidosis: Lactic acidosis can cause a high anion gap metabolic acidosis and coma and it may be due to the use of metformin in certain situations, such as high doses in very elderly and those with renal, liver, cardiac failure or dehydrated patients**(**Marshall and Bangert. 2008**)**.

1.2.3.2Long term complication of diabetes

Long term complication of diabetes fall into two groups: Micro vascular that is nephropathy, neuropathy and retinopathy. And macro vascular disease related atherosclerosis. This occurs in both types of diabetics **(**Marshall and Bangert. 2008**)**.

1.2.3.2.1Diabetic retinopathy

Diabetic retinopathy may be the most common micro vascular complication of diabetes. .The risk of developing diabetic retinopathy or other micro vascular complications of diabetes depends on both the duration and these verities of hyperglycemia. Development of diabetic retinopathy in patients with type 2 diabetes was found to be related to both severity of hyperglycemia and presence of hypertension, and most patients with type1diabetes develop evidence of retinopathy within20 years of diagnosis. Retinopathy may begin to develop as early as 7years before the diagnosis of diabetes inpatients with diabetes type 2 (Fowler. 2008).

1.2.3.2.2Diabetic nephropathy

Diabetic nephropathy is a clinical syndrome characterized by the following:

- Persistent albuminuria (>300 mg/d or >200 μg/min) that is confirmed on at least 2 occasions 3-6 months apart
- Progressive decline in the glomerular filtration rate (GFR)
- Elevated arterial blood pressure

Proteinuria was first recognized in diabetes mellitus in the late 18th century. In the 1930s, Kimmelstiel and Wilson described the classic lesions of nodular glomerulosclerosis in diabetes associated with proteinuria and hypertension.

By the 1950s, kidney disease was clearly recognized as a common complication of diabetes, with as many as 50% of patients with diabetes of more than 20 years having this complication.

Currently, diabetic nephropathy is the leading cause of chronic kidney disease in the United States and other Western societies. It is also one of the most significant long-term complications in terms of morbidity and mortality for individual patients with diabetes. Diabetes is responsible for 30-40% of all end-stage renal disease (ESRD) cases in the United States.

Generally, diabetic nephropathy is considered after a routine urinalysis and screening for microalbuminuria in the setting of diabetes. Patients may have physical findings associated with long-standing diabetes mellitus.

Good evidence suggests that early treatment delays or prevents the onset of diabetic nephropathy or diabetic kidney disease.

Regular outpatient follow-up is keyin managing diabetic nephropathy successfully.

Recently, attention has been called to atypical presentations of diabetic nephropathy with dissociation of proteinuria from reduced kidney function. Also noted is that microalbuminuria is not always predictive of diabetic nephropathy (Ekinci*et al*., 2013).

1.2.3.2.2.1Pathophysiology

Three major histologic changes occur in the glomeruli of persons with diabetic nephropathy. First, mesangial expansion is directly induced by hyperglycemia, perhaps via increased matrix production or glycation of matrix proteins. Second, thickening of the glomerular basement membrane (GBM) occurs. Third, glomerular sclerosis is caused by intraglomerular hypertension (induced by dilatation of the afferent renal artery or from ischemic injury induced by hyaline narrowing of the vessels supplying the glomeruli). These different histologic patterns appear to have similar prognostic significance.

In addition to the renal hemodynamic alterations, patients with overt diabetic nephropathy (dipstick-positive proteinuria and decreasing glomerular filtration rate (GFR) generally develop systemic hypertension. Hypertension

is an adverse factor in all progressive renal diseases and seems especially so in diabetic nephropathy. The deleterious effects of hypertension are likely directed at the vasculature and microvasculature.

Evidence suggests that hypertension associated with obesity, metabolic syndrome, and diabetes may play an important role in the pathogenesis of diabetic nephropathy. Central obesity, metabolic syndrome, and diabetes lead to increased blood pressure (Hall *et al*., 2004).

1.2.3.2.2.2Prognosis of diabetic nephropathy

Patients in whom proteinuria did not develop have a low and stable relative mortality rate, whereas patients with proteinuria have a 40-fold higher relative mortality rate. Patients with type 1 DM and proteinuria have the characteristic bell-shaped relationship between diabetes duration/age and relative mortality, with maximal relative mortality in the age interval of 34-38 years(Rosolowsky*et al.,* 2011).

1.2.3.2.2.3 Glomerular filtration

Glomerular filtrationis an ultra-filtrate of plasma that is it has a similar composition to plasma except that it is almost free of large proteins. This because the endothelium provides a barrier to red and white blood cell, and abasement membrane although permeable to water and low molecular weight substance, is largely impermeable to macromolecules. This impermeability is related to both molecular size and electrical charge. Protein with molecular weight lower than that of albumin (68 KDa) are filterable, negatively charge molecules are less easily filtered than these bearing a positive charge. Almost all protein in the glomerular filtrate is reabsorbed, with the result that normal urinary protein excretion is <150 mg/24 h.

The normal glomerular filtration rate (GFR) is approximately 120ml/min equivalent to a volume of about 170 L/24h (Marshall *et al.,* 2012).

1.2.3.2.2.4 The biochemical investigation of renal function

Disease affecting the kidney can selectively damage glomerular or tubular function, but isolated disorders of tubular function are relatively uncommon. In acute and chronic renal failure, there is effectively a loss of whole nephrons and, as the process off filtration is essential to the formation of urine, tests of glomerular function are almost invariably required in the investigation and management of any patient with renal disease. The principal function of the glomeruli is to filter water and low molecular weight components of the blood while retaining cells and high molecular weight components (Marshall *et al*., 2012).

1.2.3.2.2.5 Measurement of glomerular filtration rate

An estimate of the GFR can be made by measuring the urinary excretion of substance that is completely filtered from the blood by the glomeruli and is not secreted, reabsorbed or metabolized by the renal tubules.

Clearance= $\frac{U \times V}{P} \boldsymbol{ml/min}$ (Marshall *et al.,* 2012).

Creatinine clearanceis higher than true GFR. The difference is of little significance when the GFR is normal, but when the GFR is low (<10 ml/min), tubular secretion make major contribution to creatinine excretion and creatinine clearance significantly overestimates the GFR. The effect of creatinine breakdown in the gut also become significantly when the GFR is very low. Certain drugs, decrease creatinine secretion and thus can reduce creatinine clearance. Thus measurements of creatinine clearance are potentially unreliable and no longer recommended in routine practice. Alternative methods should be used if a reliable calculation of GFR is required.

There are two main alternative approaches to determining the GFR in clinical practice. These are to use exogenous markers of clearance or derive an estimated GFR (eGFR) from the plasma creatinine concentration. GFR can be

measured by measuring the disappearance from the blood of a test substance that completely filtered by the glomeruli and neither secreted nor reabsorbed by tubules following a single injection. This approach has the advantage that a urine collection is not required (Marshall *et al.,* 2012).

1.2.3.2.2.6Assessment of diabetic nephropathy

The most tests used to assess renal function are those that assess either the GFR or the integrity of the glomerular filtration barrier, and estimate of the GFR can be made by measuring the urinary excretion of substance that is completely filtered from the blood by the glomeruli and is not secreted, reabsorbed or metabolized by the renal tubule (clearance), experimentally inulin (plant polysaccharide) and creatinine are used(Marshall andBangert 2008).

In clinical practice, kidney function is estimated rather than measured. Glomerular filtration rate estimated using serum creatinine (eGFRcr) is the most common approach; however, creatinine is influenced by age, muscle mass, sex, and race (Ferguson and Waikar 2012).

Given these limitations, serum Cystatin C has beenproposed as an alternative filtration marker (Stevens *et al.,* 2008).

1.3Cystatin Cor Cystatin 3

Cystatin C was first described as 'gamma-trace' in 1961 as a trace protein together with other ones (such as beta-trace) in the cerebrospinal fluid and in the urine of patients with renal failure(Grubb andLofberg.1982).

Cystatin C has a low molecular weight (approximately 13.3 kilodaltons), and it is removed from the bloodstream by glomerular filtration in the kidneys. If kidney function and glomerular filtration rate decline, the blood levels of cystatin C rise. Serum levels of cystatin C are a more precise test of kidney function (as represented by the glomerular filtration rate, GFR) than serum creatinine levels(Roos*et al*., 2007; Dharnidharka *et al*., 2002).

Cystatin C is a non-glycosylated, basic protein (isoelectric point at pH 9.3). The crystal structure of cystatin C is characterized by a short alpha helix and a long alpha helix which lies across a large antiparallel, five-strandedbeta sheet. Like other type 2 cystatins, it has two disulfide bonds. Around 50% of the molecules carry a hydroxylatedproline. Cystatin C forms dimers (molecule pairs) by exchanging subdomains; in the paired state, each half is made up of the long alpha helix and one beta strand of one partner, and four beta strands of the other partner (Janowski *et al*., 2001).

Cystatin Cis a low-molecular-weight protein produced by nucleated cells. It is freely filtered by the glomerulus, reabsorbed, and catabolized by the proximal tubule. Produced at a constant rate, levels remain stable if kidney function is normal. Plasma concentrations appear to be unaffected by gender, race, age, and muscle mass. Studies have shown measurement of Cystatin C to be at least as useful as serum creatinine and creatinine clearance in detecting early changes in kidney function. A rise in cystatin C is often detectible before there is a measureable decrease in GFR or increase in creatinine. Cystatin C can be measured by immunoassay methods (Bishop*et a*l., 2010).

1.4Rationale

Diabetes, the most common disease in Sudan, is having an increasing impact on rates of morbidity and mortality in Sudan.The spread of sedentary lifestyles and adoption of western dietary habits–high in refined carbohydrates and fat – are driving an increase in the number of people with obesity-related type 2 diabetes. Knowledge of the diabetes epidemic in Sudan is limited. The most recent data come from a small-scale study that was carried out in 1996. The results of the study indicated a prevalence of 3.4%.But recent estimates place the diabetes population at around one million – around 95% of whom have type2 diabetes.

There was high prevalence of diabetes in our Sudanese communities and the ignorance of its victims in terms of prevention and its attendant complications such as diabetic nephropathy.Serum cystatin C is an alternative to serum creatinine for estimating glomerular filtration rate (GFR), since Cystatin C is less influenced by age and muscle mass.

1.5Objectives

1.5.1 General objective

- To evaluate serumCystatin Clevel among type 2 diabetes mellitus patients in Khartoum state.

1.5.2 Specific objectives:

-To estimate cystatin Cand HbA1c in study groups.

-To compare mean concentration of cystatin Cin case versus control group.

-To compare mean concentration of cystatin C in control with uncontrolled diabetes type 2 patients.

-To correlate between cystatin C and study variables (BMI, age, gender and duration of the disease).

Chapter two

Material and Methods

2.1 Materials

2.1.1 Study Design

Descriptive analytic cross-sectional study, carried out in Khartoum state during the period of March to August, 2015.

2.1.2 Study Area

This study was carried out in Khartoum state.

2.1.3 Study Population

This study includes 120 individual, classified as 60 diabetes mellitus type 2 patients as case and 60 healthy individual as control group.

2.1.4 Inclusion criteria

Patients with diabetes mellitus type 2 as test group and healthy people as control group who admitted for routine chick up.

2.1.5 Exclusion criteria

Patients with renal diseases have been excluded for this study.

2.1.6 Collection of Samples

Samples were collected under septic conditions by using dry, plastic syringes(5ml) in plane containers and (3ml) in EDTA containers from each participants. Serum was obtained by left of samples at room temperature for clotting, and then they were centrifuged at 4000 rpm, and then stored in -20° until the analysis. While blood samples in EDTA containers immediately analyzed.

2.1.7 Ethical Considerations

Study was approved from ethical committee of the Sudan University of Science and Technology, verbal informed consent was obtained and all participants were informed by the aims of the study.

2.2 Methods

(1) Estimation of cystatin C:

Principle

The test uses a sandwich immune –detection method, such that the detection antibody in a detection buffer binds to cystatin C in sample and antigen-antibody complexes are captured to another cystatin C antibody that has been immobilized on a test strep as sample mixture migrates through nitrocellulose matrix. Thus the more cystatin Cantigen in a sample, the more antigen-antibody complexes was accumulated on a test strip. Signal intensity of fluorescence on detection antibody reflects the amount of antigen captured and is processed by ichroma Reader to show the cystatin C concentration in a specimen.

Procedure

- Serum sample (10 µl) was transferred into detection buffer tube, mixed through shaking 10 times.
- From sample mixture (75µl)was taken and dispensed into sample well on the test cartridge and leaved at room temperature for 10 minute for sample loaded.
- The test cartridge inserted into ichroma™ Reader for scanning.
- The button (select) was pressed to start scanning process and the test result read on display screen.

(2) Estimation of HbA1c:

Principle

ichroma HbA1c is based on florescence immunoassay technology, specifically the sandwich immune-detection method.

Whole blood is added to the mixture of hemolysis buffer and detection buffer, which result in hemolysis of red blood cells. Such that by mixing detecting buffer with blood specimen in test tube, the florescence-labeled detector anti-HbA1c antibody in buffer binds to HbA1c antigen in blood specimen. The

sample mixture is loaded and migrates on the matrix of test cartilage; the complexes of detector antibody and HbA1c are captured to anti-HbA1c sandwich pair antibody that has been immobilized on test matrix. As a result, the higher concentration of HbA1c produces a higher florescence signal from HbA1c-antibody complexes. The signal is interpreted and the result display on ichroma Reader in units of %(NGSP).

Procedure

- From hemolysis buffer(100 µl) was added into detection buffer tube.
- Whole blood (5µl) was added to detection buffer tube then shacked 15 times.
- From sample mixture (75 µl) was taken and dispensed into sample well on the test cartridge.
- Test cartridgeinserted into ichamber for 12 minutes then inserted into ichroma™ Reader for scanning.
- The button (select) was pressed to start scanning process and the test result read on display screen.

(3) Calculation of BMI

BMI obtained by calculation according to formula:

$$\text{weight(kg)} \div \text{hight}^2\text{(m)}$$

Chapter three

Results

3. Results

A hundred and twenty randomly samples were collectedto evaluate the level of cystatin C, HbA1c and BMI among study groups, then classified as 60 healthy apparently as control group and 60 type 2 DM as case,males account 23(38.33%) and female 37 (61.67%) with ratio 0f 1:1.6, and participants average age is (51±11SD) years.

Table 3.1 Shows mean concentration of cystatin C in case (1.47 ± 0.849) versus control group (0.83 ± 0.215), with*P*-value = 0.000

Figure 3.1 Shows mean concentration of cystatin C in HbA1c good control (1.10 ± 0.198) and poor control group (1.53 ± 0.910), result expressed as (mean ± SD) and significance increase in serum cystatin C level in poor control groupis considered as *P*-value < 0.004

Figure 3.2 Personal correlation results shows positive correlation between cystatin C and age with R-value0.417 and *P*-value 0.000

Figure 3.3 Personal correlation results shows no correlation between cystatin C and gender with R-value 0.051and *P*-value 0.580

Figure 3.4 Personal correlation results shows positive correlation between cystatin C and duration of DM type 2 with R-value 0.272 and *P*-value 0.0.036

Figure 3.5 Personal correlation results shows positive correlation between cystatin C and BMI with R-value 0.260 and*P*-value 0.004

Figure 3.6 Personal correlation results shows positive correlation between cystatin C and HbA1c with R-value 0.340 and*P*-value 0.008

3.1 Mean concentration of cystatin C in case and control groups:

Variables		Mean ±SD	*P*-value
Cystatin C (mg/l)	Case	1.47 ± 0.849	0.000
	Control	0.83 ± 0.215	

Table 3.1 Shows mean concentration of cystatin C in case versus control groups, results expressed as (Mean±SD) and significance difference considered as *P*-value < 0.05.

3.1Mean concentration of cystatin C in HbA1c good control and poor control groups

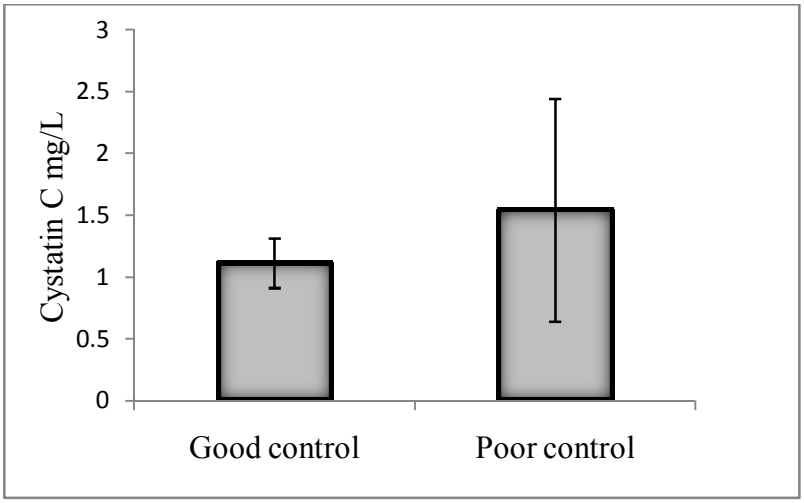

Figure 3.1 Shows mean concentration of cystatin C in good control and poor control groups, result expressed as (Mean±SD) and significance deference considered as *P*-value <0.05.

3.2 Correlation of cystatin C and age:

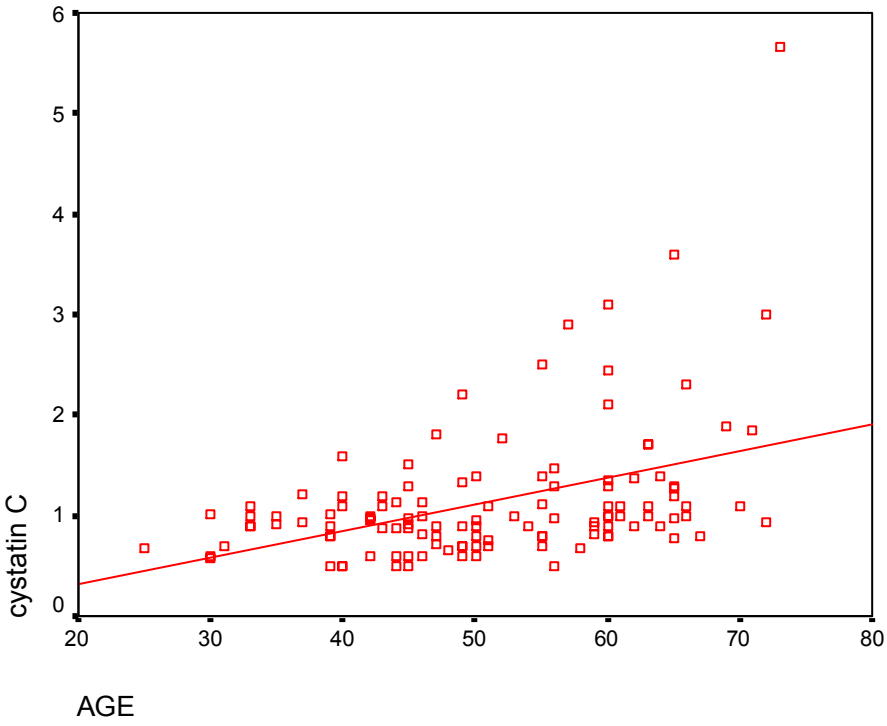

Figure 3.2 Shows personal correlation result of cystatin C versus age

R-value 0.417 = positive correlation.

P-value 0.000 = strength of correlation.

3.3 Correlation of cystatin C and gender:

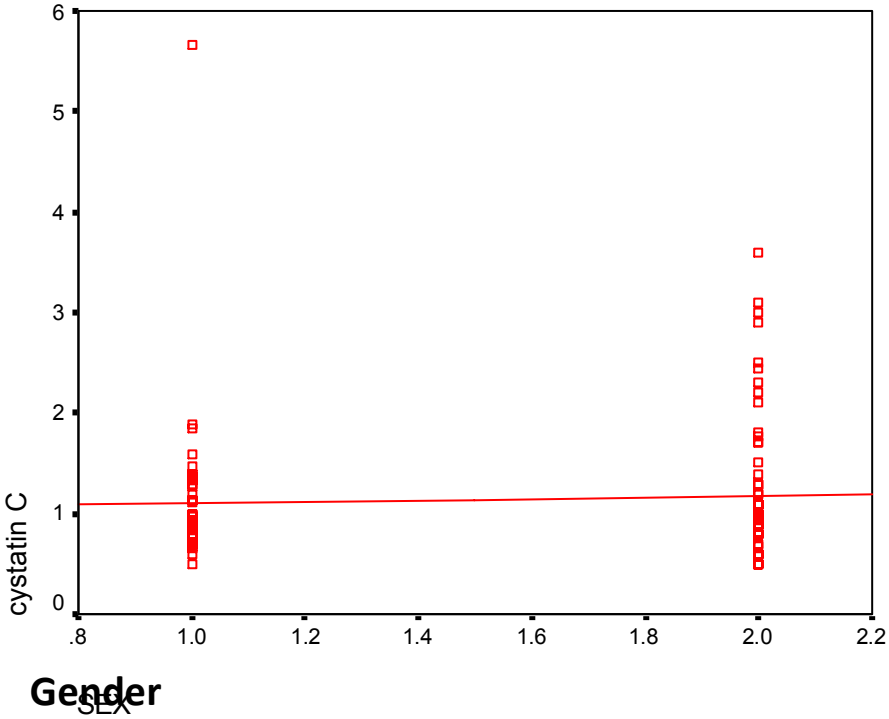

Figure 3.3 Shows personal correlation result of cystatin C versus gender

R-value 0.051 = no correlation.

P-value 0.580 = strength of correlation.

3.4 Correlation of cystatin C and duration of diabetes mellitus:

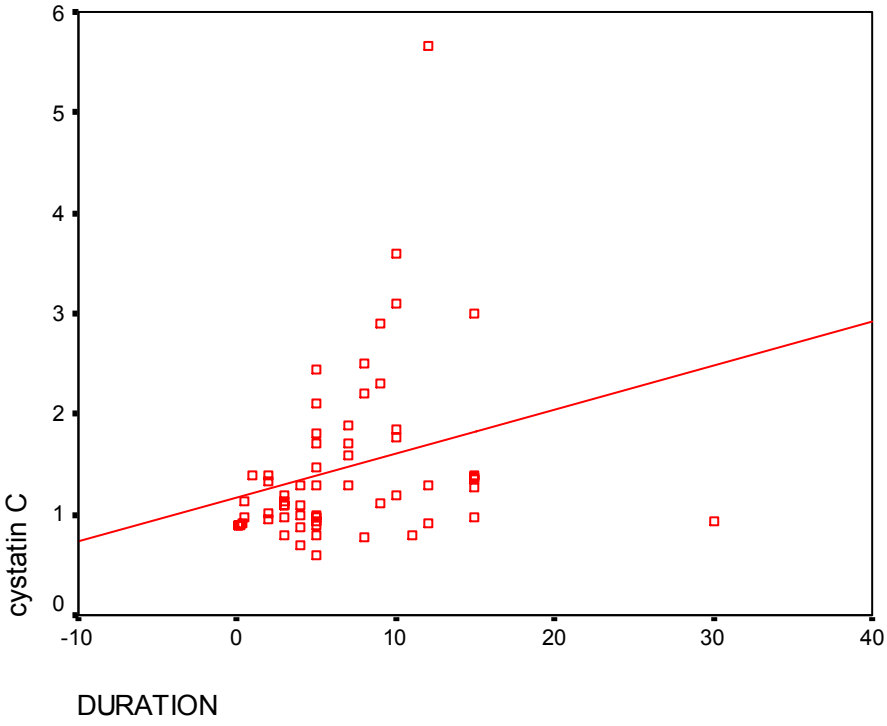

Figure 3.4 Shows personal correlation result of cystatin C versus duration of diabetes mellitus

R-value 0.272 = positive correlation.

P-value 0.036 = strength of correlation.

3.5 Correlation of cystatin C and BMI:

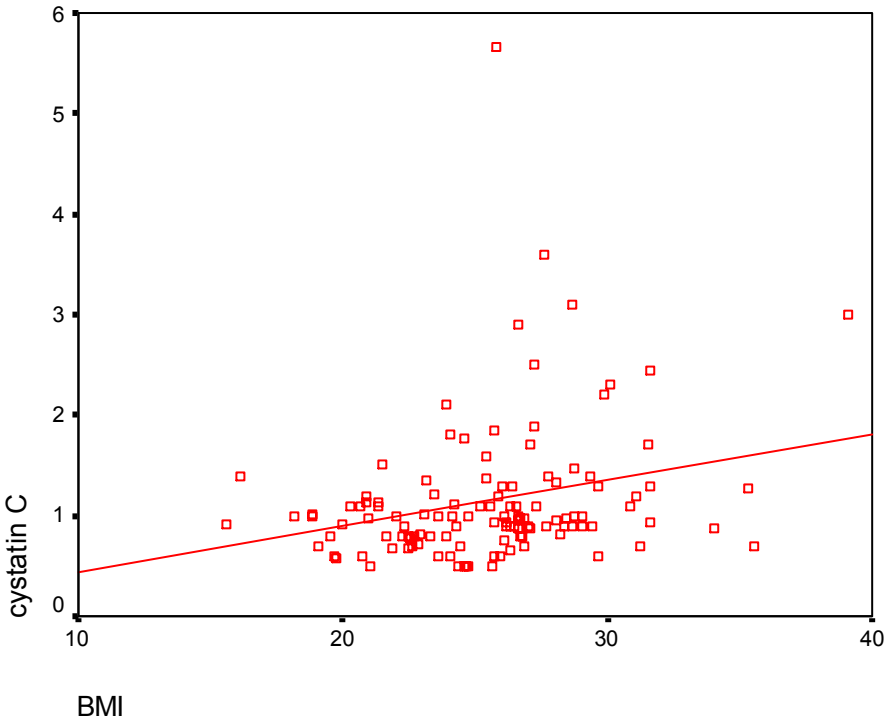

Figure 3.5 Shows personal correlation result of cystatin C versus BMI

R-value 0.260 = positive correlation.

P-value 0.004 = strength of correlation.

3.6 Correlation of cystatin C and HbA1c:

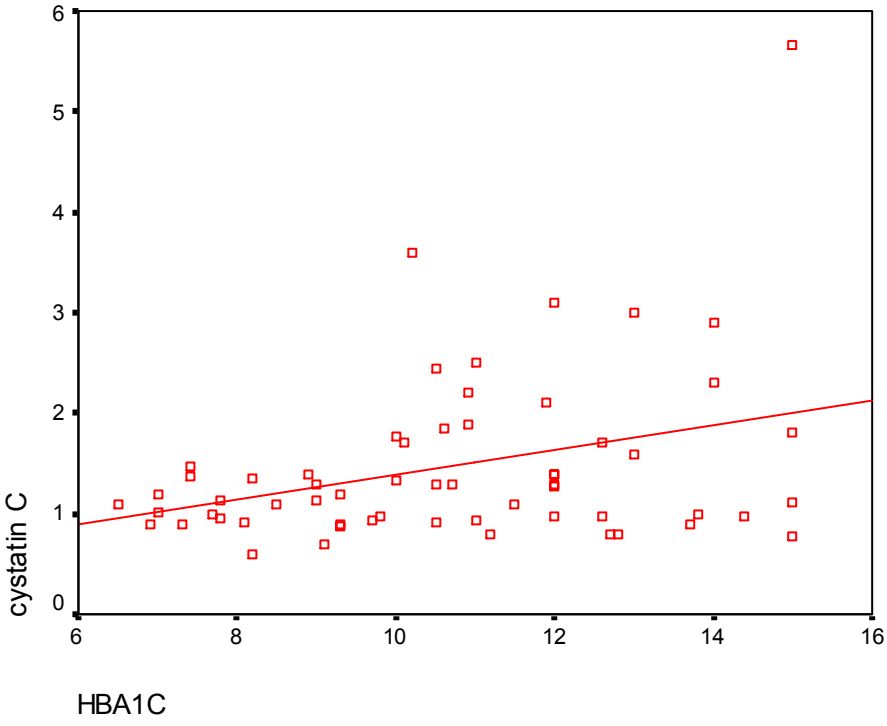

Figure 3.6 Shows personal correlation result of cystatin C versus HbA1c

R-value 0.340 = positive correlation.

P-value 0.008 = strength of correlation.

Chapter four

Discussion, conclusion, and recommendations

4.1 Discussion

Serum cystatin C recently was proposed as a promising alternative marker of GFR owing to better specificity and sensitivity for detecting mildly decreased GFR (Pei *et al*., 2012; Jeon*et al*., 2011) Accordingly the present study carried out to evaluate serum cystatin C level among type 2 DM.

The results of frequency showed that,DM is common in females thanmales with percentage (61.67%) females to (38.33%) males with ratio of 1:1.6.

The results of present study revealed that, there was significant increase inmean concentration of serum cystatin C in type 2 DM when compared with control group with *p*-value 0.000, our finding agreed with previous report that,sera homocystine and Cystatin C levels increased consistently with the development and progression of DN(Wang*et al*., 2013). Serum cystatin C used with high sensitivity and specificity as marker for GFR and severity of diabetes nephropathy, which indicate that, serum cystatin C could be useful early predictor marker for nephropathy in type 2 DM patients.

Researcher previous report stated that,cystatin C results indicate a clear relationship of declining renal function with poor glycemic control (shahid *et al*., 2012), according to previous fact our results found that, there was significant increase in mean serum cystatin C level in poor control type 2 DM patients in comparison with good control with *P*-value 0.004. Consequently high urine osmolality in high blood glucose level causes polyuria and thus hemoconcentration which may result in high serum cystatin level.

In fact that, median cystatin C levels increased steeply with age (Anna, 2008), the results of person's correlation of present study found positive correlation between serum cystatin C and age of type 2 DM patients, with R-value 0.417 and *P*-value0.000.

The present study showed no correlation between serum cystatin C and gender with R-value 0.051 and *P*-value 0.580,agree with previousreport that,serum cystatin C levels were not gender-related (Masatomo*et al*., 2009).

In fact that,the results of the previous study indicated that the risk of DN increases with the duration of DM and patients with type 2 DM are likely to suffer from DN within four years (Rao*et al*., 2014), which agreed with our finding that, the level of serum cystatin C positively correlate with duration of type 2 DM, with R-value0.272 and *P*-value 0.036.In patients with type 2 DM, the long-term hyperglycemia induced glomerular disease and significantly affected cystatin C excretion therefore, DN should be screened.

The results of present study provide experimental evidence that serum cystatin C level positively correlate with BMI with R-value 0.260 and *P*-value 0.004, our finding confirmed by previous report that,there is an association exists between higher BMI and elevated serum cystatin C(Muntner*et al*., 2008), BMI associated with higher lipids which may cause defect in cellular permeability consequently affect GFR.

Present study revealed that, there was positive correlation between serum cystatin C and HbA1c with R-value 0.340 and *P*-value 0.008 whichagree with previous studythat, HbA1c levels were also significantly high in all the patientswith nephropathyas compared to controls (Shahid *et al*., 2012).

4.2 Conclusion

The study concludes that,

1- Serum cystatin C level is higher in type 2 DM specially uncontrolled DM patients.
2- Positivecorrelation between serum cystatin C and age, duration, BMI and HbA1C.
3- As serum cystatin C a marker for GFR it could be useful predictor marker for early detection of diabetes nephropathy especially for obese and uncontrolled DM patients.

4.3 Recommendation

- Diabetes mellitus patients should be monitoring for DN using serum Cystatin C every 3 month.
- DM patients should increase awareness about importance of control blood glucose and its body weight.

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Appendixes

Revision date : July 01, 2015

ichroma™ HbA1c

INTENDED USE

ichroma™ HbA1c is a fluorescence Immunoassay (FIA) for the quantitative determination of HbA1c(Hemoglobin A1c) in human whole blood. It is useful as an aid in management and monitoring of the long-term glycemic status in patients with diabetes mellitus.

For *in vitro* diagnostic use only.

INTRODUCTION

Glycated protein is formed post-translationally through the slow, nonenzymatic reaction between glucose and amino groups on proteins. HbA1c is a clinically useful index of mean glycemia during the preceding 120 days, the average life span of erythrocytes. Carefully controlled studies have documented a close relationship between the concentrations of HbA1c and mean glycemia. HbA1c is considered as a more reliable parameter in monitoring glycemia over the glycemic reading with the conventional glucometer.

PRINCIPLE

The test uses a sandwich immunodetection method; the detector antibody in buffer binds to antigen in sample, forming antigen-antibody complexes, and migrates onto nitrocellulose matrix to be captured by the other immobilized-antibody on test strip.

The more antigen in sample forms the more antigen-antibody complex and leads to stronger intensity of fluorescence signal on detector antibody. Instrument for ichroma™ tests displays the content of glycated hemoglobin in terms of percent of the total hemoglobin in blood.

COMPONENTS

ichroma™ **HbA1c** consists of 'Cartridges', 'Detection Buffer Tubes', 'Hemolysis Buffer Vial' and an 'ID chip'.

- The cartridge contains a test strip, the membrane which has anti human HbA1c at the test line, while rabbit IgG at the control line.
- Each cartridge is individually sealed in an aluminum foil pouch containing a desiccant. 25 sealed cartridges are packed in a box which also contains an ID chip.
- The detection buffer contains anti human HbA1c-fluorescence conjugate, anti rabbit IgG-fluorescence conjugate, bovine serum albumin (BSA) as a stabilizer and sodium azide in phosphate buffered saline (PBS) as a preservative.
- The detection buffer is pre-dispensed in a separate tube. 25 detection buffer tubes are packaged in a box and further packed in a Styrofoam box with ice-pack for the shipment.
- The hemolysis Buffer contains nonionic detergent and sodium azide as preservative in PBS.

WARNINGS AND PRECAUTIONS

- For *in vitro* diagnostic use only.
- Carefully follow the instructions and procedures described in this 'Instruction for use'.
- Use only fresh samples and avoid direct sunlight.
- Lot numbers of all the test components (cartridge, ID chip and detection buffer) must match each other.
- Do not interchange the test components between different lots or use the test components after the expiration date, either of which might yield misleading of test result(s).
- Do not reuse. A detection buffer tube should be used for processing one sample only. So should a cartridge.
- The cartridge should remain sealed in its original pouch before use. Do not use the cartridge, if is damaged or already opened.
- Frozen sample should be thawed only once. For shipping, samples must be packed in accordance with the regulations. HbA1c Sample with severe hemolytic and hyperlipidemia cannot be used and should be recollected.
- Just before use, allow the cartridge, detection buffer and sample to be at room temperature for approximately 30 minutes
- ichroma™ HbA1c as well as the instrument for ichroma™ tests should be used away from vibration and/or magnetic field. During normal usage, it can be noted that instrument for ichroma™ tests may produce minor vibration.
- Used detection buffer tubes, pipette tips and cartridges should be handled carefully and discarded by an appropriate method in accordance with relevant local regulations.
- The mixture of Detection Buffer and Hemolysis buffer must be used within 1 hour after mixing.
- An exposure to larger quantities of sodium azide may cause certain health issues like convulsions, low blood pressure and heart rate, loss of consciousness, lung injury and respiratory failure.
- ichroma™ HbA1c will provide accurate and reliable results subject to the following conditions.
  - Use ichroma™ HbA1c should be used only in conjunction with instrument for ichroma™ tests.
  - Any anticoagulants other than EDTA, heparin sodium, sodium citrate should be avoided.

STORAGE AND STABILITY

- The cartridge is stable for 20 months (while sealed in an aluminum foil pouch) if stored at 4 - 30°C.
- The detection buffer pre-dispensed in a tube is stable for 20 months if stored at 2 - 8°C.
- The hemolysis buffer dispensed in a vial is stable for 20 months if stored at 4 - 30°C.
- After the cartridge pouch is opened, the test should be performed immediately.

LIMITATIONS OF THE TEST SYSTEM

- The test may yield false positive result(s) due to the cross-reactions and/or non-specific adhesion of certain sample components to the capture/detector antibodies.
- The test may yield false negative result. The non-responsiveness of the antigen to the antibodies is most common where the epitope is masked by some unknown components, so as not to be detected or captured by the antibodies. The instability or degradation of the antigen with time and/or temperature may cause the false negative as it makes antigen unrecognizable by the antibodies.
- Other factors may interfere with the test and cause erroneous results, such as technical/procedural errors, degradation of the test components/reagents or presence of interfering substances in the test samples.
- Any clinical diagnosis based on the test result must be supported by a comprehensive judgment of the concerned physician including clinical symptoms and other relevant test results.
- The test conditions for ichroma™ HbA1c are as follow.
  - Temperature : 20~30°C
  - Humidity : 10~70%

양식-GE02-15 (Rev. 02)

MATERIALS SUPPLIED

REF CFPC-38

Components of **ichroma™ HbA1c**

- Cartridge Box:
  - Cartridges 25
  - ID Chip 1
  - Instruction For Use 1
- Box containing Detection Buffer tubes
  - Detection Buffer Tubes 25
- Hemolysis Buffer Vial
  - Hemolysis Buffer Vial (3 mL) 1

MATERIALS REQUIRED BUT SUPPLIED ON DEMAND

Following items can be purchased separately from **ichroma™ HbA1c**.

Please contact our sales division for more information.

- Instrument for ichroma™ tests
  - **ichroma™ Reader** REF FR203
  - **ichroma™ D** REF 13303
- **ichroma™ Printer** REF FPRR007
- **ichroma™ HbA1c Control** REF CFPO-6
- **5 µL Capillary tube** REF CFPO-19

SAMPLE COLLECTION AND PROCESSING

The sample type for **ichroma™ HbA1c** is human whole blood.

- It is recommended to test the sample within 24 hours after collection.
- Samples may be stored for up to a week at 2-8°C prior to being tested.
- If testing will be delayed more than a week, samples should be frozen at -70°C or below. Samples stored frozen at -70°C or below for 3 months showed no performance difference.
- Once the sample was frozen, it should be used one time only for test, because repeated freezing and thawing can result in the change of test values.

INTERPRETATION OF TEST RESULT

- Instrument for ichroma™ tests calculates the test result automatically and displays HbA1c concentration of the test sample in terms of % (NGSP), mmol/mol (IFCC), mg/dL (eAG).
- The cut-off (reference range)
  - NGSP (%) : 4.5~6.5 %
  - IFCC (mmol/mol) : 26~48 mmol/mol
- Working range
  - NGSP (%) : 4~15 %
  - IFCC (mmol/mol) : 20.2~140.4 mmol/mol
  - eAG (mg/dL) : 68.1~383.8 mg/dL

QUALITY CONTROL

- Quality control tests are a part of the good testing practice to confirm the expected results and validity of the assay and should be performed at regular intervals.
- The control tests should be performed immediately after opening a new test lot to ensure the test performance is not altered.
- Quality control tests should also be performed whenever there is any question concerning the validity of the test results.
- Control materials are not provided with **ichroma™ HbA1c**. For more information regarding obtaining the control materials, contact Boditech Med Inc.'s Sales Division for assistance.

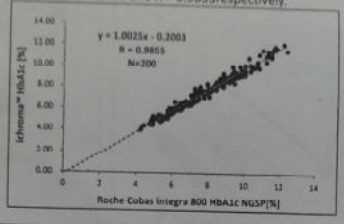
(Please refer to the instruction for use of control material.)

PERFORMANCE CHARACTERISTICS

- **Specificity**: There, in test samples, are biomolecules such as Gentisicacid (200mg/ml), Bilirubin (20mg/ml), Triglyceride (3000mg/dl), Ascorbic acid (5mg/dl) and Glucose (300mg/ml) in higher concentration than their normal physiological levels. But this doesn't interfere with the **ichroma™ HbA1c** test measurements, nor occurs any significant cross-reactivity.
- **Precision**: The intra-assay precision was calculated by one evaluator, who tested different concentration of control standard ten times each with three different lots of **ichroma™ HbA1c**. The inter-assay precision was confirmed by 3 different evaluators with 3 different lots, testing ten times each different concentration.

HbA1c (%)	Intra-assay			Inter-assay		
	Mean	SD	CV(%)	Mean	SD	CV(%)
5.4	5.34	0.08	1.58	5.34	0.15	2.82
8.4	8.39	0.15	1.90	8.41	0.17	2.04

- **Comparability**: HbA1c concentrations of 200 clinical samples were quantified independently with **ichroma™ HbA1c** and Roche Cobas integra800 as per prescribed test procedures. Test results were compared and their comparability was investigated with linear regression and coefficient of correlation (R). Linear regression and coefficient of correlation between the two tests were Y=1.0025X - 0.2003 and R = 0.9855respectively.



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Note: Please refer to the table below to identify various symbols

	Read instructions for use
	Use by
LOT	Batch code
REF	Catalog number
	Caution
	Manufacturer
	Authorized representative of the European Community
IVD	In vitro diagnostic medical device
	Temperature limit
	Do not reuse
CE	This product fulfills the requirements of the Directive 98/79/EC on in vitro diagnostic medical devices

For technical assistance; please contact:

Boditech Med Inc.'s Technical Services

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E-Mail: mail@obelis.net

CE

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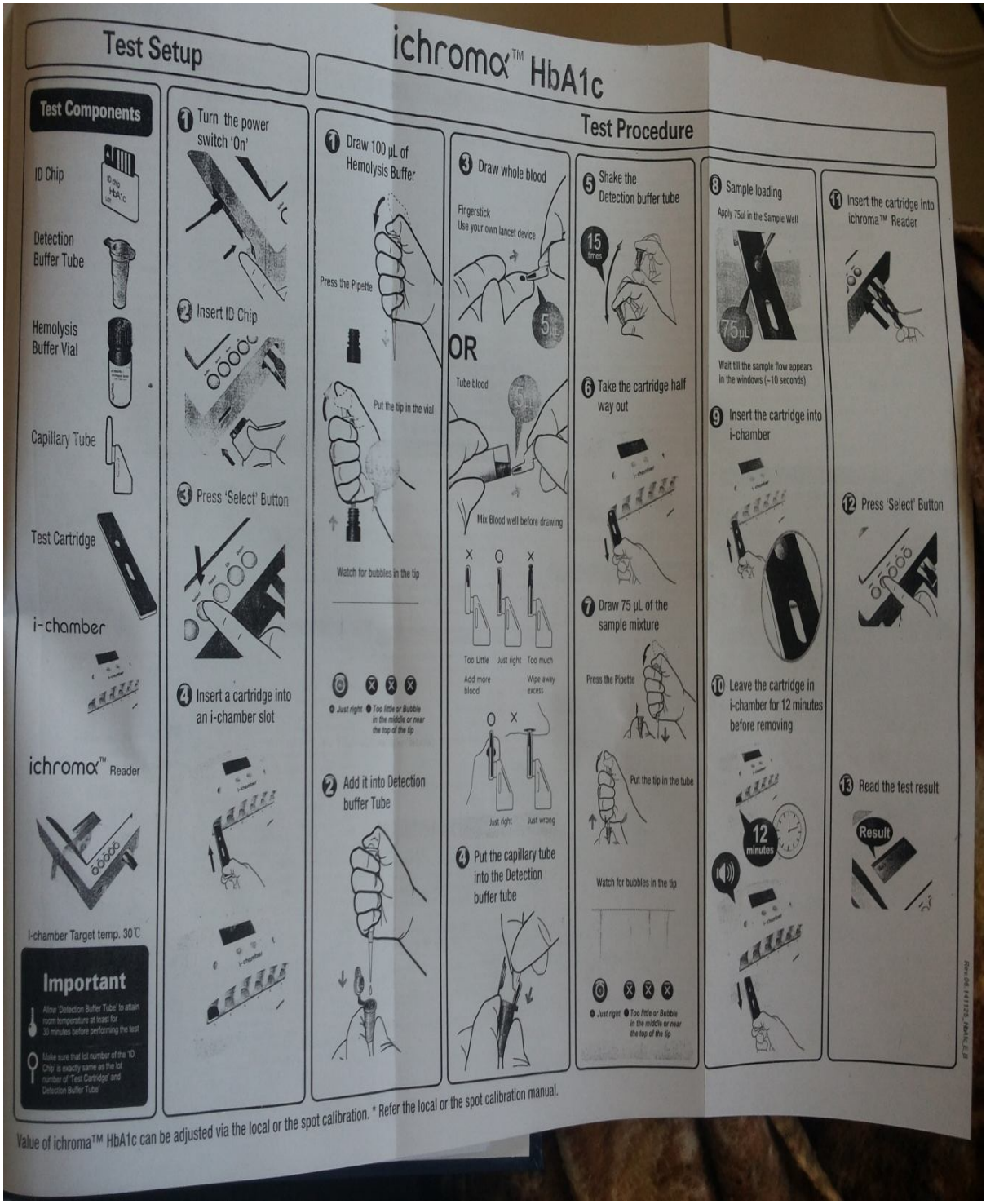
Test Setup
ichroma™ HbA1c
Test Procedure
Test Components
ID Chip
Detection Buffer Tube
Hemolysis Buffer Vial
Capillary Tube
Test Cartridge
i-chamber
ichroma™ Reader
i-chamber Target temp. 30℃
Important
Allow 'Detection Buffer Tube' to attain room temperature at least for 30 minutes before performing the test
Make sure that lot number of the 'ID Chip' is exactly same as the lot number of 'Test Cartridge' and 'Detection Buffer Tube'
1 Turn the power switch 'On'
2 Insert ID Chip
3 Press 'Select' Button
4 Insert a cartridge into an i-chamber slot
1 Draw 100 µL of Hemolysis Buffer
Press the Pipette
Put the tip in the vial
Watch for bubbles in the tip
Just right
Too little or Bubble in the middle or near the top of the tip
2 Add it into Detection buffer Tube
3 Draw whole blood
Fingerstick
Use your own lancet device
5
OR
Tube blood
5 µL
Mix Blood well before drawing
Too Little
Just right
Too much
Add more blood
Wipe away excess
Just right
Just wrong
4 Put the capillary tube into the Detection buffer tube
5 Shake the Detection buffer tube
15 times
6 Take the cartridge half way out
7 Draw 75 µL of the sample mixture
Press the Pipette
Put the tip in the tube
Watch for bubbles in the tip
Just right
Too little or Bubble in the middle or near the top of the tip
8 Sample loading
Apply 75ul in the Sample Well
75µL
Wait till the sample flow appears in the windows (~10 seconds)
9 Insert the cartridge into i-chamber
10 Leave the cartridge in i-chamber for 12 minutes before removing
12 minutes
11 Insert the cartridge into ichroma™ Reader
12 Press 'Select' Button
13 Read the test result
Result
Value of ichroma™ HbA1c can be adjusted via the local or the spot calibration. * Refer the local or the spot calibration manual.

SudanUniversity for Science and Technology

College of Graduate Studies

Clinical Chemistry

Questionnaire

Name: ……...NO ( )

Age: ……...years

Sex: Male ☐ Female ☐

Weight: ……..……kg

Height: ……..meter

Type IIdiabetic patients: ☐

Duration of diabetes mellitus: ……years

Kidney diseases: ☐

Other diseases: ……..

Serum cystatin C: ……..mg/l

HbA1c: …… %

BMI: ……

Date: ……