Estimation of Glomerular Filtration Rate
Using Cystatin C in Diabetic Patient for Possible Development
of Chronic Kidney Disease in
Asmara, Eritrea

Rustom Zeratsion Woldu Foto

B.Sc in Clinical Laboratory Sciences    University of Asmara
Eritrea, 2002

A Dissertation
Submitted for the partial fulfillment of the requirements for the degree of
M.Sc in clinical chemistry
Faculty of medical laboratory sciences,
Department of clinical chemistry
University of Gezira, Wad medeni, Sudan
February 2015
Estimation of Glomerular Filtration Rate
Using Cystatin C in Diabetic Patient for Possible Development
of Chronic Kidney Disease in
Asmara, Eritrea

Rustom Zeratsion Woldu

Supervision Committee:

<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr Shamsedein Mohammed Ahmed</td>
<td>Main Supervisor</td>
<td>..................</td>
</tr>
<tr>
<td>Dr Omer Mohammed Abdelagi</td>
<td>Co-supervisor</td>
<td>..................</td>
</tr>
</tbody>
</table>

Date: February, 2015
Estimation of Glomerular Filtration Rate
Using Cystatin C in Diabetic Patient for Possible Development of Chronic Kidney Disease in Asmara, Eritrea

Rustom Zeratsion Woldu

Examination Committee:

<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Shamsedein Mohammed Ahmed</td>
<td>Chair Person</td>
<td>............</td>
</tr>
<tr>
<td>Dr. Omer Balla Ibrahim Abddelgader</td>
<td>External Examiner</td>
<td>............</td>
</tr>
<tr>
<td>Dr. Abo baker Hassan Ataelmola</td>
<td>Internal Examiner</td>
<td>............</td>
</tr>
</tbody>
</table>

Date of Examination: 15/02/2015
I hereby declare this dissertation entitled “Estimation of glomerular filtration Rate in Diabetic Patient for Possible Development of Chronic Kidney Disease in Asmara, Eritrea” submitted by me under supervision and guidance of Dr. Shamsedein M. Ahmed, Assistant professor of clinical chemistry, University of Gezira for partial fulfillment for the award of the degree of MASTERS IN CLINICAL CHEMISTRY at Gezira University, Wad-Medeni, Sudan is original and done by my own effort, that it was not submitted in part or full for any degree and that all the sources that i have used have been indicated and acknowledged by means of complete references.
Dedication

To my father To my mother
To my brothers, sisters, and friends
Acknowledgment

The Eritrean Ministry of Health for giving me this opportunity of participating on this master’s program.

I wish to express my deep sincere thanks to my supervisor Dr. Shamsedein Mohammed Ahmed, and Dr Omer Mohammed Abdelbagi for their valuable support, advice, suggestions, and continuous encouragement. Working with them was a privilege.

I would like to acknowledge Prof. Altyeb M. Abdelmalik the support and encouragement as well as the carnage and hospitality to make this golden opportunity into reality.

Dr. Bakri Yousif, Dean, Faculty of Medical Laboratory sciences, University of Gezira, Department heads, Instructors, administrative staff and students of this Master’s program for their cooperation in making the study successful.

I am grateful to the help and support offered by the staff of Wad medeni Renal Hospital Lab especially Amid and Mohamed for helping me in my practical. Also I am indebted to MR's Abir, stuff of Clinical Chemistry for her guidance and fruit full advice.

I should acknowledge the help and support received from Dr. Asmerom Tesfamariam for his suggestions during the topic choosing, correction, valuable advice as well as unconditional support. Mr. Rezene and Dr Habte and all stuff of diabetic clinic as well as Mr. Asmerom Sium, Head of NHL, and other NHL stuff, Mr. Sereke Head of ONRHL Lab. and Mr. Amanuel Tekle head of HNRHL and Mr. Abream stuff of HNRHL, and Mr. Kbreab Petros for their assistance during sample collection and Mr. Ykealo, Stuff of ONRHL Dep"l clinical chemistry, Mr. Hermon NHL clinical chemistry lab. for participating on sample analysis using sophisticated autoanalizers.

I am very grateful to the patients who participated in the study from the three States; also we thank the local heath authority in Gezira state.

My thanks are extended to all those who help me and were not mentioned in this acknowledgment.
Estimation of Glomerular Filtration Rate
Using Cystatin C in Diabetic Patient for Possible Development
of Chronic Kidney Disease in
Asmara, Eritrea

Rustom Zeratsion Woldu

Abstract

Renal insufficiency in one of the most common complication in diabetic patients and in clinical practice renal function status is assessed using serum creatinine or its derivative eGFR. Recently Cystatin C is believed to be more sensitive for assessing renal function. A cross sectional descriptive study on 100 known 51 female ad 49 male diabetic patients whom are on regular follow up at diabetic clinic in Eritrea from Oct 2014 to Jan 2015. The aim of the study was to evaluate the renal function status and glycemic control of diabetic patients based on serum creatinine, Cystatin C, and HbA₁C, respectively. Demographic data was analyzed using structured questioner and serum creatinine, Cystatin C, HbA₁C and fasting blood glucose was determined. Data was calculated using SPSS version 19. Their age range from 12-89years old with mean (SD) 56(16.3)years and the serum creatinine ranges from 0.5-1.5 mg/dl and only 2 diabetic patients had abnormal serum creatinine, on the other hand serum Cystatin C ranges from 0.5 to 2.5mg/dl and about 50 diabetic patients with high level above normal reference value. About 47 cases has poor glycemic control (HbA₁C > 7.6%). Serum creatinine based GFR had shown about 75% of the cases fall on the normal range (>90ml/min/1.73m²) However, based on serum Cystatin C only 22% of the cases fall on normal range and the rest were distributed into minimal, moderate, and high risk group stage. The study concluded that, serum Cystatin C based assessment is more sensitive and accurate to evaluate the kidney function status in diabetic patients and the study recommend that it is high time to introduce it in clinical practice to monitor the diabetic patient for possible risk and early detection of renal complication and further study can be performed in large sample size.
قياس معدل التشخيص الكببي لدى المرضى المصابين بداء السكري لمعرفة أمكانية تطور أمراض الكلى المزمنة في اسمرا، اريتريا

رستم زراتيون ولدو فوتو

ملخص الدراسة

يعتبر القصور الكلوي من أكثر المضاعفات شيوعا عند مرضى السكري ويستخدم قياس الكرياتينين في المصل وقياس معدل التشخيص الكببي في المعامل لقياس وظائف الكلى وتحديداً داخل قياس السيستاتين في المصل لإثارة أكثر حساسية. أجريت هذه الدراسة الوصفية التخطيطية على 100 شخص مصاب بداء السكري 51 امرأة و49 رجل يحضرون عيادة السكري في اريتريا في الفترة من أكتوبر 2014 إلى يناير 2015.

هندت الدراسة لمعرفة وظائف الكلي قياس مستوى الكرياتينين والسيستاتين سي في المصل لدى المرضى المصابين بالسكري وأيضا معرفة مستوى التحكم في السكر عن طريق قياس الهيمولوبيين المرتبط بالجلوكوز والسكري الساري. اخذت عينات دم وريدى لأجراء الاختبارات المعملىة في جوانين دم تحتوي على مواد مائعة للتجلط وأخرى من غير مواد. تم قياس الكرياتينين والهيمولوبيين المرتبط بالجلوكوز والدم الساري بطرق أزمنية والسيستاتين سي بطريقة مناعية إشعاعية. حلت الانتظار لمساءلة باستخدام برنامج الحزم الإحصائية للعلوم الأساسية 19 وكان العمر من 12 - 89 سنة. ومستوى الكرياتينين بين 0.5 إلى 1.5 مجم/ديس لوك و 50 هنالك عينتين أخرى من المعالم الطبيعي، ومستوى السيستاتين سي يتراوح بين 0.5 إلى 2.5 مجم/ديس و50 شخص اعلى من المعالم الطبيعي. و47 شخص مستوي التحكم في الجلوكيز لديهم غير جديد. و75 شخص كان معدل التشخيص الكببي باستخدام الكرياتينين في المصل في المعالج الطبيعي (90 مل/ دقيقة/1.73 متر²) متوسط 30.9 ملم/ دقيقة/1.73 متر². و22 شخص كان معدل التشخيص الكببي عند استخدام مستوي السيستاتين في السيرير في المعالج الطبيعي والباقي وزعوا على مراحل أمراض الكلي المزمنة. تلخصت الدراسة التي أن السيستاتين سي أكثر حساسية من قياس الكرياتينين ودقة في تقدير وظائف الكلي عند المرضى المصابين بداء السكري. أوصت الدراسة بانحل قياس السيستاتين سي كاختبار روتيني في المعالج وإجراء دراسة أخرى باختيار حجم عينة أكبر.
# Table of contents

<table>
<thead>
<tr>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supervision committee</td>
<td>ii</td>
</tr>
<tr>
<td>Examination committee</td>
<td>iii</td>
</tr>
<tr>
<td>Declaration</td>
<td>iv</td>
</tr>
<tr>
<td>Dedication</td>
<td>v</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>vi</td>
</tr>
<tr>
<td>Abstract in English</td>
<td>vii</td>
</tr>
<tr>
<td>Abstract in Arabic</td>
<td>viii</td>
</tr>
<tr>
<td>Table of content</td>
<td>ix</td>
</tr>
<tr>
<td>List of tables</td>
<td>xii</td>
</tr>
<tr>
<td>List of figures</td>
<td>xiii</td>
</tr>
<tr>
<td>List of abbreviation</td>
<td>xvi</td>
</tr>
<tr>
<td>List of abbreviation</td>
<td>xv</td>
</tr>
</tbody>
</table>

## Chapter one

1. Introduction  
1.1 General Information  
1.1.1 Diabetics as major risk factor for kidney disease  
1.1.2 The glomerular filtration rate  
1.2 Justifications  
1.3 Objectives  
1.3.1 General objectives  
1.3.2 Specific objective  

## Chapter Two

2.1 The Literature review  
2.1.1 Kidney Anatomy and Physiology  
2.1.1.1 Kidney anatomy  
2.1.1.2 Kidney physiology  
2.1.1.2.1 Glomerular filtration  
2.1.1.2.2 Tubular function  
2.1.1.2.3 Loop of Henle  
2.1.1.2.4 Distal Convoluted tubule  
2.2 Disorders of Kidney  
2.2.1 Immunologic /Non-Immunologic  
2.2.2 Acute Renal Disease  
2.2.2.1 Type of Acute Renal Disease  
2.2.3 Chronic Kidney Disease  
2.2.3.1 Stages of progression  
2.3 Biochemical Testes  
2.3.1 Measurement of Clearance  
2.3.1.1 Exogenous Substances  
2.3.1.1.1 Inulin
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3.1.1.2 Creatinine Clearance measurement</td>
<td>16</td>
</tr>
<tr>
<td>2.3.2 estimation of glomerular filtration rate (GFR)</td>
<td>17</td>
</tr>
<tr>
<td>2.3.2.1 Creatinine</td>
<td>17</td>
</tr>
<tr>
<td>2.3.2.2 Urea</td>
<td>18</td>
</tr>
<tr>
<td>2.3.2.3 Blood Urea Nitrogen (BUN)</td>
<td>18</td>
</tr>
<tr>
<td>2.3.2.4 Low molecular weight proteins as GFR markers</td>
<td>19</td>
</tr>
<tr>
<td>2.3.2.4.1 Cystatin C</td>
<td>19</td>
</tr>
<tr>
<td>2.4 Treatment of chronic kidney disease</td>
<td>22</td>
</tr>
<tr>
<td>2.5 Diabetic Mellitus</td>
<td>22</td>
</tr>
<tr>
<td>2.5.1 Definition of Diabetics</td>
<td>22</td>
</tr>
<tr>
<td>2.5.2. Types of Diabetics</td>
<td>23</td>
</tr>
<tr>
<td>2.5.2.1 Type i Diabetics</td>
<td>23</td>
</tr>
<tr>
<td>2.5.2.2 Type ii Diabetics</td>
<td>23</td>
</tr>
<tr>
<td>2.5.2.3 Gestational Diabetics</td>
<td>24</td>
</tr>
<tr>
<td>2.5.2.4 Pre-Diabetics</td>
<td>24</td>
</tr>
<tr>
<td>2.5.3 Sign and Symptoms of Diabetics</td>
<td>24</td>
</tr>
<tr>
<td>2.5.4 Temporary Hypoglycemia</td>
<td>25</td>
</tr>
<tr>
<td>2.6 Hemoglobin A1C (HbA1C)</td>
<td>25</td>
</tr>
<tr>
<td>2.6.1 Hemoglobin A1c (Glycosylated hemoglobin) test</td>
<td>25</td>
</tr>
<tr>
<td>2.6.2 Importance of HbA1C test</td>
<td>25</td>
</tr>
<tr>
<td>2.6.3 Normal (health) level of HbA1c</td>
<td>26</td>
</tr>
<tr>
<td>2.7 Diagnosis of Diabetics</td>
<td>26</td>
</tr>
<tr>
<td>2.7.1 Fasting blood Glucose test</td>
<td>26</td>
</tr>
<tr>
<td>2.7.2 Diagnosis of diabetics by HbA1C test</td>
<td>26</td>
</tr>
<tr>
<td>2.7.3 Hemoglobin A1C level for diabetic patient</td>
<td>27</td>
</tr>
<tr>
<td>2.8 Country Background</td>
<td>28</td>
</tr>
<tr>
<td>2.8.1 Diabetic mellitus in Eritrea</td>
<td>28</td>
</tr>
</tbody>
</table>

Chapter Three

3 Methodology | 29
3.1 Study design | 30
3.2 Study population | 30
3.2.1 Inclusion criteria | 30
3.2.2 Exclusion criteria | 30
3.3 Ethics consideration | 30
3.4 Data collection | 30
3.5 Sample collection | 30
3.5.1 Measurement of serum creatinine | 31
3.5.2 Hemoglobin A1C measurement | 31
3.5.3 Serum Cystatin C measurement | 32
3.6 Materials | 33
3.6.1 Materials Reagents and equipments | 33
3.7 Data analysis and calculations | 34
3.7.1 eGFR calculation by Cockroft-Gault equation for age below 18 years | 34
3.7.2 Equations used to calculate eGFR specific to sex | 34
3.7.2.1 Creatinine equation by EP-CKD 2009 | 34
3.7.2.2 Cystatin C equation by EP-CKD 2012 | 35
2-9-3 Creatinine/Cystatin C equation by EP-CKD 2012(Mix) | 35

Chapter Four
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 Result and Discussions</td>
<td>36</td>
</tr>
<tr>
<td>4.1 Results</td>
<td>37</td>
</tr>
<tr>
<td>4.1.1 Characteristics of participants</td>
<td>37</td>
</tr>
<tr>
<td>4.1.2 Estimated eGFR and group distribution</td>
<td>39</td>
</tr>
<tr>
<td>4.1.3 Concordance between estimated GFR</td>
<td>41</td>
</tr>
<tr>
<td>4.2 Discussions</td>
<td>44</td>
</tr>
<tr>
<td>Chapter Five</td>
<td></td>
</tr>
<tr>
<td>5 Conclusion and Recommendations</td>
<td>46</td>
</tr>
<tr>
<td>5.1 Conclusion</td>
<td>47</td>
</tr>
<tr>
<td>5.2 Recommendation</td>
<td>48</td>
</tr>
<tr>
<td>Reference</td>
<td>49</td>
</tr>
</tbody>
</table>
List of tables

Table 2.1: Combine Kidney Function Stage (stage 1-5) with description of kidney damage (albuminuria) and clinical diagnosis to specify CKD fully (e.g., Stage 2 CKD with microalbuminuria, secondary to diabetic kidney disease). NKF 2013.

Table 4.1: Summary of participants characteristics parameters according age categories in diabetic patients at two diabetic clinics in Eritrea.

Table 4.2: provides average (SD) of all participants respective according to age category of eGFR using (EP-CKD 2009, EP-CKD SCy C2012 and EP-CKD Mix)ml/min/1.73m², at two diabetic clinics in Eritrea.

Table 4.3: provides the estimated GFR value of all participants according to age group distribution, along with possible risk stage range depending on GFR estimate value based on three epidemiological collaborated formulas used as reference for calculation the eGFR ml/min/1.73m², at two diabetic clinics in Eritrea.

Table 4.4: Spearman and Pearson correlation 1 in bold shows significant correlation in two tail test. the closer to 1 the best correlation between the two as shown between EP-CKD SCy C 2012 and EP-CKD Mix 2012 at two diabetic clinics in Eritrea.


Table 4.6, Test of linearity/Two-Tailed Test): provides average (SD) of all participants respectively according to age category of eGFR using (EP-CKD 2009, EP-CKD SCy C2012 and EP-CKD Mix)ml/min/1.73m², at two diabetic clinics in Eritrea.
List of figures

Figure 2.1: Anatomy of kidney...............................................................6
Figure 2.2: Glomerular filtration system.................................................9
Figure 2.3: The nephron and its main functional parts..............................9
Figure 2.4: The structure of human Cystatin C......................................20
Figure 2.5: Schematic diagram of eGFR reading for CKD stages.............21
Figure 2.6: The Eritrea country picture show six zonal administration.....28
Figure 3.1: diagram representation of test principle in ichroma™ reader testing..............................................................33
Figure 4.1: Sammary of % of eGFR three EP-CKD formula based on creatinine and Cystatin C in Bar graph.................................................41
Figure 4.2: Regretion graph showing linearity between EP-CKD SCy2012 & EP-CKD Mix.................................................................42
List of annexure

1. Demography and screening questionnaire ........................................... 54
2. Sample collection Form ........................................................................ 56
3. List of tables .......................................................................................... 57
   1. Table 4.7 EP-CKD SCy C 2012 category age category Cross table....... 57
   2. Table 4.8 Estimated GFR for overall participants with in respective age
group category .............................................................................................. .58
   3. Table 4.9 Cross tabulation Cystatin C level distribution ..................... 58
   4. Table 4.10, Chi-Square Tests correlation of Cystatin C level to Duration
category ...........................................................................................................
   5. Table 4.11 Chi-Square Tests of SCr level to duration category ............ 58
   6. Table 4.12 EP-CKD 2012MIX category * Age category Cross table provides
the percentage of estimated GFR for overall participants ......................... 59
   7. Table 4.13, The normal eGFR value: in adults, the normal eGFR value
is more than 90 (According the NKF 2014) .............................................. 59
   8. Table 4.4 provides average (SD) of all participants respective to age
group .............................................................................................................. 60

4. Test principle and procedure of creatinine measurement .................... 61
5. Test principle and procedure of Cystatin C ichromaTM reader ............ 61
4. Test principle and procedure for direct enzymatic test HA1c ............. 65
5. Index of figure of BD test tube containers use and test pictures ........... 66
6. Test process in DXC 600 Roche analyzer .............................................. 67
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKI</td>
<td>Acute Kidney Injury</td>
</tr>
<tr>
<td>ADH</td>
<td>Antidiuretic Hormone</td>
</tr>
<tr>
<td>ARF</td>
<td>Acute Renal Failure</td>
</tr>
<tr>
<td>BTP</td>
<td>β-Trace Protein</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic Kidney Disease</td>
</tr>
<tr>
<td>Cr-51 EDTA</td>
<td>Chromium 51 Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Cys C</td>
<td>Cystatin C</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
</tr>
<tr>
<td>DHS</td>
<td>Demographic and Health Survey</td>
</tr>
<tr>
<td>DKD</td>
<td>Diabetic Kidney Disease</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ENDA</td>
<td>Eritrean National Diabetic Association</td>
</tr>
<tr>
<td>EP-CKD</td>
<td>Epidemiology Cooperation Chronic Kidney Disease</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-Stage Renal Disease</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimation of Glomerulus Filtration Rate</td>
</tr>
<tr>
<td>NKF</td>
<td>National Kidney Federation</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerulus Filtration Rate</td>
</tr>
<tr>
<td>HZRHL</td>
<td>Halibet Zonal Referral Hospital Laboratory</td>
</tr>
<tr>
<td>IDF</td>
<td>International Diabetics Federation</td>
</tr>
<tr>
<td>IFCC</td>
<td>International Federation of Clinical Chemistry</td>
</tr>
<tr>
<td>KDIGO</td>
<td>Kidney Disease Improving Global Outcomes</td>
</tr>
<tr>
<td>KEEP</td>
<td>Kidney Early Evaluation Program</td>
</tr>
<tr>
<td>MOH</td>
<td>Ministry of Health</td>
</tr>
<tr>
<td>NHL</td>
<td>National Health Laboratory</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>NKDEP</td>
<td>National Kidney Disease Education Program</td>
</tr>
<tr>
<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
</tr>
<tr>
<td>ONRHL</td>
<td>Orotta National Referral Hospital Laboratory</td>
</tr>
<tr>
<td>PREVEND</td>
<td>Prevention of Renal and Vascular End-Stage Disease</td>
</tr>
<tr>
<td>PETIA</td>
<td>Enhanced Turbidimetric Immunoassay</td>
</tr>
<tr>
<td>RRT</td>
<td>Renal Replacement Therapy</td>
</tr>
<tr>
<td>SCr</td>
<td>Serum Creatinine</td>
</tr>
<tr>
<td>SCy</td>
<td>Serum Cystatin C</td>
</tr>
<tr>
<td>Tc-99m DTPA</td>
<td>Technetium-99m Diethylene Triamine Pentaacetic Acid</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>HMIS</td>
<td>Health Management Information System</td>
</tr>
</tbody>
</table>
CHAPTER ONE

INTRODUCTION
1.1 General introduction

Chronic kidney disease (CKD) is associated with a decrease in kidney function that is often progressive. It affects millions of adults in the world, and its prevalence is rising, particularly in the elderly (National kidney Foundation, 2002). CKD can be seen with a variety of conditions, including diabetes and high blood pressure. Diabetic kidney disease is a decrease in kidney function that occurs in some people who have diabetes. It means that the kidneys are not doing their job as well as they once did to remove waste products and excess fluid from the body. These wastes can build up in your body and cause damage to other organs. (Michael A. Ferguson1 and Sushrut S. Waikar. 2012) The risk factors have been linked to increased risk of developing this disease: high blood pressure, poor blood glucose (sugar) control, inherited tendency and diet control. Early detection of kidney dysfunction can help to minimize the damage. This is important because, in early stages, there may not be noticeable symptoms of kidney disease until as much as 30-50% of kidney function is lost. (Bethesda, MD 2009)

A wide variety of tests can be done to tell if a person has developed kidney disease or not, but measuring GFR is considered the most accurate way to detect changes in kidney status, however, measuring it directly is complicated, requires experienced personnel, and is typically performed only in research settings (Nickolas TL, Frisch GD, Opotowsky AR, et al. 2004). These were not very sensitive tests because it did not begin to change until the patient develops more severe disease. Because of this, the eGFR is usually used, and was calculated based on serum creatinine. With the creatinine test, a reasonable estimate of the actual GFR can be determined. Furthermore, to guarantee quality and a precise eGFR determination, other substances, such as cystatin C, are being explored. (Bethesda, MD, 2000)

1.1.1 Diabetes as major risk factor for kidney disease

Diabetes Mellitus often called ‘Diabetics’ or same times ‘Sugar’ is a condition that occur when the body an unable to make enough insulin or when the body cannot use normal amount of insulin properly( NKF. 2013). Insulin is a hormone that regulates the amount of sugar in the body. A high blood sugar level can cause problems to many parts of the body. The most common type of diabetes is type1 and type 2. Type 1 account 10% of the cases and usually occur in childhood, whereas Type2 is the most common type of diabetes and usually occur in people above 45years. Uncontrolled diabetic can cause damage to many parts of the body, especially
Kidney, Heart, Eyes and Nerves. The diabetic may damage the blood vessel of the kidney; the first sign of kidney damage is the presence of albumin in urine. (McClellan WM et al 1991)

1.1.2 The glomerular filtration rate (GFR)

The glomerular filtration rate (GFR) is the most widely used test of renal function and assessment of kidney disease, which is the rate at which the kidneys filter impurities from the blood stream. (Michael L. Bishop et al 2010) It is calculated by measuring the volume of plasma that can be cleared of a particular substance within a unit of time. The gold standard for GFR measurement requires intravenous injections of exogenous radio labeled substances such as Cr-51-EDTA and Tc-99m-DTPA, or iodinated agents such as iohexol. These procedures are invasive, risky, and time consuming. Alternatively, endogenous substances freely filtered by the glomerulus with minor absorption and secretion by the renal tubules have been used for estimating GFR (Bethesda, MD, 2000). Estimation of glomerular filtration rate (eGFR) is generally performed using serum creatinine. Despite its application, it must be adjusted for factors such as muscle mass, age, gender, diet and race which limit its clinical reliability. Decreased GFR (GFR<60 ml/min per 1.73m2) has been associated with increased mortality, cardiovascular adverse events, hospitalizations, fractures, and unsuccessful aging. International guidelines recommend using creatinine-based equations to estimate GFR, particularly the modification of diet in renal disease equation.(McClellan WM et al 1991, DeOreo PB 1997, Giatras I, Lau J 1997)

However, all creatinine-based estimating equations have limitations due to non GFR determinants of serum creatinine, largely muscle mass, which cannot be accounted for entirely by age, sex, and race. This is a particular problem was among the elderly, non-white populations, and in the range of mildly reduced GFR, where equations have bias. Therefore, the clinician’s reliance on creatinine-based equations for estimating GFR and the risk associated with low GFR could cause misclassification of patients who may be at high risk of CKD and its complications. (Pereira BJ, 2000). Cystatin C is independent of these factors and can provide a more precise calculation of GFR combination with creatinine or alone, without needing adjustment like serum creatinine.

It is a low molecular weight (13.4 kDa) protein produced at a constant rate by all human cells containing a cell nucleus(Laterza OF, Price CP, Scott MG. 2002). It has a stable production rate unaffected by inflammatory processes, gender, age, diet and nutritional status. It is freely filtered through the glomerular membrane, and is reabsorbed and almost entirely catabolised in the proximal tubules. Kidney dysfunction will cause a variation in its level and can thus be used to
give an immediate and accurate measurement of GFR. The reference interval for Ichroma™ It assay is between 0.52 and 1.0 mg/L. In patients with kidney dysfunction the its level may be raised above 1.1 mg/L. Extensive, well-documented studies comparing serum creatinine and Cystatin C as markers of GFR have demonstrated its superiority over creatinine. (Brady H.R, et al 2000)

In particular, it respond more quickly to changes in GFR than creatinine. Normal serum Cystatin C level is the same for men, women and children, and it does not have the “blind-area” from which creatinine suffers. Early signs of kidney disease are general and the use of this as a marker is important to confirm the onset of kidney disease. It cannot be stressed too highly that renal disease and renal failure are irreversible conditions giving a seriously reduced quality of life. (Cotran R.S.et al, 1999)
1.2 Justifications

Despite the increasing burden of chronic diseases like diabetics, in the world and the fact that they have now become diseases of poor people in most settings, the needs of these patients have remained largely unmet (National kidney Foundation, 2002). Recent studies showed that, 50% of the total disease burden in 2005 and were associated with higher estimated death rates in low and middle income countries than in high income countries.

In the world 387 million people have diabetes; by 2035 this will rise to 592 million. The number of people with type 2 diabetes is increasing in every country, and 77% of people with diabetes live in low- and middle-income countries this includes African countries. The greatest number of people with diabetes are between 40 and 59 years of age. 175 million people with diabetes are undiagnosed, diabetic caused 4.9 million deaths in 2014; Every seven seconds a person dies from diabetes. In Africa, 76% of deaths due to diabetes are in people under the age of 60.( IDF Diabetic Atlas 2013)

The total number of patients with diabetes, one of the most common chronic diseases, is expected to climb from 110 million in 2000 to 317 million by 2030 (according to the World Health Organization (WHO) estimates 2014).

According to Eritrean National Diabetes Registry record, the prevalence was estimated to be 2.2% based on patient information in 2004. while, The prevalence of hypertension in the general population was 16%. More than 80% of the hypertensive persons were not aware of their condition. No significant rural/urban or sex difference was seen in hypertension prevalence. This time, according the physicians in diabetic clinic the number of registered diabetic patient estimated to reach 7000-10,000(Berhane Seyum, et al. 2009).

In practice, serum or plasma creatinine are the most widely used endogenous markers of glomerular filtration. The sensitivity of serum creatinine in the detection of CKD is poor and it will fail to identify half of the patients with crucial stage 3 CKD (GFR of 30-59 ml/min/1.73m2) as serum creatinine concentration may not change until approximately 50% of the kidney function has been lost. Due to the many other problems encountered with measurements of creatinine and its use as a GFR estimate. (Bethesda, MD. 2009)
Cystatin C has been proposed as an alternative marker of renal function. Studies have shown that it may be more sensitive in identifying mild reductions in kidney function than serum creatinine (Pereira BJ, 2000, Brady H.R et al 2000).

1.3 Objectives

1.3.1 General objective
To assess the renal function status and glycemic control of known diabetic patients in regular follow up at diabetic clinics in Eritrea.

1.3.2 Specific objectives

- To determine serum Cystatin C for diabetic patients without previous history of renal complication in Eritrea.
- To determine serum creatinine concentration for the study group.
- To estimate glomerulus filtration rate (eGFR) using epidemiological collaborated formulas for chronic kidney disease (EP-CKD) based on serum Cystatin C and creatinine test results.
- To introduce Cystatin C as a base line screening tool of renal function status in clinical practice and ratify the diabetic management guidelines in Eritrea.
CHAPTER TWO

THE LITERATURE REVIEW
2.1 Kidney anatomy

The kidneys are paired, bean-shaped organs located retroperitoneal on each side of the spinal column. Macroscopically, a fibrous capsule of connective tissue enclosed each kidney, when dissected longitudinally, an outer region called the cortex and an inner region called the medulla (see Fig. 2.1). Pelvis, a basin like cavity at the upper end of the ureter into which newly formed urine passes. The bilateral ureters are thick-walled canals, connecting the kidneys to the urinary bladder. The functional unit of kidney, nephron seen only microscopically. Each kidney contains approximately 1 million nephrons in which each apparatus comprised of five basic parts. (Michael Bishop et al, 2010) These includes; The glomerulus, a capillary tuft surrounded by the expanded end of a renal tubule known as Bowman's Capsule. each is supplied by an afferent efferent arterioles carrying blood in and out respectively. The proximal convoluted tubule, located in the cortex. The long loop of Henle, composed of the thin descending limb, which spans medulla, and the ascending limb, which is located in both medulla and cortex. The distal convoluted tubule, located in the cortex. The collecting duct, formed by two or more distal convoluted tubules. (Burton D Rose. et al. 2011)

![Figure 2.1](image)

**Figure 2.1**, The anatomy of kidney showing its main functional unit nephron and blood supply system.

2.2 Kidney physiology
The kidney performs a multitude of essential functions to maintain homeostasis. In clinical medicine, glomerular filtration rate (GFR) provides the best index of overall kidney function, and proteinuria adds additional information on renal and nonrenal prognosis. There are three basic renal processes (fig. 2.2) (Burton D Rose, et al. 2011).

**Figure 2.2** The three main process of nephron, glomerular filtration, tubular reabsorption and secretion.

### 2.1.2.1, Glomerular filtration

The glomerulus is the first part of the nephron and function to filter incoming blood. Glomeruli are tiny filters in the kidney nephron that allow waste product to be removed from the blood while preventing loss of important constituents. *(NFK, 2005)* Several factors facilitate filtration. One factor is high pressure in the glomerular capillaries, which is the result of their position between two arterioles. *(Carol Porth 2014).*
The semipermeable glomerular basement membrane, which has a molecular size cutoff value of approximately 66,000Da. About molecular size of albumin, this means that water, electrolyte and dissolved solutes, like glucose, amino acids low molecular-weight proteins, urea, and creatinine, pass freely through the basement. Other, plasma constituents, such as albumin; many plasma proteins; cellular elements; and protein bound substance such as lipids and bilirubin are large enough to be filtered. In addition, because the basement membrane is negatively charged, negatively charged molecules, such as proteins, are repelled. The glomerulus filters out 126ml-130 ml of an essential protein free, cell free fluid, called glomerular filtrate. The volume of blood filtered per minute per the glomerular filtration rate (GFR), and its determination is essential in evaluating renal function. see Fig. 2.3(Carol Porth 2014).

2.1.2.2, Tubular function

The tubules are where next part of nephron, where the bulk of each valuable substance return buck to the circulation. Thus, 75% of water, sodium, and chloride; 100% of the glucose; almost all amino acids, vitamins, and proteins; varying amount of urea, uric acid, ions such as magnesium, calcium, potassium, and bicarbonates are reabsorbed. (Burton D Rose. et al. 2011) Almost all (98%-100%) uric acid, a waste product, is actively reabsorbed and secreted at the distal end of proximal tubule. When the substance move from the tubular lumen to peritubular capillary plasma, the process is called tubular reabsorption.(Carol Porth 2014) The plasma concentration above which the substance appears in urine is known as renal threshold, and it's determination useful in tubular function assessment. A second function of the proximal tubule is to secret products of kidney tubular cell metabolism, such as hydrogen ions, and drugs like penicillin. (Michael Bishop et al, 2010 )

2.1.2.3, Loop of Henle (countercurrent multiplier system)
the loop of henle is the site where, reabsorption of water, sodium, and chloride facilitated. The interaction of water leaves descending loop and sodium and chloride leave ascending loop produce hypoosmolar urine as it leaves the loop and called *Countercurrent multiplier system*. (Carol Porth 2014).

2.1.2.4, Distal convoluted tubule
The function of this tubule is to affect small adjustment to achieve electrolyte and acid-base homeostasis. These occur under the hormonal control both Antidiuretic hormone (ADH) and aldosterone. (Carol Porth 2014).

2.2 Disorders of kidney
Disorders throughout the body can affect renal function and produce abnormalities in the urinalysis. Considering that the major function of the kidneys is filtration of the blood to remove waste products. It becomes evident that the kidneys are consistently exposed to potentially damaging substances.

Renal disease is often classified; as being *glomerular, tubular* based on the area of the kidney primarily affected. In this study, the most commonly encountered disorders will be covered in relation to the affected areas of the kidney, keeping in mind that some overlap will occur.(Susan K. Strasinger 5th Ed 2008)

2.2.1, Immunologic / Non-immunologic causes
Kidney disorders are immunologic (immuneorigin) and non-immunologic, The majority of the disorders associated with the glomerulus were of immune origin, resulting from immunologic disorders throughout the body, including the kidney. Immune complexes formed as a result of immunologic reactions and increased serum immunoglobulins, such as immunoglobulin A (IgA), circulate in the bloodstream and are deposited on the glomerular membranes. (Cotran R.S.et al. 2000) Components of the immune system, including Complements, Neutrophil, Lymphocytes, Monocytes, and Cytokines, are then attracted to the area, producing changes and damage to the membranes. Depending on the immune system mediators involved, damage may consists of cellular infiltration or proliferation resulting in thickening of the glomerular basement membrane,
and complement-mediated damage to the capillaries and basement membrane (Woodson BW. et al. 2013).

Non-immunologic causes of glomerular damage include exposure to chemicals and toxins that also affect the tubules, disruption of the electrical membrane charges as occurs in the nephrotic syndrome, deposition of amyloid material from systemic disorders that may involve chronic inflammation and acute-phase reactants, and the basement membrane thickening associated with diabetic nephropathy. (Gansevoort RT, et al. 2006)

2.2.2. Acute renal disease

Acute renal failure (ARF), is characterized by sudden loss of the ability of the kidneys to excrete wastes, concentrate urine, conserve electrolytes, and maintain fluid balance, is a frequent clinical problem, particularly in the intensive care unit, where it is associated with a mortality of between 50% and 80%. (Robert W. Schrier, 2004)

The most common indicator of acute renal failure is azotemia, (an accumulation of nitrogenous wastes in the blood). In acute renal failure the glomerular filtration rate (GFR) is decreased. As a result, excretion of nitrogenous wastes is reduced and fluid and electrolyte balance cannot be maintained. Persons with acute renal failure often are asymptomatic, and the condition is diagnosed by observation of elevations in blood urea nitrogen (BUN) and creatinine. (1) (Levy E.M., Viscose 1996)

2.2.2.1 Types of acute renal failure

Acute renal failure can be caused by several types of conditions, including a decrease in blood flow without ischemic injury; ischemic, toxic, or obstructive tubular injury; and obstruction of urinary tract outflow. The causes of acute renal failure commonly are categorized as pre-renal (55% to 60%), post-renal (<5%), and intrinsic (35% to 40%). (Brady H.R.2000)

1. Pre-renal failure: Pre renal failure is the most common form of acute renal failure, is characterized by a marked decrease in renal blood flow. It is reversible if the cause of the decreased renal blood flow can be identified and corrected before kidney damage occurs. Causes of pre-renal failure include profound depletion of vascular volume (e.g., hemorrhage, loss of extracellular fluid volume), impaired perfusion caused by heart failure and cardiogenic shock, and decreased vascular filling because of increased vascular capacity (e.g., anaphylaxis or sepsis). Elderly persons are particularly at risk because of their predisposition to hypovolemia and their high prevalence of renal vascular disorders. Some vasoactive mediators, drugs, and diagnostic agents, Examples include hypercalcemia, endotoxins, and radiocontrast agents such as those used for cardiac catheterization. (Brady H.R.2000)
2. **Post-renal failure:** Post-renal failure is results from obstruction of urine outflow from the kidneys. The obstruction can occur in the ureter (i.e., calculi and strictures), bladder (i.e., tumors or neurogenic bladder), or urethra (i.e., prostatic hypertrophy). Prostatic hyperplasia is the most common underlying problem. Because both ureters must be occluded to produce renal failure, obstruction of the bladder rarely causes acute renal failure unless one of the kidneys already is damaged or a person has only one kidney.

*The treatment of acute post-renal failure*; consists of treating the underlying cause of obstruction so that urine flow can be re-established before permanent nephron damage occurs.

3. **Intrinsic renal failure:** Intrinsic or intrarenal renal failure results from conditions that cause damage to structures within the kidney—glomerular, tubular, or interstitial. Injury to the tubules is most common and often is ischemic or toxic in origin. The major causes of intrarenal failure are ischemia associated with pre-renal failure, toxic insult to the tubular structures of the nephron, and intra-tubular obstruction. Acute glomerulonephritis and acute pyelonephritis also are intrarenal causes of acute renal failure. Acute tubular necrosis (ATN), is the most common cause of intrinsic renal failure, which is characterized by destruction of tubular epithelial cells with acute suppression of renal function. (Carol M. Porth 2011)

### 2.2.3 Chronic renal failure

Unlike acute renal failure, chronic renal failure represents progressive and irreversible destruction of kidney structures. As recently as 1965, many patients with chronic renal failure progressed to the final stages of the disease and then died. The high mortality rate was associated with limitations in the treatment of renal disease and with the tremendous cost of ongoing treatment. In 1972, federal support began for dialysis and transplantation through a Medicare entitlement program. Technologic advances in renal replacement therapy (i.e., dialysis therapy and transplantation) have improved the outcomes for persons with renal failure. In the United States, there are approximately 400,000 persons with end-stage renal disease who are living today, a product of continued research and advances in treatment methods. (National Kidney and Urological Information Center. (2001))

Chronic renal failure can result from a number of conditions that cause permanent loss of nephrons, including diabetes, hypertension, glomerulonephritis, and polycystic kidney disease. Typically, the signs and symptoms of renal failure occur gradually and do not become evident until the disease is far advanced. This is because of the amazing compensatory ability of the kidneys. As kidney structures are destroyed, the remaining nephrons undergo structural and functional hypertrophy, each increasing its function as a
means of compensating for those that have been lost. It is only when the few remaining nephrons are destroyed that the manifestations of renal failure become evident.

Regardless of cause, chronic renal failure results in progressive deterioration of glomerular filtration, tubular reabsorptive capacity, and endocrine functions of the kidneys. All forms of renal failure are characterized by a reduction in the GFR, reflecting a corresponding reduction in the number of functional nephrons. (Carol M. Porth 2011)

**2.3.3.1 Stages of progression;**

The progression of chronic renal failure usually occurs in four stages: diminished renal reserve, renal insufficiency, renal failure, and end-stage renal disease. (Cotran R. S 1999) CKD has been defined as decreased kidney function and/or kidney damage persistent for at least 3 months. Kidney dysfunction is indicated by a glomerular filtration rate (GFR) of less than 60 ml/min/1.73 m2, while kidney damage most frequently is manifested as increased urinary albumin excretion.2 Within this framework, CKD has been categorized into five stages determined by Scr/Scy (blind are) see table 2.1 below.

- **a, Diminished renal reserve:** Diminished renal reserve occurs when the GFR drops to approximately 50% of normal. At this point, the serum BUN and creatinine levels still are normal, and no symptoms of impaired renal function are evident. This is supported by the fact that many persons survive an entire lifetime with only one kidney. Because of the diminished reserve, the risk for development of azotemia increases with an additional renal insult, such as that caused by nephrotoxic drugs.

- **b, Renal insufficiency:** Renal insufficiency represents a reduction in the GFR to 20% to 50% of normal. During this stage, azotemia, anemia, and hypertension appear. Signs and symptoms of renal insufficiency do not begin to appear until more than 50% of the function in both kidneys is lost. As nephrons are destroyed, the remaining nephrons compensate for those that are lost by filtering more solute particles from the blood. Because the solute particles are osmotically active, they cause additional water to be lost in the urine. One of the earliest symptoms of renal insufficiency is isosthenuria or polyuria with urine that is almost isotonic with plasma.4 Conservative treatment during this stage includes measures to retard the deterioration of renal function and assist the body in managing the effects of impaired function. Because the kidneys have difficulty eliminating the waste products of protein metabolism, a restricted-protein diet usually produces fewer symptoms and slows progression of renal failure. The few remaining nephrons that constitute the functional reserve of the kidneys can be easily disrupted; at that point, renal failure progresses rapidly.
**c. Renal failure:** Renal failure develops when the GFR is less than 20% of normal. At this point, the kidneys cannot regulate volume and solute composition, and edema, metabolic acidosis, and hyperkalemia develop. These alterations affect other body systems to cause neurologic, gastrointestinal, and cardiovascular manifestations.

**d. End-stage renal disease:** End-stage renal disease (ESRD) occurs when the GFR is less than 5% of normal. Histologic findings of an end-stage kidney include a reduction in renal capillaries and scarring in the glomeruli. Atrophy and fibrosis are evident in the tubules. The mass of the kidneys usually is reduced. At this final phase of renal failure, treatment with dialysis or transplantation is necessary for survival. (Carol M. Porth 2011)

Table 2.1, Combine Kidney Function Stage (stage 1-5) with description of kidney damage (albuminuria) and clinical diagnosis to specify CKD fully (e.g., Stage 2 CKD with microalbuminuria, secondary to diabetic kidney disease).NKF 2013.

<table>
<thead>
<tr>
<th>Kidney damage stage</th>
<th>Description</th>
<th>GFR</th>
<th>Other findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal or minimal damage with normal GFR</td>
<td>&gt;90</td>
<td>Protein or albumin in urine are high, cell or cast seen in urine</td>
</tr>
<tr>
<td>2</td>
<td>Mild decrease in GFR</td>
<td>60-89</td>
<td>Protein or albumin in urine are high, cell or cast seen in urine</td>
</tr>
<tr>
<td>3</td>
<td>Moderate decrease in GFR</td>
<td>30-59</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Severe decrease in GFR</td>
<td>15-29</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Kidney failure</td>
<td>&lt;15</td>
<td></td>
</tr>
</tbody>
</table>

* The GFR number tells doctor how much kidney function. As chronic kidney disease progresses, the GFR number decreases.

**2.3 Biochemical tests**

The clinical chemistry offers Several tests available that assess various aspect of nephron function, including glomerular filtration and proximal and distal tubular secretion and reabsorption. These biochemical markers are measured in serum, urine, and other body fluids. (Wendy A, & Jean Brickrll 2007)
2.3.1 Measurements of clearance

2.3.1.1 Exogenous substance
Measured GFR using urinary or plasma clearance of exogenous filtration markers is considered the gold standard for evaluation of kidney function but is not routinely available because of the complexity of measurement protocols. (Lesley A. Stevens 2009)
Characteristics for Measurement of clearance is a substance that can be completely eliminated into the urine per unit of time but not reabsorbed or secreted. (Tietz N.W. 2006).

2.3.1.1.1. Inulin
Inulin is a polymer of fructose found in tubers, is an exogenous substance that fulfills the characteristics outlined above. The classic method for using Inulin clearance to measure GFR described by Homer Smith requires early morning testing in a fasting state, oral fluid loading to promote diuresis, bladder catheterization to ensure complete urine collection, continuous Inulin infusion at a constant rate, and multiple urine and blood collections once a steady state has been achieved (Gansevoort RT., et al 2006).

Inulin clearance is then calculated from the plasma concentration, urine concentration, and urine flow rate. Inulin clearance is still regarded as the gold standard for the measurement of GFR, although it is rarely used clinically because of the restricted availability of Inulin and invasiveness of the procedure. Currently, Inulin measurement is not offered in most clinical laboratories. Therefore, clearance-based protocols that use other markers are currently employed when measured GFR is desired. (Rock RC. et al. 2002) Timed urine collections may be performed to estimate creatinine clearance, which is an approximation of GFR. Typically, a 24-h urine collection is performed with a single blood draw shortly before or after the collection to measure serum creatinine. Shorter timed collections may be appropriate for hospitalized individuals with rapidly changing renal function (Narva A. et al, 2007).

The major concern with 24-h urine collections from outpatients is the possibility of over- or under-collections, which substantially limits their reliability. Plasma clearance methods may be employed in the assessment of GFR. Testing typically involves the injection of an exogenous marker in a single bolus dose and measuring the plasma disappearance of the marker by using serial blood draws over a period of several hours. These methods obviate the need for a urine collection and are typically completed in a shorter period of time than conventional timed urine clearance measurement. Markers currently in use include a number of radioactive [99m Tc– diethylenetriamine pentaacetic acid (DTPA), 51 Cr-EDTA, 125 I-iothalamate] and nonradioactive (iohexol and iothalamate) substances. Single-injection methods to measure plasma clearance of each of these markers have been compared against urinary clearance of
Inulin (Jones CA, 2007, Nissenson AR., 2001) for the measurement of GFR. Radionuclide markers have the advantage of ease of measurement, which must be balanced against the disadvantage of radiation exposure and the requirement for facilities to appropriately store and dispose of radioactive materials. The use of unlabeled iothalamate and iohexol eliminate the issues related to radiation (Jones CA et al, 2007).

2.3.1.2 Creatinine Clearance and GFR
Calculation of creatinine clearance has become the standard laboratory method to determine GFR. This value is derived by mathematically relating the serum creatinine concentration to the urine creatinine concentration excreted during a period of time, usually 24 hours. A 24 hours urine specimen ideally collected at the mead point and a serum creatinine used. the concentration of creatinine in both serum and urine is measured by applicable methods (Dr Patrick Garrett 2009)

\[
\frac{U_{cr} \text{ (mg/dl)} \times V_{cr} \text{ (ml/24hour)}}{P_{cr} \text{ (mg/dl x 1440minutes/24hour)}} \times \frac{1.73}{A}
\]

Eq. 2.1 Creatinine Clearance measurement.

where: \(C_{cr}\) is creatinine clearance, \(U_{cr}\) is urine creatinine clearance, \(V_{cr}\) is urine volume excreted in 24hours, \(P_{cr}\) is serum creatinine concentration, and 1.73/A normalization factor.

2.3.2, Estimation of glomerular filtration rate(eGFR)

2.3.2.1, Creatinine
Creatinine is formed at a relatively constant rate as a result of the non-enzymatic dehydration of muscle creatine and is therefore roughly proportional to muscle mass. Creatinine is freely filtered by the glomerulus and is not reabsorbed by the renal tubules; however, it is secreted at variable rates. Drugs such as cimetidine and trimethoprim inhibit tubular secretion of creatinine. More problematic is the fact that tubular secretion of creatinine is increased proportionally relative to its glomerular filtration as kidney function declines, resulting in a significant overestimation of true GFR. As a result, an increase in serum creatinine may not be observed until a substantial decrease in GFR has occurred. Additional limitations to the use of serum creatinine to estimate GFR arise from the substantial variability in between-person and within-person creatinine generation. In an attempt to account for this variation, several serum creatinine–based equations have been developed to estimate GFR, the most notable being the Cockcroft–Gault, Modification of Diet in Renal Disease (MDRD), and CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equations for adults and the Schwartz equation for children. Although these equations generally increase the reliability of estimating the GFR, they all have limitations. For example, the MDRD equation is known to underestimate the GFR, particularly at lower creatinine concentrations, whereas the Cockcroft–Gault and Schwartz equations have been
shown to overestimate the GFR, especially at a lower creatinine concentrations. (Patrick Garrett 2009). Lastly, the equations do not account for differences that may occur as a result of unusually high or low muscle mass, extreme diets (vegan or excessive meat consumption), or ethnic variation of groups not included in their derivation. Historically, considerable variability existed with respect to serum creatinine measurement, generally resulting in less accurate estimation of GFR when serum creatinine concentrations were within or slightly above the reference interval(Myers GL, 2006 ). In 2008, the National Kidney Disease Education Program (NKDEP) in collaboration with the IFCC and the European Communities Confederation of Clinical Chemistry launched the Creatinine Standardization Program to reduce interlaboratory variability in creatinine assay calibration (NKDEP 2011).

Creatinine is the measure of Kidney function, measures the level of creatinine in blood and uses the result in a formula to calculate and reflect how well the kidneys are functioning.(Henry JB,2001) It is directly proportional to net filtration pressure and affected by factors such as; total surface area available for filtration, filtration membrane permeability, and net filtration pressure. (Dr Patrick Garrett 2009).

Kidney function estimates are central to the diagnosis and management of chronic kidney disease, and these estimates are usually based on the creatinine blood test. However, the creatinine test can miss early losses of kidney function, particularly in patients with low muscle mass. (Patrick Garrett 2009).

Additional limitations to serum creatinine for GFR measurement is the “creatinine blind area.” Serum creatinine values are not a direct measure of GFR, which can be reduced as much as 50% while the serum creatinine value is still within the normal range. Typically, serum creatinine remains within the normal range until 50% of renal function is lost. The overestimation of creatinine results in the "creatinine-blind area” in the GFR range 40-90 ml/min/1.73 m2.( Patrick Garrett 2009)

i, C & G ii, MDRD iii, CKD-EPI III formulas:

**i, GFR calculated according to C&G formula:**

\[
\frac{[140-\text{Age (years )}] \times \text{body weight (Kg)}}{[0.815 \times \text{serum creatinine (μmol/L )}]}
\]

1

The correction factor of 0.85 was for women.

**ii, GFR calculation factor to MDRD formula:**

\[
175 \times \text{serum creatinine (mg/dl)}^{-1.154} \times \text{age (year)}^{0.203}
\]

2
The correction factor of 0.742 was used for women

iii, GFR calculation factor to CKD-EPI formula

\[
\text{GFR} = a \times \text{serum creatinine (mg/dl)}^{c} \times (0.993)^{\text{age}}
\]

Eq 2.2 Estimation of glomerular filtration equations.

2.3.2.2 Urea

Historically, urea was the first marker used to formally assess kidney function. Urea is the major form of nitrogenous waste in the body. It is the product of protein and amino acid metabolism and eliminated almost entirely via urinary excretion. Urea, although it is freely filtered by the glomerulus, approximately 40% reabsorbed by tubules and for this reason, it does not provide a full clearance assessment. (Myers GL, 2006)

2.3.2.3 Blood urea nitrogen (BUN)

Blood urea nitrogen (BUN) quantification was eventually introduced into clinical medicine as a diagnostic test in the early 1900. In addition, increased BUN concentrations may be seen with increased dietary protein intake, hypercatabolism, corticosteroid use, or gastrointestinal bleeding. Therefore, interpretation of BUN concentrations needs to be carefully considered in the clinical context. (Myers GL 2006)

2.3.2.4 Low molecular weight proteins as GFR markers

Measured concentrations of several low molecular weight proteins, including 2β-microglobulin, Cystatin C, and -trace protein (BTP), have been evaluated as potential markers of GFR. In general, these proteins are freely filtered by the glomerulus, reabsorbed and catabolized, but not secreted by the renal tubules. As a result, reduction in GFR are associated with increased plasma concentration of 2β-Microglobulin is an 11.8-kDa protein that is the light chain of the MHC I molecule expressed on the cell surface of all nucleated cells. It dissociates from the heavy chain in the setting of cellular turnover and enters the circulation as a monomer. 2β-Microglobulin is filtered at the glomerulus and almost entirely reabsorbed and catabolized by proximal tubular cells (Miyata T, Jadoul 1998). Unlike creatinine, serum concentrations appear to be largely independent of age and muscle mass (Filler G, Prism, 2002); however, there does not appear to be a clear advantage of 2β-microglobulin over serum creatinine in detecting small changes in GFR (Donadio C. Serum Am. J 2002). A major factor limiting the utility of 2β-microglobulin as
a marker of renal function is its nonspecificity, because serum 2β-microglobulin concentrations are known to increase in several malignancies and inflammatory states (Delanghe JR., 2009).

**2.3.2.4.1 Cystatin C**

Cystatin C is a 122 amino acid low molecular weight protein sequence, (Fig2.5) which was the human Cystatin C was determined in 1981 (Ryan TP, Sloand JA. et al 2007) and since it did not display any significant homology with the sequences of any protein of the superfamilies known then, it was evident that it belonged to a new protein superfamily (NKF 2011).

![Figure 2.5. Amino acid sequence and schematic structure of human Cystatin C. The shaded area marks the inhibitory site for papain-like cysteine proteases, which does not overlap with the inhibitory site for mammalian legumains comprising inter alia the Asn 39 residue. The arrow indicates the Leu6 8 residue, which is replaced with a Gln residue in the cerebral hemorrhage producing cystatin](image)

Cystatin C is a ubiquitous protein secreted at a constant rate by almost all nucleated cells in the body and is filtered solely by the glomerulus, fully reabsorbed, and catabolized but not secreted by the renal tubes. Unlike creatinine, serum Cystatin C concentration appears to be independent of age, sex, and muscle mass (Kissmeyer L, Kong 1999). It may be more reliable than serum creatinine–based methods in estimating GFR, particularly in those individuals with a mild reduction in GFR, in whom changes in serum creatinine are typically not observed (the so-called creatinine blind range of GFR) (Levey AS, Atkins R 2007). Cystatin C may also be superior to creatinine in estimation of mortality and cardiovascular outcomes (Ferris M, Hogan SL 2007). Cystatin C has been reported to rise faster than creatinine after a fall in GFR, enabling earlier identification of AKI (Carol M. Porth 2014, Michael L. Bishop, 2010). Several Cystatin C–based equations to estimate GFR appear to be simpler and more accurate than creatinine-based equations (Wendy A, & Jean Brickrll, 2007).
A new, international study from the Chronic Kidney Disease Prognosis Consortium found that use of blood levels of Cystatin C to estimate kidney function – alone or in combination with creatinine strengthens the association between kidney function and risks of death and end-stage renal disease (Ferris M, Hogan SL 2007).

Enhanced sensitivity for acute and chronic kidney disease, independent of age, sex, race, lean muscle mass and diet, early detection of kidney damage due to adverse effects of potentially nephrotoxic medications such as contrast media, cancer therapeutics or certain antibiotics, Cystatin C is a strong predictor of incident CVD. Early detection and treatment improves patient outcomes in renal disease. Cystatin C concentration is not influenced by the above factors and provides an accurate assessment of the immediate renal condition. Circulating Cystatin C concentrations may be affected by corticosteroid administration and thyroid dysfunction (Fried L 2009). In addition, it should be noted that there are ongoing concerns related to the lack of standardization in Cystatin C measurement. White et al. (de Jong PE, 2006). As a whole, serum markers appear to provide adequate assessment of GFR in most clinical situations. There are many advantages to their utilization, primarily related to low technical demand of testing as well as demonstrated ability (serum creatinine and BUN) and potential (cystatin C and BTP) to provide rapid assessment. For research settings, or clinical circumstances in which estimation of GFR by serum markers is likely to be inaccurate or when precise GFR measurements are required for clinical decision-making (e.g., clearance for kidney donation in an individual with borderline eGFR), clearance-based techniques can be used to provide a more accurate estimation of true GFR (Levey AS, Atkins R 2007).
2.4 Treatment for CKD
The treatment of chronic renal failure (ESRD) can be divided into two types: conservative management of renal insufficiency and renal replacement therapy with dialysis or transplantation. Conservative treatment consists of measures to prevent or retard deterioration in remaining renal function and to assist the body in compensating for the existing impairment. Interventions that have been shown to significantly retard the progression of chronic renal insufficiency include dietary protein restriction and blood pressure normalization. Various interventions are used to compensate for reduced renal function and correct the resulting anemia, hypocalcemia, and acidosis. These interventions often are used in conjunction with dialysis therapy for patients with ESRD. (Carol M. Porth 2011)

2.5 Diabetics mellitus
The long-term effects of diabetes mellitus include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation, Charcot joints, and features of autonomic dysfunction, including sexual dysfunction. People with diabetes are at increased risk of cardiovascular, peripheral vascular and cerebrovascular disease.

2.5.1 Definition of diabetics
The term diabetes mellitus describes a metabolic disorder of multiple aetiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The effects of diabetes mellitus include long-term damage, dysfunction and failure of various organs. (WHO, 2001)
Glucose is the body's primary energy source. After a meal, carbohydrates usually are broken down into glucose and other simple sugars. This causes blood glucose levels to rise and stimulates the pancreas to release insulin into the bloodstream. Insulin is a hormone produced by the beta cells in the pancreas. It regulates the transport of glucose into most of the body's cells and works with glucagon, another pancreatic hormone, to maintain blood glucose levels within a narrow range. If someone is unable to produce enough insulin, or if the body's cells are resistant to its effects (insulin resistance), then less glucose is transported from the blood into cells. Blood glucose levels remain high and the body's cells "starve." This can cause both acute and chronic problems depending on the severity of the insulin deficiency. (NIDDK, 2010 data)

Acute hyperglycemia can be a medical emergency. The body tries to rid the blood of excess glucose by flushing it out of the system with increased urination. This process can cause dehydration and upset the body’s electrolyte balance as sodium and potassium are lost in the urine. With severe insulin deficiency, glucose is not available to the cells and the body may attempt to provide an alternate energy source by metabolizing fatty acids. This less efficient process leads to a buildup of ketones and upsets the body's acid-base balance, producing a state known as ketoacidosis. Left unchecked, acute hyperglycemia can lead to severe dehydration, loss of consciousness, and even death.

Glucose levels that rise over time and become chronically elevated may not be initially noticed. The body tries to control the amount of glucose in the blood by increasing insulin production and by eliminating glucose in the urine. Symptoms usually begin to arise when the body is no longer able to compensate for the higher levels of blood glucose. Chronic hyperglycemia can cause long-term damage to blood vessels, nerves, and organs throughout the body and can lead to other conditions such as kidney failure, loss of vision, strokes, cardiovascular disease and circulatory problems in the legs. Damage from hyperglycemia is cumulative and may begin before a person is aware that he or she has diabetes. The sooner that the condition is detected and treated, the better the chances of minimizing complications. (Henry JB, 2001)

According to the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, 2010 data), about 26 million people in the United States have diabetes, but as many as 7 million of them are not yet aware that diabetes is affecting their health. (NIDDK, 2010 data)

2.5.2 Types of diabetes

There are three main types of diabetes: type 1, type 2, and gestational diabetes.

2.5.2.1 Type I diabetes
Type I diabetes, which is called insulin dependent or juvenile diabetes, makes up about 10% of the diabetes cases in the United States. Most cases, it was diabetes are diagnosed in those under the age of 30. Symptoms often develop abruptly and the diagnosis is often made in an emergency room setting. The affected person may be seriously ill, even comatose, with very high glucose levels and high levels of ketones (ketoacidosis). It diabetics make very little or no insulin. Any insulin-producing beta cells they do have at the time of diagnosis are usually completely destroyed within 5 to 10 years, leaving them entirely reliant on insulin injections to live. (Tietz 2006)

The exact cause of type 1 diabetes is unknown, but a family history of diabetes, viruses that injure the pancreas, and autoimmune processes, in which the body's own immune system destroys the beta cells, are all thought to play a role. It may have more severe medical complications sooner than other diabetics. About 40% of those with type 1 diabetes will develop serious kidney problems leading to kidney failure by the age of 50.

2.4.2.2 Type II diabetes

Type II diabetes is used to be known as non-insulin dependent diabetes or adult onset diabetes. Those affected do make their own insulin, but it is either not in a sufficient amount to meet their needs or their body has become resistant to its effects. At the time of diagnosis, people with type 2 diabetes will frequently have both high glucose levels and high insulin levels, but they may not have any symptoms. About 90% of diabetes cases in the United States are type 2. It generally occurs later in life, in those who are obese, sedentary, and over 45 years of age. Factors associated with diabetes include: Obesity, Lack of exercise, family history of diabetes, Prediabetes, gestational diabetes or baby weighing more than 9 pounds, high blood pressure, High triglycerides, high cholesterol, and decreased HDL level, ethnicity such as; African-American, Hispanic-American, Native American, Asian-American, Pacific Islander (Henry JB, 2001)

2.5.2.3 Gestational diabetes

Gestational diabetes is a form of hyperglycemia seen in some pregnant women, usually late in their pregnancy. The cause is unknown, but it is thought that some hormones from the placenta increase insulin resistance in the mother, causing elevated blood glucose levels. Most women are screened for gestational diabetes between their 24th and 28th week of pregnancy. If gestational diabetes is found and not addressed, the baby is likely to be larger than normal, be born with low glucose levels, and be born prematurely. The hyperglycemia associated with gestational diabetes usually goes away after the baby's birth, but both the women diagnosed with gestational diabetes and their babies are at an increased risk of eventually developing type 2 diabetes. A woman who
has gestational diabetes with one pregnancy will frequently experience it with subsequent pregnancies. (Henry JB, 2001)

2.5.2.4 Pre-diabetes
Pre-diabetes is a term for impaired fasting glucose or impaired glucose tolerance. It is characterized by glucose levels that are higher than normal, but not high enough to be diagnostic of diabetes. Recent data suggest that at least 79 million adults in the U.S. had pre-diabetes in 2010. Usually those who have pre-diabetes do not have any symptoms but, if nothing is done to lower their glucose levels, they are at an increased risk of developing diabetes within about 10 years. Experts are recommending that everyone who has any of the risk factors for type 2 diabetes be tested for pre-diabetes. (Henry JB, 2001)

2.5.3 Signs and symptoms
The signs and symptoms of diabetes are related to high glucose levels (hyperglycemia), low glucose levels (hypoglycemia), and complications associated with diabetes. The complications can be related to lipid production, damage to blood vessels (vascular and microvascular), organ damage - for example, kidney (diabetic nephropathy), nerve (diabetic neuropathy), and eye (diabetic retinopathy) damage - and/or to the slower healing associated with diabetes. Type 1 diabetics are often diagnosed with acute severe symptoms that require hospitalization. With pre-diabetes, early type 2 diabetes, and gestational diabetes, there usually are no symptoms.

Symptoms of type 1 and type 2 diabetes with hyperglycemia include:- increased thirst, increased urination, increased appetite (with type 1, weight loss is also seen), fatigue, Nausea, vomiting, abdominal pain (especially in children), blurred vision, slow-healing infections, numbness, tingling, and pain in the feet, erectile dysfunction in men, absence of menstruation in women, Rapid breathing (acute), decreased consciousness, coma (acute), symptoms of impending hypoglycemia.

2.5.4 Temporary hypoglycemia
Temporary hypoglycemia is the diabetic may be caused by the accidental injection of too much insulin, not eating enough or waiting too long to eat, exercising strenuously, or by the swings in glucose levels seen with "brittle" diabetes. Hypoglycemia needs to be addressed as soon as it is noticed as it can rapidly progress to unconsciousness.

Symptoms of temporary hypoglycemia include: sudden severe hunger, headache, anxiety, confusion, sweating, trembling, weakness, double vision, convulsions, & coma.

2.6 Hemoglobin A1c (HbA1c)
Hemoglobin A<sub>1c</sub> is a form of hemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods of time. It is formed in a non-enzymatic glycation pathway by hemoglobin's exposure to plasma glucose.

### 2.6.1 HbA1c (glycosylated hemoglobin) test

The HbA1c test (also called glycosylated hemoglobin level) is a laboratory blood test which measures average blood glucose over the previous weeks and gives an indication of a longer-term blood glucose control. The test is used as a regular monitoring tool if you have been diagnosed with diabetes. It may also be used as one of several screening measures in the general population to look for elevated blood glucose levels, which are suggestive of diabetes.

### 2.6.2 Importance of HbA1c test

Most diabetes specialists and GPs have a lot of confidence in this test and will use it to help show people with type 1 or type 2 diabetes how they are going with their blood glucose management. The HbA1c measures how much glucose has become stuck onto your red blood cells. Red blood cells have a lifespan of about six weeks and so the test gives a good indication of what your overall blood glucose levels have been throughout that time. The HbA1c level is not directly equivalent to blood glucose levels. For example, an HbA1c level of 13% means that your average blood glucose level for the past six weeks has been around 18-19 mmol/L (IFCC 2011)

### 2.6.3 Normal (healthy) level of HbA1c

Target HbA1c levels will vary from person to person, A doctors should work out a safe target HbA1c for their patients. A general range for HbA1c levels [equivalent IFCC values in square brackets] is: (British Medical Journal 2000)

- Less than or equal to 7% [up to 53mmol/mol] is a very healthy HbA1c level
- Between 7% and 8% [54 - 64mmol/mol] is a fair HbA1c level and needs work to improve
- Between 8% and 10% [65 - 86mmol/mol] indicates your blood glucose levels are much too high
- Above 10% [87mmol/mol or higher] indicates your blood glucose levels are extremely high.

A diabetic patient taking insulin and the HbA1c level less than 6.2% [44mmol/mol] this almost certainly indicates that they are having lots of low blood glucose levels ('hypos' or hypoglycemia). HbA1c levels this low is not safe when you are taking insulin.

### 2.7 Diagnoses of diabetes

#### 2.7.1 Fasting blood glucose tests
The fasting test should be conducted on two separate occasions to ensure consistent results and in order to avoid a false diagnosis. This is the case as increased blood glucose levels may be as a result of Cushing’s syndrome liver or kidney disease, eclampsia and pancreatitis. However many of these conditions are often picked up in lab diagnostic tests.

The results of a fasting test with respect to glucose levels in the body are as follows:

- **Normal**: 3.9 to 5.5 mmol/l (70 to 100 mg/dl)
- **Pre-diabetes or Impaired Glucose Tolerance**: 5.6 to 7.0 mmol/l (101 to 126 mg/dl)
- **Diagnosis of diabetes**: more than 7.0 mmol/l (126 mg/dl)

### 2.7.2 Diagnosing diabetes using HbA1c

Diabetes may be defined as having an HbA1c > 6.5% / 48mmol/l (Pulse 2010). So,

- >6.5% / 48mmol/l = diabetes
- <6.0% / 42mmol/l = not diabetic
- in between....6.0-6.5% / 42-48mmol/l...maybe this is 'pre-diabetes' or 'at risk of diabetes'.
- >6.6% / 48mmol/l = chronic diabetes

HbA1c may be normal in acute stages, such as in pregnancy or sudden onset type 1, or young children, or HIV, so blood glucose testing should be used.

### 2.7.3 Hemoglobin A1c level for diabetic patient

Values vary from laboratory to laboratory but below is a common value system for hemoglobin A1c Characterized as Normal if Less than 6.5%, Excellent if 6.5%-7.5%, Good if 7.5%-8.5%, Fair if 8.5%-9.5% and Poor if it is Greater than 9.5%.
2.8 Country background
Eritrea is situated in the horn of Africa and lies between 16°30’ and 43°20’ east longitude and between 12°42’ and 18°2’ north latitude. It is bordered by Sudan to the north and north west, Ethiopia to the south, Djibouti to the south east and the Red Sea to the east. Its area is approximately 124,000 square kilometers, including the Dahlak Archipelago and the islands in the Red Sea. (See figure 2.7).

Since 1996, the country has been divided into six administrative Zobas (Zones). It is further divided in to 58 Sub Zobas, Kebabis (group of villages) and Adis (villages), with an estimated 1500 villages overall. The total population is about 6.1 million (The World Bank, World Development Indicators 2013 | UNDP, Human Development Report 2013) with an annual growth rate of 3% and it remains largely rural (more than 90%). Eritrea regained independence in 1991 (ratified by a referendum in 1993) following a 30 year war with Ethiopia. There are two official languages (Tigrigna and Arabic) and nine major ethnic groups. The most recent Demographic and Health Survey (DHS 2002) produced the following estimates: Crude birth rate to be 32/1000; infant mortality ratio to be 48/1000, and under five mortality rate to be 93/1000. These place the country in the pre-health transition stage, with a life expectancy of 51 years (World Bank 1999). Literacy is estimated to be only 10% for women and 20% for men.

2.8.1 Diabetic mellitus in Eritrea
The Eritrea National Diabetic Association (ENDA) was found by interested group in 1996, It become a member of the International Diabetic Federation (IDA) since 2003. Likewise become one of the 32 countries and 19 sub Saharan member countries in IDA AFR region, where 19million people had the disease and expected to reach 41.9million by 2030(IDF 2014 publication ). According to the latest WHO data published in April 2011,Diabetes Mellitus death in Eritrea reached 741 or 2.98% of the total deaths, which ranks Eritrea number 66 in the world. The number of registered diabetic patient may reach 7000, but no clear and transparent record can be found. Some of the main focus to increase knowledge and control skill among diabetic patients and care takers and likewise increase awareness the public at large and diabetic patients in particular in the prevention and control of diabetics and it's complications.( ENDA 2014 annual report )
CHAPTER THREE

METHODOLOGY
3.1 Study design
This is a cross-sectional descriptive study, conducted in Halibet National Referral Hospital and Hazhaz National Referral Hospital which are located in capital city Asmara, The sample was collected in Halibet National Referral Hospital and Hazhaz National Referral Hospital and the sample processed and analyzed in Eritrea and Sudan Wad-medeni renal hospital laboratory from October 1, 2014 to January 10, 2015.

3.2 Study population
A 100 registered diabetic patients of whom 51 female and 49 male, coming from all parts of the country for a routine follow up to two National Diabetic Clinics within national hospitals were included. The diabetic patients with type I & type II, of both gender and different age group, and the duration of disease with more than 3 years and less than 32 years, but without known kidney problem or decreased function.

3.2.1, Inclusion criteria;
Diabetic patients registered in one of the diabetic clinic for follow up with no known previous history of kidney disease or hypofunction and followed for more than three years.

3.2.2, Exclusion criteria;
Diabetic patients with previous history of chronic kidney disease, Thyroid disorder or whom under corticosteroid therapy, individuals with history of continuing smoking or Hypertensive.

3.3 Ethical consideration
The study obtained an ethical clearance from the MOH research ethical committee. all volunteers was informed and the aim of the study was given to the patients verbally, nature risk and benefits of the study and written consent was collected. Anonymity will be maintained by using a form, which bear no name and will be linked with the sample by standard number. The results of the participants will be kept confidential.

3.4 Data collection
A standardized structured questionnaire was used to collect the demographic data and history of diseases.

3.5 Sample collection
Blood sample from diabetic patients whom fulfilled inclusion criteria of the study was collected using gold standard BD vacutainer® product. Five milliliter (5ml) venous blood was collected in plain BD vacutainer serum tube container for creatinine, Cystatin C and fasting blood glucose tests. Likewise 4.5ml venous blood collected on the same application in a separate BD test tube container with K3EDTA 3.6mg as anticoagulant for HbA1c test from the same patients.
3.5.1 Measurement of serum creatinine

Enzymatic method quantitative determination of serum creatinine was done using Roche reagent in Roche UniCel DXC 600 clinical chemistry auto analyzer.

- Each test tube (two for each patient) was labeled prior to collection.
- 10 ml sample on plane tube and 4.5 ml on K3EDTA was collected from each patient.
- Transported within one hour to NHL every time after collection.
- Centrifuged for 20 min at 3800 RPM, and placed in respective pre-labeled separate plane 5 ml tube transported to respective area.
- Aliquot of the serum and whole blood of each patient was taken to ONRHL for comparison.
- Testing cared out on this respective hospital labs. immodestly and results collected on the same day.
- 1.5 ml serum aliquot was kept in separate plastic tube and defreeze in -20°C⁰ for overseas Cystatin C test.

3.5.2 Hemoglobin A1c measurement

Direct enzymatic HbA1c assay using Hitachi 902 chemistry analyzer.

Whole blood sample collected in K3EDTA anticoagulant from the patients were transported.

1. Reagent preparation:
   Reagents (1), (2), (3) and (4) are provided ready to use. HbA1C Standards (S1-S4): reconstitute with 2.0 ml of distilled water.
   a. Hemolysate preparation:
      The calibrators do not require pretreatment.
      The reagents and each blood sample was brought to room temperature prior testing and 10 μL pre-mixed whole blood and reagent (1) 1000 μL was taken using micropipette into specific pre-labeled a test tube for each patient sample. Then shaken thoroughly, with checking not to form foam. The Hemolysate prepared, was used after color had changed from red to brownish-green in approximately 3 minutes.
   b. Testing procedure: see appendix 1
      Applications for automated systems are available on request.
      • Hemoglobin denaturant reagent R1
      • Total hemoglobin reagent R2
      • HbA1c R3: antibody reagent
      • HbA1c R4: Agglutinator reagent
      • HbA1c/Hb calibration
      • HbA1c quality control
• General laboratory equipment
Assay detailed procedure was provided on annexure 3

c. Procedure:
Refer to Hitachi operator’s manual for user defined applications.
Place R2 (Hb reagent) on reagent plate 1, R3 (A1c antibody reagent) on reagent plate 1 and R4 (A1c agglutination reagent) on reagent plate 2.

d. Calculation:
The calculation of the hemoglobin A1c concentration is generated using the following equation:

\[
\text{HbA1c } \% = \frac{\text{[HbA1C g/dl]}}{\text{[Hb g/dl]}} \times 100
\]

Limitations – interference:
The assay gives accurate and precise results for a range of total hemoglobin varying between 7g/dl and 23g/dl. Patients with severe anaemias (total hemoglobin < 7g/dl) and those with polythemia (total hemoglobin > 23g/dl) should not be assayed by this method. Any case of shortened red cell survival such as hemolytic anemia or other hemolytic disease, pregnancy, recent significant blood loss etc. will result in a decrease in % glycated hemoglobin.

Reference range:
• >6.5% / 48mmol/l = diabetes
• <6.0% / 42mmol/l = not diabetic

3.5.3 Cystatin C measurement

a. Test principle:
Fluorescence immunoassay for the quantitative in vitro determination of Cystatin C in human serum and plasma using ichroma®. It is a sandwich immune detection method, such that the detection antibody in a detection buffer binds to Cystatin C in a sample and antigen-antibody complexes are captured to another Cystatin C antibody that has been immobilized on the test strip as sample mixture migrates through a nitrocellulose matrix. the more Cystatin C antigen in a sample, the more antigen-antibody complexes are accumulated on the test strip.
The signal intensity of the fluorescence on detection antibody reflects the amount of antigen captured and is processed by ichroma™ reader to show the Cystatin C concentration on the sample.

- Roche SYNCHRON® System UniCel DXC 600
- ichroma™

Reference level of ichroma™ on health individuals

<table>
<thead>
<tr>
<th>Age range</th>
<th>Ref. Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-50 years old</td>
<td>0.56 - 0.90 mg/L</td>
</tr>
<tr>
<td>51-70 years old</td>
<td>0.58 - 1.09 mg/L</td>
</tr>
</tbody>
</table>

3.6 Materials
For fluorescence immunoassay for the quantitative in vitro determination of Cystatin C in human serum and plasma using ichroma™ and enzymatic method reagent from Roche, and automated clinical chemistry analyzers testing of creatinine concentration.

3.6.1 Materials, Reagents and equipments
- One 1/2 kg Cotton roll
- Three BD Alcohol swab of 100/pack
- One medium size glove of 100/pack
- Plane BD test tube container of 5ml without anti-coagulant around 150 test tubs.
- The same number of BD 4.5ml K3EDTS test tube container
- Plane test tube free of 5ml capacity.
- Ten vacationer holder cup of 10.22mm
- Two BD standard vacutainer needles of 100/pack
- Tornicator BD pack of 10 each two.
- Five sample container rack
- Micro pipette graduated (5-20)μl
Micro pipette graduated to (10-100) μl
Micro pipette tip capacity of 200 μl. and 100 μl, enough for the whole test.
Sample plastic cup 13x 75, of 0.5ml
Creatinine reagent pack
Hemoglobin A1c reagent pack for all the test
Quality control materials
Test cartridge box for cystatin c five (25 cartridge per box)
Box containing detection buffer tube five (25 tube per box)
Reserved rack for sample use on DXC 600 serious
Centrifuge Allegra x 12 from Beckman coulter with rack of 24 hole.
Mixer for whole blood on HbA1c testing.
Beck Man UniCel DXC 600 Synchron clinical chemistry auto analyzer.
Ichroma™ semi automated reader
Hitachi 902 automated chemistry analyzer.

3.7 Data calculations and analysis

3.7.1 eGFR was calculated using the Cockroft-Gault equation for age below 18 years. [Cockroft D.W. 1994]

\[
eGFR = (140 - \text{age}) \times \text{weight (kg)} \times 0.85 \text{ (for females)} \\
\frac{1}{72 \times \text{serum creatinine (mg/dl)}}
\]

3.7.2 Equations used for calculation to GFR specific to sex

Age specific equations used in calculation of eGFR where epidemiology collaboration equations with exception to patients with age less than 18 years.

3.7.2.1 Creatinine equation EP-CKD 2009

<table>
<thead>
<tr>
<th>Basic Equation</th>
<th>SCr mg/dl</th>
<th>SCy mg/dl</th>
<th>eGFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female &lt; 0.7</td>
<td>144 × (Scr/0.7) – 0.329 × 0.993 Age [× 1.159 if black]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female &gt; 0.7</td>
<td>144 × (Scr/0.7) – 1.209 × 0.993 Age [× 1.159 if black]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male ≤ 0.9</td>
<td>141 × (Scr/0.9) – 0.411 × 0.993 Age [× 1.159 if black]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male &gt; 0.9</td>
<td>141 × (Scr/0.9) – 1.209 × 0.993 Age [× 1.159 if black]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### 3.7.2.2 Cystatin C equation EP-CKD 2012

<table>
<thead>
<tr>
<th>Basic Equation</th>
<th>SCr mg/dl</th>
<th>SCy mg/L</th>
<th>eGFR Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Female or Male</td>
<td>≤0.8</td>
<td>133 × (Scys/0.8)−0.499 × 0.996Age [× 0.932 if female]</td>
<td></td>
</tr>
<tr>
<td>- Female or Male</td>
<td>&gt;0.8</td>
<td>133 × (Scys/0.8)−1.328 × 0.996Age [× 0.932 if female]</td>
<td></td>
</tr>
</tbody>
</table>

### 3.7.2.3 Creatinine/Cystatin C equation EP-CKD 2012

<table>
<thead>
<tr>
<th>Basic Equation</th>
<th>SCr mg/dl</th>
<th>SCy mg/L</th>
<th>eGFR Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Female</td>
<td>≤0.7</td>
<td>≤0.8</td>
<td>130 × (Scr/0.7)−0.248 × (Scys/0.8)−0.375 × 0.995Age [× 1.08 if black]</td>
</tr>
<tr>
<td>- Female</td>
<td>&gt;0.7</td>
<td>≤0.8</td>
<td>130 × (Scr/0.7)−0.248 × (Scys/0.8)−0.711 × 0.995Age [× 1.08 if black]</td>
</tr>
<tr>
<td>- Female</td>
<td>&gt;0.7</td>
<td>&gt;0.8</td>
<td>130 × (Scr/0.7)−0.601 × (Scys/0.8)−0.375 × 0.995Age [× 1.08 if black]</td>
</tr>
<tr>
<td>- Male</td>
<td>≤0.9</td>
<td>≤0.8</td>
<td>135 × (Scr/0.9)−0.207 × (Scys/0.8)−0.375 × 0.995Age [× 1.08 if black]</td>
</tr>
<tr>
<td>- Male</td>
<td>&gt;0.9</td>
<td>≤0.8</td>
<td>135 × (Scr/0.9)−0.601 × (Scys/0.8)−0.711 × 0.995Age [× 1.08 if black]</td>
</tr>
<tr>
<td>- Male</td>
<td>&gt;0.9</td>
<td>&gt;0.8</td>
<td>135 × (Scr/0.9)−0.601 × (Scys/0.8)−0.711 × 0.995Age [× 1.08 if black]</td>
</tr>
</tbody>
</table>

To convert the values for serum creatinine mg/dl to micromoles per liter, multiply by 88.4.

- The CKD-EPI creatinine equation (2009) that we developed previously can be expressed as a single equation: \[ 141 \times \min(\text{Scr/}k, 1)\alpha \times \max(\text{Scr/}k, 1)−1.209 \times 0.993\text{Age} \times 0.918 \text{ if female} \] 
  \[ \times 1.018 \text{ if black}, \] where Scr is serum creatinine, k is 0.7 for females and 0.9 for males,
- \( \alpha \) is −0.329 for females and −0.411 for males, min is the minimum of Scr/\( k \) or 1, and max is the maximum of Scr/\( k \) or 1.
- The CKD-EPI Cystatin C equation (2012) can be expressed as a single equation: \[ 133 \times \min(\text{Scy/}k, 1)−0.499 \times \max(\text{Scy/}k, 1)−1.328 \times 0.996\text{Age} \times 0.932 \text{ if female}, \] 
  where Scy is serum Cystatin C, min indicates the minimum of Scy/k or 1, and max indicates the maximum of Scy/k or 1.
- The CKD-EPI Creatinine–Cystatin C equation (2012) can be expressed as a single equation: \[ 135 \times \min(\text{Scr/}k, 1)\alpha \times \max(\text{Scr/}k, 1)−0.601 \times \min(\text{Scy/}k, 1)−0.375 \times \max(\text{Scy/}k, 1)−0.711 \times 0.995\text{Age} \times 0.969 \text{ if female} \times 1.08 \text{ if black}, \] where Scr is serum creatinine, Scy is serum Cystatin C, k is 0.7 for females and 0.9 for males, \( \alpha \) is −0.248 for females and −0.207 for males, min indicates the minimum of Scr/\( k \) or 1, and max indicates the maximum of Scr/\( k \) or 1. (Filler G, Prime F. 2002)
CHAPTER FOUR

RESULT AND DISCUSSION
4.1, Results

4.1.1 Characteristics of participants
The study includes 100 known diabetic patients without previous history of renal disease, in which 51 females and 49 males. Table 4.1 summarizes characteristics study group according to age categories. Their age range from (12 - 89) years old with an average 56 years with standard error of 1.63, and the majority of the study group was adults above 20 years and only 3% was in the range (12-20) years old. The duration of morbidity range (3 - 32) years with mean (SD) 9.5 (6.6) years. Serum creatine and Cystatin C level was in the range of (0.4 - 1.9)mg/dl and (0.5 - 2.5) mg/dl, respectively. About 98% of the patients had normal serum creatinine level, however, only 50% had normal level of serum Cystatin C finding. Glycemic control was assessed using fasting blood glucose and HbA1C, and about 56% had FBG below 181mg/dl and the rest ranged from of (182 - 429) mg/dl. The HbA1C ranges (5.5%-14%) and about 31% of the patients had targeted level of HbA1C (5.5% - 7.4%), and the rest of the study group had poor control which ranges (7.5% - 14%).

Among all participants, the average of serum cystatin C and serum creatinine 1.10 (0.30) mg/l and 0.74(0.20)mg/dl ranges (0.5-2.29)mg/dl and (0.4-1.9)mg/dl respectively. Male and female, average serum creatinine and serum Cystatin C (0.84 and 0.63 mg/dl), ranges[(0.5-1.9)mg/dl & (0.4-1.0)mg/dl] and (1.11 and 1.07 mg/dl), ranges [(0.5-1.4)mg/dl &(0.5-2.29)mg/dl], respectively.
Table 4.1 Summary of participants characteristics parameters according age categories in diabetic patients at two diabetic clinics in Eritrea.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Age category in years</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;20</td>
<td>20-29</td>
</tr>
<tr>
<td>Creatinine level (mg/dl)</td>
<td>0.00-1.30</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>&gt;1.31</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Cystatin C level (mg/dl)</td>
<td>0.52-1.10</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.11-1.70</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1.71-2.50</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>HbA1c level (%)</td>
<td>&lt; 6.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6.5-7.4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7.5-8.4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>8.5-9.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>&gt;9.5</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Duration interval (years)</td>
<td>&lt; 5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5-10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>11-15</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>16-20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>&gt;20</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>
4.1.2. Estimated GFR according to age group distribution:
Subjects were divided into seven age category based on the NKF 2013 recommendation and the results is summarized in table4.2. The eGFR value for less than 20 years show normal average (SD) in EP-CKD 2009 SCr (104(48.1) ml/min/1.73m²) however, the eGFR average (SD) was 78(26) ml/min/1.73m² using EP-CKD 2012 SCy equation, likewise, eGFR average (SD) was 93(37) ml/min/1.73m² based on EP-CKD 2012 mix. The estimated GFR using EP-CKD SCr 2009 for age group 20-29 was 114 ml/min/1.73m² and slightly higher on the rest of the age groups. The average (SD) eGFR values using EP-CKD 2012 SCy alone shows dramatically low for all age groups,( 78(26), 88(28.5), 88(19), 76(28.7), 75(21.8), 64(21.1), and 64 (21.1) )ml/min/1.73m² for age group (<20, 20-29, 30-39, 40-49, 50-59, 60-69, and ≥70) years of age respectively. Likewise, The average (SD) results of eGFR EP-CKD SCy/SCr mix on all age groups shows low average value, except for the age group 30-39 years of age which was 100 ml/min/1.73m² as compared to respective accepted value from NKF2011.(See below for fully described in table 4.3).
Table 4.3 shows estimated GFR value of overall participants on according to age group distribution. From overall study participants 75% show low possible risk to develop CKD based on EP-CKD SCr2009 equation, however, 21% and 4% had (60-89,& 30-59) eGFR ml/min/1.73m² respectively. When EP-CKD SCy C2012 was taken to estimate GFR was as follows: 22%, 41%, 35%, and 4% that was (>90, 60-89, 30-59 and 15-29) eGFR ml/min/1.73m² respectively. According to the EP-CKD Mix the eGFR result was 34%, 57%, and 9% had (>90, 60-89 and 30-59) eGFR ml/min/1.73m² respectively. For respective percentage of all age group distribution with in respective category (see on index, table 4.6).
Table 4.2 provides average (SD) of all participants respective according to age category of eGFR using (EP-CKD 2009, EP-CKD SCy C2012 and EP-CKD Mix)ml/min/1.73m², at two diabetic clinics in Eritrea.

<table>
<thead>
<tr>
<th>eGFR ml/min/1.73m²</th>
<th>Age category in years</th>
<th>&lt;20</th>
<th>20-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
<th>60-69</th>
<th>≥70</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ep-ckd 2009 Scr</strong></td>
<td>Average</td>
<td>104</td>
<td>114</td>
<td>117</td>
<td>100</td>
<td>105</td>
<td>95</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>(48.1)</td>
<td>(12.6)</td>
<td>(10.2)</td>
<td>(14.7)</td>
<td>(12.3)</td>
<td>(11.0)</td>
<td>(16.4)</td>
</tr>
<tr>
<td><strong>Ep-ckd 2012 Scy</strong></td>
<td>Average</td>
<td>78</td>
<td>88</td>
<td>88</td>
<td>76</td>
<td>75</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>(26)</td>
<td>(28.5)</td>
<td>(18.97)</td>
<td>(28.7)</td>
<td>(21.8)</td>
<td>(21.1)</td>
<td>(21.5)</td>
</tr>
<tr>
<td><strong>Ep-ckd 2012 Scr &amp; Scy mix</strong></td>
<td>Average</td>
<td>93</td>
<td>98</td>
<td>100</td>
<td>87</td>
<td>88</td>
<td>78</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>(37)</td>
<td>(18.2)</td>
<td>(13.3)</td>
<td>(23.1)</td>
<td>(15.6)</td>
<td>(17.7)</td>
<td>(19.4)</td>
</tr>
</tbody>
</table>
Table 4.3: Provides the estimated GFR value for all participants according to age group distribution, along with possible risk stage range depending on GFR estimate value based on three epidemiological collaborated formulas used as reference for calculation the eGFR ml/min/1.73m², at two diabetic clinics in Eritrea.

<table>
<thead>
<tr>
<th>eGFR</th>
<th>Age group category</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;20</td>
<td>20-29</td>
</tr>
<tr>
<td>EP-CKD Scr 2009</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥90</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>60-89</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30-59</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>EP-CKD Scy 2012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥90</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>60-89</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>30-59</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15-29</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>EP-Mix 2012 (SCr/Scy)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥90</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>60-89</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>30-59</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>
4.1.3 Concordance between estimates of GFR:
As was shown in table 4.4 eGFR of all tested mean difference, significantly correlated one another and was indicated in bold. The creatinine based eGFR EP-CKD Scr 2009 was weak when correlated to EP-CKD Scy C 2012 (r = 0.468), but had a better correlation with EP-CKD2012 mix (r = 0.755). However, the correlation between EP-CKD Scy c 2012 and EP-CKD Mix 2012 was remarkable (r = 0.922). In addition, the result on persons correlation elaborate clearly the correlation between (EP-CKD Scr 2009 Vs CKD Scy C2012, EP-CKD Scr2009 Vs EP-CKD Mix 2012 and EP-CKD Scy C2012 Vs EP-CKD Mix 2012) with values r = (0.414, 0.687 and 0.937) and at (p< 0.0001) respectively.

The regression coefficient indicates the linearity of eGFR between EP-CKD Scr 2009, EP-CKD Scy C2012, and EP-CKD Mix 2012. Where, table 4.5 summarizes briefly the values of linearity relationship by interception point and slope coefficient. However, EP-CKD Scy C2012 intercepts with EP-CKD Mix at 22.44, with upper and lower boundary (17.4 & 27.5) respectively and a positive slope r=0.853 close to one (1) as compared to linearity coefficient and slope obtained on creatinine based eGFR.

Table 4.4 Spearman and Pearson correlation 1 in bold shows significant correlation in two tail test. the closer to 1 the best correlation between the two as shown between EP-CKD Scy C 2012 and EP-CKD Mix 2012 at two diabetic clinics in Eritrea.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>EP-CKD Scr 2009</td>
<td>1</td>
<td>0.468</td>
<td>0.755</td>
<td>1</td>
<td>0.414</td>
<td>0.687</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EP-CKD Cy C 2012</td>
<td>0.468</td>
<td>1</td>
<td>0.922</td>
<td>0.414</td>
<td>1</td>
<td>0.937</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EP-CKD Mix 2012</td>
<td>0.755</td>
<td>0.922</td>
<td>1</td>
<td>0.687</td>
<td>0.937</td>
<td>1</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Table 4.5 regression summary (linearity) of eGFR (ml/min/1.73m²) between EP-CKD SCr 2009, EP-CKD SCy C 2012, and EP-CKD Mix 2012, with respective mean, SD, min, and max, in addition to slope (r) and interception point at two diabetic clinics in Eritrea.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
<th>Std. deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP-CKD SCr 2009</td>
<td>51.000</td>
<td>147.000</td>
<td>97.690</td>
<td>18.324</td>
</tr>
<tr>
<td>EP-CKD SCy C 2012</td>
<td>22.000</td>
<td>134.000</td>
<td>71.690</td>
<td>23.528</td>
</tr>
<tr>
<td>EP-CKD Mix 2012</td>
<td>45.000</td>
<td>144.000</td>
<td>83.600</td>
<td>20.271</td>
</tr>
</tbody>
</table>

Model coefficients:

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
<th>Lower bound 95% (Mean)</th>
<th>Upper bound 95% (Mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(SCr Vs SCy C)</td>
<td>Slope coefficient (r)</td>
<td>1.782</td>
<td>0.894</td>
</tr>
<tr>
<td>Intercept EP-CKD</td>
<td>-29.532</td>
<td>-59.432</td>
<td>0.368</td>
</tr>
<tr>
<td>(SCr Vs Mix)</td>
<td>Slope coefficient (r)</td>
<td>1.158</td>
<td>0.846</td>
</tr>
<tr>
<td>Intercept EP-CKD</td>
<td>22.444</td>
<td>17.408</td>
<td>27.480</td>
</tr>
<tr>
<td>(SCy C Vs Mix)</td>
<td>Slope coefficient (r)</td>
<td>0.853</td>
<td>0.785</td>
</tr>
</tbody>
</table>

Fig 4.2 Regression diagram between EP-CKD SCy C 2012 to EP-CKD Mix 2012 and residuals to show linearity as well distribution of their individual values.
Table 4.6. Test of linearity/Two-Tailed Test): provides average (SD) of all participants respectively according to age category of eGFR using (EP-CKD 2009, EP-CKD SCy C2012 and EP-CKD Mix)ml/min/1.73m$^2$, at two diabetic clinics in Eritrea.

<table>
<thead>
<tr>
<th>Max(Cusum)(observed value)</th>
<th>12.422</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max(Cusum) (critical value)</td>
<td>13.581</td>
</tr>
<tr>
<td>P-value</td>
<td>0.091</td>
</tr>
<tr>
<td>Alpha</td>
<td>0.05</td>
</tr>
</tbody>
</table>

**Test interpretation**


- As the computed p-value is greater than the significance level alpha=0.05, one canot reject the null hypothesis H0. The risk to reject the null hypothesis H0 id true is lower than 9.13%. 
4.2 Discussion:
The study revealed about 98% of the patient had normal serum creatinine level, however, only 50% had normal serum Cystatin C level the rest 50% with abnormal Cystatin C. This suggests that there is a great variation in detecting the renal disease using the two methods. There is similar studies which support our finding (Ramanathan K. and Padmanaban G. 2011 ), which claim Cystatin C is more sensitive for assessing of renal damage. In our setting, diabetic patients are monitored, based on the old traditional practice, using serum creatinine alone or in combination with urine albumin and BUN.

Even though our sample size is small, it revealed that serum creatine is dependent on age and sex (p=0.01) which is statistically significant, however, Cystatin C is not depend on sex and age (p=0.98 and 0.248), respectively. This finding is consistent with the studies of (Ching-Wei Tsai, 2014 and Lesley A Stevens, et al. 2008).

Many previous studies provided evidence that validate Cystatin C has superiority over serum creatinine as a marker of renal impairment (Brown WW. et.al, 2007, V Viswanathan. et. al, 2005 ,and Lesley A Stevens. et. al 2008). This can be explained by the fact that elimination exclusively via renal filtration is a further essential requirement for a GFR marker. While cystatin C dose as well, creatinine can be alternatively secreted via the tubules system. This alternative elimination pathway compensates for a decrease in GFR and keeps the serum creatinine level unchanged until GFR has declined to 60 ml/min/1.73m². Creatinine level only increase if the capacity of the alternate tubular secretion pathway is fully used; this is why there is a “creatinine-blind range” limiting the sensitivity and precision of creatinine in the normal and slightly reduced GFR range (Fliser D, et al 2006).

The ideal GFR marker should not be influenced by age, sex, body weight, or other patient criteria allowing easy result interpretation. Cystatin C shows only a minor, not clinically relevant, difference between men and women. Creatinine levels differ considerably between both sexes, requiring a separate reference range. As creatinine levels are directly related to the lean body mass (= muscle mass), lower concentrations are found in women compared to men, as well as elderly compared to younger individuals. The decrease of muscle mass with aging can mask the decrease of renal function with aging when considering creatinine levels for renal function testing. Therefore, all creatinine-based formulas (but not Cystatin C-based formulas) require age and sex (and / or race) to compensate for these factors. (Carola Wagner et al 2010)

The study showed that Cystatin C, had constant relationship with and GFR; the chi square describes decline of renal function with aging is reflected by increasing Cystatin C level in elderly in a sensitive manner as explained on table 4.1which agree with study of (A, Malm J et al.2004) and Larsson A. 2004); Up from one year of age, Cystatin C level have reached adult level, whereas creatinine level increase as long as the muscle mass is growing such as in children and teenagers.
this study showed a strong correlation between Cystatin C and GFR \( (r = 0.937) \) as compared with creatinine and GFR \( (r= 0.687) \) and supports as marker for early detection of renal disease. Similar findings was observed in late studies of (Kyhse-Andersen,1995).

Furthermore, our study showed a strong association between serum Cystatin C level and the morbidity duration as well as glycemic control( HbA1C), however the serum creatinine does not. As the duration of morbidity increases the serum Cystatin C level increases (appendix 1, table 4.9). Though, the glycemic control indicates in to its best management, the serum Cystatin C level increases in every category as the morbidity of the disease duration increase. An early detection of renal complication in diabetic patients using accurate assessment of GFR is becoming essential for follow up and management. A reduction in GFR to less than 60 ml per minute per 1.73 m² for 3 months or longer is a diagnostic criteria for chronic kidney disease and is associated with an increased risk of complications and mortality (Gansevoort RT. et. al, 2011).

As our study showed that, out of 75 cases which are considered normal/minimal based on creatinine GFR, 50 of the cases are reclassified to moderate/moderately high risk stage on Cystatin C based eGFR. Similarly, recent study by Richard J. et. al, 2011 from Australia; has highlighted the limitations of existing creatinine-based methods of estimating GFR on diabetic patients. In his findings, patients with normal or hyperfiltering range of renal function was grossly underestimated when creatinine based MDRD formula used as estimating tool, by approximately 10% to 40%. Another study by Perkins BA. et. al, 2005 has focused on underestimated and overestimated GFR based on creatinine Cockroft-Gault formula. This wide variation in estimated GFR by the Cockroft-Gault formula, most likely relates to the characteristics of the different population studied, especially their differences in body weight or muscle mass. In addition, longitudinal studies of (Perkins BA. el at, 2005 and Premaratne E. et.al, 2008) demonstrated that, both the MDRD and Cockroft-Gault formulae also significantly underestimate the rate of decline in GFR when measured using a reference method, Cystatin C based formulas. In other studies using a Cystatin C-based calculation of eGFR, 42% of the study participants were reclassified from a creatinine-based eGFR of 45 to 59 ml/min/1.73 m² to higher eGFR lower risk stage, while 14% of participants with a creatinine based eGFR of 60 to 89 ml/minute/1.73 m² were reclassified to a higher risk stage. Recent study of Stevens LA. et. al, 2010 has showed that, on eGFR reference levels in the range 90–119 ml/min/1.73m², bias was reduced from 10.0 ml/min/1.73m² of MDRD equation to only 1.9 ml/min/1.73m² when had estimated by the EP-CKD serum Cystatin C equation.

..
CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS
5.1 Conclusion

Our study revealed that Cystatin C is more sensitive and accurate in assessing the kidney function status in diabetic patients. Moreover, from this study it is concluded that; Cystatin C is independent of age and sex unlike serum creatinine. The GFR estimated by serum creatinine showed, most to fall on stage 1 and stage 2 of study patients in the normal range of eGFR (>90 ml/min/1.73m²), while, serum Cystatin C based eGFR reclassify those cases to fall in moderate and moderately high risk stage to develop CKD.

Based on this finding, it is concluded that Cystatin C is an ideal marker for evaluating the kidney function and early detection of any renal insufficiency in diabetic patients.
5.2 Recommendations

The study recommended that:


2. All the individuals whom are at risk to develop renal impairment should be screened annually using serum Cystatin C.

3. Serum Cystatin C can be used as an ideal marker for monitoring patients with renal disease or disorder whom are under treatment. An early detection can avoid any possible risk, unnecessary complications, reduce mortality due to CKD, and for early therapeutic interventions to slow or prevent the progression toward ESRD and improve the quality of life of patients. It is high time to introduce screening tool in addition to the existing follow up and for effective management, and slowing progression toward ESRD.
REFERENCES


Carol M. Porth, 2014; Essentials of Pathophysiology, Concepts of Altered health state, 2014 Ch. 24, pp 433-446,


**Stevens LA** Schmid CH, Greene T, Zhang YL, Beck GJ, Froissart M, et al. 2010. Comparative performance of the CKD Epidemiology Collaboration (CKD-EPI) and the Modification of Diet in Renal Disease (MDRD) Study equations for estimating GFR levels above 60 ml/min/1.73m2. Am J Kidney Dis 2010;56:486-95


Annexure 1:

Diabetes patients history questionnaire

Date: _______________ P/Code: _______________

1. Patient identification

Full Name: ________________________________

Sex: Male ☐ Female ☐ Age: ___ years National I.D. Number: ______________

Physician Name: __________________________ Date of sample collected _____________

Patient R.I.D. number: _____________________ Citizenship: ______________ Hospital name: __________________________ Body height: ____m Body weight: ____Kg

(if known)

Address:

Zone: ____________________________ Sub-Zone: ____________________________

Town/Village: _________________________ Street: ______________ House No: ________

Workplace/School: _____________________ Occupation/Profession: ______________

Tel: Residential/Neighbor): _____________ Mobile: ______________

2. Health status

- How long ago were you told by a doctor that you had diabetes? _____ years
- Which type of diabetes did your doctor say that you have?
  - ☐ insulin-dependent diabetes, type 1 diabetes.
  - ☐ non insulin-dependent diabetes, type 2 diabetes.
- Have you ever attended a hospital due to complications diabetic? ☐ Yes ☐ No
(If "Yes", what was the complication and when? __________________________. ____________)

- Are you now taking diabetes pills?  ☐ Yes ☐ No
- Are you now taking insulin?  ☐ Yes ☐ No
- Are you now under treatment other than diabetes with insulin?  ☐ Yes ☐ No
- Are you now under corticosteroid therapy?  ☐ Yes ☐ No
- Are you now diagnosed with hypothyroidism / hyperthyroidism?  ☐ Yes ☐ No
- Did you smoke cigarette?  ☐ Yes ☐ No
- If yes, are you now smoking cigarette now?  ☐ Yes ☐ No

3. Physical Assessment
   • General Health Status:
     ______________________________________________________
     ______________________________________________________
     ______________________________________________________
   • Examination done by: ________________________  Sign: ____________________

   • I would like to confirm that, I fully understand and freely participating on this research study and express my willing by my signature.
     Patient Name: ________________________  Sign: ________________

   ✓ Questioner filled by: ________________________
     Sign: ____________________
Annexure 2

Index of Tables 1;

Table 4.7 **EP-CKD Scy C 2012 category** * Age category Cross table* provides the percentage of estimated GFR for overall participants with in respective age group category distribution, eGFR ml/min/1.73m² calculation. in two diabetic clinics, in Eritrea.

<table>
<thead>
<tr>
<th>EP-CKD2012 Scy C category</th>
<th>Age Group category</th>
<th>Count</th>
<th>% within EP Scy category</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;90</td>
<td>1.00</td>
<td>1</td>
<td>4.5%</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>2</td>
<td>9.1%</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>4</td>
<td>18.2%</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>4</td>
<td>18.2%</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>6</td>
<td>27.3%</td>
</tr>
<tr>
<td></td>
<td>6.00</td>
<td>3</td>
<td>13.6%</td>
</tr>
<tr>
<td></td>
<td>7.00</td>
<td>2</td>
<td>9.1%</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>22</td>
<td>100.0%</td>
</tr>
<tr>
<td>60-89</td>
<td>1.00</td>
<td>2</td>
<td>4.9%</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>2</td>
<td>4.9%</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>5</td>
<td>12.2%</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>4</td>
<td>9.8%</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>9</td>
<td>22.0%</td>
</tr>
<tr>
<td></td>
<td>6.00</td>
<td>14</td>
<td>34.1%</td>
</tr>
<tr>
<td></td>
<td>7.00</td>
<td>5</td>
<td>12.2%</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>41</td>
<td>100.0%</td>
</tr>
<tr>
<td>30-59</td>
<td>1.00</td>
<td>0</td>
<td>.0%</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>0</td>
<td>.0%</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>0</td>
<td>.0%</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>6</td>
<td>17.1%</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>7</td>
<td>20.0%</td>
</tr>
<tr>
<td></td>
<td>6.00</td>
<td>9</td>
<td>25.7%</td>
</tr>
<tr>
<td></td>
<td>7.00</td>
<td>13</td>
<td>37.1%</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>35</td>
<td>100.0%</td>
</tr>
<tr>
<td>15-29</td>
<td>1.00</td>
<td>0</td>
<td>.0%</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>0</td>
<td>.0%</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>0</td>
<td>.0%</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>0</td>
<td>.0%</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>0</td>
<td>.0%</td>
</tr>
<tr>
<td></td>
<td>6.00</td>
<td>2</td>
<td>100.0%</td>
</tr>
<tr>
<td></td>
<td>7.00</td>
<td>0</td>
<td>.0%</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>2</td>
<td>100.0%</td>
</tr>
<tr>
<td>Total</td>
<td>Count</td>
<td>3</td>
<td>4.0%</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>9</td>
<td>9.0%</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>14</td>
<td>14.0%</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>22</td>
<td>22.0%</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>28</td>
<td>28.0%</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>20</td>
<td>20.0%</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>100</td>
<td>100.0%</td>
</tr>
</tbody>
</table>
Table 4.8 EP-CKD2009 Scr category * Age category Cross tabulation provides the percentage of estimated GFR for overall participants with in respective age group category distribution, eGFR ml/min/1.73m² calculation. data generated using SPSS19

<table>
<thead>
<tr>
<th>EP-CKD2009 Scr category</th>
<th>Age Group category</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.00</td>
<td>2.00</td>
</tr>
<tr>
<td>&gt;90 Count</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>% within EP Scr category</td>
<td>2.7%</td>
<td>5.3%</td>
</tr>
<tr>
<td>60-89 Count</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>% within EP Scr category</td>
<td>.0%</td>
<td>.0%</td>
</tr>
<tr>
<td>30-59 Count</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>% within EP Scr category</td>
<td>25.0%</td>
<td>.0%</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>% within EP Scr category</td>
<td>3.0%</td>
<td>4.0%</td>
</tr>
</tbody>
</table>

Table 4.9 Cross tabulation Cystatin C level distribution into five duration categories in two diabetic clinics, in Eritrea.

<table>
<thead>
<tr>
<th>Cystatin c level</th>
<th>Duration interval in years</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;5</td>
<td>6 - 10</td>
</tr>
<tr>
<td>&gt;90</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>60-89</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>30-59</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>37</td>
</tr>
</tbody>
</table>
### Table 4.10, Chi-Square Tests

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
<th>df</th>
<th>Asymp. Sig. (2-sided)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson Chi-Square</td>
<td>19.994*</td>
<td>8</td>
<td>.010</td>
</tr>
<tr>
<td>Likelihood Ratio</td>
<td>17.428</td>
<td>8</td>
<td>.026</td>
</tr>
<tr>
<td>Linear-by-Linear Association</td>
<td>11.224</td>
<td>1</td>
<td>.001</td>
</tr>
<tr>
<td>N of Valid Cases</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

.7 cells (46.7%) have expected count less than 5. The minimum expected count is .12.

### Table 4.11 Chi-Square Tests

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
<th>df</th>
<th>Asymp. Sig. (2-sided)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson Chi-Square</td>
<td>9.964*</td>
<td>4</td>
<td>.041</td>
</tr>
<tr>
<td>Likelihood Ratio</td>
<td>7.293</td>
<td>4</td>
<td>.121</td>
</tr>
<tr>
<td>Linear-by-Linear Association</td>
<td>.636</td>
<td>1</td>
<td>.425</td>
</tr>
<tr>
<td>N of Valid Cases</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. 17 cells (68.0%) have expected count less than 5. The minimum expected count is .54.

### Table 4.12 EP-CKD 2012Mix category * Age category Cross table

<table>
<thead>
<tr>
<th>EP Mix</th>
<th>Age Group category</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCr/SCy category</td>
<td>&lt;20</td>
<td>20-29</td>
</tr>
<tr>
<td>&gt;90</td>
<td>Count</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>% within EP Mix category</td>
<td>5.9%</td>
</tr>
<tr>
<td>60-89</td>
<td>Count</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>% within EP Mix category</td>
<td>.0%</td>
</tr>
<tr>
<td>30-59</td>
<td>Count</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>% within EP Mix category</td>
<td>11.1%</td>
</tr>
<tr>
<td>Total</td>
<td>Count</td>
<td>3</td>
</tr>
</tbody>
</table>
The normal eGFR value: in adults, the normal eGFR value is more than 90 (According the NKF 2014) eGFR declines with age, even in people without kidney disease.

<table>
<thead>
<tr>
<th>Age Range in years</th>
<th>eGFR value</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-29</td>
<td>116</td>
</tr>
<tr>
<td>30-39</td>
<td>107</td>
</tr>
<tr>
<td>40-49</td>
<td>99</td>
</tr>
<tr>
<td>50-59</td>
<td>93</td>
</tr>
<tr>
<td>60-69</td>
<td>85</td>
</tr>
<tr>
<td>&gt; 70</td>
<td>75</td>
</tr>
</tbody>
</table>

Table 4.4 provides average (SD) of all participants respective to age group distribution of eGFR using (EP-CKD 2009, EP-CKD Scy C2012 and EP-CKD Mix)ml/min/1.73m². data generated using SPSS19
Annexure 3

3.5.1.1. Test principle and procedure of creatinine measurement

1. Test principle

Creatinine reagent was intended for the quantitative determination of creatinine concentration in human serum, plasma or urine. Used to measure the creatinine concentration by a modified rate Jaffé method (Newman, D. J. 1992, Tietz, N. W1994, NCCLS 1990). Serum creatinine combines with picrate in an alkaline solution to form a creatinine-picrate complex. The SYNCHRON® System(s) automatically proportions the appropriate sample and reagent volumes into the cuvette from manually supplied in appropriate rack position. The ratio used is one part sample to 11 parts reagent for serum.

Creatinine + picrate acid → alkaline solution → creatinine picrate (a red colored complex)

The system monitors the change in absorbance at 520 nanometers. This change in absorbance is directly proportional to the concentration of creatinine in the sample and is used by the system to calculate and express creatinine concentration.

Reference value of serum creatinine:
Male 0.9–1.3 mg/dl
Female 0.6–1.1 mg/dl

2. Test procedure

1. Each individual entered with specific identification, age, name, sex, location, sample type, time, sample position and label into the analyzer.
2. Specific test selected was ordered using the specific key menu on the display for all patients.
3. Sample aliquot was placed in their respective sample rack (Reserve® rack) in appropriate volume of cup (0.5ml cup) as identified earlier.
4. The sample rack with specific identification number was placed on autoloader with the rack bar code facing to the right in increasing order.
5. Quality control samples where run the same way as patient sample and examined and recorded.
6. System run started after making all necessary reagent confirmed in place with educate volume.
7. Finally test results were collected and documented in excel sheet for each patient.

N.B Fasting blood glucose was run from the same sample at the same time using appropriate separate specific glucose reagent.
b. Test procedure for Cystatin C

Components and reagents of ichroma™ includes;

i. ID chip.

ii. Detection buffer tube 25 per pack. which contains fluorochrome-labeled anti cystatin c antibody, fluorescence labeled anti-chicken IgY, bovine serum albumin as stabilizer and sodium azide in PBS as preservative.

iii. Test cartridge, containing both murine antibodies against human Cystatin C and chicken IgY have been immobilized at the test line and the control line, respectively.

iv. ichroma™ reader (FR203)

As described in the test insert, all contents where checked for integrity and expire date. Each serum sample aliquot, and test cartridge, and detection buffer was brought to room temperature prior testing.(20-30°C, for 30min, and <70% humidity).

The ichroma™ reader turned on, then the provided specific ID chip was inserted into its ID chip slot port. select button was Pressed. A 10μl of serum was transferred to the tube containing detection buffer using transfer pipette, mixed by shaking 10-15 times. After closing the lid of the detection buffer, and then 75μl of the sample mixture was dispensed into the sample well of the test cartridge. The sample loaded test cartridge was left for 10min to room temperature. then, scanned by loading it into the cartridge holder of the ichroma™ reader and select button was pressed to start scanning process. Finally the results were read from the screen of ichroma™ reader.

Test Principle and Procedure 1

Direct Enzymatic HbA1c Assay

Assay Principle

The Direct Enzymatic HbA1c test is an enzymatic assay in which lysed whole blood samples are subjected to extensive protease digestion with Bacillus sp protease. This process releases amino acids including glycated valines from the hemoglobin beta chains. Glycated valines then serve as substrates for specific recombinant fructosyl valine oxidase (FVO) enzyme, produced in E. coli. The recombinant FVO specifically cleaves N-terminal valines and produces hydrogen peroxide. This, in turn, is measured using a horseradish peroxidase (POD) catalyzed reaction and a suitable chromagen. No separate measurement for total Hemoglobin (Hb) is needed in this Direct Enzymatic HbA1c Assay. The HbA1c concentration is expressed directly as %HbA1c by use of a suitable calibration curve in which the calibrators have values for each level in %HbA1c.

Reagent Composition
Lysis Buffer  
CHES, pH 8.7  =  100 mM  
Triton-X-100  =  1 %  
SDS  =  0.45 %  
Redox Agents  =  0.5 mM  
Reagent R1a 
MES pH 7.0  =  5 mM  
Proteases  =  4 KU/ml  
Triton-X-100  =  0.5%  
Redox agents  =  >10µM  
Reagent R1b 
MES pH 6.3  =  1 mM  
Redox agent  =  <3 mM  
Reagent R2 
Tris pH 8.0  =  15 mM  
FVO enzyme  =  >10 U/ml  
POD  =  90 U/ml  
Chromagen  =  0.8 mM  

**Assay Procedure**

Whole Blood Bench Top Lysis Procedure  
1) Dispense 250 µL of Lysis reagent in a sample cup or an Eppendorf microfuge tube.  
2) Prior to testing, whole blood samples should be mixed by gentle inversion at least 5 times to resuspend settled erythrocytes. Accuracy of the assay will be affected if whole blood is not thoroughly mixed prior to testing. Add 20 µL of fully resuspended whole blood sample to the Lysis buffer in the sample cup or microfuge tube. Mix gently with a suitable pipettor without creating foam and incubate at room temperature (25°C) for 10min to completely lyses the red blood cells. Complete Lysis is observed when the mixture becomes a clear dark red solution without any particulate matter. Incubate the samples longer as needed to ensure complete Hemolysate preparation. The lysate, thus prepared, is ready for use in the Direct Enzymatic HbA1c assay steps and is stable up to 4 hours at room temperature.  
3) The calibrators and controls should be treated exactly as patient samples and used per instructions on labeling.  
4) Direct Enzymatic HbA1c assay reagents are comprised of Redox balanced components. mixed reagent blank or water as blank sample should not be used in this assay. For instruments that require a zero calibrator, please order Direct Enzymatic HbA1c Assay blank solution (IB46119-S0V).
Annexure 4
Index of Figures 1
Figures 1: Beckon Dixon (BD) Vacutainer test tubes (SRS, Microtainer, and K$_3$EDTA,) of different range tube container with vacutainer needle and needle holder.
Figure 2: Roche Hitachi 902, and Beck Man Coulter UNI Cell DXC 600 Chemistry Analyzers
Figure 3: ichromaTM™-Reader

Annexure 5
Index of diagrams;
Diagram 1: for DxC 600 sample processing summary schematic diagram.
Sample Processing

Start Here

Remove visible blood from the top of capped tubes using a cotton tipped swab moistened with DI water.

Validated CTS primary tube?

Yes

Adequate Sample Volume?

No

Remove cap

Adequate Sample Volume?

Yes

Is sample programmed at the LIS?

No

Use the "Low Volume Sample Processing" flowchart

Is there a readable bar code?

Yes

Select the Samples from the menu bar

No

Select Next F10

Place container in the appropriate rack. Ensure that bar code label, if present, is visible through the rack slot. Verify that the tube is seated properly.

Enter the Sample ID. Enter rack and position.

Select Next F10

Load the prepared racks on the auto loader with the rack bar code facing to the right.

Press the <RUN> button

Enter the Sample ID. Enter rack and position if readable bar code is not available.

Select the Chemistries.

If needed, select the STAT check box, Sample Type, Sample Comment and/or Demog F2

Select Next F10