SERUM CYSTATIN C AS A MARKER IN THE ASSESSMENT OF RENAL FUNCTION IN PATIENTS WITH RETINOPATHY AND MILD TO MODERATE DIABETIC NEPHROPATHY

BY

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Thesis submitted to the University of Sri Jayewardenepura for the award of the degree of Master of Philosophy in Biochemistry on 8th of April 2014.
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Dedication

I dedicate this thesis to

my husband, parents,

teachers and very close friends
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A

ACEi Angiotensin converting enzyme inhibitor
ACR Albumin to creatinine ratio
ADA American Diabetes Association
AER Albumin excretion rate
ANOVA Analysis of Variance
ARB Angiotensin receptor blockers
AUC Area under the curve

B

BMI Body mass index

C

CKD Chronic kidney disease
CG Cockcroft–Gault equation
51Cr-EDTA 51Cr-ethylenediaminetetra-acetic acid
CVD Cardiovascular disease
CysC Serum cystatin C

D

DR Diabetic retinopathy
DXA Dual energy X-ray absorptiometry

E

eGFR Estimated glomerular filtration rate
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<td>G</td>
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<td>HSD</td>
<td>Honestly Significant Difference</td>
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<td>K</td>
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<td>L</td>
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<td>Non proliferative diabetic retinopathy</td>
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$^{99m}$Tc-DTPA  $^{99m}$Tc-diethyleneethaminepenta-acetic acid

U

UAC  Urine albumin concentration

UAE  Urine albumin excretion

UKPDS  United Kingdom Prospective Diabetes Study

W

WHR  Waist to Hip Ratio
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Serum Cystatin C as a marker in the assessment of renal function in patients with retinopathy and mild to moderate diabetic nephropathy
Nadeeja Niranjali Wijayatunga

ABSTRACT

Introduction: Serum cystatin C (CysC) has been described as a promising marker of GFR. However there are no literature on performance of CysC in nephropathy and retinopathy (DR), in Sri Lankan type 2 diabetic patients (T2DM).

Objectives: To determine correlation between serum CysC and Serum Creatinine (SCr), Albumin to Creatinine Ratio (ACR), and estimated glomerular filtration rate based on Modification of Diet for Renal Disease study equation (eGFR-MDRD) in both T2DM patients and healthy adults, to compare CysC levels in T2DM subjects with mild to moderate diabetic nephropathy with age and gender matched healthy individuals and also to identify the correlation of DR in those selected T2DM patients with Cys, SCr, eGFR-MDRD and ACR.

Methods: Sixty one T2DM patients with possibility of mild to moderate renal impairment, and 118 apparently healthy adults, between 30-60 years were enrolled. Out of the 118 healthy adults, 61 were age and gender matched with the T2DM patients. Retinopathy status was assessed by slit lamp examination. SCr and CysC and urine creatinine and albumin were measured. ACR and eGFR-MDRD, eGFR- Chronic Kidney Disease Epidemiology Collaboration (CKD EPI) were calculated.

Results: CysC significantly correlated with SCr and eGFR –MDRD in both T2DM patients and healthy adults but was stronger in the T2DM patients. CysC significantly
correlated with ACR severity categories only in T2DM patients with a significant stepwise increase of CysC according to degree of albuminuria. The T2DM patients (n=61) had higher CysC than the matched controls. Both T2DM patient groups with moderate diabetic nephropathy (n=21) and microalbuminuria (n=22) had higher CysC than the respective matched healthy control groups (p<0.05). Only the ACR categories showed a significant correlation with DR categories (P<0.05). CysC cut off value for the diagnosis of moderate renal impairment was 0.98mg/L (sensitivity of 85.7%, specificity of 82.5%) and for albuminuria (ACR >30mg/g) was 0.96 mg/L (sensitivity of 73.3% and a specificity of 80.6%). Only CysC could differentiate the moderately increased chronic kidney disease (CKD) risk prognosis category from the absent/low CKD risk category (p < 0.05). The reference intervals for the healthy adults for < 50 years of age for male and females are 0.62 – 1.01 mg/L and 0.54 – 0.90 mg/L respectively while for > 50 years of age for males and females are 0.65 – 1.12 mg/L and 0.60 – 1.01 mg/L respectively.

**Conclusion:** CysC may be used as a reliable renal function marker in T2DM patients with mild to moderate diabetic nephropathy and it is also useful in early detection of poor prognosis in CKD. ACR may be able to predict DR status in T2DM patients. Our study suggests that screening for low GFR with CysC in a low-risk population is probably not worthwhile. In healthy adults, gender based reference intervals for less than 50 year and more than 50 years of age are suggested.
1. INTRODUCTION

1.1 General Introduction

Global prevalence of diabetes mellitus is predicted to increase rapidly from 4% in 1995 to 5.4% by the year 2025 due to increasing population growth, aging, urbanization and increasing prevalence of obesity and sedentary life [1]. Due to the insidious onset of type 2 diabetes mellitus (T2DM), about one fourth of the patients will be having at least one of the complications of diabetes at the time of diagnosis. A much higher prevalence of diabetic complications are observed in Asians than the Europeans [2]. Retinopathy and nephropathy are two important microvascular complications in diabetic patients with hyperglycemia. Eventually 20-40 % of T2DM patients develop CKD (Diabetic nephropathy) over 15 years and it is the leading cause of end stage renal failure (ESRF)[3, 4]. With the global epidemic of T2DM , the incidence of retinopathy is also expected to rise to higher levels[5]. Diabetic retinopathy is the leading cause of blindness in people of working age and the risk of a diabetes patient becoming blind is approximately 29 times more than a non-diabetic of the same age. Of the type 2 diabetics, 20% may have diabetic retinopathy at the time of diagnosis of diabetes for the first time[6].

Recent evidence suggests that there is a strong association between the prevalence of diabetes nephropathy and retinopathy [6]. Thus this highlights the importance of early identification of diabetic nephropathy and retinopathy, in the management and prevention of aggravation of these complications of T2DM.
1.2 Chronic Kidney Disease (CKD)

The rise in CKD and renal failure rate worldwide is associated with ageing, diabetes and other chronic non communicable diseases [7]. According to the literature, 30.3% of CKD is associated with diabetic nephropathy suggesting diabetic nephropathy as the leading cause of CKD[1]. Similarly it has also been reported that main contributing factor for CKD in Western province of Sri Lanka was diabetic nephropathy [8].

Patients with CKD have an increased risk of renal failure, cardio vascular disease (CVD) and other complications [7]. Anemia, hypertension, malnutrition, bone disease, and a decreased quality of life are some of the complications associated with CKD. Thus early detection of renal impairment will enable the clinicians to treat the patients and manage them more effectively [9].

1.3 Diabetic Nephropathy

In 2011, the International Diabetes Federation declared that there are 366 million diabetics worldwide and projected it to rise to 552 million by 2030. Most of the people with diabetes are from low- and middle-income countries [10]. Similarly the prevalence of T2DM in Sri Lanka is also projected to rise. In 1993 it was 5.02%, while in 2005 it rose to 10.3 % and one in every 5 adults who are more than 20yrs of age have either diabetes, or pre-diabetes. The projected prevalence of diabetes by year 2030 is 13.9% [11, 12]. In diabetic nephropathy, glomerular sclerosis, tubular and interstitial changes occurs and it is the leading cause of CKD, ESRF and is also associated with increased mortality worldwide [4, 13]. The prevalence of diabetic nephropathy is more among
Africans, Asians and Native Americans than in Caucasians [13]. South Asian T2DM patients develop diabetes at a younger age and have a higher loss of GFR (1.45 times higher than in Europeans) [14]. Diabetes was the most common (44%) cause for CKD in Sri Lanka [8, 15]. Thirty three percent of Sri Lankan type 2 diabetics had nephropathy [16].

1.4 Diabetic Retinopathy

Diabetic retinopathy is a major cause of blindness in the age group 20–60 years in the world and causes socio-economic problems for the patient as well as for the country [17]. More than 60% of T2DM patients will have DR after 10 years of onset of diabetes[5]. The earliest clinical signs of DR are present in nearly 80% after 20 years of T2DM [18]. During their life time 10% of diabetics will develop macular oedema [6]. Results from Sri Lanka show that retinopathy DR was present in 21.2% of T2DM patients [16].

Several mechanisms have been suggested for the occurrence of microvascular complications due to hyperglycaemia leading to retinopathy. Increased activity of protein kinase C (PKC) and formation of advanced glycation end products (AGEs) play a major role than polyol accumulation or oxidative stress in causing DR[19]. Treatment is most effective when DR is detected at an early stage when vision impairment has not occurred. The fundus can be examined by ophthalmoscopy, using a slit lamp or by retinal photography [17]. In April 2002, the Global Diabetic Retinopathy Group at the International Congress of Ophthalmology in Sydney, Australia developed the diabetic retinopathy severity scale (Table 1) [20].
Table 1. International Clinical Diabetic Retinopathy Disease severity scale [20]

<table>
<thead>
<tr>
<th>Proposed disease severity level</th>
<th>Findings observable upon dilated ophthalmoscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>No apparent retinopathy</td>
<td>No abnormalities</td>
</tr>
<tr>
<td>Mild NPDR</td>
<td>Microaneurysms only</td>
</tr>
<tr>
<td>Moderate NPDR</td>
<td>More than just microaneurysms but less than severe NPDR</td>
</tr>
<tr>
<td>Severe NPDR</td>
<td>Any of the following:</td>
</tr>
<tr>
<td></td>
<td>&gt;20 intra-retinal hemorrhages in each of 4 quadrants</td>
</tr>
<tr>
<td></td>
<td>Definite venous beading in 2+ quadrants</td>
</tr>
<tr>
<td></td>
<td>Prominent intra-retinal microvascular</td>
</tr>
<tr>
<td></td>
<td>abnormalities in 1+ quadrant</td>
</tr>
<tr>
<td></td>
<td>And no signs of proliferative retinopathy</td>
</tr>
<tr>
<td>PDR</td>
<td>One or more of the following:</td>
</tr>
<tr>
<td></td>
<td>Neovascularization</td>
</tr>
<tr>
<td></td>
<td>Vitreous/pre-retinal hemorrhages</td>
</tr>
</tbody>
</table>
The risk of significant progression is less for the “no apparent retinopathy,” and “mild NPDR” levels while the “severe NPDR” level has a high risk for progression to PDR which is the most severe form of retinopathy (Figure 1) [20].

Proliferative retinopathy may occur in about 10% of patients with type 2 diabetes after 15 years and if untreated, it has a poor prognosis. New vessel formation may lead to vitreous hemorrhage, tractional retinal detachments or neovascular glaucoma resulting in visual loss [18].

Figure 1. Photographs of retinopathy

a. Non-Proliferative Diabetic Retinopathy (NPDR) and b. Proliferative Diabetic Retinopathy (PDR) [17].
When diabetic retinopathy is in and around the macula, it is called as “diabetic maculopathy”, which may cause marked visual impairment [17]. Clinically significant macular oedema (CSME) is associated with high risk of visual loss [17]. Diabetics are also at a significantly increased risk of developing cataract [17]. In T2DM patients soon after diagnosis of diabetes, a comprehensive eye examination is required. If diabetic retinopathy is present, annual or more frequent eye examinations are recommended and if there is no evidence of DR, then eye examinations should be performed every 2 years [21].

1.5 Association between Diabetic Nephropathy and Retinopathy

The prevalence of diabetic retinopathy and nephropathy is more in South Asians at the time of diagnosis of T2DM in comparison to Europeans [2]. CKD is a risk factor for prevalent retinopathy while proteinuria is a risk factor for incident diabetic retinopathy [22]. Presence of diabetic retinopathy is significantly associated with progression of diabetic nephropathy [23]. Serra et al 2002 reported that, in a group of proteinuric T2DM patients, all those with diabetic retinopathy had diabetic glomerulopathy while diabetic glomerulopathy was present only in 74% of the patients without retinopathy [24].
1.6 Investigations currently used in the assessment of Diabetic Nephropathy in clinical practice

The two main manifestations of CKD in people with T2DM are a reduction in GFR or the presence of albuminuria / proteinuria. In type 2 diabetic nephropathy renal impairment and albuminuria are believed to be 2 different, complimentary manifestations, thus this mandates assessment for renal impairment as well as for albuminuria [25].

There was lack of effective screening / assessment of diabetic nephropathy till recent past in Sri Lanka and screening for diabetic nephropathy was done by annual assessment of urine albumin levels and serum creatinine (SCr) levels. However according to the new guidelines by the National Institutes for Clinical Excellence (NICE-2008), American Diabetes Association(ADA-2014) and Kidney Disease improving Global Outcomes (KDIGO -2012) it is recommended to measure annual urine ACR and estimated Glomerular Filtration Rate (eGFR) for more accurate assessment of diabetic nephropathy[21, 26, 27].

1.6.1 Glomerular Filtration Rate (GFR)

The human kidney contains $10^6$ capillary units called glomeruli [28]. By definition, GFR is the sum of filtration rate of all functioning nephrons of the kidney and it is accepted as the best overall index of kidney function and it varies according age, gender and body size [9, 29]. GFR is assessed by measuring clearance of a particular substance from plasma. GFR is expressed as the rate of clearance of plasma by the glomerulus per
minute and is corrected for standard body surface area (ml/min/1.73m²) [30]. The reference range of GFR in young individuals varies from 80-130 ml/min/1.73m² [13]. Several endogenous and exogenous markers are used to estimate GFR. An ideal marker of GFR is described as a marker with stable concentrations in plasma, freely filtered at the glomerulus, not reabsorbed, metabolized nor secreted by renal tubules and without any extra renal elimination from the body. But so far, such an ideal GFR marker is not found [29, 30].

1.6.2. Hierarchical arrangement of GFR markers

According to the hierarchical arrangement of GFR markers, continuous infusion of Inulin with urinary clearance method is considered as the “Gold” standard. Inulin (molecular mass -5000 Da) is freely filterable at the glomerulus; not reabsorbed, secreted, or metabolized by the renal tubule; notbound to plasma proteins; nontoxic; and physiologically inert. However, this method is not widely used in clinical practice due to the necessity for an intravenous infusion and cumbersome chemical assay required [28]. The “Silver” standard methods measures plasma clearance following a single bolus of Inulin, ⁵¹Cr-ethylenediaminetetra-acetic acid(⁵¹Cr-EDTA), ⁹⁹ᵐTc-diethylenetriamine-penta-acetic acid (⁹⁹ᵐTc-DTPA), ¹²³I-iothalamate, and Iohexol. The endogenous markers of GFR such as SCr and CysC belong to “Bronze” standard methods. The formerly popular blood urea and creatinine clearance did not belong to any of the gold, silver or bronze categorization [31].
1.6.3 Measured Glomerular Filtration Rate (mGFR)

Plasma clearance of both non-radioisotopic and radioisotopic labeled markers are used to measure GFR. Inulin, and lohexol are non-radioactive markers, whilst $^{123}$I-iothalamate, $^{99m}$Tc-DTPA and $^{51}$Cr-EDTA are radioisotopic markers[31]. Even though measured GFR is considered to be more accurate, it is costly, time consuming, labour intensive, not entirely free of risk to patients and can be performed only in specialized laboratories [32].

1.6.4 Endogenous Markers of Glomerular Filtration Rate

Substances which are formed in the body such as blood urea, SCr and several low molecular weight proteins which are potential markers of GFR such as CysC, β2-microglobulin and β-trace protein, are categorized as endogenous markers of GFR and the serum levels of these are measured [32, 33]. SCr and CysC are completely filtered out by the glomeruli [32]. Those are used to calculate the eGFR using different formulae. eGFR is considered as a useful first step in the detection, evaluation and management of CKD [34].

1.6.4.i Blood Urea

Urea is a byproduct of protein breakdown and was historically the first marker used to assess renal function. But due to the poor sensitivity and specificity it has limited value[1, 31].
1.6.4.ii. Serum Creatinine

Creatinine replaced blood urea in mid 1900’s and remains as the most commonly used marker [33]. Creatine is synthesized primarily in the liver and released into circulation. It is phosphorylated into phosphocreatine which is a high energy compound which provides energy during metabolic process of muscle contraction in the muscles. Muscle contains 98% of the total body creatine of which 60-70% is phosphocreatine, and the rest is free creatine. A proportion of free creatine (1-2%/day) is non enzymatically and irreversibly converted to creatinine which is its anhydride waste product[28, 31]. Thus creatinine is a non-protein nitrogenous metabolite produced at a constant rate in the body. It has a molecular weight of 113 Da, physiologically inert and is not protein bound. Therefore it is freely filtered at the glomerulus and not metabolized by the kidney demonstrating some characteristics of an ideal GFR marker[31].

The amount of creatinine produced by a person per day is relatively constant but it is proportional to the muscle mass [31] Thus SCr values are affected by sex, age, ethnicity, muscle mass, chronic illness and meat consumption and will also be altered in people with amputations and in vegetarians [3, 32, 35].Reference interval for SCr using Jaffe methods are 0.9-1.3mg/dL (80- 115 μmol/L ) in men and 0.6-1.1 mg/dL ( 53-97 μmol/L) in women [31]. But the use of a single reference range for SCr values to detect renal impairment will be incorrect [9].

A substantial fraction of creatinine excretion occurs by the kidney as about 15% is secreted at proximal tubule, leading to a normal individuals’ creatinine clearance exceeding inulin clearance by 10- 40% [28, 32]. Furthermore a decrease in GFR to
around 40 ml/min/1.73m² does not cause an increase in SCr above the normal upper limit and also in CKD, the increase of SCr measured is only about 30-50% of what could be expected for the actual GFR. This is because, extra glomerular elimination of creatinine (16-66%) increases via tubular secretion and intestinal elimination as kidney function decreases. Therefore the relationship between GFR and SCr is a reciprocal non-linear one [33]. This may cause SCr levels to be within the normal range even with a GFR close to 60ml/min/1.73m² and this is called as the “creatinine blind range” [36]. Hence, if previous records are not available, a concentration within the normal range cannot be interpreted correctly [32].

Serum creatinine levels can be measured by the colorimetric Jaffé assay, enzymatic methods, high-performance liquid chromatography (HPLC) and the gold standard isotope dilution mass spectrometry (IDMS). Due to the cost effectiveness and convenience, Jaffé method is commonly used despite the lack of specificity by this method when measuring creatinine levels. Non creatinine chromogens such as bilirubin, ascorbic acid, acetic acid, pyruvate, glucose and proteins can cause colour reactions with this method leading to 15-25% overestimation of SCr levels. In order to overcome this, manufacturers have adjusted the calibrations of the assays and have produced “compensated” versions of Jaffé method [3, 28, 36, 37]. With this an additional source of error was introduced into the mathematical prediction of GFR due to lack of standardization for creatinine measurement since there were variations between assays [38]. Thus in 2008, the National Kidney Disease Education Program (NKDEP) in collaboration with the International Federation of Clinical Chemistry(IFCC) and the European community’s Confederation of Clinical Chemistry launched the creatinine
Standardization Program in order to reduce inter laboratory variability in creatinine assay calibration [33]. Even though reference methods using IDMS can measure the SCr accurately, it is available only in a few laboratories [36]. Thus it has been recommended that the calibration of creatinine assays to be traceable to an international reference creatinine method such as IDMS [39].

In Sri Lanka, SCr is the most widely used index for non-invasive assessment of GFR. However not all the laboratories in the Sri Lanka are using standardized methods in measurement of SCr. Even though measured GFR is more accurate and is the gold standard, it is not practical to use it for all the patients in the clinical setup as it is expensive, invasive and cumbersome.

1.6.4.iii. Serum Creatinine based estimated Glomerular Filtration Rates

Estimated glomerular filtration rate is reported in milliliters per minute and corrected for standard body surface area [ml. min⁻¹. (1.73 m²)⁻¹] [33]. Several SCr based equations were developed to estimate GFR and to increase the reliability. The most used equations are the Cockcroft–Gault (CG), MDRD, CKD EPI equations for adults and Schwartz equation for children. However these equations do have limitations since those cannot correct the errors that may occur as a result of unusually high or low muscle mass, extreme diets (vegans or excessive meat consumption) or ethnic variations of groups [33].
1.6.4.iii (a) Cockcroft–Gault Equation

This equation was developed in 1973 using data from 249 healthy men who had creatinine clearances in the range of 30 to 130 ml per minute [9].

The estimating equation is,

\[
eGFR = (140 - \text{age in years}) \times (\text{weight in kg/72} \times \text{SCr in mg/dl})
\]

\[
\times 0.85 \text{ (if the subject is female)}
\]

Since the values are not adjusted for body-surface area, a comparison with normal values for eGFR requires measurement of height, computation of body-surface area, and adjustment to 1.73 m² [9].

There are few limitations for the use of this equation which was formulated using outdated Jaffé assay for SCr measurement. Therefore it must be used with caution when using current creatinine methods to calculated eGFR [36]. The other limitation is that it tends to overestimate GFR particularly at lower SCr concentrations [33].

1.6.4.iii (b) Modification of Diet in Renal Disease Study Equation

The MDRD study equation was developed in 1999 using data from 1628 patients with CKD and the initial equation used age, gender, ethnicity, SCr, urea nitrogen, and albumin to calculate eGFR. The advantage of not using weight to calculate eGFR is that it is less prone to errors from fluid overload and obesity [40].

In 2000, a more simplified 4 variable MDRD study equation was formulated.

\[
GFR = 186 \times (Scr)^{-1.154} \times (age)^{-0.203} \times 0.742 \text{ (if the subject is female)}
\]

\[
or \times 1.212 \text{ (if the subject is black)}
\]

[9]
This equation was re-expressed in 2005 for use with a standardized serum creatinine assay.

\[ \text{GFR} = 175 \times (\text{standardized Scr})^{-1.154} \times (\text{age})^{-0.203} \times 0.742 \text{ (if the subject is female)} \]

or \[ \times 1.212 \text{ (if the subject is black)} \]  

[9]

The MDRD study equation has been evaluated in various populations, including blacks, whites, and Asians with non diabetic kidney disease, patients with diabetes and kidney disease, patients with diabetes without kidney disease, kidney-transplant recipients, and potential kidney donors [9, 36].

MDRD equation tends to underestimate the GFR above 60ml/min/1.73m², especially at lower SCR concentrations because the MDRD equation was developed exclusively using data from patients with CKD [32, 36]. However the performance of re-expressed MDRD study equation was improved after calibration to standardized creatinine and provides reasonably accurate estimates when eGFR was less than 60ml/min/1.73m²[41]. The 4 variable MDRD study formula has been recommended by the NKDEP for estimation of GFR and studies have shown MDRD to be superior to CG equation [42]. However, in order to overcome any errors, the NKDEP recommends that eGFR values above 60ml/min/1.73m² to be reported as “more than 60ml/min/1.73m² (>60ml/min/1.73m²)” and not as an exact number and the values less than 60ml/min/1.73m² are to be rounded up to the nearest whole number [33, 37]. Therefore a problem exists in diagnosis of incipient diabetic nephropathy due to the fact that in early stages of diabetic nephropathy with renal hypertrophy and hyper filtration, the
GFR may be normal or raised [32]. Currently in Sri Lanka, MDRD equation is the widely used equation to calculate eGFR using SCr.

1.6.4.iii.(c) Chronic Kidney Disease Epidemiology Collaboration Equation

In 2009, CKD EPI equation was developed using SCr. CKD EPI has proved to be more accurate than the commonly used MDRD equation and has a lower bias, mainly for eGFR more than 60ml/min/1.73m² [43]. It has less sensitivity but higher specificity for detection of GFR more than 60ml/min/1.73m². With the CKD EPI equation one could report eGFR as a numerical value even above eGFR of 60ml/min/1.73m² unlike for MDRD study equation. [44]. Thus, CKD EPI equation would be more appropriate for estimation of GFR in public health and general practice rather than in nephrology practice because this equation cause over estimation at lower GFRs. CKD EPI equation has been expressed for use with creatinine assays traceable to IDMS [44].

2009 CKD-EPI creatinine equation:

$$eGFR = 141 \times \min(SCr/k, 1)^{a} \times \max(SCr/k, 1)^{-1.209} \times 0.993^{\text{Age}} \times [1.018 \text{ if female}] \times [1.159 \text{ if black}]$$

where, SCr (mg/dl), k is 0.7 for females and 0.9 for males, a 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 [27].
1.6.4.iv. Twenty four hour Urine Creatinine Clearance

Urine creatinine clearance is also used to assess GFR. The creatinine concentration in a timed / 24 hours urine collection and in a blood sample is needed for the calculation [1]. Creatinine clearance overestimates GFR by 10-40% as creatinine is secreted by proximal convoluted tubule and by small intestine. Thus it would underestimate the severity of the renal impairment at lower GFR [29]. Also errors occur due to inaccuracies in collection of urine by the patients. Thus, it is not categorized in the hierarchical arrangement and it is considered as an imperfect marker of GFR and it is no longer recommended routinely to estimate the level of kidney function [9, 31, 42].

1.6.5 Albuminuria

Albumin is negatively charged at physiological pH and is repelled by the anionic residues in endothelial cell, glomerular basement membrane or podocytes [45]. Even though there are high amount in blood only a very small amount of protein is excreted in the urine by normal individuals. In a normal person the proteinuria level is less than 150mg/d, which consists of 120mg/d tubular proteins and no more than 30mg/d of albumin [46, 47]. About 1-2 mg/min (about 2 g/d) of albumin is normally filtered by glomeruli but 99% of those filtered albumin is reabsorbed and degraded by the proximal convoluted tubule. Thus only a very small amount, less than 5 μg/min (7mg/g), is excreted in urine in a normal individual [45].
But in kidney damage, persistently high protein excretion is detected. In most of the CKD due to diabetes, glomerular disease, and hypertension, increased levels of albumin in urine is detected. Therefore urine albumin is a sensitive marker for CKD [47].

Screening for urine albumin excretion can be performed by ACR in a random spot collection or in a 24 hour or timed urine collections. Measurement of only albumin in spot urine using immunoassay or dipsticks, may lead to false values due to variations in urine concentration. The 24 hour sample may result in errors due to incomplete urine collection[21]. The use of ACR or total protein to creatinine ratio will correct variability due to concentration of urine by hydration, diuretics, osmotic diuresis, concentration defects and variations in urinary flow rates. The urinary albumin is divided by urine creatinine concentration with the assumption that creatinine excretion is constant and is about 1 gm per day across measurements[9, 33]. At present reference methods for measurement of urine albumin are not available[33].

The urine albumin level may increase with urinary tract infections, posture, following exercise, fever, congestive cardiac failure, hyperglycemia, marked hypertension, menstruation. Also it may be affected by patient’s state of hydration and the method of sample collection [42, 47, 48]. ACR is affected by the level of creatinine excretion in addition to the level of albumin excretion because, creatinine excretion reflects creatinine generation by muscle mass and to a lesser extent, dietary intake [41]. Because of the variability, albuminuria is confirmed by high levels in at least 2 out of 3 sample collections, within a period of 3 - 6 months [21].

The newer nomenclature uses the terms persistent albuminuria and normal albuminuria and does not use the terms microalbuminuria or macroalbuminuria (Table 2).
Table 2. Definitions of abnormalities in albumin excretion according to the 2014 American Diabetes Association guidelines [21]

<table>
<thead>
<tr>
<th>Category</th>
<th>Spot collection (µg/mg creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>Increased urinary albumin excretion*</td>
<td>≥ 30</td>
</tr>
</tbody>
</table>

*Historically the ratios between 30 and 299 was called as microalbuminuria and 300 or greater was called as macroalbuminuria.

Since 7% of T2DM patients have microalbuminuria at the time of diagnosis of diabetes, screening for albuminuria is required at the first visit and must be repeated annually [13]. Microalbuminuria has an increased probability of progressing to CKD [45]. And it is detected in 20-40% of diabetics 10 to 15 years after the onset of type 2 diabetes. In diabetics with microalbuminuria, decline in GFR varies from one patient to another and only in 20-40% of patients does it progress to macroalbuminuria or overt nephropathy 15 to 20 years after the onset of type 2 diabetes. [49]. Nevertheless, spontaneous remission of microalbuminuria also occurs in more than 50% of diabetics [45]. Decline in GFR is lower in normoalbuminuric patients than in proteinuric patients [50]. According to the United Kingdom Prospective Diabetes Study (UKPDS) 25% of newly diagnosed T2DM patients develop diabetic nephropathy by 10 years and the rate of deterioration from one stage to the other was 2-3% per year. [51]. Preceding
albuminuria was not observed in 51% of those who developed renal impairment and only 24% of the patients who developed albuminuria later developed renal impairment [52]. Similarly, in NHANES III (Third National Health and Nutrition examination Survey) renal impairment was detected in 30% of patients without albuminuria or retinopathy [13]. Diabetics with macroalbuminuria face a 19 times more rapid decline in renal function compared to diabetics without albuminuria [53]. Microalbuminuria reflects diffuse endothelial injury [35]. Since there is an association between the level of albuminuria, progression to CKD and cardiovascular events, it is considered as a biomarker of adverse outcomes [45].

1.6.6 Serum Cystatin C

On the quest for a better marker to estimate GFR, CysC was discovered. It was discovered in 1961 and since 1985 CysC has been described as a promising endogenous marker of GFR [36]. CysC testing is currently available in few of the private sector hospitals however it is not routinely used in clinical practice in Sri Lanka as it is novel and expensive.

1.7 Assessment of Chronic Kidney Disease

According to the latest 2012 KDIGO guideline, CKD is classified based on cause, GFR category, and albuminuria category as given below (Table 3, Table 4) [27].
Table 3. GFR categories [27].

<table>
<thead>
<tr>
<th>GFR category</th>
<th>GFR (ml/min/1.73 m²)</th>
<th>Terms</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>≥ 90</td>
<td>Normal or high</td>
</tr>
<tr>
<td>G2</td>
<td>60–89</td>
<td>Mildly decreased</td>
</tr>
<tr>
<td>G3a</td>
<td>45–59</td>
<td>Mildly to moderately decreased</td>
</tr>
<tr>
<td>G3b</td>
<td>30–44</td>
<td>Moderately to severely decreased</td>
</tr>
<tr>
<td>G4</td>
<td>15–29</td>
<td>Severely decreased</td>
</tr>
<tr>
<td>G5</td>
<td>&lt;15</td>
<td>Kidney failure</td>
</tr>
</tbody>
</table>

*Relative to young adult level

It is recommended by the KDIGO 2012 guideline and by the NKDEP for clinical laboratories to measure SCr using a specific assay with calibration traceable to the international standard reference materials (SRM) and with minimal bias compared to IDMS reference methodology and to use a GFR estimating equation using SCr (eGFR-SCr) rather than relying on the SCr concentration alone. The NICE guidelines recommends the use of revised four-variable MDRD equation while the KDIGO 2012 guideline suggests using SCr based 2009 CKD-EPI equation [27, 54].
Table 4. Albuminuria * categories[27]

<table>
<thead>
<tr>
<th>Categ</th>
<th>AER (mg/24 hours)</th>
<th>ACR (approximate equivalent)</th>
<th>Terms</th>
</tr>
</thead>
<tbody>
<tr>
<td>ory</td>
<td>&lt;30</td>
<td>&lt;3</td>
<td>Normal to mildly increased</td>
</tr>
<tr>
<td>A2</td>
<td>30 - 300</td>
<td>3 - 30</td>
<td>Moderately increased*</td>
</tr>
<tr>
<td>A3</td>
<td>&gt;300</td>
<td>&gt;30</td>
<td>Severely increased</td>
</tr>
</tbody>
</table>

*Relative to young adult level.

For the diagnosis of CKD markers of kidney damage or decreased GFR must be present for a duration of more than 3 months (Table 1.5).

Progressive CKD will be defined based on decline in GFR category accompanied by a 25% or greater drop in eGFR from baseline and rapid progression is defined as a sustained decline in eGFR of more than 5 ml/min/1.73 m²/year [27].
Table 5. Criteria for Diagnosis of CKD

Either of the following should be present for more than 3 months[27].

<table>
<thead>
<tr>
<th>Markers of kidney damage</th>
<th>Albuminuria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(AER ≥ 30 mg/24 hours; ACR ≥ 30 mg/g)</td>
</tr>
<tr>
<td>(one or more)</td>
<td>([≥ 3 mg/mmol])</td>
</tr>
</tbody>
</table>

Urine sediment abnormalities
Electrolyte and other abnormalities due to tubular disorders
Abnormalities detected by histology
Structural abnormalities detected by imaging
History of kidney transplantation

Decreased GFR

GFR < 60 ml/min/1.73 m2 (GFR categories G3a-G5)

The ADA and NICE guidelines recommend annual testing of urine for albumin (ACR) in all type 2 diabetic patients starting at diagnosis. Irrespective of albumin measurement SCr should also be measured annually and the eGFR calculated[55, 56].

Until recent times, clinical decision making in CKD was based solely on GFR. The 2012 KDIGO guideline has proposed a staging system for the prediction of prognosis of CKD using GFR and albuminuria categories (Figure 1.2). The GFR and albuminuria grid reflects the risk of progression of CKD, which is graded by intensity of coloring (green, yellow, orange, red, deep red) (Figure 2).
Green - Low risk (if no other markers of kidney disease, no CKD
Yellow - Moderately increased risk
Orange - High risk
Red - Very high risk

<table>
<thead>
<tr>
<th>A1</th>
<th>A2</th>
<th>A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal to mildly increased</td>
<td>Moderately increased</td>
<td>Severely increased</td>
</tr>
<tr>
<td>&lt;30mg/g &lt;3mg/mmol</td>
<td>30-300mg/g 3-30mg/mmol</td>
<td>&gt;300mg/g &gt;30mg/mmol</td>
</tr>
</tbody>
</table>

Figure 2. Prognosis of CKD by GFR and albuminuria category [27]

The KDIGO 2012 guideline has suggested the use of CysC or mGFR to confirm specific circumstances when eGFR based on SCr is less accurate. In instances when eGFR based on SCr is between 45–59 ml/min/1.73 m² and not having markers of renal damages such as albuminuria, the guideline suggests the use of CysC. If eGFR calculated by both SCr and CysC are less than 60 ml/min/1.73 m², the diagnosis of CKD is confirmed [27].
1.8 Justification

With a high projected diabetes prevalence of 13.9% for the year 2030 in Sri Lanka, in future, there will be more T2DM patients with diabetic nephropathy and retinopathy [12]. Early diagnosis and proper treatment of diabetic nephropathy before the progression to end stage renal failure would reduce the cost of dialysis and transplantation and would also have a significant effect on patients’ wellbeing. Similarly, early detection of retinopathy would reduce the progression to blindness. To diagnose and monitor renal functions in T2DM patients, assessment of GFR and albuminuria are the methods currently used in Sri Lanka. Earlier studies have shown that CysC is a reliable marker of GFR in patients with mild to moderate renal impairment[3]. Recently a “3-dimensional” approach to the diagnosis and staging of CKD has been proposed using albuminuria and eGFR based on SCr and CysC [57]. Also the latest guideline on testing for CKD has suggested the use of CysC based eGFR as a confirmatory test in patients when the diagnosis of CKD is doubtful [27]. Even though many studies have been carried out in Caucasian patients, CysC not been evaluated extensively by research in Sri Lanka. According to the literature there had been no studies carried out in Sri Lanka in T2DM patients to find out the association between CysC concentrations with eGFR and albuminuria, nor studies had been done to identify the association between diabetic retinopathy and CysC, SCr, ACR. Hence, from clinical point of view, more information on CysC in T2DM with mild to moderate nephropathy and association of renal function markers with diabetic retinopathy would help the medical practitioners when planning out investigations for the diagnosis,
management of their patients. Thus, our aim was to explore the possibility of utilizing CysC in Sri Lankan T2DM patients, by studying the associations between CysC, SCr, eGFR and ACR in T2DM patients with mild to moderate nephropathy. Furthermore in screening the association of retinopathy with CysC, SCr, eGFR and ACR would assist in the clinical assessment of diabetic nephropathy.
1.9 Objectives

General Objectives:

1. To identify the correlation between serum cystatin C and serum creatinine, albuminuria, and estimated glomerular filtration rate calculated using MDRD study equation and also to compare serum cystatin C levels in healthy individuals and subjects with type 2 diabetes.

2. To identify the correlation of diabetic retinopathy with serum cystatin C and selected renal function tests in diabetic patients.

Specific Objectives:

1. To identify correlation between serum cystatin C and serum creatinine.

2. To identify correlation between serum cystatin C and urinary albumin excretion.

3. To identify correlation between serum cystatin C and estimated GFR using MDRD formula.

4. To compare serum cystatin C levels in diabetic patients with a sample of normal people.

5. To identify the correlation between diabetic retinopathy and serum cystatin C, serum creatinine and albuminuria in diabetic patients.
2. LITERATURE REVIEW

2.1 Cystatin C structure and metabolism

Cystatin C is a low molecular weight protein with a molecular mass of 13 kD [3, 58]. In 1961 Jorgen Clausen was first to report the occurrence of a “cerebrospinal fluid-specific” protein [59]. CysC was initially known as inter alia γ-trace, post-γ-globulin, and gamma-CSF protein. Subsequently it’s amino acid sequence was determined in 1981 [60]. CysC has a non glycosylated polypeptide chain composed of 122-amino acids and is positively charged due to an isoelectric point of 9.3 [60, 61]. The crystal structure of CysC has been identified. Its structure shows a long α helix running across a large, five-stranded antiparallel β-sheet and has an ellipsoid shape with axes of about 30 Å (Figure 3) [60]. Human CysC has four characteristic disulfide-paired cysteine residues. Human CysC is a monomer in its native functional state in biological fluids, whereas significant amounts exists as dimers in pathological states [59].

The half-life of CysC is 20 minutes [60]. It is found in all major human biological fluids such as urine, human plasma, CSF, ascitic and pleural fluid [61, 62]. However the levels of CysC are highly variable; it is present in micro molar levels in cerebrospinal fluid and semen but in serum, saliva and tears it is found in much lower concentrations [58].

Cystatin belongs to a super family of cysteine protease inhibitors which are divided into 3 main families; type 1 cystatins include stefins A and B which are intracellular; type 2 cystatins include, cystatin C, D, E, F, G, S, SN,SA and these are extracellular; whilst the type 3 cystatins include the kininogens and are intravascular. These are a group of
potent, non-covalent, competitive inhibitors of mammalian lysosomal cysteine proteinases like lysosomal cathepsins B, H, L and S [59, 60]. Thus, usually CysC has a protective function, preventing connective tissue from destruction by intracellular enzymes leaking from dying cells or being misrouted for secretion from malignant cells. Furthermore CysC is suggested to be involved in defense mechanisms against microbial infections and to have an antiviral function [61].

![Figure 3. Models for Cystatin C monomer (a) and dimer (b).][63]

The production of CysC occurs at a constant rate by all nucleated cells of the body, and it is encoded by CST3 gene in chromosome 20. This gene has some features of a housekeeping gene, ubiquitously expressed at moderate levels [3, 59, 61, 62]. However it is not an acute phase protein [58].

Cystatin C is almost completely cleared from the circulation by the glomerular ultrafiltration. It is freely filtered by the glomerulus because of its low molecular weight and positive charge [59]. Then it is almost completely reabsorbed and degraded by the
proximal tubules. Thus the concentration of cystatin C excreted in urine is very small and negligible under normal conditions (0.03-0.3 mg/l). Plasma or serum concentrations are a good measure of GFR even though urine clearance of cystatin C cannot be used for that purpose[3, 61].

2.2 Factors affecting Serum Cystatin C Levels

Knowledge of non GFR determinants of CysC helps the clinicians in the interpretation of serum levels of CysC. Several earlier studies have shown that CysC range in the normal population is constant after the age of 1 year. There is no circadian rhythm and CysC is not affected by extra renal factors such as age, gender, diet and muscle mass like SCr[1, 58]. CysC correlates with decrease in GFR due to aging and in people more than 80 years of age, more than 50 % have abnormal CysC values [32, 64].

However, in contrast to the popular belief, in the study carried out by Knight et al 2004, CysC was significantly associated with increased age, male gender, increased weight and height, current smoking, and high CRP levels even after adjustment for creatinine clearance levels [65]. CysC also increases independently of GFR in patients with malignancies, with excessively elevated rheumatoid factor and in hyperthyroidism. Low or medium doses of glucocorticoids (20-60mg prednisolone) do not affect CysC levels, but larger doses (methyl prednisolone 500mg) increase CysC levels. [36]. Higher CysC levels (or lower eGFR- CysC) are also observed with decreased physical activity, higher triglycerides, high levels of low density lipoprotein (LDL) cholesterol, low levels of high density lipoprotein (HDL) cholesterol, and obesity [66]. Also an association between CysC with BMI and fat mass has been suggested [67]. Even though
obesity is associated with high levels of CysC, CysC based eGFR is more sensitive and specific in overweight and obese subjects than SCr based eGFR equations [68, 69]. However, in a population of hypertensive individuals, only diabetes was the independent predictor of high CysC levels, whereas other variables, such as age, gender, obesity, dyslipidemia and smoking, were not [70]. Furthermore, according to some studies, CysC is not associated with inflammation [71, 72].

2.3 Measurement Methods of Serum Cystatin C

CysC levels are not significantly affected by various storage conditions. It is stable at room temperature for at least 7 days, for 1 to 2 months at -20°C and for at least 6 months at -80°C. It can withstand at least seven freeze/thaw cycles. There will not be any adverse effects when blood is left unseparated for up to 24 hours [73].

Initially, CysC was measured using physio-chemical properties like electrophoretic mobility and papain inhibition [59]. The first immunoassay for the measurement of CysC was developed in 1979 by Lofberg and Grubb and later radio-, fluorescent, and enzymatic immunoassays were developed [74]. Following the sandwich enzyme immunoassay developed to measure CysC in 1993, further development in measurement techniques lead to the introduction of a particle enhanced turbidimetric immunoassay (PETIA). This is a fully automated, with fixed interval and shorter analytical time turbidimetric measurement. In 1997, particle enhanced nephelometric immunoassay (PENIA) was introduced [3]. In both these automated immunoassays, latex or polystyrene particles coated with CysC antibodies are utilized [75]. Both
PETIA and PENIA methods have a faster turnaround time and higher precision since these methods are not interfered by other substances unlike the Jaffé method [3]. CysC reference material (ERM-DA471/IFCC) was produced in 2010 by the IFCC working group for standardization in collaboration with the Institute of Reference Materials and Measurements, since various CysC measurement procedures resulted in results which were not uniform [76].

2.4 Reference Ranges

The use of different standardization procedures and the use of different methods in the measurement of CysC, has resulted in different reference intervals [61]. A high value of CysC in present at birth but as the renal function matures it rapidly declines within the first year [60]. Some studies have suggested the use of one reference interval after the age of 1 year [73] while, some studies have recommended reference intervals using the age 50 years for dichotomizing and having different reference ranges for above and below 50 years in adults [61] (Table 6).
Table 6. Different reference ranges for Serum Cystatin C

<table>
<thead>
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<th>Authors</th>
<th>Method of CysC measurement</th>
<th>Reference ranges</th>
<th>References</th>
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<tr>
<td>Norlund L et al 1997</td>
<td>PETIA</td>
<td>20-50 years 0.70 - 1.21</td>
<td>[77]</td>
</tr>
<tr>
<td>Randers E et al 1999</td>
<td>Not</td>
<td>&gt;50 years 0.84 - 1.55</td>
<td></td>
</tr>
<tr>
<td>Finney H et al 2000</td>
<td>PENIA</td>
<td>&lt;50 years 0.53 ± 0.92</td>
<td>[78]</td>
</tr>
<tr>
<td>Ognibene A et al 2006</td>
<td>PENIA</td>
<td>&lt;45 years &lt; 0.95</td>
<td>[79]</td>
</tr>
<tr>
<td>Ichihara K et al 2007</td>
<td>PENIA</td>
<td>30-50 years 0.60 - 0.95</td>
<td>[80]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Males 0.60 - 0.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Females 0.55 - 0.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>51-75 years</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Both 0.64 - 1.05</td>
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</table>
2.5 Serum Cystatin C as a marker of GFR

CysC demonstrates the some important features of an “ideal” endogenous marker to estimate GFR as it is freely filtered at the glomerulus, almost completely reabsorbed, not secreted by renal tubules and fully catabolized in the renal tubules [74]. In addition, CysC is considered as a high potential endogenous marker of GFR due to its independence from height, age, gender, and body composition as well as acute phase reactions in the body. Therefore renal function is the main determinant of its serum concentration [60]. Patients with abnormal CysC levels (≥1mg/l) , but without renal impairment based on SCr (eGFR more than 60ml/min/1.73m²) have been proposed to be called as patients with “preclinical kidney disease” [36].

2.6 Advantages of Serum Cystatin C over Serum Creatinine

Like SCr, CysC also shows an inverse, non linear relationship with GFR. However, when GFR falls to 40- 70 ml/min/1.73m² the proportional increase of CysC is higher than SCr [32]. Thus when there is only a mild reduction in GFR, and changes in SCr is not observed (“creatinine blind” range) but there will be changes in CysC levels. Hence, CysC may be able to identify the gradient of kidney function among patients who are not diagnosed as having clinical kidney disease by conventional definition (Figure 4) [33].

CysC is a better marker of GFR than SCr in children, elderly, myaesthenics, leg amputees, paraplegics, patients with malignancies and chronically ill patients in whom, SCr may be unreliable due to low muscle mass. Also CysC is useful to assess GFR in
cirrhotic patients as SCr values are unreliable in them due to reduction in creatinine metabolism in the liver [3, 32, 81].

**Figure 4.** Concentrations of serum creatinine and Cystatin C according to GFR [32].

Proportional increase in serum creatinine (black) and serum Cystatin C (gray) with decreasing GFR.

### 2.7 Serum Creatinine and Creatinine based eGFR Equations

SCr is the most widely used marker in assessment of renal function since 1900 [33]. GFR is currently considered the best overall measure of kidney function and eGFR is an accurate, inexpensive and widely available method to estimates GFR [38]. According to the guidelines, SCr alone should not be used to assess renal function and it is recommended to use a SCr based equation to calculate eGFR [27]. The CG, MDRD, and CKD EPI equations are SCr based equations used to calculate eGFR in adults. The
performance of these equations varies depending on the population. MDRD equation can adequately predict the GFR in CKD and diabetic nephropathy patients and has better performance than CG equation [82]. Even though eGFR calculated using both C-G and MDRD study equations correlated well with measured GFR, MDRD equation had better diagnostic accuracy for moderate and severe renal impairment in diabetic patients when studied using ROC curves [83]. Both the MDRD and CG equations are less accurate in early kidney disease since they have a tendency to underestimate GFR [82].

Unlike CG equation which relies on weight, the MDRD equation is adjusted for body surface area. Thus it will be taking into account the variations in muscle mass with certain diseases or amputations. And also, MDRD equation is more accurate in older aged, obese patients and in diabetics[82]. A population based study in Pakistan, CG and MDRD formulae demonstrated better agreement with 24 hour urinary creatinine clearance than serum creatinine alone [84]. MDRD equation has been evaluated in many populations including African, American, European and Asian patients with or without diabetes/kidney disease, kidney transplant recipients and potential kidney donors. This equation has reasonable accuracy in CKD patients but less accurate in populations without kidney disease. The standardized MDRD Study equation provided reasonably accurate eGFR in the CKD-EPI pooled data set when estimated GFR (eGFR) was less than 60 mL/min/1.73 m², whereas higher eGFRs were with lower precision and higher bias [41]. In 2009, the British National Formulary revised its drug dosage guidance for adjustment in renal disease calculate to eGFR using the MDRD study equation [85]. Chronic Kidney Disease Epidemiology collaboration has
recommend the use of re-expressed MDRD equation for estimation of clinically significant GFR less than 60 ml/min/1.73m² without considering the racial adjustments in multiracial Asian populations [86]. In a study performed in India, 24 hour creatinine production was calculated using CG, modified MDRD, CKD-EPI equations and was compared with estimated creatinine production calculated using Rule formula where age and muscle mass of the patient was used. Muscle mass was measured using dual energy X-ray absorptiometry (DXA). MDRD derived 24 hour creatinine production showed the best correlation with DXA measured muscle mass. Thus even though the SCr based equations were developed in the white population, this supports the use of standardized MDRD equation in the Indian population [87].

In patients with eGFR more than 60 mL/min/1.73 m², the 2009 CKD-EPI-SCr equation has a significantly less bias, higher accuracy and precision than MDRD equation [35, 43]. The CKD-EPI-SCr equation reclassifies people at lower risk of CKD and death into higher eGFR categories, suggesting a more accurate categorization [88] also it provides more accurate risk prediction of mortality than the MDRD study equation [89].

2.8 Comparison of Serum Cystatin C and Serum Creatinine

Over the past 25 years, CysC has been studied both in diabetics, non-diabetics and CKD patients and have tested for the correlation of GFR and CysC or the reciprocal of CysC to assess the feasibility of using it as a surrogate marker in different populations. Grubb et al 1985, was the first to report that both CysC and SCr correlated similarly to GFR which was determined by 51Cr-EDTA clearance among 135 patients with various renal
pathologies [74]. In most of the studies, the degree of correlation between a reference GFR procedure and CysC or the reciprocal CysC concentration was superior or at least equivalent to SCr or reciprocal SCr [3, 73, 90].

Serum cystatin C levels were compared with 24 hour creatinine clearance in some studies. In patients with various nephropathies, a significant positive correlation of CysC was also reported with SCr and a significant negative correlation with creatinine clearance[91]. However, in one study, CysC didn’t perform better than SCr in predicting creatinine clearance, when parameters such as age, weight and gender were incorporated into the models [65].

According to the literature, many studies have been carried out to assess the place of CysC in T2DM for the detection of nephropathy. Detection of very early reduction in renal function will optimize prevention, and treatment strategies in diabetic nephropathy. In early diabetic nephropathy CysC more closely follow the measured GFR than SCr. CysC is considered as more a reliable marker of GFR in patients with mildly to moderately impaired kidney function since as it is more sensitive for small changes in GFR and it is identified as a useful marker for the follow up of diabetic patients [3, 64]. CysC is with superior diagnostic accuracy than SCr and has a higher area under the curve (AUC) for receiver operating curves (ROC) analysis when measured GFR is used [74]. However, CysC is a more sensitive parameter than SCr for the detection of an incipient nephropathy in diabetes with normal SCr as the AUC of the ROC was better for CysC and eGFR-MDRD than for SCr at the cut off value of 80 mL/min for GFR EDTA [92]. The reciprocal of CysC showed a significantly stronger association with mGFR than did SCr or eGFG-CG. When mGFR decreased from 120 to
20 mL/min/1.73 m², CysC increased more significantly than SCr [93]. Maclsaac et al. 2006 found that estimated GFR calculated with CysC had the same predictive potential when compared with the SCr based MDRD and C-G equations in diabetic patients. They used plasma clearance of 99mTc-DTPA to measure GFR [94]. The maximum diagnostic accuracy of CysC was significantly better than SCr and eGFR-CG in discriminating between type 2 diabetic patients with normal GFR more than 80 mL/min per 1.73 m² and those with reduced GFR less than 80 mL/min/1.73 m² [93]. As the GFR decreased, mean CysC concentrations showed step-by-step statistically significant increments. At 90 mL/min/1.73 m² and 75 mL/min/1.73 m² cut-points, diagnostic efficiencies of CysC were 89% and 92% respectively and better than SCr [95]. There is an argument on performance of CysC at various GFR levels. In one study, CysC performed better than SCr in diagnosing mild diabetic nephropathy (GFR <80 ml/min/1.73 m²) but did not have a major advantage over age adjusted SCr in the evaluation of GFR < 60 ml/min/1.73 m². A significant difference was not observed between CysC and SCr in their correlations with mGFR [96]. In another study, at the upper threshold for mild CKD (GFR of less than 90 ml/min per 1.73 m²) CysC has greater diagnostic accuracy than SCr but CysC had similar performance to eGFR based on SCr (MDRD and CG equations) [97, 98]. However, CysC had the better diagnostic accuracy than the creatinine-based methods for detection of moderate CKD at GFR < 60 ml/min per 1.73 m², and the cut-off level of CysC was 1.10 mg/l [97]. Hojs et al. 2006 also demonstrated that in CKD stages 2–3 with mild to moderate renal impairment (GFR 30–89 ml/min/1.73 m²), CysC had a significantly higher diagnostic accuracy than SCr and eGFR-CG in the analysis of ROC curve (cut-off for GFR 60 ml/min/1.73 m²).
However a significant difference in diagnostic accuracy was not found between CysC and eGFR calculated from the MDRD formula [99].

In diabetics with eGFR less than 60 ml/min/1.73m², a strong correlation was observed for eGFR calculated with CysC based Grubb eGFR equation and with MDRD equation than with CG equation. In patients with normal renal function with eGFR above 60 ml/min/1.73m², there was a much weaker correlation between eGFR based on Grubb and MDRD equation [100]. Similarly, in Japanese T2DM patients, a good correlation existed between CysC and eGFR -MDRD and CysC was identified to be a good screening tool for CKD stage 3 and more, as it had a large AUC in ROC analysis [101]. Contrary to popular belief, Oddoze C 2001 found that CysC was not superior to SCr, or eGFR calculated using CG equation in detecting early renal failure in a study in diabetics with early renal impairment, where ⁵¹Cr- EDTA was used as a gold standard to measure GFR[102].

The diagnostic performance of CysC in comparison with SCr and the diagnostic sensitivity and specificity of CysC for the detection of a decreased GFR were analyzed by several meta-analyses over the past two decades. In the meta-analysis by Dharnidharka K et al 2002, reciprocal of CysC had a greater AUC in ROC plot than reciprocal of SCr and also reciprocal of CysC showed a superior correlation coefficients [103]. A similar study carried out in 2007, found that CysC could strongly rule in renal impairment (at mGFR of 60-79ml/min/1.73m²) in whom it is suspected, when CysC concentration falls within the range of 0.9-1.4 mg/l, while SCr had only a moderate ability to rule in renal impairment [104]. Both of the above meta-analyses contained all spectrums of diseased patients. However, a good correlation between
CysC, SCr and the measured GFR was demonstrated in another meta-analysis carried out in 2011 with a larger sample size. Furthermore CysC was found to be more sensitive but less specific than SCr in the estimation of GFR[105].

As the renal complications are more prevalent among T2DM patients with nephropathy, monitoring of renal functions is important to detect any further deterioration. In a four year cohort study of renal function in thirty T2DM Pima Indians with a GFR more than 20 ml/min/1.73m² showed a close relationship between longitudinal trends in mGFR by iothalamate clearance and trends in renal function estimated from CysC. The trends in 100/CysC and iothalamate clearance strongly correlated than the trends between 100/SCr, eGFR by CG and MDRD equation poorly correlated with trends in iothalamate clearance [106]. Hence CysC may be useful for early identification of patients who are at risk of developing CKD during the period of follow up.

The presence of high CysC increased the risk of progression to pre-diabetes by three folds, over a follow up of six years in the participants of the Western New York Health Study who were healthy at the initiation of the study[107]. It has been shown that the T2DM patients irrespective of albuminuria, had higher CysC concentration than non-diabetic controls in [108, 109]. Similarly, hypertensive, T2DM patients had higher CysC levels than hypertensive, normoalbuminuric patients without diabetes [70]. However, a significant difference in SCr was not detected between non diabetic controls and diabetics with normoalbuminuria. SCr was elevated only in the diabetics with proteinuria and not in normoalbuminuric diabetic patients [108].

Over the years, several CysC based equations were developed and majority were found to be more accurate than SCr based equations, while some studies found that the
performances to be similar [36]. CysC based eGFR had been compared with measured GFR in several studies. In most of the studies, eGFR-Cys had better correlation with mGFR than SCr based equations [3, 36].

Serum CysC levels and eGFR-CysC were compared with SCr and eGFR-SCr in T2DM in past studies. CysC demonstrated a gradual increase as the GFR decreased from mild to moderate renal impairment in a cross sectional study in 103 Taiwanese T2DM patients. However SCr significantly increased only from eGFR stage 2 to 3. [110]. On analysis of ROC curve, CysC had a higher diagnostic accuracy than SCr in for stage 1 and stage 2 (eGFR cut off values of 90 and 60 ml/ min respectively) [110]. An increasing trend in CysC levels was observed when eGFR (calculated using CG equation) was more than 90, between 60 and 90 and less than 60 ml/min [108]. The correlation between CysC and eGFR-CG was stronger than SCr and eGFR-CG. [108]. This suggests CysC to be a better marker than SCr in moderate renal impairment in diabetics. The sensitivity and diagnostic accuracy of CysC was better than 24 hour urinary creatinine clearance, and both were better than SCr [111].

Many equations have been proposed to calculate eGFR using CysC. The latest CKD-EPI equation using CysC was developed in 2012 by studying 5352 persons including those with kidney disease and healthy volunteers and validated in 1119 persons. This equation was developed using newly established international reference standard for CysC [112].

Equations using both SCr and CysC to calculate eGFR appear to be better than eGFR equations using either one of them alone [33]. The 2012 CKD-EPI-CysC and 2009 CKD-EPI-SCr had similar performance whilst the best estimation of GFR was
calculated using the combined CKD-EPI-SCr-CysC equation. This equation also improved staging of CKD near the threshold (in GFR between 45-75 ml/min/1.73m² [90]. Hence, the combined equation may be useful as a confirmatory test for chronic kidney disease [113]. The use of CysC and SCr combination equation for estimating GFR in a multiethnic Asian population with CKD does not require ethnicity coefficients because the derived coefficients are very close to each other [114]. However, the combined equation should not be used if the eGFR-SCr and eGFR-CysC differ by more than 40% [90]. The approach in Sweden is to take the average of eGFR-SCr and eGFR-CysC if those eGFR are within 40% of each other and if the difference of those eGFR is more than 40%, the clinicians have to choose the estimate least likely to be biased and more likely to be correct based on the clinical features of the patient [90].

A longitudinal analysis from Reasons for Geographic and Racial Differences in Stroke (REGARDS) cohort demonstrated that eGFR-CysC improves CKD definition and risk stratification relative to eGFR -SCr [90]. End stage renal disease risk stratification significantly improved in young or middle-aged diabetic patients who were in GFR categories 1-3 (based on routine measurements of SCr and eGFR calculated using MDRD / CKD EPI equations), by a secondary assessment of renal function based on CysC [115].

Taking into account all the latest research outcomes, the latest KDIGO guideline suggests measuring CysC to calculate eGFR using 2012 CKD-EPI-CysC equation to confirm in patients who will be diagnosed as CKD solely based on eGFR-SCr of 45-60 ml/min/1.73m², without other manifestations of CKD such as albuminuria [27].
Table 7. CKD-EPI equations based on Cystatin C or both Serum Creatinine and Serum Cystatin C [27].

<table>
<thead>
<tr>
<th>Serum creatinine (mg/dL)</th>
<th>Serum cystatin C (mg/L)</th>
<th>CKD-EPI cystatin C equations</th>
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<tr>
<td>Female/ male ≤ 0.8</td>
<td></td>
<td>$133 \times (\text{CysC}/0.8)^{-0.499} \times 0.996^{\text{Age}} \times 0.932 \text{ if female}$</td>
</tr>
<tr>
<td>Female/ male &gt;0.8</td>
<td></td>
<td>$133 \times (\text{CysC}/0.8)^{-1.328} \times 0.996^{\text{Age}} \times 0.932 \text{ if female}$</td>
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<th>CKD-EPI SCr-CysC equations [x1.08 if black]</th>
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<tr>
<td>Female &gt; 0.7 &gt; 0.8</td>
</tr>
<tr>
<td>Female ≤ 0.7 ≤ 0.8</td>
</tr>
<tr>
<td>Female &gt; 0.7 &gt; 0.8</td>
</tr>
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<td>Male ≤ 0.9 ≤ 0.8</td>
</tr>
<tr>
<td>Male &gt; 0.9 &gt; 0.8</td>
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<tr>
<td>Male ≤ 0.9 ≤ 0.8</td>
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<td>Male &gt; 0.9 &gt; 0.8</td>
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Thus, clinicians can use CysC to confirm and reassure the low risk subset of patients with eGFR-Scr less than 60ml/min/1.73m²[90].

Early detection and treatment of CKD would reduce the progression of kidney damage. KDIGO conference in Amsterdam (2006) recommends for all countries to have a targeted CKD screening program for high risk patients with diabetes, hypertension, cardiovascular disease, etc [116]. CysC is more like a secondary test in the evaluation of GFR. Therefore the value of using CysC for screening would depend on the prevalence of CKD in that population [90]. Even though CysC concentration is measured as an automated test it is much more expensive than SCr. Hence cost effectiveness and the outcome has to be weighed when using CysC for screening. Shilpak et al 2013 suggested three potential strategies to decide on using CysC as screening test. Those are, persons with borderline eGFR-Scr, persons at high risk of CKD and persons with conditions known to make SCr levels insensitive for detecting CKD (eg. unpredictable muscle mass, chronic liver disease) [90]. Therefore, CysC has a value in screening to detect occult CKD in T2DM patients as the prevalence of reduced GFR is higher and early treatment would improve the outcome.

2.9 Association between Serum Cystatin C and Albuminuria

CysC levels and eGFR–CysC were associated with albuminuria in T2DM patients. CysC level was higher in patients with albuminuric than normalalbuminuric T2DM patients, whereas SCr level was not significantly different between groups [117]. CysC levels show a step wise increase from normo to micro to macroalbuminuria in T2DM patients [110, 118-121]. Both, CysC and microalbuminuria are independent risk
factors for development of CKD stage 3 [122]. Both SCr and CysC are significantly higher in macrolbuminuric type 2 diabetic patients with renal dysfunction than macrolbuminuric type 2 diabetic patients with normal renal function, the microalbuminuric, and the normoalbuminuric group [111, 123]. ROC analysis indicate that the CysC is better to distinguish microalbuminuria or macroalbuminuria than SCr [109, 123] also CysC is the most sensitive and specific marker of macroalbuminuria [109]. However Viswanathan S. et al 2005, did not observe any differences in CysC levels between normoalbuminuric and albuminuric T2DM patients [108].

A significant positive stronger correlation was observed for CysC with albuminuria /ACR than SCr or eGFR based on SCr in T2DM patients [110, 121]. Macroalbuminurics had a significantly lower eGFR-CysC than the normoalbuminurics diabetics [118].

CysC reflect the progression of albuminuria and both CysC and eGFR-CysC were predictors of the development of microalbuminuria [118]. In a retrospective study with a 3 year follow up, albuminuria level for each patient corresponded with the annual change in CysC based eGFR but not with SCr based eGFR calculated using MDRD equation. Thus CysC may reflect the trend of albuminuria more accurately than SCr enabling early detection of DN [124]. In a hospital based cohort study with a 3 year follow up of T2DM patients, CysC based eGFR was superior to SCr and eGFR-MDRD in reflecting the trend of albuminuria[125]. Findings of another cohort study with a 10year follow up of diabetics with a mean age of 78 years, suggested CysC based eGFR and ACR had additive effects on mortality risk [126].
2.10 Prediction of End stage renal failure by renal function markers

In a meta-analysis of 9 general population and 8 cohorts of patient by Gansevoort et al 2011 found that lower eGFR and higher albuminuria were independent risk factors for ESRD, progression of CKD and acute kidney injury. The hazard ratios for eGFRs of 60, 45, 15 ml/min/1.73m² were 4, 29, 454 respectively when compared with an eGFR of 95ml/min/1.73m². Furthermore the hazard ratios for ACR values of 30, 300 and 1000mg/g were 5, 13, 28 respectively [127]. Diabetics with macroalbuminuria have an estimated 19 times more rapid decline in renal function compared with those without albuminuria. Similarly those with microalbuminuria also have 5 times rapid decline of eGFR compared with people with normoalbuminuria [53].

Patients who were placed in a higher stage by eGFR - CysC than eGFR- SCr had a significantly higher risk of ESRD than those with and those placed in a lower stage by eGFR-CysC than by eGFR- SCr had a lower risk for ESRD [115]. The risk for ESRD is only 2.6-fold elevated in patients with eGFR- SCr less than 60 ml/min per 1.73m² and normal eGFR-CysC , whereas it is 23.8-fold elevated in cases in which eGFR-CysC is also decreased [128].

Subjects without CKD but with CysC more than 1mg/L, had a 4 times higher risk of developing CKD after 4 years [3]. After a median follow-up of 4.6 years, in a cohort of 26 643 US adults, the risk of incident end-stage renal disease was higher among those with CKD defined by all three markers; eGFR less than 60ml/min/1.73m² whencalculated with CKD-EPI-SCr and CKD-EPI-CysC and ACR more than 30 mg/g. Evidence suggests that combining CysC, SCr and ACR to assess renal functions
enhances the predictive accuracy of renal function for all-cause mortality and end-stage renal disease [129]. Therefore it may be more beneficial to use a 3-dimensional “triple marker method” using all 3 markers in appropriately selected patients.

2.11 Prediction of cardiovascular risk and mortality by renal function tests

According to the UKPDS 64 study, cardio vascular disease is the most common cause of death at all stages of diabetic nephropathy and the risk increases as the severity of nephropathy increase [51]. A meta-analysis by Nitsch et al 2013, which included 46 cohorts from Europe, North and South America and Australasia, confirmed that risk of all-cause and cardiovascular mortality and end stage renal disease increased with lower eGFR and higher albuminuria in both sexes [130].

Obese diabetic patients with metabolic syndrome have higher levels of CysC than controls unlike SCr / eGFR SCr [131]. Many studies have shown that CysC level is a better predictor of adverse events than SCr or GFR estimated using SCr in elderly or cardiac populations [41]. Higher levels of CysC is also shown to be associated with increased mortality and cardiovascular outcomes like myocardial infarction, heart failure [3]. CysC has shown stronger associations than eGFR-SCr with cardiovascular disease, hypertension, infection risk, heart failure, frailty, and all-cause mortality[90].

Being a smoker, having decreased physical activity, higher triglycerides, higher LDL cholesterol, lower HDL cholesterol, and being obese which care cardiovascular risk factors, were associated with higher CysC levels or lower eGFR-CysC [66]. Increased Framingham risk scores (which gives the 10 year cardiovascular risk) were related to lower eGFR-CysC but not with lower mGFR or lower eGFR SCr [66]. Thus CysC is a
better predictor of CVD than SCr. This could be due to the link between CysC and inflammation as there is an association of CysC and inflammatory markers such as C-reactive protein and interleukin-6 [132]. Another possible interpretation is that SCr is not so sensitive to detect modest reductions like CysC and these modest reductions in GFR may predict CVD[132].

Cystatin C–estimated GFR and urine ACR are additive in terms of mortality risk in elderly diabetics. Furthermore, CysC–estimated GFR also predicts mortality more strongly than SCr based estimated GFR [133]. However in advanced CKD, eGFR-CysC, eGFR-SCr and mGFR all have very similar associations with mortality [90].

2.12 Renal functions and diabetic retinopathy (DR)

Presence of diabetic retinopathy is significantly associated with progression of diabetic nephropathy [23]. In the Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) study, patients with diabetic retinopathy had higher albuminuria and lower glomerular filtration rate (GFR) than those without. Also the diagnosis of diabetic retinopathy at baseline of the study was associated with more proteinuria, lower GFR, and a higher risk for ESRD and death in type 2 diabetic patients [134]. In a population-based cohort of 3,280 community-dwelling adults of Malay ethnicity aged 40–80 years, retinopathy was positively associated with both eGFR and micro/macroalbuminuria [135]. Microalbuminuria and SCr are associated with proliferative diabetic retinopathy [136, 137].

Diabetic retinopathy correlated more with the albuminuric CKD phenotypes than with the nonalbuminuric phenotypes [138]. Diabetic retinopathy and macro-albuminuria
shows a strong correlation[139]. Patients with T2DM with microalbuminuria and without renal impairment (eGFR more than or equal to 60ml/min/1.73m²) had a significantly greater risk for development and progression of diabetic retinopathy than patients with moderate renal impairment (eGFR between 30-60ml/min/1.73m²) and normoalbuminuria. This highlights that microalbuminuria has a greater ability in predicting the development and progression of diabetic retinopathy compared with moderate decline in GFR among type 2 diabetic patients[140]. However, microalbuminuria detected using urine dipstick (Micral test) was not associated with diabetic retinopathy in type 2 diabetic patients [141]. Retinopathy was shown only to be associated with poor glycemic control and high systolic and diastolic blood pressures and not with renal functions in T2DM patients in Sri Lanka [16].

Increased levels of CysC are associated with several eye conditions. High levels of CysC increase the risk for specific types of age-related cataract[142]. Also CysC is associated with the incidence of early age related macular degeneration and exudative age related macular degeneration[143]. In Asian Indians with T2DM, higher CysC levels were present in patients with microalbuminuria and diabetic retinopathy than those with microalbuminuria but without retinopathy. However eGFR-MDRD was not significantly different between those two groups [121]. In a study in China, the severity of diabetic retinopathy was positively associated with both albuminuria and CysC. Moreover, CysC was an independent risk factor. With ROC analysis the optimal cutoff value of CysC was 1.25mg/L in predicting sight threatening diabetic retinopathy with a specificity of 87.6%. A CysC value higher than 1.25mg/L predicted a 11 fold risk of severe retinopathy in T2DM [144].
3. MATERIALS AND METHODS

3.1. Chemicals and Reagents

3.1.1 Special Chemicals and Reagents

Konelab™/ T series Cystatin C assay reagent and Konelab™/ T series Albumin MST assay reagents with quality controls and calibrators from Thermo Scientific Oy, Clinical Diagnostics Finland and creatinine kinetic method assay reagents with quality controls for serum and urine creatinine and multicalibrator from Biolabo SA, France were used. All reagents were compatible to Kone 20XT auto analyzer, Thermo Scientific Fischer, Clinical Diagnostics Finland.

3.1.2 Water

Deionized water was used in all experiments.

3.2 Selection of Subjects

3.2.1 Selection of cases (T2DM patients)

Sixty one previously diagnosed type 2 diabetes patients between the ages of 30-60 years who can converse in Sinhala or English were recruited as cases from the diabetic clinic of Colombo South Teaching Hospital, Kalubowila and National Diabetes Center, Rajagiriya. Inclusion criteria for the cases were, possibility of having renal impairment as they had at least one eGFR value between 30 - 60ml/min/1.732m² when calculated using past 3 consecutive SCr values from the clinic records. Estimated GFR was calculated using the revised MDRD formula.
Exclusion criteria for the cases were, previously diagnosed renal disease other than diabetic nephropathy, hypertension, chronic lung disease, liver disease, cardiovascular diseases (other than hypertension), rheumatoid arthritis, hyperthyroidism, hypothyroidism, pregnancy, females with menstruation at the time of urine collection, history of fever or infection within one week, vaccination or immunization in previous 3 weeks and smokers smoking more than 10 cigarettes per day[145]. Subjects with features of hypo or hyperthyroidism, peri-orbital oedema, pitting ankle edema, jaundice, signs of arthritis, pyrexia and infected wounds were also excluded after a general examination. Also those with BMI more than 30kg/m² were excluded from the study.

3.2.2 Selection of Controls (healthy adults)

Altogether 118 apparently healthy persons were recruited for the study as control subjects from Colombo South Teaching hospital and Family Practice Center, Faculty of Medical Sciences, University of Sri Jayewardenepura. Out of them, 61 subjects were matched for age (± 1 year) and sex with the cases. Exclusion criteria for the controls were previously diagnosed diabetes, renal disease, hypertension, chronic lung disease, liver disease, cardiovascular disease, rheumatoid arthritis, hyperthyroidism, hypothyroidism, pregnancy, menstruating females at the time of urine collection, history of fever or infection within one week, received vaccination or immunization in previous 3 weeks, persons on drugs that may influence renal function or CysC concentrations (i.e. antihypertensive, diuretics, anti-inflammatory agents, hypoglycemic agents, anticonvulsants, anti-cancer or anti-viral drugs and antibiotics) and smokers smoking more than 10 cigarettes per day[145]. Subjects with features of hypo or hyperthyroidism, peri-
orbital oedema, pitting ankle edema, jaundice, signs of arthritis, pyrexia and infected wounds were also excluded after a general examination as well as those with BMI more than 30 kg/m². Random blood glucose levels were checked using One Touch Ultra glucometer and those with values more than 200mg/ dl [146] and those with blood pressure more than 140/90 were excluded.

3.3 Data Collection

3.3.1 Questionnaire

An interviewer based questionnaire was administered to all subjects (cases and controls) recruited to the study by the researcher (Appendix 1).

The questionnaire comprised of two sections; one for individual identification, socio-demographic factors and to ascertain risk factors and the other section was to record anthropometric measurements, blood pressure, RBS, and assessment of eyes.

3.3.2 Summary of the data collected and definitions in the type 2 diabetes patients

3.3.2.1 Data collected by interviewing the cases

1. Age (to the nearest completed year)
2. Sex – Male / Female
3. Ethnicity – Sinhala/Tamil/ Muslim/ Burgher
4. Level of education – Less than Ordinary level/ Ordinary level/ Advanced level/ University degree level
5. Duration of diabetes (to the nearest completed month)
6. Past medical history – Hypertension (systolic blood pressure ≥160 , diastolic blood
pressure $\geq 90$, or on anti-hypertensive treatment)

Hyperlipidemic if – already diagnosed, on lipid lowering drugs

7. Family history of - Diabetes, hypertension, ischemic heart disease, hyperlipidemia.

8. Smoking - Current smokers were defined as those who smoked tobacco during the previous 12 months and included those who have stopped smoking within the past year. Former smokers were categorized as “stopped smoking” if they stopped smoking more than one year back. Nonsmokers were defined as those who had not smoked any tobacco at any stage in life.

3.3.2.ii Data collected from clinic and past records

1. Drug history was recorded from clinic records.

2. Last 5 fasting blood glucose values and the last post prandial blood glucose value

3. Last 3 SCr values

4. Albuminuria/proteinuria values

5. Last lipid profile data

6. Diabetic retinopathy stage – Defined as “No retinopathy, Non Proliferative Diabetic Retinopathy (NPDR), Proliferative Diabetic Retinopathy (PDR)”

7. Whether treatment was given or not

8. Presence of maculopathy, macular oedema, cataract, glaucoma
3.3.3 Summary of the data collected and definitions in the apparently healthy subjects

3.3.3.1 Data collected by interviewing the controls

1. Age (to the nearest completed year)
2. Sex – Male / Female
3. Ethnicity – Sinhala/Tamil/ Muslim/ Burgher
4. Level of education – Less than Ordinary level/ Ordinary level/ Advanced level/ University degree level
5. Family history of - Diabetes, hypertension, ischemic heart disease, hyperlipidemia.
6. Smoking - Current smokers were defined as those who smoked tobacco during the previous 12 months and included those who have stopped smoking within the past year. Former smokers were categorized as “stopped smoking” if they stopped smoking more than one year back. Nonsmokers were defined as those who had not smoked any tobacco at any stage in life.

3.3.4 Data collected by measurements in both diabetic patients and healthy adults

1. Systolic blood pressure (mmHg)
2. Diastolic blood pressure (mmHg)
3. Height (cm)
4. Weight (kg)
5. Waist circumference (cm)
6. Hip circumference (cm)
3.3.5 Height, weight and Body Mass Index

Height was measured using a stadiometer and recorded to the nearest 0.1 cm with the head positioned in the Frankfort Horizontal Plane, with the individual standing barefoot with heels together, both heels touching the base of the vertical board, the medial borders of the feet at an angle of about 60° and the scapulae and buttocks in contact with the vertical board. Weight was measured with a digital scale, with accuracy of 0.1 kg. Body Mass Index was calculated as weight in kilograms divided by height in squared meters (kgm$^2$).

Body Mass Index (BMI) = Weight (kg) / Height (m$^2$)

3.3.6 Waist, Hip circumferences and Waist to Hip ratio

According to the WHO guidelines, waist circumference was measured at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest, at end of normal expiration. Hip circumference was measured around the widest portion of the buttocks, with the tape parallel to the floor. Both waist and hip circumferences were measured using a plastic flexible, non-stretchable tape to the nearest 0.1 cm. For both measurements, the subject stood with feet close together, arms at the side with the body weight evenly distributed. Reading was taken to the nearest 0.1 cm. Each measurement was repeated twice; and if the measurements are within 1cm of one another, average was calculated. If not, the two measurements were repeated [147].

Waist to hip ratio was calculated by dividing the waist circumference (in cm) by the hip circumference (in cm) of the individual.

Waist to Hip ratio (WHR) = Waist circumference (cm) / Hip circumference (cm)
3.3.7 Measurement of Blood Pressure

Blood pressure was measured in the seated position after participants had rested for at least 5 minutes. The measurement was taken using the left arm at the heart level, using a mercury column sphygmomanometer (Bokang – Model BK1001) by the researcher. Two readings were taken 5 minutes apart and the mean was used for analysis.

3.3.8 Diabetic Retinopathy Assessment

Examination of the fundus of the eye was performed on the volunteers by trained medical officers or by consultant ophthalmologists working at Eye clinic. Diabetic retinopathy (DR) was assessed using the slit-lamp binocular indirect ophthalmoscopy through dilated pupils. The findings of the examination were recorded from clinic notes. Patients were categorized according to the degree of their diabetic retinopathy: Group 0: No diabetic retinopathy; group 1: Non proliferative diabetic retinopathy (NPDR) including microaneurysms, hard exudates, cotton wool spots, retinal hemorrhages; group 2: proliferative diabetic retinopathy (PDR), including new vessels, extensive neovascularization, vitreous haemorrhages, fibrovascular proliferation with or without tractional retinal detachment, patients with pan photocoagulation. The severity of diabetic retinopathy was graded based on worst eye [148].

3.4 Collection of Blood and Urine Samples

3.4.1 Collection of Blood Samples

Blood samples were collected from the participants for SCr and CysC levels by venipuncture by the researcher under aseptic conditions. 3ml of blood was collected into
plain test tubes and samples were transported to the laboratory within 6 hours after
collection and serum was separated after centrifugation at 3500 rpm for 10 minutes and
analyzed immediately or stored at -20 °C pending analysis [149, 150].

3.4.2 Collection of Urine Samples

Patients provided mid-stream urine sample collected as a spot sample in a 20 ml sterile
plastic urine collecting container and sample was analyzed within 8 hours or stored in
the refrigerator at 2-8 °C and analyzed within 7 days.

3.5 Laboratory Quantitative Analysis

All the biochemical analyses were performed at the Clinical Laboratory at the
Department of Biochemistry, Faculty of Medical Sciences, University of Sri
Jayewardenepura. For all the samples the analyses were performed in duplicate except
for CysC. For CysC, random samples were run in duplicate for double checking.
Patients detected with abnormal findings were referred for treatment.

3.5.1 Quantitative Analysis of Serum Cystatin C

3.5.1.i Cystatin C assay procedure

The PETIA method was used to analyze the concentration of serum CysC using Kone
20XT auto analyzer, Thermo Scientific Fischer, Clinical Diagnostics Finland.
Konelab™/T series Cystatin C (Code 981911) kit by Thermo Fischer Scientific
Oy, Clinical Diagnostics, Finland consists of cystatin C reagent (Microparticles coated
with anti-human cystatin C), cystatin C buffer (a solution of polymers in MOPS-
buffered saline) and specimen diluent (PBS). The reagents contain sodium azide as preservative.

1. Serum samples were thawed to room temperature and mixed thoroughly using low speed vortexing.
2. 200µl of serum was pipetted into separate sample cups and introduced into Kone 20 XT auto-analyzer and the serum cystatin C concentration measured.

3.5.1.ii Reaction Principle

The method is based on micro particle enhanced immunoturbidimetry. Micro particles coated with anti-human (rabbit) cystatin C are added to buffered samples. The increase in absorbance caused by formation of immune complexes is recorded at 540nm when the reaction has reached its end point. The change in absorbance is proportional to the amount of antigen (CysC) in solution.

3.5.1.iii Calibration

Calibration was carried out using Cystatin C calibrator (Code 981912) which is a pool of delipidated human serum enriched with recombinant human cystatin C produced in E.coli. Lot specific assigned Cystatin C calibrator value was 8.18mg/l. Calibration curve was plotted using a serial dilution of stock calibrator according to parameters recommended in the Konelab cystatin C reagent kit (Figure 5).
3.5.1.iv Traceability

The calibrator has been value assigned by turbidimetry using a precise transfer protocol ensuring traceability to a pure recombinant human cystatin C preparation where the cystatin C concentration was established by Dry Mass Determination.

![Cystatin C Calibration curve](image)

**Figure 5.** Cystatin C Calibration curve

3.5.1.v Quality Control

Two levels of cystatin C quality controls, cystatin C control (Code 981913) and cystatin C control high (code 981914) were used between assays and within assay to ensure that assay values were within ± 2SD of the specified concentration(Appendix II, Figure 25, 26)
3.5.1.vi Detection limit and measuring range

Detection limit was 0.18 mg/l whilst measuring range for serum cystatin C was from 0.44-7 mg/l.

3.5.1.vii Imprecision

This assay shows a within run percent coefficient of variation of (%CV) of 1.4% when mean is 0.7mg/l, 2.6% when mean is 1.49mg/l and 1.2% when 0.5% when mean is 71mg/l. The %CV of between runs was 1.1% for low and 0.7% for high cystatin C.

3.5.2 Quantitative Analysis of Serum and Urine Creatinine

3.5.2.i Creatinine assay procedure

Colorimetric reaction (Jaffe reaction) was used in Creatinine kinetic method of Biolabo SA, France (Ref. code 80107). The reagents consisted of vial R1 containing Base (Disodium phosphate 6.4 mmol/L and Sodium hydroxide 150 mmol/L) and vial R2 containing (Sodium dodecyl sulfate 0.75 mmol/L and Picric acid 4.0mmol/L). Reagent R1 and R2 are placed separately in the auto analyzer.

Assay of creatinine in serum samples,

1. Serum samples were thawed to room temperature and mixed thoroughly using low speed vortexing.
2. 200μl of serum was pipetted into separate sample cups and introduced into Kone 20 XT auto analyzer and measured the concentrations of sample.

Assay of creatinine in urine samples,
1. Fresh urine or urine samples stored at 2-8°C were brought to room temperature and centrifuged at 2500 rpm for 8 minutes.

2. 200μl of urine was pipetted into separate sample cups and introduced into Kone 20 XT auto analyzer and measured the concentrations of the sample.

3. Auto dilution of 1+19 with demineralized water was carried out in the auto analyzer before analysis.

3.5.2.ii Reaction Principle

Creatinine reacts with picrate ion in an alkaline medium (NaOH) and forms a red-orange color complex and is measured kinetically at 490 nm (490-510 nm), without any pretreatment step.

\[
\text{Creatinine} + \text{Picric acid} \xrightarrow{\text{OH}^-} \text{Red - orange dye}
\]

3.5.2.iii Calibration

Calibration was carried out using Biolabo Multicalibrator (Ref. code 95015). Reagent preparation was carried out by pipetting 5 ml of diluent (vial R2) into vial R1 containing lyophilised bovine serum, contents dissolved by gentle swirling and allowed to stand at room temperature for 15-30 minutes followed by storage at -20°C, away from light after pipetting 250μl aliquots for later use with only one freeze-thaw cycle. Calibration curve was plotted using a serial dilution of stock calibrator according to parameters recommended by the Biolabo creatinine reagent kit (Figure 6).
3.5.2.iv. Traceability

The multi calibrator contains the National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 914a which is a primary reference material with creatinine values assigned by the IDMS reference method.

Response (A)

![Creatinine Calibration curve](image)

**Figure 6.** Creatinine Calibration curve

3.5.2.v Quality Control

3.5.2.v.a. Quality Control for Serum Creatinine

Two levels of creatinine quality controls, Biolabo EXATROL-N (Level I) (Ref. code 95010) and Biolabo EXATROL-P (Level II) (Ref. code 95011) were used before assay of each batch of samples to ensure that assay values were within ± 2SD of the specified concentration. Reagent preparation was carried out separately by pipetting 5 ml of
diluent (vial R2) into vial R1 containing lyophilised bovine serum, contents dissolved by gentle swirling and was allowed to stand at room temperature for 15-30 minutes followed by storage at -20 °C, away from light after pipetting 250µl aliquots for later use with only one freeze–thaw cycle (Appendix II, Figure 27,28).

3.5.2.v.b. Quality Control for Urine Creatinine

Two levels of creatinine quality controls, Biolabo urine chemistry control - Level I and Level 2 (Ref. code 95012) were used before assay of each batch of samples to ensure that assay values were within ± 2SD of the specified concentration(Appendix II, Figure 29,30).

3.5.2.vi Detection limit and measuring range

Detection limit is 0.2mg/dl at 37\(^\circ\)C.

3.5.2.ii Imprecision

Creatinine assay shows a within run %CV of 3.9 and 0.8 for low and high levels respectively. The %CV of between run was 2.9% and 2.7% were considered to be for low and high values respectively.

3.5.3 Quantitative Analysis of Urine Albumin

3.5.3.i Albumin assay procedure

Immunoturbidimetric method was used to analyze the concentration of urine albumin in all samples on Kone 20XT auto analyzer.
Konelab™ / T series Albumin MST (Ref. code 981927) kit by Thermo Fischer Scientific Oy, Clinical Diagnostics, Finland consists of Albumin antiserum (rabbit), protein buffer (a solution polymer in phosphate-buffered saline) and specimen diluent (PBS). The reagents contain sodium azide as preservative and are ready to use.

3.5.3.ii Reaction Principle

Specific albumin antiserum was added to buffered samples. An increase in absorbance is caused by formation of immune complexes between albumin and specific antibody. The absorbance was measured at 450nm for urine samples when the reaction has reached the end-point. The change in absorbance was proportional to the amount of albumin in urine.

3.5.3.iii Calibration

Calibration was carried out using Albumin U calibrator (Ref. code 981877). Lot specific assigned Albumin U calibrator value was 200mg/L. Calibration curve was plotted using a serial dilution of stock calibrator according to parameters recommended by the Konelab Albumin MST reagent kit.

The Konelab 20XT auto analyzer automatically prepares a dilution series from the stock calibrator according to preset parameters and the calibration curve is generated from the measured calibrators using spline fit. The results are calculated using the calibration curve (Figure 7).
3.5.3.iv Traceability

Albumin U calibrator is a liquid human based reference preparation for immunoturbidimetric methods, traceable to the IFCC prepare primary reference material ERM-DA470k.

![Graph](Image)

**Response (A)**

<table>
<thead>
<tr>
<th>Urine albumin concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

**Figure 7.** Urine Albumin MST Calibration curve

3.5.3.v Quality Control

Two levels of urine albumin quality controls, Albumin U control (code 981878) and Albumin U control High (code 981879) were used before assay of each batch of
samples to ensure that assay values were within ± 2SD of the specified concentration (Appendix II, Figure 31,32).

3.5.3.vi Detection limit and measuring range

Measuring range for urine albumin was 5-188 mg/L and extended measuring range after secondary dilution is 5-752 mg/L. Limit of blank which represents the highest measurement result that is likely to be observed for albumin free urine sample was 2 mg/L. The lowest amount in sample that can be detected is 3 mg/L. The limit of quantification, or the lowest actual concentration in a sample that can be quantitatively determined, is 5 mg/L.

3.5.3.vii Imprecision

This assay shows a within run %CV of 1.4% with a mean value of 10 mg/L and 1% with a mean value of 33 mg/L and 0.5% with a mean value of 71 mg/L. The %CV of between runs was 7.7% with a mean of 10 mg/L, 3.8% with a mean of 33 mg/L and 2.9% with a mean of 71 mg/L.

3.6 Calculation of eGFR

3.6.1 Calculation of eGFR using Serum Creatinine based Modified Modification of Diet in Renal Disease Study Equation

The following formula was used to calculate the eGFR based on the assumption that clinic SCr values were analyzed using methods not traceable to IDMS.

\[
GFR = 186 \times (SCr)^{-1.154} \times (age)^{-0.203} \times 0.742 \text{ (if the subject is female)}
\]

or \( \times 1.212 \text{ (if the subject is black)} \)[9]
3.6.2 Calculation of eGFR using serum creatinine based re-expressed Modification of Diet in Renal Disease Study Equation

The SCr analysis of this study was carried out using an assay traceable to a standard reference material and IDMS traceable. Thus the latest re-expressed MDRD formula was used to calculate eGFR.

\[
GFR = 175 \times (\text{standardized SCr})^{-1.154} \times (\text{age})^{-0.203} \times 0.742 \text{ (if the subject is female) or } \times 1.212 \text{ (if the subject is black)}
\]  

[9]

3.6.3 Calculation of eGFR using Serum Creatinine based 2009 CKD-EPI Creatinine Equation

The SCr analysis of this study was carried out using an assay traceable to a SRM and IDMS. Thus the 2009 CKD-EPI creatinine equation was also used to calculate eGFR.

\[
eGFR = 141 \times \min(\text{SCr}/k, 1)^{0.018} \times \max(\text{SCr}/k, 1)^{-1.209} \times 0.993^{\text{Age}} \times 1.018 \text{ if female}
\]

\[\times 1.159 \text{ if black}\]

Where “SCr” is serum creatinine (in mg/dl) and “k” denotes 0.7 for females and 0.9 for males and \(\alpha\) denotes -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 (Table 8).
Table 8. CKD-EPI equations based on serum creatinine levels [27].

<table>
<thead>
<tr>
<th>Gender</th>
<th>Serum creatinine</th>
<th>Equation for estimating GFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>≤ 0.7 mg/dl</td>
<td>144 \times (SCr/0.7)^{-0.329} \times 0.993 \times \text{Age} [-1.159 if black]</td>
</tr>
<tr>
<td>Female</td>
<td>&gt;0.7 mg/dl</td>
<td>144 \times (SCr/0.7)^{-1.209} \times 0.993 \times \text{Age} [-1.159 if black]</td>
</tr>
<tr>
<td>Male</td>
<td>≤ 0.9 mg/dl</td>
<td>141 \times (SCr/0.9)^{-0.411} \times 0.993 \times \text{Age} [-1.159 if black]</td>
</tr>
<tr>
<td>Male</td>
<td>&gt; 0.9 mg/dl</td>
<td>141 \times (SCr/0.9)^{-1.209} \times 0.993 \times \text{Age} [-1.159 if black]</td>
</tr>
</tbody>
</table>

3.7 Calculation of Albumin to Creatinine Ratio

Using the urine albumin and urine creatinine levels, ACR was calculated.

ACR = Urine Albumin (mg) / Urine Creatinine (g)

3.8 Staging of Chronic Kidney Disease

CKD was classified based on GFR category, and albuminuria category according to the KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease [27].

The data from analysis of serum sample for SCr, CysC and urine for ACR were used to categorize the cases according to the GFR (Table 3) and albuminuria categories (Table 4) of the T2DM patients. Then using those GFR and albuminuria categories, they were
again grouped according to the KDIGO categories predicting the prognosis of CKD (Figure 2).

3.9 Data Processing and Analysis

Data were analyzed using Microsoft Excel, Statistical Package for Social Sciences (SPSS) version 17 (Chicago, Illinois), and SAS Enterprise Guide 5.1 version 9.3 For Windows, 2011. Results were expressed as mean ± standard deviation (SD) or median and range. Preliminary analyses were performed to assess normality, linearity. Histograms, normal Q–Q plots, Shapiro–Wilk and Kolmogorov–Smirnov tests were used to test for normality. In those tests, a $P$ value less than 0.05 was considered to indicate that the data are not normally distributed. Depending on that, parametric and non-parametric tests were used accordingly. To find correlations, Pearson correlation or Spearman rank correlation were used. Furthermore, linear regression analysis was also performed. For the comparison of CysC between albuminuria and, eGFR categories, T2DM patients were categorized in to 3 groups as normoalbuminuria (NAU), microalbuminuria (MAU) and macroalbuminuria (macroAU) according to ACR levels and were also dichotomized in to 2 groups as eGFR-MDRD more than 60 ml/min/1.73m$^2$ and eGFR between 30-60ml/min/1.73m$^2$. Either Independent t test or Mann Whitney test were used to compare two unpaired groups. For the case control study, Paired t test or Wilcoxon Signed Rank test were used to compare the two paired groups. On comparison of more than 2 groups, either one way analysis of variance (ANOVA) test with post hoc analysis using Tukey’s honestly significant difference (HSD) or Kruskal-Wallis test with post hoc analysis using Mann-Whitney test with
Bonferroni correction were used. For the comparison of proportions of categorical variables Chi square test or Fischer exact test were performed. The sensitivity and specificity of renal markers were assessed from ROC curves and AUC of CysC and SCr were calculated and compared using the trial version of a software (http://analyse-it.com/). The normal reference interval for CysC in normal, healthy adults was calculated with 95% confidence limits and the 97.5\textsuperscript{th} percentile and 2.5\textsuperscript{th} percentile formed the upper and lower reference limits respectively. To study the effect of age, the healthy controls were grouped into 30-40, 41-50, and 51-60 year age categories. Again the T2DM patients were categorized using eGFR-CKD EPI formula and then were categorized according to the latest KDIGO guideline’s classification for identification of prognosis in CKD patients to compare the differences of renal markers in early stage categories. A $p$ value less than 0.05 is defined as statistically significant.

3.10 Ethical Issues

3.10.1 Ethical Clearance

Ethical Clearance was obtained from the Ethics Review Committees of Faculty of Medical Sciences, University of Sri Jayewardenepura and of Colombo South Teaching Hospital(Appendix III).

3.10.2 Consent

Written informed consent was taken from each and every participant before the interview, examination, measurement of anthropometry and collection of blood and
urine samples. Each participant was given an information sheet regarding the research (Appendix IV,V).

3.11 Sample Size Calculation

The sample size was targeted for a case control study.

- The sample size was calculated using the below mentioned formula where difference between means were used [151].

\[
 n = \frac{2k \sigma^2}{\delta^2}
\]

\( n \) = Sample size
\( k \) = Power (k =7.8 for 80% power and 5 % 2 sided significance level)
\( \sigma \) = Standard deviation
\( \delta \) = Difference of means

With reference to the study carried out by Viswanathan V et al. (13) the calculated sample size was a minimum of 35 for each group. The sample size will be adequate to assess correlation as well (15).
4. RESULTS

4.1 Characteristics of Study Subjects

The study population consisted of 61 type 2 diabetes patients as cases, who were with at least one of the eGFR values being less than 60 ml/min/1.73m$^2$ among the last 3 clinic records at the diabetes clinic. A total of 118 apparently healthy controls were selected. Out of them, 61 were age and sex matched with the cases for comparison.

T2DM group was composed of 23 (37.7%) males and 38 (62.3%) females. A total of 53 (44.9%) males and 65 (55.1%) females were recruited as healthy controls. Majority of the participants were non-smokers (Table 9).

The results revealed that the mean duration of diabetes was 10.23 ± 6.95 years ranging from 0.5 to 35 years (Table 10). Of the T2DM patients, uncontrolled high blood pressure of 140/80 or more (According to the 2014 American Diabetes Association guidelines [21]) was present in 13 (21.3%) and 46 (75.4%) were on antihypertensive medication (Table 11). Angiotensin converting enzyme inhibitor (ACEi) drugs were used by 40 (65.6%) and Angiotensin receptor blockers (ARB) were used by 5 (8.2%). All the diabetics were on a hypoglycemic agent. Four (6.6%) patients were only on Insulin, 12 (19.7%) were both on Insulin and oral hypoglycemic agents while 45 (73.8%) were only on oral hypoglycemic agents. Majority of the patients (83.6%) were on statins (Table 11).
Table 9. Characteristics of study subjects (in percentages)

<table>
<thead>
<tr>
<th></th>
<th>Diabetics (n=61)</th>
<th>Age and sex Matched controls (n=61)</th>
<th>Total controls (n=118)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>23</td>
<td>23</td>
<td>53</td>
</tr>
<tr>
<td>Female</td>
<td>38</td>
<td>38</td>
<td>65</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sinhala</td>
<td>56</td>
<td>57</td>
<td>114</td>
</tr>
<tr>
<td>Tamil</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Muslim</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Education</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>26</td>
<td>24</td>
<td>42</td>
</tr>
<tr>
<td>Ordinary level</td>
<td>21</td>
<td>26</td>
<td>37</td>
</tr>
<tr>
<td>Advanced level</td>
<td>13</td>
<td>11</td>
<td>26</td>
</tr>
<tr>
<td>University</td>
<td>1</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td><strong>Smoking</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoked</td>
<td>44</td>
<td>47</td>
<td>83</td>
</tr>
<tr>
<td>Stopped more than 1 year back</td>
<td>15</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>Current smokers</td>
<td>2</td>
<td>8</td>
<td>19</td>
</tr>
</tbody>
</table>
Table 10. Characteristics of study subjects [Mean (SD)]

<table>
<thead>
<tr>
<th></th>
<th>Diabetics (n=61)</th>
<th>Age and sex matched controls (n=61)</th>
<th>Total controls (n=118)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>53.98 (4.89)</td>
<td>53.48 (4.87)</td>
<td>49.23 (8.26)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>128.79 (19.07)</td>
<td>120.49 (12.71)</td>
<td>118.86 (11.86)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>81.74 (8.27)</td>
<td>79.20 (7.54)</td>
<td>78.50 (7.13)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.68 (2.73)</td>
<td>23.80 (3.23)</td>
<td>23.15 (3.59)</td>
</tr>
<tr>
<td>Waist circumference(cm)</td>
<td>92.66 (8.22)</td>
<td>87.22 (8.19)</td>
<td>85.92 (8.93)</td>
</tr>
<tr>
<td>Waist to hip ratio</td>
<td>0.97 (0.06)</td>
<td>0.92 (0.05)</td>
<td>0.91 (0.06)</td>
</tr>
</tbody>
</table>
Table 11. Summary of hypoglycemic, antihypertensive and lipid lowering medications used by the type 2 diabetes patients.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Number of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACEi</td>
<td>40 (65.6%)</td>
</tr>
<tr>
<td>ARB</td>
<td>5 (8.2%)</td>
</tr>
<tr>
<td>Hydrochlorothiazide (HCT)</td>
<td>7 (11.5%)</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>8 (13.1%)</td>
</tr>
<tr>
<td>Beta blockers</td>
<td>3 (4.9%)</td>
</tr>
<tr>
<td>Insulin</td>
<td>16 (26.2%)</td>
</tr>
<tr>
<td>Metformin</td>
<td>46 (75.4%)</td>
</tr>
<tr>
<td>Sulfonylurea</td>
<td>48 (78.7%)</td>
</tr>
<tr>
<td>Glitazones</td>
<td>3 (4.9%)</td>
</tr>
<tr>
<td>Sitalgiptin</td>
<td>2 (3.3)</td>
</tr>
<tr>
<td>Statins</td>
<td>51 (83.6%)</td>
</tr>
</tbody>
</table>
The mean and SD of renal functions including SCr, CysC, and eGFR-MDRD summarized in Table 12.

**Table 12.** Mean (SD) serum creatinine, cystatin C and eGFR values in the study Population.

<table>
<thead>
<tr>
<th></th>
<th>Diabetics (n=61)</th>
<th>Matched controls (n=6)</th>
<th>All controls (n=118)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>1.04 (0.29)</td>
<td>0.84 (0.15)</td>
<td>0.87 (0.15)</td>
</tr>
<tr>
<td>Serum cystatin C (mg/l)</td>
<td>1.02 (0.37)</td>
<td>0.81 (0.11)</td>
<td>0.81 (0.12)</td>
</tr>
<tr>
<td>eGFR – MDRD (ml/min/1.73m²)</td>
<td>66.85 (16)</td>
<td>82.02 (12.70)</td>
<td>81.12 (12.18)</td>
</tr>
</tbody>
</table>

According to eGFR calculated based on SCr of the 61 T2DM patients, 40 (65.6%) had eGFR more than 60ml/min/1.73m² while 21 (34.4%) had renal impairment levels between 30 - 60ml/min/1.73m².

The patients were categorized into albuminuria groups based on the ACR values. Majority of the diabetics (51%) were normoalbuminuric while 36% and 13% had
microalbuminuria and macroalbuminuria respectively (Figure 8). However subjects in the control group had normoalbuminuria.

![Pie chart of albuminuria categories](image)

**Figure 8.** Categories of Albuminuria according ACR (mg/g) in diabetes patients

Considering the diabetic retinopathy status, out of the 61 diabetic patients, 29% and 5% had Non Proliferative Diabetic Retinopathy, (NPDR) and Proliferative Diabetic Retinopathy (PDR) respectively (Figure 9).

![Pie chart of retinopathy](image)

**Figure 9.** Retinopathy in the diabetes cases
4.2 Associations between Serum Cystatin C and Serum Creatinine, Albumin to Creatinine Ratio, and estimated GFR

4.2.1. Association between Serum Cystatin C and Serum Creatinine

There was a very strong, positive correlation between CysC and SCr in the type 2 diabetic patients \( (\rho = 0.845, n = 61, p < 0.05) \). Linear regression analysis was done and CysC was considered as the independent variable while SCr was considered as the dependent variable. An \( R^2 \) value of 0.624 indicates the 62.4 % of the variance in SCr can be explained or accounted for by CysC. Unstandardized coefficient B value of 0.626 indicates that, increase of CysC by 1 mg/L of would result in an increase of SCr by 0.626 mg/dL with a 95% confidence interval (CI) of 0.499 to 0.752 mg/dL (Figure 10).

In the healthy controls, there was a moderate positive correlation between CysC and SCr with Pearson correlation \( (r = 0.463, n = 118, p < 0.05) \). CysC was considered as the independent variable while SCr was considered as the dependent variable in the linear regression analysis and only 21.4 % of the variance in SCr can be explained or accounted for by CysC\( (R^2 = 0.214) \). Increase of CysC by 1 mg/L of would result in an increase of 0.598 mg/dL of SCr with a 95% confidence interval of 0.387 to 0.808 mg/dL (Unstandardized coefficient B = 0.598) (Figure 11).
Figure 10. Scatter plot for serum cystatin C and serum creatinine in the diabetes patients.

Figure 11. Scatter plot for serum cystatin C and serum creatinine in healthy controls.
4.2.2 Association of CysC, SCr, eGFR-MDRD with ACR

Since all the healthy controls had ACR less than 30mg/g and were considered as normoalbuminuric, and the lowest actual concentration in a sample that can be quantitatively determined by the urine albumin assay is 5mg/l, ACR was graded into 8 categories as less than 5, 5 - 9.9, 10 - 14.9, 15-19.9, 20-24.9, 25-29.9, 30 - 299.9 and more than 300 mg/g.

The correlation between of SCr, CysC and eGFR-MDRD with ACR categories were studied using Spearman correlation. CysC, SCr and eGFR-MDRD showed significant correlations with ACR severity categories in the T2DM patients. However, neither CysC nor eGFR-MDRD in healthy adults show a significant correlation with ACR categories while SCr showed a negative correlation (Table 13).

**Table 13.** Summary of correlations between CysC, SCr and eGFR-MDRD with ACR severity in type 2 diabetes patients (n=61) and healthy adults (n=118)

<table>
<thead>
<tr>
<th>Correlation between</th>
<th>In diabetics</th>
<th>In healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>CysC and ACR severity</td>
<td>0.61*</td>
<td>-0.06</td>
</tr>
<tr>
<td>SCr and ACR severity</td>
<td>0.61*</td>
<td>-0.40*</td>
</tr>
<tr>
<td>eGFR-MDRD and ACR severity</td>
<td>-0.58*</td>
<td>-0.001</td>
</tr>
</tbody>
</table>

*p value <0.05

Based on ACR values, diabetics were categorized in to 3 groups as normoalbuminuria (NAU) (n=31), microalbuminuria (MAU) (n=22) and macroalbuminuria (macroAU) (n=8) and comparison between groups was performed (Table 14).
Table 14. Comparison between the albuminuria categories in type 2 diabetes patients.

<table>
<thead>
<tr>
<th></th>
<th>NAU</th>
<th>MAU</th>
<th>MacroAU</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td></td>
</tr>
<tr>
<td>Serum cystatin C</td>
<td>0.84 (0.22)</td>
<td>1.08 (0.28)</td>
<td>1.59 (0.43)</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>(mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>0.88 (0.16)</td>
<td>1.11 (0.28)</td>
<td>1.43 (0.27)</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eGFR- MDRD</td>
<td>74.64 (13.03)</td>
<td>63.01 (16.26)</td>
<td>47.24 (7.61)</td>
<td>&lt;0.05+</td>
</tr>
<tr>
<td>(ml/min/1.73m²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|                  | Number (%)            | Number (%)            | Number (%) of        |
|                  | (% NAU patients)      | (% of MAU patients)   | MacroAU patients     |
| eGFR-MDRD        | 3 (9.7%)              | 11 (50%)              | 7 (87.5%)            |
| less than        |                       |                       |                      |
| 60ml/min/1.73m²  |                       |                       |                      |
| Serum cystatin C | 6 (19.4%)             | 11 (50%)              | 8 (100%)             |
| more than 1mg/L  |                       |                       |                      |

* Kruskal-Wallis test – with post hoc analysis using Mann Whitney test with Bonferroni correction
+ One way Anova test with post hoc analysis using Tukey’s HSD
There was a significant increase of CysC levels from normoalbuminuria to micro to macroalbuminuria on post hoc analysis using Mann-Whitney test with Bonferroni correction following the Kruskal-Wallis test ($p<0.05$). SCr also increased in a similar manner ($p<0.05$) (Table 14 and Figure 12). There was a gradual significant reduction ($p<0.05$) in eGFR-MDRD as the severity of albuminuria increased (Figure 13).

Concentration of CysC and SCr

![Graph showing concentration of CysC and SCr](image)

**Figure 12.** Association between albuminuria categories with serum cystatin C and serum creatinine in type 2 diabetes patients.
Figure 13. Association between albuminuria categories and estimated GFR calculated using MDRD equation in type 2 diabetes patients.

4.2.3 Association of CysC, SCr with eGFR-MDRD

In the diabetic patients, Spearman correlation showed a strong negative correlation between the CysC and eGFR-MDRD, \((rho = -0.820, n = 61, p < 0.05)\) and a stronger negative correlation between SCr and eGFR-MDRD in the diabetics \((rho = -0.885, n = 61, p < 0.05)\). In the T2DM patients, on linear regression analysis, 56.5% of the variance in CysC can be explained by eGFR-MDRD \((R^2 = 0.56)\). A decrease in eGFR-MDRD by 1ml/min/1.73m² would result in an increase of CysC by 0.017 mg/L with a
95% confidence interval of -0.020 to -0.013 mg/L (Unstandardized coefficient B = -0.017) (Figure 14).

Serum cystatin C (mg/L)

![Scatter plot for serum cystatin C and eGFR-MDRD in type 2 diabetes.](image)

**Figure 14.** Scatter plot for serum cystatin C and eGFR-MDRD in type 2 diabetes.

However in the T2DM patients, 72% of the variance in SCr can be explained by eGFR-MDRD ($R^2 = 0.72$) and a decrease of eGFR-MDRD by 1 ml/min/1.73m$^2$ would result in an increase of SCr by 0.015 mg/L with a 95% confidence interval of -0.017 to -0.012 mg/L (Unstandardized coefficient B = -0.015) (Figure 15).

In the healthy controls, there was a moderate negative correlation between the CysC and eGFR-MDRD ($r = -0.353$, $n = 118$, $p < 0.05$) and strong negative correlation between SCr and eGFR-MDRD ($r = -0.607$, $n = 118$, $p < 0.05$).
Serum creatinine (mg/dL)

Figure 15. Scatter plot for serum creatinine and eGFR-MDRD in type 2 diabetes.

In healthy controls, only 12.5% of the variance in CysC can be explained by eGFR-MDRD ($R^2 = 0.125$). Furthermore, a decrease of eGFR-MDRD by 1 ml/min/1.73 m$^2$ would result in an increase of CysC by 0.003 mg/L with a 95% confidence interval of -0.005 to -0.002 mg/L (Unstandardized coefficient $B = -0.003$) (Figure 16). Moreover in the healthy controls, 36.9% of the variance in SCr can be explained by eGFR-MDRD ($R^2 = 0.369$) and a decrease of eGFR-MDRD by 1 ml/min/1.73 m$^2$ would result in an increase of SCr by 0.007 mg/L with a 95% confidence interval of -0.009 to -0.006 mg/L (Unstandardized coefficient $B = -0.007$) (Figure 17).
Figure 16. Scatter plot for serum cystatin C and eGFR-MDRD in healthy controls.

Figure 17. Scatter plot for serum creatinine and eGFR-MDRD in healthy controls.
4.2.4 Comparison between type 2 diabetic patients with mild to no renal impairment and moderate renal impairment

T2DM patients were dichotomized into 2 groups as eGFR-MDRD more than 60 ml/min/1.73m² (mild/no renal impairment, n=40) and eGFR between 30-60ml/min/1.73m² (moderate renal impairment, n=21). The patients with moderate renal impairment were significantly older with higher CysC and SCr levels than those with mild/no renal impairment (p<0.05). There were significantly more albuminuric patients in the moderate renal impairment category (Table 15).

**Table 15.** Comparison between type 2 diabetes patients with mild renal impairment and moderate renal impairment.

<table>
<thead>
<tr>
<th></th>
<th>Mild/no renal impairment</th>
<th>Moderate renal impairment</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (Years)</td>
<td>52.98 (5.23)</td>
<td>55.90 (3.55)</td>
<td>&lt;0.05**</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.88 (0.16)</td>
<td>1.34 (0.24)</td>
<td>&lt;0.05**</td>
</tr>
<tr>
<td>Serum cystatin C (mg/L)</td>
<td>0.85 (0.21)</td>
<td>1.36 (0.37)</td>
<td>&lt;0.05**</td>
</tr>
<tr>
<td>eGFR-MDRD (ml/min/1.73m²)</td>
<td>76.45 (11.15)</td>
<td>48.46 (6.19)</td>
<td>&lt;0.05**</td>
</tr>
<tr>
<td>Albuminuria</td>
<td>n (%)</td>
<td>n (%)</td>
<td>&lt;0.05**+</td>
</tr>
<tr>
<td>Present (&gt;30mg/g)</td>
<td>12 (30%)</td>
<td>18 (85.7%)</td>
<td></td>
</tr>
<tr>
<td>Absent (&lt;30mg/g)</td>
<td>28 (70%)</td>
<td>3 (14.3%)</td>
<td></td>
</tr>
</tbody>
</table>

** Significant, * Not significant p value using Mann Whitney U test
++ Significant Chi square test p value
4.3 Comparisons of CysC levels in patients with type 2 diabetes and healthy individuals

Sixty one T2DM patients with mild to moderate renal impairment were compared with same number of age and sex matched healthy controls. The T2DM patients with mild to moderate renal impairment had a significantly higher family history of T2DM and hypertension than the healthy controls ($p<0.05$) (Table 16). The systolic blood pressure was significantly higher ($p<0.05$) in the diabetics but there was no significant difference in the diastolic pressure between the 2 groups ($p>0.05$). Even though the BMI was not significantly different between the 2 groups ($p>0.05$), the waist circumference and the WHR were significantly higher in the diabetics than the healthy controls ($p<0.05$) (Table 17).

**Table 16.** Comparison family history of age and sex matched type 2 diabetes patients and healthy controls.

<table>
<thead>
<tr>
<th>Presence of family history of,</th>
<th>Diabetics</th>
<th>Healthy controls</th>
<th>McNemar Test p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 2 diabetes</td>
<td>60.7%</td>
<td>4.9%</td>
<td>&lt;0.05**</td>
</tr>
<tr>
<td>Hypertension</td>
<td>44.3%</td>
<td>14.8%</td>
<td>&lt;0.05**</td>
</tr>
</tbody>
</table>

** Statistically significant
**Table 17.** Comparison of age and sex matched type 2 diabetes patients and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>Median (IQ range)</th>
<th>Median (IQ range)</th>
<th>Wilcoxon Signed Rank test</th>
<th>Z value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BMI</strong></td>
<td>24.30 (23.01-26.81)</td>
<td>23.74 (20.85-26.54)</td>
<td>-1.33</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td><strong>Systolic blood pressure (mmHg)</strong></td>
<td>125 (115-140)</td>
<td>120 (110-130)</td>
<td>-2.61</td>
<td>&lt;0.05**</td>
<td></td>
</tr>
<tr>
<td><strong>Diastolic blood pressure (mmHg)</strong></td>
<td>80 (78-90)</td>
<td>80 (75-85)</td>
<td>-1.80</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td><strong>Mean (SD)</strong></td>
<td>92.66 (8.22)</td>
<td>87.22 (8.19)</td>
<td>3.452</td>
<td>&lt;0.05**</td>
<td></td>
</tr>
<tr>
<td><strong>Waist to hip ratio</strong></td>
<td>0.97 (0.06)</td>
<td>0.92 (0.05)</td>
<td>4.853</td>
<td>&lt;0.05**</td>
<td></td>
</tr>
</tbody>
</table>

**Statistically significant**
The levels of CysC, SCr and eGFR-MDRD were compared between age and sex matched diabetic patients and healthy controls. Significantly higher levels of SCr and CysC and lower eGFR-MDRD are present in the T2DM patients than the healthy controls ($p<0.05$) (Table 18).

**Table 18.** Comparison of between type 2 diabetes cases with mild to moderate renal impairment with age and sex matched healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>Diabetics (n=61)</th>
<th>Age and sex matched healthy controls (n=61)</th>
<th>Z value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.96 (0.79-1.22)</td>
<td>0.81 (0.72-0.92)</td>
<td>-5.277</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Serum cystatin C (mg/L)</td>
<td>0.94 (0.74-1.26)</td>
<td>0.81 (0.72-0.88)</td>
<td>-3.992</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>MDRD</td>
<td>66.85 (16.50)</td>
<td>82.02 (12.70)</td>
<td>-6.67</td>
<td>&lt;0.05+</td>
</tr>
</tbody>
</table>

*Wilcoxon Signed Rank test used
+ Paired t test used
Wilcoxon Signed Rank test was used to compare the median (IQ) of T2DM patients categorized according to eGFR-MDRD and ACR categories (Table 19).

CysC was not significantly different ($p>0.05$) between the T2DM patients with no/mild renal impairment (eGFR-MDRD more than 60 ml/min/1.73m$^2$) compared to age and sex matched healthy controls, while SCr was significantly higher in the T2DM patients. However, the T2DM patients with moderate renal impairment (eGFR-MDRD between 30- 60 ml/min/1.73m$^2$) had a significantly higher SCr and CysC levels than the healthy controls ($p<0.05$).

There was no significant difference in the CysC levels between the normoalbuminuric T2DM patients and matched controls ($p> 0.05$). But, CysC levels were significantly higher in the T2DM patients with microalbuminuria than the matched controls ($p<0.05$).
Table 19. Comparison of median (IQ) of serum creatinine and cystatin C of T2DM patients according eGFR and albuminuria categories with matched healthy controls.

<table>
<thead>
<tr>
<th>Comparison of diabetics with eGFR-MDRD ≥ 60 ml/min/1.73m² with controls</th>
<th>T2DM patients</th>
<th>Healthy controls</th>
<th>Z value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>40</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.84 (0.76-0.96)</td>
<td>0.80 (0.72-0.90)</td>
<td>-2.80</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Serum cystatin C (mg/L)</td>
<td>0.79 (0.70-0.95)</td>
<td>0.80 (0.70-0.87)</td>
<td>-1.24</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Comparison of diabetics with eGFR-MDRD 30-60 ml/min/1.73m² with controls</th>
<th>T2DM patients</th>
<th>Healthy controls</th>
<th>Z value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>21</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>1.3 (1.11-1.56)</td>
<td>0.86 (0.76-0.97)</td>
<td>-4.02</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Serum cystatin C (mg/L)</td>
<td>1.35 (1.06-1.54)</td>
<td>0.81 (0.75-0.92)</td>
<td>-3.81</td>
<td>&lt;0.05*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Comparison of diabetics with normoalbuminuria (ACR &lt;30mg/g) with controls</th>
<th>T2DM patients</th>
<th>Healthy controls</th>
<th>Z value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>31</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.83 (0.77-0.96)</td>
<td>0.79 (0.72-0.89)</td>
<td>-2.42</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Serum cystatin C (mg/L)</td>
<td>0.77 (0.70-0.94)</td>
<td>0.79 (0.70-0.86)</td>
<td>-1.01</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Comparison of diabetics with microalbuminuria (ACR 30-300 mg/g) with controls</th>
<th>T2DM patients</th>
<th>Healthy controls</th>
<th>Z value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>22</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>1.10 (0.89-1.32)</td>
<td>0.80 (0.69-0.94)</td>
<td>-3.71</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Serum cystatin C (mg/L)</td>
<td>1.00 (0.86-1.28)</td>
<td>0.80 (0.72-0.90)</td>
<td>-3.08</td>
<td>&lt;0.05*</td>
</tr>
</tbody>
</table>

*Statistically significant
4.4 Association of Diabetic Retinopathy with selected Renal Function Tests

Cross tabulation of renal functions with retinopathy status was performed. Fischer’s exact test was performed instead of Chi square when the expected frequency is less than 1 or more than 20% of expected frequencies are less than or equal to 5. There was no significant difference with regard to the presence or absence of reduced eGFR and DR status. Similarly there was no significant association between CysC categories and DR categories. However, there was a statistically significant trend for the percent of T2DM patients with albuminuria (micro or macro albuminuria) to increase significantly with an increase in severity of diabetic retinopathy ($p<0.05$) (Table 20). The majority of the patients with normoalbuminuria did not have retinopathy while the majority of patients with macroalbuminuria had retinopathy in this group of diabetic patients (Figure 18).

![Figure 18. Association of degrees of albuminuria and diabetes retinopathy status.](image)

Table 20: 

<table>
<thead>
<tr>
<th>Albumin mg /g creatinine</th>
<th>Diabetic retinopathy categories</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;30mg/g</td>
<td>No DR</td>
</tr>
<tr>
<td>30-299mg/g</td>
<td>NPDR</td>
</tr>
<tr>
<td>&gt;300mg/g</td>
<td>PDR</td>
</tr>
</tbody>
</table>

Figure 18. Association of degrees of albuminuria and diabetes retinopathy status.
Table 20. Associations of renal function categories and diabetes retinopathy categories.

<table>
<thead>
<tr>
<th></th>
<th>No DR</th>
<th>NPDR</th>
<th>PDR</th>
<th>$X^2$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>According to renal impairment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eGFR-MDRD $\geq 60$ ml/min/1.73m$^2$</td>
<td>27</td>
<td>12</td>
<td>1</td>
<td>0.191</td>
<td>$&gt;0.05$</td>
</tr>
<tr>
<td>eGFR-MDRD &lt;60 ml/min/1.73m$^2$</td>
<td>13</td>
<td>6</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>According to albuminuria categories</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoalbuminuria</td>
<td>23</td>
<td>8</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microalbuminuria</td>
<td>15</td>
<td>6</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macroalbuminuria</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>According to Cystatin C levels</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystatin C $&lt; 1$mg/L</td>
<td>24</td>
<td>11</td>
<td>1</td>
<td>0.87</td>
<td>$&gt;0.05$</td>
</tr>
<tr>
<td>Cystatin C $\geq 1$mg/L</td>
<td>16</td>
<td>7</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant Fischer’s exact test p value

On comparison of the T2DM patients with and without diabetic retinopathy, there was no significant difference in the renal impairment, albuminuria and levels of SCr, CysC, eGFR-MDRD ($p>0.05$) (Table 21).
Table 21. Comparison of diabetes patients with and without diabetes retinopathy.

<table>
<thead>
<tr>
<th></th>
<th>% of patients</th>
<th>% of patients</th>
<th>Chi square value (2x2)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albuminuria (ACR≥30mg/g)</td>
<td>61.9%</td>
<td>42.5%</td>
<td>2.08</td>
<td>&gt;0.05 +</td>
</tr>
<tr>
<td>With renal impairment (eGFR-MDRD&lt;60 ml/min/1.73m²)</td>
<td>38.1%</td>
<td>65.6%</td>
<td>0.19</td>
<td>&gt;0.05+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>With DR</th>
<th>Without DR</th>
<th>Z value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median (IQ)</td>
<td>Median (IQ)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>1.07</td>
<td>0.92</td>
<td>-0.995</td>
<td>&gt;0.05*</td>
</tr>
<tr>
<td>(0.84-1.24)</td>
<td>(0.78-1.22)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum cystatin C (mg/L)</td>
<td>0.94</td>
<td>0.92</td>
<td>-0.357</td>
<td>&gt;0.05*</td>
</tr>
<tr>
<td>(0.78-1.29)</td>
<td>(0.74-1.23)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eGFR-MDRD (ml/min/1.73m²)</td>
<td>63.61</td>
<td>70.80</td>
<td>-0.812</td>
<td>&gt;0.05*</td>
</tr>
<tr>
<td>(52.41-75.17)</td>
<td>(56.07-80.90)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+ Chi square test        *Mann Whitney test performed.

95
On analysis of correlations of the various renal function markers with DR using Spearman correlation, only albuminuria severity categories (normo, micro and macroalbuminuria) showed a mild but significant correlation with DR \((\rho = 0.29, p < 0.05)\) (Table 22).

### Table 22. Summary of correlation between retinopathy and renal function markers.

<table>
<thead>
<tr>
<th>Correlations between diabetic retinopathy and,</th>
<th>(\rho) value</th>
<th>(p) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum cystatin C (mg/L)</td>
<td>0.76</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.15</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>eGFR-MDRD (ml/min/1.73m(^2))</td>
<td>-0.12</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Albuminuria categories</td>
<td>0.29</td>
<td>&lt;0.05*</td>
</tr>
</tbody>
</table>

\*\(p\) value < 0.05 is statistically significant

### 4.5 Establishing normal reference range for Serum Cystatin C in a selected sample of healthy Sri Lankan adults

In the sample of 118 healthy controls, CysC showed a normal distribution and was confirmed by the histogram, normal Q-Q plots and had a \(p\) values more than 0.05 for Kolmogorov-Smirnov and Shapiro-Wilk tests which also confirms the normal distribution of CysC. The reference interval for CysC was calculated with 95% confidence limits and the 97.5\(^{th}\) percentile and 2.5\(^{th}\) percentile formed the upper and lower reference limits of the adult normal healthy subjects respectively.
When all the 118 healthy subjects were considered, the mean CysC was 0.81 with a SD of 0.115 mg/L, with values ranging from 0.5 to 1.14 mg/L. The normal reference range for this particular group of adult Sri Lankans was with a lower limit of 0.581 (95% CI = 0.545-0.616) to an upper limit of 1.04 (95% CI = 1.004-1.075). Healthy control subjects were grouped into 10 year age groups and the differences in the mean CysC levels were compared using one way-ANOVA. When both genders were considered together, mean CysC value in the 51-60 years age group was significantly higher than 30-40 and 41-50 years age groups ($p<0.05$). However there
was no significant difference between the 30-40 years and 41-50 years age groups (p<0.05) (Figure 20, Table 23).

Mean serum cystatin C

<table>
<thead>
<tr>
<th>Age categories (years)</th>
<th>Mean serum cystatin C levels according to age categories in healthy adults.</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-40</td>
<td>0.85mg/L (SD=0.11)</td>
</tr>
<tr>
<td>41-50</td>
<td>0.77mg/L (SD=0.10)</td>
</tr>
</tbody>
</table>

On comparison of males and females using Independent t-test, males had a significantly higher mean CysC levels of 0.85mg/L (SD=0.11) than the mean value of 0.77mg/L (SD=0.10) of females (t value = -4.02, p<0.05) (Figure 21).
Table 23. Mean (SD) serum cystatin C levels according to age categories in healthy adults.

<table>
<thead>
<tr>
<th></th>
<th>30-40 years</th>
<th>41-50 years</th>
<th>51-60 years</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>24</td>
<td>28</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Serum cystatin C (mg/L)</td>
<td>0.75 (0.11)</td>
<td>0.78 (0.10)</td>
<td>0.84 (0.11)</td>
<td>&lt;0.05*</td>
</tr>
</tbody>
</table>

*Statistically significant

Figure 21. Serum cystatin C values according to the gender in healthy adults.
Since there was a significant difference in mean CysC levels after the age of 50 years, the subjects were dichotomized into 2 groups as 30-50 years and 51-60 years and mean CysC levels were compared according to gender as well using Independent t test. There were significant differences in mean CysC levels both in males and females between 30 to 50 years and 51-60 years age categories (p<0.05) (Table 24).

Table 24. Comparison of mean cystatin C levels according age and gender.

<table>
<thead>
<tr>
<th></th>
<th>30-50 years</th>
<th>51-60 years</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n)</td>
<td>(n)</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>0.82 (0.10)</td>
<td>0.89 (0.12)</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>(n=24)</td>
<td>(n=29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>0.73 (0.09)</td>
<td>0.81 (0.10)</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>(n=28)</td>
<td>(n=37)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Statistically significant

Because gender and age differences seem to affect the reference intervals in our population, 95% confidence intervals for the upper and lower reference values for cysC were calculated across various subgroups (Table 25) and considerable overlap in the central ranges were not observed.
Table 25. Reference limits and upper and lower limit confidence intervals according to age and gender.

<table>
<thead>
<tr>
<th>Percentile</th>
<th>Limits of serum cystatin C</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>31-50 years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>2.5% 0.62</td>
<td>0.54 - 0.68</td>
</tr>
<tr>
<td></td>
<td>97.5% 1.01</td>
<td>0.96 - 1.09</td>
</tr>
<tr>
<td>Females</td>
<td>2.5% 0.54</td>
<td>0.48 - 0.60</td>
</tr>
<tr>
<td></td>
<td>97.5% 0.90</td>
<td>0.86 - 0.97</td>
</tr>
<tr>
<td>51-60 years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>2.5% 0.65</td>
<td>0.57 - 0.71</td>
</tr>
<tr>
<td></td>
<td>7.5% 1.12</td>
<td>1.06 - 1.20</td>
</tr>
<tr>
<td>Females</td>
<td>2.5% 0.60</td>
<td>0.55 - 0.66</td>
</tr>
<tr>
<td></td>
<td>97.5% 1.01</td>
<td>0.96 - 1.07</td>
</tr>
</tbody>
</table>

Therefore, for this population of healthy Sri Lankan adults, reference ranges of CysC are,

For 30-50 years of age,

Males = 0.62 – 1.01 mg/L, Females = 0.54 – 0.90 mg/L

For 51-60 years,

Males = 0.65 – 1.12 mg/L, Females = 0.60 – 1.01 mg/L
4.6 Analysis of Receiver Operator Characteristics (ROC) curves for determination of diagnostic accuracy of Cystatin C in Diabetic Nephropathy

Sensitivity and specificity of SCr and CysC in identification of abnormal GFR (less than 60ml/min/1.73m²) and albuminuria were assessed using ROC curves. An ideal diagnostic test should have a cut-off point near the upper left-hand corner of the graph at a point where both sensitivity and specificity are maximized.

The eGFR of 60ml/min/1.73m² calculated using MDRD equation was used as the cut-off point for the detection of abnormal/ moderate to severe renal impairment in the T2DM patients. The ROC plots showed that an area under the curve (AUC) for CysC was 0.904 and the cut-off value of CysC of 0.98 mg/L with a sensitivity of 85.7% and a specificity of 82.5%. AUC for SCr was 0.952 and the cut-off value of SCr of 1.04 mg/dL to detect abnormal GFR was with a sensitivity of 90.5% and a specificity of 85.0% (Figure 22 and Table 26). There was no significant difference in the AUCs of SCr and CysC (p value > 0.05). Thus SCr and CysC have similar diagnostic accuracy to detect moderate renal impairment.

Table 26. Diagnostic accuracy at cut-off values of serum creatinine and cystatin C for detection of moderate renal impairment.

<table>
<thead>
<tr>
<th></th>
<th>Cut off levels</th>
<th>AUC (CI)</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum cystatin C (mg/L)</td>
<td>0.98</td>
<td>0.904 (0.827-0.980)</td>
<td>85.7</td>
<td>82.5</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>1.04</td>
<td>0.952 (0.907-0.998)</td>
<td>90.5</td>
<td>85.0</td>
</tr>
</tbody>
</table>
Figure 22. Nonparametric ROC plots to assess the diagnostic efficiency of serum cystatin C (CysC) and serum creatinine (SCr) in diagnosing moderate renal impairment in type 2 diabetes patients.

When ACR level of 30mg/g was used as the cut-off point for the detection of albuminuria, the ROC plots showed that an area under the curve (AUC) for CysC was 0.815 and the cut-off value of CysC at 0.96 mg/L with a sensitivity of 73.3% and a specificity of 80.6%. AUC for SCr was 0.791 and the cut-off value of SCr 0.97 mg/dL to detect abnormal GFR was with a sensitivity of 76.7% and a specificity of 80.6%. There was no significant difference in the AUCs of SCr and CysC (p value >0.05). Thus SCr and CysC have equal diagnostic accuracy in detection of albuminuria.
Table 27. Diagnostic accuracy at cut-off values of serum creatinine and cystatin C for detection of albuminuria at ACR > 30mg/g.

<table>
<thead>
<tr>
<th>Cut off level</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum cystatin C (mg/L)</td>
<td>0.96</td>
<td>0.815 (0.704-0.925)</td>
<td>73.3</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.97</td>
<td>0.791 (0.669-0.913)</td>
<td>76.7</td>
</tr>
</tbody>
</table>

Sensitivity

Figure 23. Nonparametric ROC plots to assess the diagnostic efficiency of serum cystatin C (CysC) and serum creatinine (SCr) in diagnosing albuminuria in type 2 diabetes patients.
4.7 Serum Cystatin C in levels in identification of Diabetic patients with moderate risk of progression of CKD

The T2DM patients were categorized using the albuminuria and eGFR-CKD-EPI formula based on SCr according to the latest KDIGO guideline’s classification for prediction of risk of CKD progression (Figure 2). The low risk group had normoalbuminuric patients with eGFR more than 60 ml/min/1.73m². The moderately increased risk group included normoalbuminuric patients with eGFR between 45-59 and micoralbuminuric patients with eGFR more than 60 ml/min/1.73m².

Serum cystatin C

(mg/L)

![Chart showing serum cystatin C levels in type 2 diabetes patients according to CKD risk categories]

**Figure 24.** Serum cystatin C levels in type 2 diabetes patients according to the CKD risk categories.
Mann-Whitney test was used to compare CysC and SCr between those two groups. CysC levels were significantly higher in the group with moderately increased CKD risk than the low/ no risk group \((U = 146.5, z = 2.028, p < 0.05)\) (Figure 24). However there was no significant difference in SCr levels between those two groups. \((U = 159, z = 1.732, p = >0.05)\). Furthermore, on analysis with Independent t test there was no significant difference for eGFR-MDRD and eGFR CKD EPI SCr between those 2 groups \((t=1.73, df=43, p>0.05)\) and \((t=1.62, df=43, p>0.05)\) respectively (Table 28).

**Table 28.** Comparison of markers of GFR between the low risk and moderate KDIGO risk group.

<table>
<thead>
<tr>
<th></th>
<th>Low risk (n=29)</th>
<th>Moderate risk (n=16)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>Mean (SD) 0.87 (0.15)</td>
<td>0.98 (0.20)</td>
<td>&gt;0.05 *</td>
</tr>
<tr>
<td></td>
<td>Median(IQ) 0.82</td>
<td>1.00 (0.76-0.94)</td>
<td>(0.79-1.15)</td>
</tr>
<tr>
<td>Serum cystatin C (mg/L)</td>
<td>Mean (SD) 0.83 (0.20)</td>
<td>0.93 (0.20)</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td></td>
<td>Median(IQ) 0.76</td>
<td>0.93 (0.70-0.94)</td>
<td>(0.79-1.05)</td>
</tr>
<tr>
<td>eGFR- MDRD (ml/min/1.73m²)</td>
<td>Mean (SD) 76.28(11.74)</td>
<td>69.54 (12.91)</td>
<td>&gt;0.05+</td>
</tr>
<tr>
<td>eGFR-CKD EPIcrea (ml/min/1.73m²)</td>
<td>Mean (SD) 84.21 (12.51)</td>
<td>77.12 (16.60)</td>
<td>&gt;0.05+</td>
</tr>
</tbody>
</table>

* Mann Whitney test  
+ Independent t test
5. DISCUSSION

Cystatin C has been introduced several years ago as a marker of renal function. However, in Sri Lanka CysC measurement is not widely used by the clinicians. To the best of our knowledge, this is the first study assessing the performance of CysC in Sri Lankan patients with mild to moderate diabetic nephropathy which will be of clinical significance. Our aim was to study the association of CysC with some of the renal function markers such as ACR, SCr, and eGFR-MDRD which are frequently and routinely used in clinical setup in Sri Lanka. Furthermore it was decided to compare CysC levels between T2DM with mild to moderate renal impairment and apparently healthy adults and also to study the association between diabetic retinopathy status and renal function markers including CysC in the same group of T2DM patients with mild to moderate renal impairment. Since there are no data on the normal reference interval values for Sri Lankan healthy adults, it was decided to find reference intervals for healthy adults as a preliminary study. In our study, 2012 KDIGO guidelines were used for categorization of CKD.

The mean duration of T2DM was $10.23 \pm 6.95$ years and ranged from 0.5 to 35 years. Presence of renal impairment in those with shorter duration of diabetes could be due delayed diagnosis of T2DM or due to the insidious onset of T2DM. All the T2DM patients in this study were with eGFR more than 30 ml/min/1.73m$^2$ and belonged to 1, 2 or 3 CKD stages. Of the diabetics 51% were with normal albumin excretion while 49% had albuminuria (ACR > 30mg/g)
Recent research has been focused on tests for the correlation of GFR and new renal function markers to assess the feasibility of using them as a surrogate marker. SCr is the most frequently used marker to evaluate renal function in clinical practice and Jaffe' method is the commonly used method to measure it. In our study we measured standardized SCr, using modified Jaffé method assay with calibration traceable to IDMS. A strong, positive correlation between CysC and SCr in the T2DM patients \( (\rho = 0.845, n = 61, p < 0.05) \) was present and 62.4% of the variance in SCr can be explained or accounted for by CysC. Furthermore an increase of 1 mg/L of CysC would result in an increase of 0.626 mg/dL of SCr (95% CI 0.499 - 0.752 mg/dL). This supports the concept that CysC and SCr have similar properties as endogenous serum markers of renal impairment in type 2 diabetes patients. However, there was only a moderate positive correlation between the two variables in the healthy adults \( (r = 0.463, n = 118, p < 0.05) \) and only 21.4 % of the variance in SCr can be explained or accounted for by CysC with increase of 1 mg/L of CysC would result in an increase of 0.598 mg/dL of SCr (95% CI 0.387 - 0.808 mg/dL) in the healthy adults. Previous studies have also shown a positive correlation between SCr and CysC in CKD and in diabetics [91, 108]. Our study found a stronger correlation between SCr and CysC than by Williems et al 2009 [92], where the correlation coefficient was 0.54. In their study all the diabetic subjects had SCr within the normal range unlike in our study. At the upper threshold for mild CKD (GFR of less than 90 ml/min per 1.73 m^2) CysC has greater diagnostic accuracy than SCr [97, 98]. This suggests a lower association between CysC and SCr at lower levels of SCr.
Measurement of albumin is recommended for the detection of early diabetic nephropathy [33]. Normalization to the urine creatinine concentration as in ACR would minimize the changes in urine albumin concentration due to changes in urinary flow rates by assuming the occurrence of constant creatinine excretion of approximately 1 gm per day across measurements [33]. ACR was graded into several groups as less than 5mg/g, 5 to 9.9 mg/g, 10 to 14.9, 15 to 19.9, 20 to 24.9, 25 to 29.9, 30 to 299.9 and more than 300 mg/g. Both CysC and SCr showed similar significant correlations with ACR categories (rho = 0.61, n =61, p< 0.05) and significant negative correlation with eGFR-MDRD (rho = - 0.58, n = 61, p < 0.05 ) in T2DM patients. However, in the healthy adults, CysC did not show a significant correlation with ACR (p> 0.05). Yang Y.S. et al 2007 [110] also found a positive correlation between CysC and ACR in T2DM patients. But they did not observe a significant correlation between SCr and ACR in contrast to our study. It is not mentioned whether they used standardized SCr in their study and if not, it would result in inaccuracies.

In the T2DM patients, there was a stepwise significant increase in CysC levels from normoalbuminuria to microalbuminuria to macroalbuminuria (p< 0.05) with mean (SD)CysC values of 0.84 (0.22), 1.08 (0.28) and 1.59 (0.43) mg/L respectively. Thus, our findings confirm previous findings in that CysC increases significantly in T2DM patients from normoalbuminuria to micro to macroalbuminuria [110, 118-120]. In Korean T2DM patients, CysC level reflects the trend in albuminuria level more accurately than SCr level [125]. In our study SCr also showed significant increase according to the increase in albuminuria severity categories while eGFR- MDRD showed a stepwise significant decrease (p < 0.05). In contrast to our findings,
Christensson et al 2004[96] did not find a difference in both CysC and SCr levels between normoalbuminuric and microalbuminuric patients while in the study reported by Yang Y.S. et al 2007 [110] revealed that CysC was significantly higher in microalbuminuric patients but not SCr.

Albuminuria is one of the most significant prognostic biomarkers of kidney disease outcomes and even cardiovascular disease and death [33]. Diabetic patients with macroalbuminuria have an estimated 19 times more rapid decline in renal function than those without albuminuria while patients with microalbuminuria have a 5 times greater decline compared with people with normoalbuminuria [53]. Even in subjects without CKD but with CysC more than 1mg/L, there is a 4 times higher risk of developing CKD in the next 4 years [3]. In our study, only 9.7% of diabetic patients with normoalbuminuria had impaired renal function (eGFR less than 60 ml/min/1.73m²) while 50% of microalbuminuric and 87.5% of macroalbuminuric patients had renal impairment. CysC levels more than 1mg/L were present in 19.4%, 50% and 100% of diabetic patients with normo, micro and macroalbuminuria respectively. This suggests a high association between elevated CysC and albuminuria. In diabetes, albuminuria and reduction in GFR are complementary/overlapping manifestations. Significant reduction in GFR does not always precede albuminuria [152]. In a 3 year follow up study, CysC based eGFR reflected the changes in albuminuria more accurately than eGFR-MDRD [125].

In the T2DM patients, a strong negative correlation was observed between the CysC and eGFR-MDRD, ($\rho = -0.820$, $n = 61$, $p < 0.05$) while a stronger negative correlation was present between SCr and eGFR-MDRD ($\rho = -0.885$, $n = 61$, $p < 0.05$). Similar
findings were presented by previous studies where a SCr demonstrated a stronger correlation with eGFR-MDRD than CysC [153]. This may be because, eGFR-MDRD is derived using SCr. On linear regression analysis, variance in CysC and SCr could be explained by eGFR-MDRD by 56.5 % and 72 % respectively. A decrease of 1ml/min/1.73m² of eGFR-MDRD would result in almost similar increase of CysC by 0.017 mg/L (95% CI of -0.020 to -0.013 mg/L) and increase of SCr by 0.017 mg/L (95% CI of -0.017 to -0.012 mg/L) in the T2DM patients. Both SCr, CysC are known to have an inverse, non linear relationship with GFR. However, when GFR falls to 40-70 ml/min/1.73m² the proportional increase of CysC is supposed to be higher than SCr [32]. However, unit decrease of eGFR-MDRD seems to increase of both SCr and CysC in a similar manner in our study and this could possibly because majority of the T2DM patients had eGFR more than 60ml/min/1.73m². A significant strong positive correlation has been identified between the reciprocal of CysC and eGFR-MDRD[101] and CysC levels also correlated with eGFR-CG in T2DM patients [110]. A study carried out by Peiris et al 2008 in Sri Lankan CKD patients, presented that CysC showed better correlation against 24 hour creatinine clearance than SCr. Also a significantly high correlation was present between CysC based estimated GFR with creatinine clearance [154].

In the present study, T2DM patients were dichotomized in to 2 groups as eGFR-MDRD more than 60 ml/min/1.73m² and eGFR between 30-60ml/min/1.73m². The diabetic patients with moderate renal impairment (eGFR between 30 and 60 ml/min/1.73m²) were significantly older and had significantly higher levels of CysC along with higher levels of SCr. There were more albuminuric patients in that group than those with
eGFR-MDRD more than 60 ml/min/1.73m$^2$. Similarly, significant increasing trend in CysC and SCr levels were noted across different stages of eGFR based on CG equation (>90, 60-90, <60 ml/min/1.73m$^2$) [108, 110].

Many previous studies in patients with diabetes or CKD have found that, CysC correlates better with measured GFR than SCr [95, 153]. However, C. Oddoze et al found a lower correlation between mGFR and CysC than SCr [102]. CysC is also shown to have a better correlation with mGFR than eGFR-MDRD did with mGFR in diabetic patients with normal GFR and the authors suggested the higher ability of CysC to detect early renal impairment in diabetes in a study carried out by Pucci et al [95]. In their study patients were divided into 6 categories according to mGFR as less than 45, 45-59.9, 60-74.9, 75-90, 90-120 and more than 120 ml/min/1.73m$^2$. A step by step statistically significant change was observed only for CysC mean values and could detect early decreases in GFR within 75-90 ml/min/1.73m$^2$. Both CysC and eGFR-MDRD reflected changed within the reference interval (>90 ml/min/1.73m$^2$). But with MDRD, renal functions were estimated with low precision in individuals with higher GFR. The bias between mGFR and MDRD tends to increase for GFR values less than 45ml/min/1.73m$^2$ and more than 90ml/min/1.73m$^2$ [95]. This could possibly be the reason for the lower but significant negative correlation was observed between CysC and eGFR-MDRD ($r = -0.353$, $n = 118$, $p < 0.05$) in the healthy adults in the present study. In them, a strong negative correlation existed between SCr and eGFR-MDRD ($r = -0.607$, $n = 118$, $p < 0.05$) which may be due to the fact that eGFR-MDRD is derived from SCr. Also in them, only 12.5% of the variance in CysC and 36.9% of variance in SCr could be explained or accounted for by eGFR-MDRD. Similar findings were
present in a previous study where eGFR was estimated using CKD EPI equation where T2DM patients showed a better correlation with the eGFR compared to the normal subjects [155]. Erikson et al. 2010 measured GFR by iohexol clearance in a representative sample of middle aged (50-62 years) general population without coronary heart or kidney disease, stroke or diabetes mellitus and found no evidence that equations based on CysC alone or in combination with creatinine provide better GFR estimates than the commonly used MDRD and CKD-EPI equation[156]. Thus our results suggest the advantage of using CysC for CKD detection in T2DM patient over the general population and also indicate that screening for low GFR with SCr and CysC in a low-risk general population is probably not worthwhile.

In the present study, a case–control study was performed with 61 T2DM patients and 61 age and sex-matched healthy controls. The T2DM patients had a significantly higher family history of T2DM and hypertension than the healthy controls (p value<0.05). Only the systolic blood pressure was significantly higher (p value<0.05) in the diabetics and they had more central obesity with significantly higher waist circumference and the WHR than the healthy controls (p value<0.05). Significantly lower eGFR-MDRD levels and significantly higher levels of SCr and CysC were present in the diabetes patients (n=61) than in the matched healthy controls (p value <0.05). On further comparison, T2DM patients with moderate renal impairment (eGFR between 30-60ml/min/1.73m²) had significantly higher levels of CysC than the age and sex matched healthy controls (p<0.05, n=21). However, T2DM patients with eGFR more than 60ml/min/1.73m² did not have significantly higher levels of CysC than the age and sex matched healthy controls (p>0.05, n=40). CysC levels were significantly higher in the T2DM patients
with microalbuminuria than the matched controls \( (p<0.05, n=22) \). But there was no significant difference in the CysC levels between the normoalbuminuric T2DM patients and matched controls \( (p>0.05, n=31) \). These findings have a clinical importance in the decision making of using CysC in the assessment of diabetic nephropathy. According to the results, CysC levels may not be elevated significantly in diabetics with normoalbuminuria and in diabetics with eGFR more than 60 ml/min/1.73 m\(^2\).

Studies have also shown that CysC level in hypertensive patients with diabetes mellitus was significantly higher compared to hypertensive patients without diabetes. In a cross sectional study with hypertensive patients who were with eGFR-MDRD levels \( \geq 60 \) ml/min/1.73 m\(^2\) and without established kidney disease, CysC levels were significantly higher among patients with diabetes than those without [70]. However, a healthy control group was not present in this study for comparison. It has been shown that hypertensive subjects who are either diabetic or non diabetic would have higher levels of CysC [155]. In another cross sectional study showed that normoalbuminuric diabetic subjects had significantly higher CysC levels than non diabetic subjects [108]. Both these studies are not case controls studies where age and sex were matched and have not excluded ischemic heart disease patients who may have higher levels of CysC as it was done our well planned study.

In our sample of T2DM, 34.4% had DR and is higher than the 21.2% prevalence of DR in newly diagnosed T2DM patients in Sri Lanka [16]. In our study, there was no significant association between DR and impaired renal function as eGFR less than 60 ml/min/1.73 m\(^2\) or by CysC \( \geq 1 \) mg/L when studied using cross tabulation \( (p>0.05) \). CysC \( \geq 1 \) mg/L was used for categorization because, in subjects without CKD but with
CysC more than 1mg/L, there is a 4 times higher risk of developing CKD after 4 years [3]. Furthermore, there was no significant difference in CysC, SCr, eGFR-MDRD levels and albuminuria on comparison of T2DM patients with DR and in those without DR. The majority of the patients with normoalbuminuria did not have retinopathy while the majority of patients with macroalbuminuria had retinopathy, in this group of diabetic patients. Moreover, there was a statistically significant trend for the diabetic patients with albuminuria (micro or macro albuminuria) to increase significantly with an increase in severity of diabetic retinopathy ($X^2 = 11.79$, $df = 4$, $n=61$, $p<0.05$). Only ACR categories (<30, 30-299.9, >300 mg/g) showed significant mild correlation with DR ($\rho = 0.29$, $p<0.05$). Retinopathy was previously shown only to be associated with poor glycemic control and high systolic and diastolic blood pressures and not with renal functions in T2DM patients in Sri Lanka [16].

Even though our study did not find an association between CysC and DR, Surendaret al, reported that Cys C values were highest in microalbuminuric T2DM subjects with DR, followed by microalbuminuric T2DM subjects without DR, normoalbuminuric T2DM subjects without DR. eGFR – MDRD was not different in microalbuminuric T2DM subjects without DR compared to T2DM subjects with DR [121]. The prevalence of site threatening DR (STDR) in the 4th quartile of CysC was higher than other three quartiles in diabetic patients. CysC levels were higher in the group of T2DM patients with STDR than those T2DM patients without DR and T2DM patients with non STDR. Also in the same study, severity of DR positively correlated with CysC and CysC was an independent risk factor for DR and STDR. On ROC analysis, CysC value of 1.25mg/L is the optimal cutoff value in predicting STDR [144]. Our findings were in-line with
previous studies, where microalbuminuric T2DM with DR and T2DM subjects with DR did not have significant differences in eGFR-MDRD levels [121]. About 60% of subjects with advanced DR had CKD, whereas about 15% of individuals without CKD could develop DR [138].

In our study, albuminuria and DR were associated and this is also supported by previous studies [157]. Diabetic subjects with microalbuminuria were around 2 times more likely to have DR than those without microalbuminuria, and almost 6 times more in the presence of macroalbuminuria [158]. DR is an indicator to predict the development of microalbuminuria [118]. However, Potisat, Srisubat et al, 2008 reported that there was no relationship between microalbuminuria and diabetic retinopathy in type 2 diabetes [141] probably due to use of urine dipsticks (Micral test II) to detect albuminuria levels which is not sensitive enough to detect albuminuria.

In the Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) study, diabetics with retinopathy had higher systolic blood pressure, albuminuria and lower GFR values compared to normal subjects suggesting that presence of any degree of diabetic retinopathy is associated with more proteinuria and a higher risk for ESRD and death in T2DM patients [134]. T2DM patients with microalbuminuria and eGFR ≥60 mL/min/1.73 m² have a significantly greater risk for development and progression of diabetic retinopathy (HR= Hazards ratio = 3.34 [95% CI 1.04–10.70]) than those normoalbuminuric T2DM with moderate renal impairment. This study suggests that microalbuminuria has a greater impact on predicting the development and progression of diabetic retinopathy compared with moderate decline in GFR among type 2 diabetic patients [140]. Albuminuria is associated with
generalized endothelial dysfunction and is a window into systemic small vessel disease [33]. This could be a reason for the association between albuminuria and DR.

Utilizing the CysC values of the 118 healthy subjects of the present study, we attempted to formulate a preliminary normal reference interval for CysC for Sri Lankan adult population. And this is the first report from Sri Lanka which would be of value in the assessment of renal impairment using CysC. The mean CysC was 0.81 with SD of 0.115 mg/L, with values ranging from 0.5 to 1.14 mg/L when all the 118 subjects were considered and the normal reference interval for this particular group of adult Sri Lankans was with a lower limit of 0.581 (95% CI = 0.545-0.616) to an upper limit of 1.04 (95% CI = 1.004-1.075). Several earlier studies have shown that CysC range in the normal population is constant after the age of 1 year and not affected by age, gender, muscle mass [1, 58]. However, in the present study, there was a step wise increase in mean(SD) CysC values 0.75 (0.11), 0.78 (0.10) and 0.84 (0.11) in 30-40 years, 41-50 years and 51-60 years respectively. The CysC levels were significantly higher in the 51-60 years age category than the other 2 categories indicating that CysC levels are higher after the age of 50 years (p<0.05). The males had a higher mean CysC level of 0.85 (0.11) mg/L than the females 0.77 (0.10) mg/L (p<0.05). Both genders showed a significantly different mean CysC levels in 30-50 years and 51-60 years age categories (p<0.05). Since a significant overlapping of the 95% CI of the upper and lower limits of the CysC reference intervals was absent, we propose different reference intervals for males and females for both below 50 years and above 50 years of age. For 30-50 years of age, reference intervals for male and females are 0.62 – 1.01 mg/L and 0.54 – 0.90 mg/L respectively. For 51-60 years of age, reference interval for males and females are
0.65 – 1.12 mg/L and 0.60 – 1.01 mg/L respectively. These reference intervals are
different and the upper limit is lower than the reference intervals mentioned in the CysC
assay kit (Kone lab™/ T series- Thermo Fischer Scientific) we used for this study.
Those suggested values of the kit were; for individuals 1-50years = 0.55 – 1.15mg/L
and for >50 years = 0.63 – 1.44 mg/L.
The high levels of CysC present at birth rapidly declines within the first year [60].
Earlier studies suggested the use of one reference interval after the age of 1 year [73]. As
observed in our study, some other studies have also recommended reference intervals
using age 50 years for dichotomizing and having different reference ranges for above
50 years and below 50 years [61]. CysC is found to remain constant from around the
age of 1 year to 50 years [60]. In the healthy subjects significantly higher levels of
CysC have been observed after the age of 50 years [96, 159]. Galteau et al 2001 [160]
claimed that CysC levels significantly increased after the age of 60 years while
Ognibene et al 2006, considered 45 years for dichotomization of age [79]. The age
related decline in GFR with impaired concentrating ability occurs due to pathologic
changes such as global glomerular sclerosis, vascular sclerosis, tubular atrophy with
reduction in cortical thickness and overall kidney size [34]. In contrast to our findings,
most of the authors in the past studies claimed no effect of gender on CysC levels [79,
161]. Some earlier studies suggested the use of a common reference range for males
and females aged 1 to 50 years as CysC concentration within this age range is
independent of age, sex, height and weight and to have gender based reference intervals
after the age of 50 year [159]. However, Ichihara et al 2007, suggested the use of
gender specific reference intervals for adults less than 50 years of age and a single
reference interval for both genders above 50 years of age [80]. In some studies even though a gender difference was present, the range of distribution of the reference intervals were the same thus allowing the use of a single reference interval for both genders [77, 78] in contrast to the present study.

Multiple studies have compared CysC and SCr as predictors of GFR. Most studies have found CysC to be a better predictor, although others have found no difference [162]. A meta-analysis by Dharnidharka et al 2002 reported that the reciprocal of CysC had a greater approximation of GFR compared with the reciprocal of SCr as measured both by a higher average correlation coefficient (0.82 vs. 0.74; p<0.001) and a higher area under the receiver operator characteristic (ROC) curve (0.93 vs. 0.84; p<0.001) [103].

Thus, Nonparametric ROC plots were performed in the present study to assess the diagnostic efficiency of CysC and SCr in diagnosing moderate renal impairment (eGFR-MDRD < 60 ml/min/1.73m²) in type 2 diabetes patients. The AUC for CysC and SCr were 0.904 and 0.952 respectively and were not significantly different (p>0.05). The cut-off value with maximum sensitivity and specificity for CysC was 0.98 mg/L with a sensitivity of 85.7% and a specificity of 82.5%. whilst for SCr it was 1.04 mg/dL with a sensitivity of 90.5% and a specificity of 85.0%. Thus SCr and CysC have similar diagnostic accuracy to detect moderate renal impairment. Many past studies have found CysC as a more precise test of kidney function than SCr (Roos et al., 2007). However, one study where GFR was derived using CG equation, greater AUC was observed for SCr than for CysC [108] while another had higher AUC for CysC than SCr [110]. In a study carried out in Sri Lankan patients by Peiris et al 2008, a sensitivity of 82% and a specificity of 68% was present at the cutoff value for CysC as 1.25mg/L.
to detect creatinine clearance of 60ml/min indicating moderate renal impairment[154]. Compared to that, our CysC cut off level of 0.98mg/L for the detection of moderate renal impairment is lower. This could be because we used the MDRD equation and not the 24 hour creatinine clearance. Also, in our study the sensitivity and specificity of CysC in the detection of moderate renal impairment at the value of 0.98mg/L are better. Another reason for this may be the fact that we selected only T2DM patients with only mild to moderate renal impairment which gives more uniformity than selecting patients with CKD due to various pathologies and at different CKD stages as in the above mentioned study.

When ACR level of 30mg/g was used as the cut-off point for the detection of albuminuria, the ROC plots showed AUCs of 0.815 and 0.791 for CysC and SCr respectively. But they were not significantly different (p > 0.05). At the cut-off value of 0.96 mg/L for CysC, was with a sensitivity of 73.3% and a specificity of 80.6% and the cut-off value of SCr 0.97 mg/dL was with a sensitivity of 76.7% and a specificity of 80.6%. Thus SCr and CysC have similar diagnostic accuracy in detection of albuminuria. However other studies have shown that patients with early diabetic nephropathy with only microalbuminuria, have a greater AUC for CysC than for creatinine clearance and SCr [111] and also, CysC had a significantly higher AUC than SCr and eGFR-CG at the cut-off value of 300 μg/mg creatinine[110].

In studies where GFR was measured, some found that CysC had better diagnostic characteristics compared to SCr for detecting moderate CKD with mGFR < 60 ml/min/1.73m² [97]. However, some did not find CysC to be a better marker at 60 ml/min/1.73m² and the authors suggested that CysC has no major advantage versus SCr
in the evaluation of GFR less than 60 mL/min/1.73 m²[95, 96]. CysC has a greater diagnostic accuracy than SCr at the upper threshold for mild CKD (> 75 and 90 mL/min/1.73 m²) [95, 97].

Early prediction of risk CKD progression is important since initiation of early treatment will reduce complications, and the need for dialysis and transplantation. According to previous studies, eGFR <60 mL/min/1.73 m² and albuminuria are predictors of all-cause mortality and cardiovascular mortality, independent of each other [85, 130]. In a meta-analysis, lower eGFR and higher albuminuria are described as risk factors for ESRD, acute kidney injury and progressive CKD in both general and high-risk populations, independent of each other and of cardiovascular risk factors.[127]. In the present study, T2DM patients (n=61) were categorized into the 4 CKD prognosis categories using the albuminuria and eGFR –CKD EPI based on SCr according to the latest KDIGO guideline’s. The low risk group constituted of normoalbuminuric patients with eGFR more than 60 ml/min/1.73 m². The moderately increased risk group included normoalbuminuric patients with eGFR between 45 – 59 ml/min/1.73 m² and microalbuminuric patients with eGFR more than 60 ml/min/1.73 m². A significantly higher CysC level was detected in the group with moderately increased CKD risk than the low/ no risk group (p = <0.05) while SCr, eGFR-MDRD, eGFR-CKD EPISCr levels were not significantly different between those two groups(p>0.05). This finding is of clinical importance as it highlights the ability of CysC in identification of moderately increased risk for CKD progression. For this part of the analysis, we used eGFR calculated using 2009 CKD EPI equation based on SCr to estimate GFR as recommended by the KDIGO 2012 guidelines [27]. CysC is linearly associated with
mortality, CVD and non-CVD outcomes. However, SCr is mainly associated with risk in individuals with more advanced kidney disease [162]. This may be the explanation for our findings with regard to early identification of risk for progression of CKD. The 2012 KDIGO guidelines suggests measuring CysC in adults with SCr based eGFR between 45–59 ml/min/1.73 m² who do not have markers of kidney damage such as albuminuria if confirmation of CKD is required [27]. Thus, clinicians can use CysC based eGFR to reassure the low risk subset of patients with SCr based eGFR<60 ml/min/1.73 m². A triple marker approach in the assessment of CKD has been suggested by Peralta et al. 2011 This is because, addition of CysC based eGFR to SCr based eGFR and albuminuria can more accurately reclassify persons and can distinguish a 3-fold risk of death and 4-fold risk of end-stage renal disease[163].

The new 2012 CKD EPI equation seems to be the best available GFR estimating equations based on CysC at present. However this equation has to be evaluated in several populations [90]. Future research is required to assess the diagnostic performance of this CysC equation in our population.

The strengths of this study are the stringent selection of subjects, use of assay method with calibration traceable IDMS to measure SCr and use of the re-expressed MDRD equation. Our focus was on directly measured CysC levels and their correlates without making assumptions about eGFR formulae based on CysC. Retinal photography which is only a screening tool, and was used in most of the earlier studies. In our study we had a comprehensive eye examination performed by trained medical officers in eye unit or
by consultant eye surgeon where DR was assessed using slit-lamp binocular indirect ophthalmoscopy through dilated pupils.

There are few limitations in this study as well to be acknowledged. We did not use a 'measured gold standard' test to compare the performance of CysC. These techniques are invasive, expensive, time consuming, involve radiation exposure and repeated blood and urine sampling which is cumbersome. However, the assumption that measured GFR is the 'gold standard' has been challenged and there is an absence of a universally recognized gold standard method of measuring GFR and different methods yield different measurements. Also, mGFR is not better than other markers of renal disease at predicting outcomes in CKD [164]. We compared CysC with eGFR-MDRD. The MDRD equation is widely used in clinical practice and is recommended by the National Kidney Foundation (NKF) (2002) and NICE guidelines [26,35]. MDRD equation demonstrates a higher accuracy in CKD stages 3–5 than in CKD stages 1 and 2 [165] because it is known to underestimate the GFR, particularly at lower creatinine concentrations [33]. The MDRD study equation expressed in terms of standardized SCr shows improved performance [34]. In a study in an Indian population, 24-hour creatinine production was derived from CG, MDRD and CKD EPI formulae were compared with the creatinine production calculated using measured muscle mass and age. Their analyses support the use of MDRD for estimating renal function in Indian populations [87] and we assumed it to be applicable to the Sri Lankans as well. We used an assay using modified Jaffe method. However, enzymatic methods are known to be more accurate [38]. Our study was performed using a CysC assay which was not standardized against the latest international CysC reference material (ERM-
This study was a cross sectional study where renal functions were studied only once. Thus we assumed the presence or absence of renal impairment and albuminuria based on a spot sample. Thus our diagnosis of diabetic nephropathy may not be very accurate. Furthermore, since it is a cross sectional study, we cannot determine the direction of the association or determine causality. We had a relatively small sample size than previous studies due to financial and time restraints and our study subjects were only between the ages of 30-60 years. Even though we assessed the reference interval for healthy adults, our healthy subjects sample is not representative of the general population and was not randomly selected. However, our reference interval values will be useful as a preliminary study.
6. CONCLUSION

Findings of this study indicates that,

1. Serum cystatin C levels significantly correlate with serum creatinine and eGFR-MDRD in both type 2 diabetes mellitus (T2DM) patients with mild to moderate diabetic nephropathy and healthy adults.

2. Serum cystatin C concentration significantly correlates with ACR in T2DM patients but not in healthy individuals.

3. T2DM patients with moderate renal impairment (eGFR between 30-60 ml/min/1.73 m²) have significantly higher levels of CysC than the age and sex matched healthy controls.

4. T2DM patients with mild/no renal impairment (eGFR more than 60 ml/min/1.73 m²) did not have significantly higher levels of CysC than the age and sex matched healthy controls.

5. T2DM patients with microalbuminuria (ACR between 30-300 mg/g) have significantly higher levels of CysC than the age and sex matched healthy controls.

6. T2DM patients with normoalbuminuria (ACR less than 30 mg/g) did not have significantly higher levels of CysC than the age and sex matched healthy controls.

7. There is a significant positive correlation between ACR and DR.
8. There is no significant association between serum cystatin C, serum creatinine and eGFR-MDRD with diabetic retinopathy (DR) in our study population.

9. This study has established separate serum cystatin C cutoff values for moderate renal impairment and for albuminuria in T2DM patients using ROC curves.

10. For the first time, reference intervals for serum cystatin C for healthy male and female Sri Lankans adults less than 50 years and more than 50 years of age for are reported. These reference values are as follows.

<table>
<thead>
<tr>
<th>Category</th>
<th>Reference Interval (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt; 50years</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.62 - 1.01</td>
</tr>
<tr>
<td>Female</td>
<td>0.54 - 0.90</td>
</tr>
<tr>
<td>Age &gt; 50years</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.65 - 1.12</td>
</tr>
<tr>
<td>Female</td>
<td>0.60 - 1.01</td>
</tr>
</tbody>
</table>

11. Serum cystatin C is useful in early detection of poor prognosis in CKD in T2DM when compared to the other markers of renal impairment.

**Recommendations**

The findings of the present study support the use of serum cystatin C in screening diabetic nephropathy. Based on the results of this study, we would like to advise the following,

1. Periodic assessment of serum CysC, eGFR and ACR for patients with T2DM

Limitations/Shortcomings of this study

1. It was not possible to select a statistically viable sample number of healthy adult males to establish a reference interval for Cys C as most of the males had at least one of the exclusion criteria and the proportion of females attending the diabetic clinic was higher than males.

2. A ‘measured gold standard’ test was not used to compare the performance of CysC. These techniques are invasive, expensive, time consuming, involve radiation exposure and repeated blood and urine sampling which is cumbersome.

3. MDRD and CKD EPI equations based on serum creatinine were used based on the assumption that those are applicable to Sri Lankans.

4. More accurate enzymatic method was not used to measure serum creatinine, but used modified Jaffe method since enzymatic methods are more expensive.

5. The serum cystatin C assay used was not standardized against the latest international CysC reference material (ERM-DA471/IFCC). This was the only assay that was supplied to Sri Lanka at the time of the research.

6. Analysis for CysC concentration in serum was not performed in duplicate due to financial constraints.

7. Due to financial and time restraints and the study subjects were only between the ages of 30-60 years.

8. The healthy subjects sample was selected by convenient sampling and is not representative of the general population.
Proposal for future research


2. Population study to investigate the association between severity of retinopathy and other markers of renal function in T2DM patients.

3. Study the performance of eGFR using the MDRD and CKD EPI formula based on CysC, SCR, or both against a gold standard method using an exogenous glomerular filtration marker in Sri Lankan population.

4. Population study to investigate the performance of cystatin C based eGFR in all stages of diabetic nephropathy in type 2 diabetes
7. REFERENCES


110. Yang, Y.-S., et al., Use of Serum Cystatin C to Detect Early Decline of Glomerular Filtration Rate in Type 2 Diabetes. Internal Medicine, 2007. 46(12): p. 801-806.


129. Peralta Ca, S.M.G.J.S. and et al., **Detection of chronic kidney disease with creatinine, cystatin c, and urine albumin-to-creatinine ratio and association**


133. de Boer, I.H., et al., Cystatin C, Albuminuria, and Mortality Among Older Adults With Diabetes. Diabetes Care 2009 32 (10): p. 1833-1838


156. Eriksen, B.O., et al., Cystatin C is not a better estimator of GFR than plasma creatinine in the general population. 2010. 78(12): p. 1305-1311.


8. APPENDIX

Appendix I

List of publications and communications from thesis


4. Wijayatunga N.N, Perera P.P.R., Wanigasuriya K., Peiris H. **Differences in estimation of Glomerular Filtration Rate using three standard formulae in selected Type 2 Diabetic patients** - Sri Lanka Medical Association 126th Anniversary Scientific Medical Congress, Sri Lanka: 2013 (Poster presentation) (S Ramachandran Prize for the best paper in Nephrology )

5. Wijayatunga N.N, Perera P.P.R., Wanigasuriya K., Peiris H. **Serum Cystatin C as a marker in the assessment of renal function in diabetic nephropathy in Sri Lankan type 2 diabetics- Preliminary study**- Sri Lanka Medical

7. Wijayatunga N.N., Perera P.P.R., Peiris H., Wanigasuriya K. Estimated Glomerular Filtration Rate calculated using IDMS-traceable MDRD equation may predict the duration of diabetes in Sri Lankan Type 2 diabetic patients- International Conference on Public Health Innovations, Sri Lanka: 2013 (Oral presentation)

8. Wijayatunga N.N., Perera P.P.R., Peiris H., Wanigasuriya K. Waist circumference and waist to hip ratio is higher in Type 2 diabetics with moderate renal impairment – Findings from a preliminary study. Annual sessions of Nutrition Society of Sri Lanka:2013 (Poster presentation)

9. Wijayatunga N.N., Perera P.P.R., Wanigasuriya K.and Peiris H. Association between duration of type 2 diabetes and body mass index in a group of female Sri Lankan type 2 diabetics with routine clinic follow up - A preliminary study. Annual Academic Sessions of Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka;2012 (Poster presentation)
Appendix II

Questionnaires – English and Sinhala
Questionnaire

For subjects

1. Number :

2. Age :

3. Sex :

4. Home town :

5. Occupation :

6. Ethnicity :

7. Level of education : I\textsuperscript{st} II\textsuperscript{nd} III\textsuperscript{rd}

8. Duration of diabetes :

9. Diabetic retinopathy diagnosed ?: Yes [ ] No [ ] Don't know [ ]

10. Past medical history :

10.1 Hypertension : Yes [ ] No [ ]

10.2 Hypercholesterolemia : Yes [ ] No [ ]

(Patients with renal diseases/chronic lung disease/liver disease/heart failure/ischaemic heart disease/rheumatoid arthritis/malignancy/hyperthyroidism/hypothyroidism will be excluded)

Etc. .................................................................

11. Family history :

11.1 Diabetes [ ]

11.2 Hypertension [ ]

11.3 Ischaemic heart disease [ ]

11.4 Hypercholesterolaemia [ ]

11.5 Diabetic nephropathy [ ]

12. Social history

12.1 Smoking status

- Never smoked [ ]

Ex smoker (stopped for >1yr) [ ]

12.2 Alcohol intake :

Yes [ ] No [ ]

Stopped [ ]

Amount .............................................................

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13. **Drug history**

- **Insulin**

  - **Oral hypoglycemic agents**
    - Metformin
    - Sulfonylurea
    - Thiazolidinediones

  - **Angiotensin Converting enzyme inhibitors**

  - **Angiotensin receptor blockers**

  - **Statins**

14. **From past records (if available)**

12.3. Last fasting blood glucose value:

12.4. Last post prandial blood glucose value:

12.5. HbA1c:

12.6. Last albuminuria/proteinuria level:

12.7. Last serum creatinine level:

12.8. Recent most calculated eGFR:

12.9. **Lipid profile**

  - Total cholesterol:
  - TAG:
  - LDL:
  - HDL:
On examination (of subjects)

1. Height (cm):
2. Weight (kg):
3. Waist circumference (cm):
4. Hip circumference (cm):
5. BMI:
7. Retinopathy severity:
   7.1 No retinopathy
   7.2 Mild non proliferative diabetic retinopathy (NPDR)
   7.3 Moderate NPDR
   7.4 Severe NPDR
   7.5 Proliferative diabetic retinopathy (PDR)
   7.6 Macular oedema
8. Cataract: Yes □ No □
Questionnaire - For controls

1. Number : .................................................................
2. Age : ......................................................................
3. Sex : .....................................................................
4. Hometown : ..............................................................
5. Occupation : ............................................................
6. Ethnicity : .................................................................
7. Level of education : $I^*$ $II^*$ $III^*$

8. Past medical history :
   8.1 Hypercholesterolaemia ............................................
   Etc: ..........................................................................

9. Family history :
   9.1 Hypertension .....................................................
   9.2 Diabetes ..............................................................
   9.3 Ischaemic heart disease ........................................
   9.4 Hypercholesterolaemia ...........................................
   9.5 Diabetic nephropathy .............................................

10. Social history
   10.1 Smoking status ..................................................
       Never smoked ....................................................
       Ex smoker (stopped for >1yr) ..............................
   10.2 Alcohol intake : 
       Yes .................................................................
       No ...............................................................
       Stopped .........................................................
   Amount ....................................................................

11. From past records
   11.1 HbA1c : ..............................................................
   11.2 Last fasting blood glucose value : ........................
   11.3 Last post prandial blood glucose : ......................
   11.4 Last serum creatinine level: ...............................
8 Lipid profile

- Total cholesterol: ..................................................
- TAG: .................................................................
- LDL: ................................................................
- HDL: ................................................................

On examination

1. Height (cm): ..............................................................
2. Weight (kg): ..............................................................
3. Waist circumference (cm): ..............................................
4. Hip circumference (cm): .................................................
5. BMI: ................................................................
6. Blood pressure: 1...................... 2...................... Mean .... 

Random blood glucose level: ......................................................

Urine microalbumin strip test: Positive □ Negative □
පුද්ගලික විස්තර අනුව ප්‍රකට කරන්නේදින්

01. අංකන:
02. වූලි:
03. අමාත්‍ය/දේශය:
04. අමාත්‍ය ප්‍රශ්න:
05. මෙම්ප්‍රශ්න:
06. මෙම්ප්‍රශ්න:
07. මෙම්ප්‍රශ්න අයිතිය: ගමාන්/නිළිය/නැගී

08. නැවික ආවරණුලින් පැසු විකුණු නිදසිණි:
08.1 පිළිතුරු විශේෂ ආවරණුලින් නිදසිණි:
08.2 විනාවන:

09. පිටිස් සමාවේදනයක් අදන් නිදසිණි:
09.1 දෙරේන්නට:
09.2 දෙරේන්න තිවිද නිදසිණි:
09.3 මාල නිදසිණි:
09.4 පිළිතුරු විශේෂ ආවරණුලින් නිදසිණි:
09.5 දෙරේන්න තිවිද කාලයක් නිදසිණි:

10. අනුවර්තනය
10.1 කුඩ උතිහ අධ්‍යාපන නිදසිණි:
10.2 අදාළ උතිහ උතිහ/විශේෂ/ජාතීන් උතිහ

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07. අවධානය අශ්‍රී ලාංකික/ඨෙක්ෂණ/මෙහෙය

08. නොවිම්බර්ස්/පියවන්/ඉමුබවිසිනි

09. මෙහෙය අවධානය අශ්‍රී ලාංකිකේය වර්තමාන වේදය පැහැදිලි ආරෝග්‍ය අදික මාර්ගය දැක්වීම?
ප්‍ර/වැම/කාම්පුන්.

10. අධික මැයි වෙනි මෙහෙය අශ්‍රී ලාංකිකේය :
10.1 අවධානය අශ්‍රී ලාංකිකේය : දෙ/වහ
10.2 අවධානය අශ්‍රී ලාංකිකේය දෙක්ෂණ අධිකිත ගණනය : දෙ/වහ
10.3 අරමාන:

11. විශේෂ අදිකිතාකරණ විදූශ අශ්‍රී ලාංකිකේය :
11.1 අවධානයේවත්
11.2 අවධානය අශ්‍රී ලාංකිකේය:
11.3 අරමාන;
11.4 අවධානය අශ්‍රී ලාංකිකේය දෙක්ෂණ අධිකිත ගණනය:
11.5 අවධානය අශ්‍රී ලාංකිකේය දෙක්ෂණ අරමාන:

12. විශේෂ බිදුර
12.1 අවධානය අශ්‍රී ලාංකිකේය:
මාර්ග අදිකිතාකරණ පැහැදිලි
12.2 අවධානය අශ්‍රී ලාංකිකේය දෙක්ෂණ/මාර්ග/කාම්පුන් අදිකිතාකරණ

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Appendix III

Consent forms- English and Sinhala
Consent form

Name(s) of the investigators:
Prof. H. Peiris, Dr. Kamani Wanigasuriya, Dr. P. P. Rasika Perera, Dr. N. N. Wijayatunga.

Contact information of the principle investigator: Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura.

Contact no: 0112758761

Address of the institution where the study is to be carried out
Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura

Title of the research project: Cystatin C as a marker in the assessment of renal function in patients with diabetic nephropathy

I ...........................................have read the information sheet and understood

   (a) What the study involves
   (b) Refusal to participate in the study will not affect my treatment or care in any way.
   (c) That I may withdraw at anytime and it will not affect me adversely in any manner

I have had an opportunity to discuss the study and ask questions and they had been satisfactorily answered.

Therefore I agree to participate in this study.

Signature of the participant ______________________________ Date: ______________________________
Full name
Postal address

I have been present while the procedure has been explained to the participant and I have witnessed his / her consent to take part in the study.

Witness’ Signature ______________________________ Date ______________________________
Full Name: ______________________________________
Address: ______________________________________
සංස්කරණ සම්බන්ධ පදනම් අදිනත්ව

ංගකරන වැනි කතාවක් කොටස්

උපකරණය මලිනු පෙද, උපත් කොළඹ, උපත් ප්‍රදේශීයව, උපත් රාජීය, උපත් ප්‍රදේශීය සංවිධානය

ප්‍රාථමික උපකරණය මලිනු පෙද - මෙම කොටස් කුරුණේකම් කොටස්, උපත් කොළඹ, උපත් ප්‍රදේශීය සංවිධානය, උපත් ප්‍රදේශීය සංවිධානය

ශිල්පී මලිනු පෙද : 0012738761

ප්‍රාථමික උපකරණයේ කතාව - මෙම කොටස් කුරුණේකම් කොටස් කුරුණේකම් කොටස්, උපත් කොළඹ, උපත් ප්‍රදේශීය සංවිධානය මෙම් කොටස් කුරුණේකම් කොටස්

(ඉහළට හා ඇති කොටස් හා ඇති කොටස් හා ඇති කොටස් කුරුණේකම් කොටස්

a) උපත් ප්‍රදේශීය සංවිධානය

b) උපත් ප්‍රදේශීය සංවිධානය කොටස් පෙමෙන් කොටස් කුරුණේකම් කොටස් සංවිධානය/මාධ්‍යසිංහලික කොටස් කුරුණේකම් කොටස්

c) මෙම් විවිධ ප්‍රාථමික උපකරණයේ කොටස් පෙමෙන් කොටස් කුරුණේකම් කොටස් සංවිධානය/මාධ්‍යසිංහලික කොටස් කුරුණේකම් කොටස්

දකුණුවෙන් අති විවිධ ප්‍රාථමික උපකරණයේ කොටස් පෙමෙන් කොටස් කුරුණේකම් කොටස් සංවිධානය/මාධ්‍යසිංහලික කොටස් කුරුණේකම් කොටස් මෙම් කොටස් කුරුණේකම් කොටස්

විකාරමය සඳහා මෙහෙයින් සහ අංගියේ රත්නය මෙහෙයින්

ඔබේ උපකරණයේ කොටස් පෙමෙන් කොටස් කුරුණේකම් කොටස්

ිතහා පීඨේපත සහ උපකරණයේ කොටස් පෙමෙන් කොටස් කුරුණේකම් කොටස්

විකාරමය සඳහා මෙහෙයින් සහ අංගියේ රත්නය මෙහෙයින්

විකාරමය සඳහා මෙහෙයින් සහ උපකරණයේ කොටස් පෙමෙන් කොටස් කුරුණේකම් කොටස්

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Appendix IV

Information sheets – English and Sinhala
**Information sheet**

I am Dr. N.N. Wijayatunga attached to Faculty of Medical Sciences, University of Sri Jayewardenepura. My current designation is lecturer (probationary). I would like to invite you to take part in the research study titled “**Cystatin C as a marker in the assessment of renal function in patients with diabetic nephropathy**”, conducted by Dr. N.N. Wijayatunga at Colombo South Teaching Hospital / Nawaloka hospitals (Pvt) Ltd.

**The title of the research:** Cystatin C as a marker in the assessment of renal function in patients with diabetic nephropathy

**Purpose of the research:** Cystatin C level in blood is a new marker of kidney function. We are hoping to identify the relationship between serum cystatin C and other routine kidney function tests and also to identify the association between diabetic retinopathy with kidney function tests in diabetic kidney disease patients.

**Participant selection:** Diabetic kidney disease patients will be selected from Colombo South Teaching Hospital diabetic and medical clinics. **Controls will be selected from volunteers at Colombo South Teaching Hospital from the wards and those who undergo routine health checks at Nawaloka hospitals (Pvt) Ltd.**

**Procedure and the duration:** I will fill an interviewer based questionnaire and obtain details regarding history of diabetes and other illnesses, drugs, family history of diseases. Previous investigations will be recorded from clinic records. **A general examination will be performed by me. In the controls a random blood glucose levels will be checked using a glucometer.** Blood pressure, height, weight, waist and hip circumference will be measured. 3ml of blood will be collected from a vein by the investigator under strict aseptic measures. Urine spot sample will be collected into a container. Eyes will be examined by an ophthalmologist in diabetic patients.

**Risks / benefits:** There will not be any risk to the participants. If any abnormality is detected, they will be referred for medical advice.

Refusal to participate in the study will not affect your treatment or care in any way. We would like to inform you that you may withdraw at anytime and it will not affect you adversely in any manner.

We are grateful for your corporation in our study.

If you have any further queries please contact: Dr. N.N. Wijayatunga

Address: Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura. **Contact no: 0112758761**
සතාවය නිළිනින් ලැබුණි ආකාරය

මාරුමාම. මෙම අපාරත්නය ස්ලිහත්වය ආධාරය කළව නොවත් ගැනීම. කොහොමද ඉතිහාසික කලාපයන් කෙරිනි අපාරත්නය විශේෂය. කොහොමද මෙම අපාරත්නය ල "නේදුරු නොදුරු අධිරාජ විශේෂීය විශේෂීය විද්‍යාත්මක පරිණාමයේ (Cystatin - C) අධිරාජය" පවුල්කමක් කරයිමේ අපත් වීමට අපාරත්නය ආදිනි. කොහොමද මෙම අපාරත්නය ද අපත් වීමට අධිරාජ විශේෂීය විද්‍යාත්මක පරිණාම විශේෂීය විද්‍යාත්මක් පරිණාමයේ අදිනුමය.

මාරුමාම අධිරාජයමන් - සතාවය අධිරාජ විශේෂීය විද්‍යාත්මක පරිණාමයේ අධිරාජයමන් දියක් පිළිතුරු.

මාරුමාම කාලාල - කාලාලයක් ද සතාවය අධිරාජ විශේෂීය විද්‍යාත්මක පරිණාමයේ මාරුමාම කාලාලයක්. කාලාලයක් ද සතාවය අධිරාජ විශේෂීය විද්‍යාත්මක පරිණාමයේ මාරුමාම කාලාලයක් ද සතාවය අධිරාජ විශේෂීය විද්‍යාත්මක් පරිණාමයේ මාරුමාම කාලාලයක් දියක් පිළිතුරු.

මාරුමාම අධිරාජයමන් - කාලාලයක් ද සතාවය අධිරාජ විශේෂීය විද්‍යාත්මක පරිණාමයේ අධිරාජයමන් දියක් පිළිතුරු.

මාරුමාම කාලාලයක් - කාලාලයකෝ ද සතාවය අධිරාජ විශේෂීය විද්‍යාත්මක පරිණාමයේ මාරුමාම කාලාලයක්.

මාරුමාම අධිරාජයමන් - කාලාලයකෝ ද සතාවය අධිරාජ විශේෂීය විද්‍යාත්මක පරිණාමයේ අධිරාජයමන් දියක් පිළිතුරු.

මාරුමාම කාලාලයකෝ - කාලාලයකෝ ද සතාවය අධිරාජ විශේෂීය විද්‍යාත්මක පරිණාමයේ මාරුමාම කාලාලයකෝ.

මාරුමාම අධිරාජයමන් - කාලාලයකෝ ද සතාවය අධිරාජ විශේෂීය විද්‍යාත්මක පරිණාමයේ අධිරාජයමන් දියක් පිළිතුරු.
Appendix V

Ethics Review Committee letters from USJP and CSTM
ERC meeting date : 26th January 2012

Dr. NN Wijeyatunge
Department of Biochemistry, faculty of Medical Sciences, University of Sri Jayewardenepura

Application No: 548/11 - Cystatin C as a marker in the assessment of renal function in patients with Diabetic nephropathy (alterations)

Your letter dated 13th January 2012 was received at the ERC meeting on 26th January 2012 and the committee has accepted the alterations to your proposal.

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The ethical approval is effective from 26th January 2012. Please note that ethical approval would be withdrawn if any alteration is made to the project without obtaining prior written consent from the ethics review committee.

Chairperson
Dr. C. A. Wanigatunge

Secretary
Dr. Vathsala Jayasuriya

Address all correspondence to: Secretary, Ethics Review Committee, Department of Community Medicine, Faculty of Medical Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka.

Tel: 94-11-2758586, Fax 94-11-2811480, erc.fms.usjp@gmail.com
Ethical Clearance for Research Proposal No – 189

This is to inform you that the Ethical Clearance Committee held on 09th September 2011 has granted clearance for the above proposal no 189.

A copy of the letter from Ethical Review Committee is attached herewith for your reference.

Dr. L. J. Methandirange
MBBS, MSc. (Med. Admin)
Deputy Director
Colombo South Teaching Hospital
Kalubowila.
Figure 25. The Lever-Jenning chart for serum Cystatin C Quality Control

Figure 26. The Lever-Jenning chart for serum cystatin C Quality Control High
EXATROL-P creatinine concentration (mg/dL)

![Diagram of EXATROL-P creatinine concentration with data points and control limits](image)

Figure 27. The Lever-Jenning chart for serum creatinine Quality Control—EXATROL-P

EXATROL-N creatinine concentration (mg/dL)

![Diagram of EXATROL-N creatinine concentration with data points and control limits](image)

Figure 28. The Lever-Jenning chart for serum creatinine Quality Control—EXATROL-N
Urine L1 creatinine concentration (mg/dL)

Figure 29. The Lever-Jenning chart for urine creatinine Quality Control Level 1 (L1)

Urine L2 creatinine concentration (mg/dL)

Figure 30. The Lever-Jenning chart for urine creatinine Quality Control Level 2 (L2)

Albumin concentration (mg/L)
Figure 31. The Lever-Jenning chart for urine albumin Quality Control

Albumin concentration (mg/L)

Figure 32. The Lever-Jenning chart for urine albumin Quality Control High