CHAPTER ONE

1.0 BACKGROUND

The red blood cell distribution width (RDW or RCDW) is a measure of the variation of red blood cell (RBC) width that is reported as part of a standard complete blood count. Red blood cells have a standard size of 6–8 µm. Certain disorders, however, cause a significant variation in cell size. Higher RDW values indicate greater variation in size. Normal reference range for RDW is 11–15% in both males and females, hence all values above 15% are considered to be elevated (Grant B. et al., 2009).

The "Width" in RDW is sometimes thought of as "misleading," since RDW is a measure of deviation of the volume of RBCs, and not directly the diameter. However, "width" refers to the width of the volume curve (distribution width), not the width of the cells.

RDW is calculated using the following formula:

$$\text{RDW} = \left( \frac{\text{Standard deviation of MCV}}{\text{mean MCV}} \right) \times 100$$

It has been known for some time that elevated RDW predicts poor outcome in both diseased and normal populations (Patel et al., 2009).

Recently, elevated red blood cell distribution width (RDW) was identified and proposed to be of diagnostic importance in diabetic nephropathy (DN) (Perlstein et al., 2009).

Diabetic nephropathy (nephropatia diabetica), also known as Kimmelstiel - Wilson syndrome and intercapilary gromerulonephritis, is a progressive disease caused by angiopathy of capillaries in the kidney glomeruli.

After many years of diabetes the delicate filtering system in the kidney becomes destroyed, initially becoming leaky to blood proteins such as albumin which are then lost in urine and this forms a basis for diagnosing DN by estimating the amount of albumin in urine (Gaspari et al., 2007).

DN is more likely to occur if the blood glucose is poorly controlled and rises above the expected normal threshold level of 6.6mmol/l. Diabetic nephropathy is characterized by nephritic syndrome and nodular glomerulosclerosis. It is mostly due to long standing diabetes mellitus (Lin. C. et al., 2012).
Currently diabetic nephropathy is often diagnosed by clinical screening and laboratory estimation of albumin levels in urine (albuminuria), (normal range 20 – 29 mg/l), Serum Creatinine levels (normal value is 63 µmol/l to 120 µmol/l), Urea (1.0 to 8.3mmol/l), plasma protein levels (normal range 60 – 80 g/l). Values above these parameters indicated in brackets are considered to be high and are used as diagnostic indicators of DN (Ichinose.K. et al., 2007).

In addition to the above existing methods used in diagnosing DN, RDW would enhance the diagnosis of DN and prevent further mortality and morbidity in patients with diabetes type2 if the pattern of association between DN and RDW is established.

According to the World Health Organization (WHO) April 2010 report on complications of diabetes, diabetic nephropathy is a leading cause of kidney failure and consequent death in Africa, Europe and USA.

Audit reports done at Kabwe General Hospital in 1995, 2009, 2010 and 2011 all showed that Diabetic nephropathy (DN) was on the increase with a range of 21% to 28% representing more than 20% of diabetes type 2 patients being diagnosed with DN (Kabwe General Hospital audit report., 2011).

This study therefore wishes to assess RDW as a marker for diabetic nephropathy.

1.1 Statement of the problem

There is an increasing number of DN cases in the world (Kikkawa et al., 2012). Starting in the second half of the 20th century, the prevalence of non-insulin-dependent (type 2) diabetes increased substantially in many populations and ethnic groups, including African Americans, Native Americans, Mexican Americans and Pacific Islanders.

Around 3.2 million deaths every year are attributable to complications of diabetes and this translates into six deaths every minute. (Grant et al., 2011).

Kabwe General Hospital (KGH) indicated an increase in the number of diabetic nephropathy cases as shown in the audit report mentioned above where more than 20% of patients with type2 Diabetes were also found to have DN.

Diabetes mellitus is a common metabolic disease worldwide affecting 150 million people in 2000 and was predicted to rise to 220 million in 2010. (Zimmet et al., 2011).
Diabetes and its associated complications such as nephropathy, retinopathy and neuropathy have become a public health problem of considerable magnitude (Schwartz et al., 2008).

DN causes most of the excess morbidity and mortality in diabetes mellitus. Adults with diabetes are at a 2-to 4-fold increased risk of renal complications relative to those without diabetes (Fox et al., 2009). Renal diseases accounts for up to 80% of premature excess mortality in diabetic patients (Winner et al., 2010). Because of the huge premature morbidity and mortality associated with this disease, prevention and interventions of these complications has become a key issue (Tsuboi. S, et al., 2013).

1.2 Study justification

This study assessed the possibility of RDW as a marker for DN in type 2 diabetes mellitus patients at Kabwe General Hospital. This Hospital was chosen because of its role as a referral centre for central province of Zambia.

Diabetes and its complications such as nephropathy have increased worldwide at an alarming rate (Mogensen et al., 2010). Currently in Zambia, RDW test is not used as part of the management of type 2 diabetes mellitus patients. In most cases, the prognosis is not good when diabetic patients suffer from cardiovascular, macro or microvascular angiopathies. Therefore, early detection of diabetic complications such as nephropathy is very critical for the administration of preventive therapy in type 2 diabetes patients.

In the nationally representative study done by Felker and colleagues in Asia, RDW was found to be a strong predictor of mortality in both middle aged and older adults with DN. Summary measures of global model fit, model calibration, and model discrimination further showed that RDW significantly improved mortality risk prediction. The magnitude and robustness of these associations indicated that RDW may be an age-associated biomarker that is prognostic in adults aged 45 and older. The results obtained from this study would be used as a basis to advocate for the inclusion of RDW test in the general management of type 2 diabetes mellitus patients at Kabwe General Hospital and other Hospitals under the Ministry of Health (Kabwe General Hospital audit report. 2011).

Red blood cell distribution width (RDW) was reported to be a risk marker of morbidity and mortality for renal and cardiovascular diseases in various study populations (Tonelli et al., 2008). This study proposes to evaluate RDW as a marker of nephropathy in a representative sample of the adult diabetes population at Kabwe General Hospital in Zambia.
This will result in early commencement of treatment in those patients suspected to have diabetic nephropathy and hence reduce on deaths occurring as a result of diabetic nephropathy. Urea, creatinine and urine albumin have been widely used as markers for DN in type 2 diabetes mellitus patients. However, these assays are very expensive and tedious to perform hence cannot be done routinely in most laboratories in Zambia. The total cost of each of them is ZMK 2000 while the cost of RDW test is ZMK 10 per sample and it is always produced as part of the full blood count test (this was determined by the Ministry of health commodity price list). Hence if RDW is included as a biomarker, the cost of diagnosing DN will be reduced. It was therefore important that a study was done to determine suitability of RDW as a marker for DN in type 2 diabetes mellitus using Creatinine, urea and urine albumin as gold standards. This is very cardinal especially that RDW can easily be done in most laboratories because its cheap, non toxic and reagents are readily available and would enable clinicians to identify or get a clue about type 2 diabetes mellitus patients at risk of developing diabetic nephropathy by simply looking at the level of RDW from a full blood count report.

1.3 Research question: Is RDW a good bio marker of diabetic nephropathy?

1.4 Null hypothesis: (Ho hypothesis) – RDW cannot be used as a biomarker of diabetic nephropathy.

1.5 General objective:

To assess red blood cell distribution width (RDW) as a bio marker of diabetic nephropathy in type 2 diabetes mellitus patients reviewed at Kabwe General Hospital.

1.6 Specific objectives:

1. To estimate the prevalence of diabetic nephropathy in diabetes type 2.

2 To describe and characterize RDW variations in patients with Diabetes nephropathy and those without.

3 To determine the distribution of RDW among male and female participants with DN.

4 To determine suitability of RDW as a marker for diabetic nephropathy in type 2 diabetes mellitus patients using renal profiles (urea, creatinine and urine albumin) as a gold standard.
CHAPTER TWO

2.0 LITERATURE REVIEW

Diabetic nephropathy is kidney disease that is a complication of diabetes. It can occur in people with type 2 diabetes, the diabetes type that is most common and is caused by resistance to insulin, or in people with type 1 diabetes, the type that more often begins at an early age and results from decreased insulin production. Diabetic nephropathy is caused by damage to the tiniest blood vessels. When small blood vessels begin to develop damage, both kidneys begin to leak proteins into the urine. As damage to the blood vessels continues, the kidneys gradually lose their ability to remove waste products from the blood. Currently, diabetic nephropathy is the leading cause of chronic end stage kidney disease in the United States and other Western societies (Silveiro et al., 2008). It is also one of the most significant long-term complications in terms of morbidity and mortality for individual patients with diabetes. Diabetes is responsible for 30-40% of all end-stage renal disease (ESRD) cases in the United States (Quinn et al., 2009).

The natural history of diabetic nephropathy differs according to the type of diabetes and whether microalbuminuria (defined as >30 mg but <300 mg albumin in the urine per day) is present. If untreated, 80% of people who have type 1 diabetes and microalbuminuria will progress to overt nephropathy (i.e. proteinuria characterized by >300 mg albumin excreted daily), whereas 20–40% of those with type 2 diabetes over a period of 15 years will progress to nephropathy, this was demonstrated by Nielsen and his colleagues more than a decade ago. A clear early predictor of the disease progression was increasing systolic blood pressure even within the pre hypertensive range. Among patients who have type 1 diabetes with nephropathy and hypertension, 50% will go on to develop end-stage renal disease within a decade (Raile et al., 2010). Mortality among dialysis patients with diabetes is 22% higher in the first year following the initiation of dialysis and 15% higher at 5 years than that among dialysis patients without diabetes (Remuzzi et al., 2011). There is no study available regarding RDW and its possible use in the screening of diabetic nephropathy in type 2 diabetes mellitus patients in Zambia. Many related studied have only been conducted in Europe, America and Asia (Cook. N.R et al., 2008).
2.1 Risk factors and mechanisms for diabetic nephropathy

Multiple factors and mechanisms contribute to the development and outcomes of diabetic nephropathy such as an interaction between hyperglycemia-induced metabolic and hemodynamic changes and genetic predisposition which sets the stage for kidney injury (Ziyadeh et al., 2012). Hemodynamic factors are the activation of various vasoactive systems such as the rennin – angiotensin – aldosterone and endothelin systems. In response, secretion of pro fibrotic cytokines, such as transforming growth factor $\beta_1$ (TGF-$\beta_1$) is increased and further hemodynamic changes occur such as increased systemic and intra glomerular pressure. Metabolic pathway involvement among other features leads to non enzymatic glycosylation, increased protein kinase C (PKC) activity and abnormal polyol metabolism. Findings from various studies supported an association between increased secretion of inflammatory molecules such as cytokines, growth factors and metalloproteinases and development of diabetic nephropathy (Raptis E and Viberti G, 2011). Oxidative stress also seems to play a central part in the development of DN (Singh et al., 2008).

According to Raptis AE and Viberti G, Studies that have used inhibitors of the pathways involved in genesis of diabetic nephropathy have shed light on the pathogenesis of this condition. Other factors include age and ethnicity which are two main non modifiable risk factors of DN (Wong et al., 2012). Life styles such as obesity and lack of physical activity and to some extent poor social economic status have contributed to the development of DN (Wolf and Ziyadeh. 2012).

2.2 Epidemiology

The International Diabetes Federation (IDF) estimated that 285 million people around the world have diabetic nephropathy. This number was expected to rise to 560 million within 20 years. Each year an additional 8-10 million people develop diabetes. In addition, there were more than 400 million people worldwide with pre-diabetes. According to IDF, the peak incidence of diabetic nephropathy is 3% per year beginning 10-20 years after the initial diagnosis of diabetes. IDF also states that, Approx. 38,000 people with diabetes develop kidney failure every year and that about 20-30% of patients with type 1 or type 2 diabetes develop evidence of nephropathy. IDF further states that In the US, diabetic nephropathy accounts for approx. 40% of all new cases of End Stage Renal Disease, and is the most common cause of ESRD (Banno. S. et al., 2008).
2.3 Pathogenesis

The development process of DN involves a combination of factors such as dietary end products, genetics, cytokine and oxygen reactive species among others.

2.3.1 Glycosylation

Glycosylation of tissue proteins is one of the most important factors that contribute to the development of diabetic nephropathy and other microvascular complications. Details are that, in chronic hyperglycemia, some of the excess glucose combines with free amino acids on circulating or tissue proteins, this non enzymatic process affects the glomerular basement membrane and other matrix components in the glomerulus and initially leads to formation of reversible early glycosylation end products and, later, irreversible advanced glycosylation end products. These advanced products are involved in the pathogenesis of diabetic nephropathy by altering signal transduction via alterations in the level of soluble signals, such as cytokines, hormones and free radicals. Circulating levels of advanced glycosylation end products are raised in people with diabetes, particularly those with renal insufficiency since they are normally excreted in the urine (Makita et al., 2011). The net effect is tissue accumulation of advanced glycosylation end products (in part by cross-linking with collagen) that contributes to the associated renal and microvascular complications (Singh et al., 2008). Advanced glycosylation end products (AGE) interact with the AGE receptor and nitric oxide concentrations which are reduced in a dose-dependent manner (Hogan et al., 2012).
2.3.2 Nitric Oxide changes in diabetic nephropathy

![Nitric Oxide changes in diabetic nephropathy diagram](image)

Fig.2.1: Schematic representation of Nitric Oxide changes in diabetic nephropathy and various pathophysiologic mechanisms which trigger such changes (Makita et al. 2011).

2.3.3 Cytokines

Each cytokine has several different effects, for example IL-1 alters the expression of chemotactic factors and adhesion molecules alters intra glomerular hemodynamics (by affecting mesangial cell prostaglandin synthesis) which increase vascular endothelial cell permeability and increases hyaluron production by renal tubular epithelial cells (which in turn increases glomerular cellularity) (Jones et al 2011). IL-6 has a strong association with the development of glomerular basement membrane thickening as well as possible relationships with increased endothelial permeability and mesangial cell proliferation. IL-18 induces the production of other inflammatory cytokines, such as IL-1, interferon \( \varpi \) and tumor necrosis factor and is associated with endothelial cell apoptosis. Tumor necrosis factor has the widest variety of biological activities and effects that contribute to development of diabetic nephropathy. Importantly though, it causes direct renal injury as a cytotoxin, as well as affecting apoptosis, glomerular hemodynamics, endothelial permeability and cell to cell adhesion. It also plays an important part in the early hypertrophy and hyper function of diabetic nephropathy (DiPetrillo K and Gesek FA 2012).
2.3.4 Oxidative stress

Metabolic activity within the nephron produces a large amount of reactive oxygen species that are counterbalanced by a large number of antioxidant enzymes and free radical scavenging systems. Reactive oxygen species mediate many negative biological effects, including peroxidation of cell membrane lipids, oxidation of proteins, renal vasoconstriction and damage to DNA. Hyperglycemia tips the balance towards production of reactive oxygen species, most of which is generated in the mitochondria (Nishikawa et al 2009).

Hyperglycemia specifically induces oxidative stress, even before diabetes becomes clinically apparent. Concentrations of markers of DNA damage induced by reactive oxygen species are higher in patients with more-severe nephropathy (i.e. proteinuria versus microalbuminuria). Furthermore, histological analysis of human kidney biopsy specimens has detected products of glyco-oxidation (combined products of glycation and protein oxidation) and lipoxidation in the mesangial matrix and glomeruli, whereas these lesions are much less common in specimens from individuals without diabetes (Suzuki et al 2009).
2.3.5 Role of reactive Oxygen species in diabetic nephropathy

Figure 2.2 scheme of pathogenesis of diabetic nephropathy (Suzuki et al., 2009)
2.4 Routine blood test of kidney function

The usual blood test which checks that the kidneys are working properly measures the level of urea, creatinine, urine albumin and certain dissolved salts.

Urea is a waste product formed from the breakdown of proteins. Urea is usually passed out in the urine. A high blood level of urea (‘uremia’) indicates that the kidneys may not be working properly, or that the patient may be dehydrated (have low body water content).

Creatinine is a waste product made by the muscles. Creatinine passes into the bloodstream, and is usually passed out in urine. A high blood level of creatinine indicates that the kidneys may not be working properly. Creatinine is usually a more accurate marker of kidney function than urea (Osterby et al., 2009). A urine micro albumin test is a test to detect very small levels of a blood protein (albumin) in urine. The urine albumin test is used to detect early signs of kidney damage in people who have a risk of kidney disease.

Healthy kidneys filter waste from the blood and keep the healthy components, such as proteins like albumin. Kidney damage can cause proteins to leak through the kidneys and leave the body through urine. Albumin (al-BYOO-min) is one of the first proteins to leak when kidneys become damaged.

Urine albumin tests are recommended for people with an increased risk of kidney disease, such as those with type 1 diabetes, type 2 diabetes or high blood pressure.

2.5 Pathology

Diabetes causes unique changes in kidney structure. These changes include Classic glomerulosclerosis which is characterized by increased glomerular basement membrane width, diffuse mesangial sclerosis, hyalinosis, microaneurysm, and hyaline arteriosclerosis (Mauer et al., 2011). Tubular and interstitial changes are also present (Katz et al., 2012). Areas of extreme mesangial expansion called Kimmelstiel-Wilson nodules or nodular mesangial expansion were observed in 40–50% of patients developing proteinuria (Kimmestiel et al., 2009). Micro- and macro albuminuric patients with type 2 diabetes had more structural heterogeneity than patients with type 1 diabetes (Osterby et al., 2009). Evaluated by electron microscopy, the severity of glomerular lesions is related to GFR and UAE and to diabetes duration, degree of glycemic control and genetic factors (Rudberg et al., 2010). Nonetheless, there is an important overlap in mesangial expansion and the presence of symptoms during urination which suggests urinary tract disorders such as obstruction, infection, or stones. Skin rash or arthritis may indicate systemic lupus erythematosus or cryoglobulinemia. Presence of risk factors for parenterally transmitted disease may raise
the suspicion of kidney disease associated with HIV, hepatitis C or hepatitis B. History of proteinuria and/or hypertension during childhood or pregnancy may suggest other glomerulonephritis. Also, family history of kidney disease may indicate the presence of polycystic kidney disease or other genetic diseases (Levey et al., 2012). Imaging of the kidneys, usually by Ultrasonography, should be performed in patients with symptoms of urinary tract obstruction, infection, or kidney stones or with a family history of polycystic kidney disease (Levey et al., 2012). The criteria for renal biopsy are not well established, but in type 1 diabetes the presence of proteinuria in association with short diabetes duration and/or rapid decline of renal function, especially in the absence of diabetic retinopathy, have been used (Mauer et al., 2003). In patients with type 2 diabetes, the criteria are less clear. The proportion of non diabetic renal lesions in proteinuric type 2 diabetic patients seems to vary according to the criteria used to perform the biopsy and to the ethnic background of the patient. When absence of retinopathy was the biopsy criterion in 49 proteinuric Caucasian patients with type 2 diabetes, only 12% had non diabetic glomerulonephritis (Christensen et al., 2011). On the other hand, other nephropathies, isolated or superimposed onto diabetic glomerulosclerosis, were observed in 46 and 19%, respectively, of 68 Chinese patients with type 2 diabetes. Proteinurias 1 g/24 h, renal involvement in the absence of retinopathy, or unexplained hematuria were the reasons for performing a biopsy (Wong et al., 2012). Patients with non diabetic glomerulosclerosis had a better prognosis than those with diabetic glomerulosclerosis alone or in association with other nephropathies (Wong et al., 2012). However, the real benefit of identifying and treating non diabetic renal lesions in patients with diabetes remains to be established.

2.6 Diagnosis

Screening for diabetic nephropathy must be initiated at the time of diagnosis in patients with type 2 Diabetes, this is because 7% of them already have microalbuminuria at that time. For patients with type 1 diabetes, the first screening has been recommended at 5 years after diagnosis (American Diabetes Association, Diabetes Care 27, 2010). The prevalence of microalbuminuria before 5 years in this group can reach 18%, especially in patients with poor glycemic and lipid control and high normal blood pressure levels (Stephenson et al., 2012). Furthermore, puberty is an independent risk factor for microalbuminuria (Schultz et al., 2009). Therefore, in type 1 diabetes, screening for microalbuminuria might be performed 1 year after diabetes diagnosis, especially in patients with poor metabolic control and after the onset of puberty. If microalbuminuria is absent, the screening must be repeated annually for both type 1 and 2 diabetic patients (American Diabetes Association, Diabetes Care 27, 2010). The first step in the screening and diagnosis
Of diabetic nephropathy is to measure albumin in a spot urine sample collected either as the first urine in the morning or at random, for example, at the medical visit. The results of albumin measurements in spot collections may be expressed as urinary albumin concentration (mg/l) (Gross et al., 2011) or as urinary albumin to-creatinine ratio (mg/g or mg/mmol) (Eknayan et al., 2012). The cutoff value of 17 mg/l in a random urine specimen had a sensitivity of 100% and a specificity of 80% for the diagnosis of microalbuminuria when 24-hour timed urine collection was the reference standard (Zelmanovitz et al., 2011). This value is similar to the cutoff value of 20 mg/l recommended by the European Diabetes Policy Group (European Diabetes Policy Group: 2010).

Samples must be refrigerated if they are to be used the same day or the next day, and one freeze is acceptable before measurements (Mogensen et al., 2010).
CHAPTER THREE

3.0 RESEARCH DESIGN AND METHODOLOGY.

3.1 Study design: Prospective descriptive study

3.1.0 Selection of participants

The study was a comparative study involving male and female consenting diabetes type2 adult patients above the age of 18 years attending a routine diabetes clinic every Wednesday at Kabwe General Hospital. Convenience sampling method was used to select participants. Cases in this study were defined as diabetes type2 patients with nephropathy and Controls were defined as non diabetic healthy individuals. Nephropathy is a medical condition affecting the kidneys resulting in the leakage of protein molecules such as albumin and metabolic products such as Creatinine and Urea. Nephropathy is confirmed by detecting elevated amounts of Urea (> 8.3 mmol/l), Creatinine (>120 µmol/l) and albuminuria (>30 mg/l). The control group was recruited from the same population and had the same characteristics as cases but without diabetes mellitus.

3.2.0 Case definition

Cases were type2 Diabetes mellitus patients with nephropathy while controls were healthy adult male and female participants.

3.2.1 Inclusion criteria

The study included all type 2 diabetes patients referred for diagnosis and routine diabetes checks with characteristics listed below:

- Diabetes type2
- Normal mean cell volume (MCV)
- Normal mean cell hemoglobin concentration (MCHC)
- Normal hemoglobin level (Hb)
- Normal Haematocrit (Hct)
• Red Cell Distribution Width (RDW)
• Adults above 18 years
• Willing to participate by providing informed consent
• not transfused in the last 2 to 3 weeks

3.2.2 Exclusion criteria
• pregnancy
• aged below 18 years
• transfused within 2 to 3 weeks
• All those that for any reason refused to participate in the study despite meeting the inclusion criteria.

3.3.0 Research site
The study was conducted at Kabwe General Hospital, a second level Hospital situated in the central province of Zambia and a referral centre for the central province with the bed capacity of 400. The study involved indigenous Zambian adults with diabetes type2 living in Kabwe, an urban town in Central Province of Zambia.

3.3.1 Study population
It was limited to adults above 18 years attending the diabetes clinic at Kabwe General Hospital outpatient department (OPD) from December 2012 to May 2013.

3.3.2 Target population: all patients with diabetes type2

3.4 Ethical considerations
Ethics approval was obtained from the University of Zambia Biomedical Research Ethics Committee (Assurance No.FWA00000338, IRB00001131 of IOR0000774). Written permission was obtained from Kabwe General Hospital. The information sheet about the study was given to patients, translated in Bemba
which is the local language or read out to them in cases where they could not read. Patients were informed about the study and given an option to decide if they did not want to take part in the study.

The purpose of the study was explained to all the study participants and those that declined to participate in the study were not forced, but were assured of their protected privileges and other benefits such as being managed by clinicians as per standard treatment and guidelines. The respondents were interviewed individually in a private room and only 4mls of blood and urine specimens were collected from them.

Privacy and confidentiality was maintained by using codes for the patients instead of names in the report and data was kept in a locked cabinet and keys kept by the researcher. The respondents were thus assured of utmost confidentiality. Patient’s consent to be included in the study was obtained. Patient’s comfort and dignity during and after the procedure was paramount. The well being and prompt definitive management of the patient was first before the research.

3.5 Study procedures

3.5 .0 Sample size Calculation

This was calculated using the formula below;

\[ N = \left[ u \sqrt{\pi_1 (1-\pi_1) + \pi_0 (1-\pi_0) + v \sqrt{2\pi (1-\pi)}} \right]^2 \]

\[
\left( \pi_0 - \pi_1 \right)^2
\]

- \( N = \) size of each group
- \( \pi_0, \pi_1 \) Proportions, \( \pi = \text{Avg of the proportions} \)
- \( u = 1.28 \) for 90% power, 0.84 for 80% power

\( V = Z \text{ statistic} = 1.96 \text{ if } \alpha = 0.05 \)

It is expected that:

- 20% of Type 2 Diabetes Mellitus patients have nephropathy
- About 3% of the residents of Kabwe have Type 2 Diabetes Mellitus.
The sample size calculated was 166 which was increased by 10% to 183 for Type 2 Diabetes Mellitus patients with DN and control participants to cater for non response and missing data.

Based on an expected Type 2 Diabetes Mellitus prevalence of 3% in Zambia and 20% Diabetic nephropathy in individuals with Type 2 Diabetes Mellitus, 183 participants (cases and controls) were enrolled in order to have 80% power to detect 17% difference in Diabetic nephropathy (20%) in Type 2 Diabetes Mellitus patients, using $\alpha = 0.05$

122 cases were recruited, 73 females and 49 males.
61 controls were recruited, 19 females and 42 males.

3.5.1 Variables and indicators of measurements.

Renal profiles (Urea, Creatinine and Urine albumin) were used as a standard marker for diabetic nephropathy and these were the independent variable. Diabetic nephropathy is a medical term in which there is abnormal increased tendency for the kidneys to release proteins in urine. The red blood cell distribution width (RDW) was used as a dependent variable. Potential confounding variables for the study included recent blood transfusion, bacterial infection, Cancer, Vitamin B12 deficiency anemia, hemolytic anemia, iron and folate deficiency anemia.

Vitamin B12 deficiency, hemolytic anemia, iron and folate deficiency were controlled during statistical analysis of the results.

Recent blood transfusion, cancer and bacterial infection were controlled by excluding individuals with these conditions from the study.

Type 2 diabetes mellitus patients were considered to have nephropathy if their levels of urea were above 8.3 mmol/l, creatinine levels above 120 µmol/l and urine albumin above 30 mg/l.

Red blood cell distribution width (RDW) was also estimated from each participant by drawing 2ml of blood into the EDTA container and analyzing the sample using ABX ES 60 hematology analyzer and all RDW levels above 15% were considered as being high or abnormal.
Table 3.1 Study Variables and their cut off points in diabetic nephropathy.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent Variables</th>
<th>Cut off Point</th>
<th>Indicator</th>
</tr>
</thead>
<tbody>
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<td>RDW</td>
<td>Creatinine</td>
<td>63 – 120 µmol/l</td>
<td>normal</td>
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<tr>
<td></td>
<td>Urea</td>
<td>1.0 – 8.3 mmol/l</td>
<td>Normal</td>
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<td></td>
<td>Urine albumin</td>
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<tr>
<td>Age</td>
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</tr>
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</table>
3.6 Data Collection

3.6.1 Questionnaire and Laboratory request forms

Information relating to diabetes type 2 and nephropathy was obtained from the prospective participants at Kabwe General Hospital using the questionnaire. Structured interview schedule was used to collect qualitative data. The interview schedule captured demographic variables, knowledge on common diabetes symptoms and factors associated with these symptoms. The schedule had close-ended questions. These close-ended questions helped capture specific and guided responses. The questionnaire was administered in the simple English language and translated into local language (Bemba) for those who did not understand English.

Demographic data from patients was collected as per standard clinical practice. Two (2) Research Assistants were recruited and trained over a period of 7 days in collection of data. Data was collected over a period of 3 months.

Questionnaires were piloted on the first ten participants. All questions which were not clear were revised accordingly.

Each participant’s sample was accompanied by a laboratory request form when being taken for analysis.

Hematology request forms were used for Red blood cell distribution (RDW) test while clinical chemistry request form was used for Blood Urea, Blood Creatinine and urine albumin tests.

3.6.2 Sample collection

Blood amounting to 4ml was collected from each eligible patient using the vein puncture method.

The blood was then immediately dispensed into an Ethylene Diamine tetra Acetic acid (EDTA) and sodium fluoride containers in quantities of 2ml in each container. A tube inversion was done for a couple of times to ensure proper mixing of the anticoagulant (EDTA and sodium fluoride) with blood to prevent clotting.

The two blood specimens were then stored in the refrigerator at the temperature of 2 degrees Celsius.
After blood collection, patients were provided with a urine container each and asked to submit about 4ml of urine for an on spot albuminuria check using the multistrix, the rapid albumin diagnostic method and the results entered on the information sheet that was provided.

At the time of testing, Samples for RDW, Glucose, Creatinine and Urea determination were removed from the refrigerator and brought to room temperature (22 – 25 degrees Celsius).

Blood samples for RDW estimation were put on the blood mixer at an appropriate time to ensure thorough mixing of the blood and the anticoagulant while carrying out quality control checks on ABX Micros 60 hematology Analyzer while samples for glucose, Urea and Creatinine estimation were centrifuged and loaded on the Cobas Integra 400+ Chemistry analyzer In batches of 30 for sample analysis.

All samples were processed using Kabwe General Hospital Laboratory equipment

### 3.6.3 Laboratory Tests

Laboratory tests were all done according to the Ministry of health laboratory quality control manual in order to ensure uniformity, consistency, reliability and reproducibility of all the laboratory test results that have been produced in this study. Good laboratory Practice (GLP) principles were also observed.

Blood specimens collected and stored in two different anti coagulated containers were processed and analyzed by using two different types of laboratory equipment.

### 3.6.4 Hematology laboratory Tests

### 3.6.5 Red Blood cell Distribution Width Estimation.

The 2ml of venous blood was collected in EDTA container for the determination of RDW levels in the specimen by using a fully - automated hematology analyzer called ABX micros 60 supplied by Horiba company. Miniclean and Minilyse reagents manufactured by Roche Company limited of German were adopted for use with ABX Micros 60. RDW levels were grouped into low RDW level (< 11%), normal (11 – 15 %) and high RDW level (> 15 %).
3.6.6 Principle of the Test

The Red blood cell distribution width was determined, according to the RBC count. The red blood cells pass through a Micro-aperture that generates electronic pulses, as the cells pass through it. These pulses are then grouped according to size, Threshold, and calculated to form a Histogram (Distribution curve). This distribution curve is then used to calculate the distribution of the Red blood cells as a percentage of the curve. This curve is then used in determining the RBC size abnormalities as in Anisocytosis. This was also a calculation of the RBC Histogram.

• Calculations were as follows:

\[
\text{RDW (\%)} = \frac{K \times SD}{MCV}
\]

K: Calibration Coefficient for RDW.
SD: Standard Deviation according to statistical studies on cell distribution within the RBC Histogram.
MCV: (Mean Cell Volume) of the erythrocytes.

3.6.7 Procedure

The detailed procedure used for RDW estimation was provided by the ABX Micros Manufacturer (Horiba group of companies) as shown in the appendix 13 Page 105

3.6.8 Chemistry Laboratory Tests

3.6.9 Blood Glucose Estimation

Venous blood for glucose estimation was collected in Sodium fluoride containers. The samples were then refrigerated at 2 degrees Celsius during which period the plasma separated from the whole blood.

Samples were then analyzed in batches of 30 so as to minimize wastage of reagents.

Random blood glucose was determined by using the Cobas Integra 400+ automated chemistry analyzer.

The COBAS INTEGRA Analyzer uses Glucose HK cassettes (GLUC2) which contain an in vitro diagnostic reagent system intended for use on COBAS INTEGRA systems for the quantitative determination of the glucose concentration in the hemolysate.
Glucose levels were grouped into low glucose level (< 3.3 mmol/l), normal (3.3 – 7.8 mmol/l) and high glucose level (> 11.0 mmol/l)

3.6.10 Principle of the Test

The test Principle uses an enzymatic reference method with hexokinase. Hexokinase (HK) catalyzes the phosphorylation of glucose by ATP to form glucose-6-phosphate and ADP. To follow the reaction, a second enzyme, glucose-6-phosphate dehydrogenase (G6PDH) is used to catalyze oxidation of glucose-6-phosphate by NADP+ to form NADPH

3.6.11 procedure

The procedure used for glucose estimation was provided by the equipment/ reagent manufacturer as shown in appendix 9 page 98

3.6.12 Blood Urea Estimation

Venous blood for Urea estimation was collected in sodium fluoride containers, the samples were immediately centrifuged and Plasma separated and stored at 2 degrees Celsius. Samples were analyzed in batches of 30 so as to minimize on reagent wastage.

Urea estimation was done by using the Cobas Integra 400+ fully automated Chemistry analyzer.

3.6.13 Test principle

Urea determination involves a Kinetic test with urease and glutamate dehydrogenase. Urea is hydrolyzed by urease to form ammonium and carbonate. In the second reaction 2-oxoglutarate reacts with ammonium in the presence of glutamate dehydrogenase (GLDH) and the coenzyme NADH to produce L-glutamate. In this reaction two moles of NADH are oxidized to NAD for each mole of urea hydrolyzed.

3.6.14 Test procedure

The procedure used for Urea estimation was provided by the manufacturer as shown in the appendix 11 Page 102

3.6.15 Creatinine estimation

Venous blood for Creatinine estimation was collected in sodium fluoride containers, the samples were immediately centrifuged and Plasma separated and stored at 2 degrees Celsius. Samples were analyzed in batches of 30 so as to minimize on reagent wastage.
Creatinine estimation was done by using the Cobas Integra 400+ fully automated Chemistry analyzer.

The cassette COBAS INTEGRA Creatinine plus ver.2 (CREP2) contains an in vitro diagnostic reagent system intended for use on COBAS INTEGRA systems for the quantitative determination of the creatinine concentration in serum, plasma, and urine.

3.6.16 Procedure

The detailed procedure used for Creatinine estimation was provided by the equipment / reagent manufacturer as shown in appendix 10 Page 100

3.6.17 Calculation

COBAS INTEGRA analyzers automatically calculate the analyte concentration of each sample.

3.6.18 Urine Albumin Estimation

4ml of Urine specimen for albumin estimation was collected in a plain container. The dip stick coated with reagent was then dipped into Urine and the results were obtained from the protein pad as indicated below

3.9.15 multi reagent dipstick

![Figure 3.4 determination of albumin in Urine (Ziyadeh et al., 2008)](image)

3.6.19 Principle of the test

The protein pad on the multi reagent dipstick (Multistix®, illustrated above) is based on the "protein error of pH indicator dyes". Basically, the test is dependent on the ability of amino groups in proteins to bind to and alter the color of acid-base indicators, even though the pH is unchanged. The reaction is extremely sensitive to albumin (as it contains the most amino groups), but is much less sensitive to globulins. It is insensitive to Bence-Jones proteins. Generally this differential sensitivity is not a significant problem (nearly all cases of significant proteinuria involve albuminuria).
3.6.20 Interpretation

The urine protein results were interpreted in context with the urine specific gravity and pH. Normal urine contained little protein; negative to trace reactions were usual in concentrated urine. A trace to 1+ reaction in very dilute urine was suggestive of significant proteinuria. A dipstick protein reaction > 2+ in concentrated or dilute urine indicated significant proteinuria.

3.7 Sex and age distribution of participants

The sex and age distribution of participants were categorized and each category’s frequency was determined.

3.7.1 Association between red blood cell distribution width and diabetic nephropathy in type 2 diabetes mellitus patients

RDW which was a continuous variable was recorded in the SPSS in order to categorize it into two. Patients who had RDW >15% were categorized as having high RDW and those who had RDW <15% were regarded as normal. Thereafter, a Chi – square test of independence was done to determine the proportion of RDW in type 2 diabetes mellitus patients and control subjects.

3.7.2 RDW levels in male and female patients with DN and the control participants

RDW levels were determined and categorized as normal or high in all male and female patients with DN and controls depending on whether RDW results were below 15% or above 15% respectively.

The proportions of participants who had raised RDW (>15%) between male and female DN patients were analyzed using the Chi – square test to determine if the association between RDW and DN was dependent on gender. The strength of association was further determined by the use of logistic regression to determine odds ratios.

3.7.3 Determining the association between age and raised RDW in patients with DN

Age of DN patients with high RDW (>15) was categorized and each category’s frequency was determined and compared among different categories to determine if the differences were statistically significant.
3.7.4 Assessment of red blood cell distribution width (RDW) as a biomarker of diabetic nephropathy (DN) in type 2 diabetes mellitus patients

The assessment of RDW as a biomarker of DN was done by estimating the levels of RDW in type 2 diabetes mellitus patients with DN and those without DN as controls.

RDW is a derived factor from the red blood cell indices which is produced as part of the full blood count.

Diabetic nephropathy was determined by finding high levels of Creatinine >120µmol/l, Urea > 8.3mmol/l and Urine albumin >30mg/l (in the confirmed and non confirmed cases).

The mean results of RDW levels were calculated and compared in patients with DN and controls.

The independent T – test was used to evaluate if the differences were statistically significant.

3.7.5 Suitability of RDW as a marker of DN

The sensitivity, specificity, positive and negative predictive values for RDW as a marker for DN were determined. Sensitivity (positivity in disease) refers to the proportion of participants who have the target condition (reference standard positive) and give positive test results. Specificity (negativity in health) is the proportion of participants without the target condition and gives negative test results. Positive predictive value is the proportion of positive results that are true positive (with the target condition) whereas negative predictive value is the proportion of negative results that are true negatives (which do not have target condition).

Urea, Creatinine and Urine albumin concentrations were used as proxy standard method for determining DN and these were compared to RDW results for each patient. Cross tabulation tables obtained from SPSS between Urea, Creatinine and Urine albumin results in comparison to RDW were transferred to CEBM (centre for evidence based medicine) statistics calculator for the calculation of sensitivity, specificity, positive and negative predictive values.

3.8 Data management and Statistics

Raw data and results from patients were edited for consistency and legibility on a daily basis. The close ended responses were pre-coded before the interview to ensure easy entry and analysis of data using SPSS Computer Software and CEBM. Hematological and chemistry data was entered on the data
sheet and was used for analysis. Results obtained from the analyzing machines were tabulated in the data sheets. All the parameters were normally distributed and hence reported as the mean +/- standard deviation. The significance of the differences between patients and controls for normally distributed parameters were determined using the independent samples T – test for continuous variables and the Chi – square test for categorical variables. Risk factors and patient attributes associated with diabetic nephropathy in diabetes type 2 were determined by logistic regression analysis. Odds ratios and their 95% confidence intervals were reported.

Sensitivity, specificity and positive predictive values for RDW as a marker for diabetic nephropathy were calculated from a 2 by 2 table computed in CEBM statistics calculator. P – Values of less than 5% were taken as significant.
CHAPTER FOUR

4.0 RESULTS

4.1 Presentation of Data and Data analysis

Figure 4.1: Distribution of participants according to sex.

Figure 4.1 shows that there were more female than male participants with diabetic nephropathy enrolled in this study (50.3% and 49.3% respectively).
Figure 4.2: Age distribution of respondents

Figure 4.2 shows that there were more participants between the age group of 35 – 55 years.
Figure 4.3: Association between RDW levels and DN.

Figure 4.3 above shows that RDW was higher in patients with diabetic nephropathy compared to the control participants ($X^2 = 96.93$), $p = 0.004$. 
Figure 4.4 shows that the mean RDW was higher in female type 2 diabetes mellitus patients with Diabetic nephropathy than male participants. ($t$-value 7.00), $p$ – value 0.002.
Figure 4.5 shows that an abnormal RDW was high in the age group 35 – 55 years of type 2 diabetes mellitus patients with diabetes nephropathy.
### 4.2 Assessment of RDW as a biomarker of diabetic nephropathy in type 2 diabetes mellitus patients.

Table 4.1 Comparison of mean renal profiles in patients with diabetic nephropathy and control subjects. (Independent T – test parameters).

<table>
<thead>
<tr>
<th></th>
<th>Status</th>
<th>Number</th>
<th>Mean</th>
<th>Std deviation</th>
<th>T - Value</th>
<th>P - Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine (µmol/L)</td>
<td>Control DN</td>
<td>61</td>
<td>250</td>
<td>2.1</td>
<td>5.00</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>DN</td>
<td>122</td>
<td>730</td>
<td>4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>Control DN</td>
<td>61</td>
<td>2.2</td>
<td>1.5</td>
<td>8.26</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>DN</td>
<td>122</td>
<td>4.2</td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine albumin (mg/L)</td>
<td>Control DN</td>
<td>61</td>
<td>12.2</td>
<td>2.9</td>
<td>5.41</td>
<td>0.168</td>
</tr>
<tr>
<td></td>
<td>DN</td>
<td>122</td>
<td>12.4</td>
<td>3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RDW</td>
<td>Control DN</td>
<td>61</td>
<td>14.7</td>
<td>3.8</td>
<td>7.58</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>DN</td>
<td>122</td>
<td>32.2</td>
<td>4.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1 shows that an abnormal RDW (p – Value 0.001) was more likely to be associated with abnormal blood urea (p – Value 0.02) and creatinine (p – value 0.03) than urine albumin (p – Value 0.168).

Figure 4.1 further reveal that the mean creatinine concentration for type 2 diabetes mellitus patients (750 ± 4.0 µmol/l ) was significantly higher than control participants (250 ± 2.1 µmol/l ) t – value 5.00; P – value = 0.003.

The mean urea concentration for type 2 diabetes mellitus patients (4.2 ± 4.2 mmol/l) was significantly higher than control participants (2.2 ± 1.5 mmol/l). t – Value = 8.26; p – value 0.002.

The mean urine albumin concentration for type 2 diabetes mellitus patients (12.4 ± 3.3 mg/l) was higher than the control participants (12.2 ± 2.9 mg/l) but the difference was not significant t – value 5.41; p – value 0.168.

The mean RDW for type 2 diabetes mellitus patients (32.2 ± 4.2 %) was significantly higher than the control participants (14.7 ± 3.8 %). t – value 7.58; p – value 0.001.

The above table (Figure 4.1) also indicated that high RDW levels correlated with high creatinine and high urea levels and not with urine albumin levels, supporting the use of RDW as a suitable biomarker of diabetic nephropathy.
4.3: Suitability of RDW as a marker of diabetic nephropathy in diabetes mellitus patients using renal profiles (Creatinine, Urea and Urine albumin) as a standard.

Table 4.2 Comparison of renal profile results and RDW test results in patients with diabetic nephropathy

<table>
<thead>
<tr>
<th>RDW test</th>
<th>Renal profiles</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diabetic nephropathy</td>
<td>No diabetic nephropathy</td>
</tr>
<tr>
<td></td>
<td>Creatinine &gt;120µmol/l, Urea &gt;8.3mmol/l, Urine albumin &gt;30mg/l</td>
<td>Creatinine &lt;120µmol/l, Urea &lt; 8.3mmol/l, Urine albumin &lt; 30mg/l</td>
</tr>
<tr>
<td>High tests</td>
<td>TP</td>
<td>FP</td>
</tr>
<tr>
<td></td>
<td>67</td>
<td>2</td>
</tr>
<tr>
<td>Low tests</td>
<td>FN</td>
<td>TN</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>48</td>
</tr>
<tr>
<td>Total</td>
<td>TP + FN</td>
<td>FP + TN</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>50</td>
</tr>
</tbody>
</table>

Key: TP: True positive = test positive in actually positive cases, TN: True negative = test negative in actually negative cases, FP: false positive = test positive in actually negative cases, FN: false negative = test negative in actually positive cases.

4.4 Calculations

(a) Sensitivity = TP/TP + FN x 100 = 67/72 x 100 = 93%

(b) Specificity = TN/FP + TN x 100 = 48/50 x 100 = 96%

(c) PPV = TP/TP + FP x 100 = 67/69 x 100 = 97%

(d) NPV = TN/TN + FN x 100 = 48/53 x 100 = 91%

(e) Efficiency = TN + TP/TP + FN + FP + TN x 100 = 115/122 x 100 = 94%
Table 4.3 reliability of RDW as a biomarker of diabetic nephropathy.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDW</td>
<td>93</td>
<td>96</td>
<td>97</td>
<td>91</td>
<td>94</td>
</tr>
</tbody>
</table>

Table 4.3 shows that RDW was a reliable marker of diabetic nephropathy due to its high sensitivity, specificity, PPV, NPV and efficiency (93%, 96 %, 97%, 91% and 94 %) respectively.

Table 4.2 above illustrates the cross tabulations of renal profiles (Creatinine, Urea and Urine albumin) results in comparison to the RDW test results. It shows that 72 (59%) of the participants had diabetic nephropathy ( had high results; Creatinine >120µmol/l, Urea >8.3mmol/l, Urine albumin >30mg/l). Out of these results, 67 (55%) participants results were true positive, implying that both the renal profile tests and RDW were abnormal. 2 (2%) of the results were false positive test results with the RDW test implying that patients did not actually have diabetic nephropathy because the renal profile results were low (Creatinine <120µmol/l, Urea < 8.3mmol/l, Urine albumin < 30mg/l). 5 ( 4%) of the participants results were false negative, implying that RDW test results were negative but the participants had diabetic nephropathy in the actual fact. 48 (39%) negative RDW test results were true negative because both renal profile tests and RDW results were low, implying that patients did not have DN.

The cross tabulation of RDW and renal profile tests further support that RDW is a suitable and reliable biomarker for the diagnosis of diabetic nephropathy.
CHAPTER FIVE

5.0 DISCUSSION OF FINDINGS

5.1 Assessment of Red blood cell distribution Width as a biomarker of diabetic nephropathy in type 2 diabetes mellitus patients.

5.2 Age and sex distribution of participants

A total of 183 subjects aged between 15 – 65 years participated in the study out of which 91 (49.7%) were males and 92 (50.3%) were females. One hundred and twenty two (122) participants were type 2 diabetes mellitus patients (cases) and 61 were control subjects. The number of controls was less than that of cases because it was difficult to recruit controls than cases for various reasons, some doubted the benefit of giving out blood when they were not ill while others refused to participate because of traditional and religious reasons. The majority of participants 67 (37%) were in the age range of 45 – 54, the mean age was 49 years.

This study revealed that patients aged 35 years and above were at risk of developing DN than those who were below 35 years old. These results were consistent with the study done by (Tsuboi et al., 2013) who found that the risk group was above 65 years. However, the reduction in the risk age group of DN observed in this study may be due to a number of factors including HIV, poor glycemic control, lack of exercises and poor knowledge on the complications of diabetes mellitus to some extent. On the other hand, the risk of DN with increasing age could be attributed to changes that occur in the glomerular basement membrane as a result of aging tilting the scale to DN in older patients.

5.3 Association between red blood cell distribution width and diabetic nephropathy in type 2 diabetes mellitus patients

This study reveals that diabetic nephropathy in type 2 diabetes mellitus patients was related to the level of RDW among type 2 diabetes mellitus patients. Respondents with diabetic nephropathy had a higher proportion of RDW 84 (69 %) than control participants 18 (29 %). The difference was significant $X^2 = 96.93$, $p = 0.004$. This result correlates with Al-Najjar (2009) who found an association of 28%. This shows that there is a significant correlation between RDW and diabetic nephropathy among type2 diabetes mellitus patients in Zambia as compared to the general population.

Diabetic nephropathy may influence erythropoiesis, circulatory half life and deformability of erythrocytes, promoting anisocytosis and thus elevating RDW levels (Weils et al., 2009). High level of RDW was also associated with the metabolic syndrome (Sánchez et al., 2010), leading to postulate a possible effect of an
underlying diabetic nephropathy state (which typically occurred in diabetes and metabolic syndrome) on increased destruction of erythrocytes. Similar finding was previously reported by Acosta et al. (2010), where they reported shortened half life of red blood cells in diabetes mellitus. However, the Relationship between RDW and diabetic complications has not been investigated (Malandrino et al., 2010). So the present study was done to study the relation between RDW and diabetic nephropathy in patients with type 2 diabetes mellitus and its possible use as a marker for DN.

5.4 Comparison of mean high RDW between male and female participants
The mean RDW in female patients with DN (30.0 ± 3) was significantly higher than in the male patients (25 ± 3.5), P – Value 0.002.

The differences revealed in the levels of RDW between males and females correlate very well with results obtained by (Patel et al 2010) among Mexican adult patients with DN in which RDW was high in females than males. The main reason why females with diabetic nephropathy tend to have high RDW than males with the same condition in type 2 diabetes mellitus is still unclear.

5.5 Association between age and high RDW in type 2 diabetes mellitus patients with diabetic nephropathy
This study reveals that the proportion of participants who had high RDW differed significantly among different age groups in type 2 diabetes mellitus patients. The proportion of type 2 diabetes mellitus patients who had higher RDW was in the age range of 35 – 55 years 91 (74%). Results obtained in this study correspond to those obtained by Quinn et al., (2008) who reported a correlation between old age and diabetic nephropathy. These results were also consistent with the study done by (Tsuboi et al., 2013) who found that ≥65 years was the most vulnerable age group. Old age tends to decreased resistance in both the afferent and efferent arterioles of the glomerulus. These early hemodynamic changes facilitate albumin leakage from the glomerular capillaries and overproduction of mesangial cell matrix, as well as thickening of the glomerular basement membrane and injury to podocytes resulting in diabetic nephropathy and consequent raise in RDW levels (Malandrino et al., 2013). The pick incidence of diabetic nephropathy (40 years) observed in this study, could be associated with the occurrence of early complications which lead to early death as indicated by the decline in the study participants above the age of 55 years.

5.6 Assessment of RDW as a biomarker of diabetic nephropathy in type 2 diabetes mellitus patients
It was observed in this study that RDW of patients with diabetic nephropathy (32.2 ± 4.2) was significantly higher in patients with diabetic nephropathy than in the control participants (14.7 ± 3.8), P – Value 0.001.
These results are consistent with (Jannuzzi et al., 2010) who found a correlation between RDW and diabetic nephropathy in type 2 diabetes mellitus patients. The results obtained are also similar to those of (Patel et al., 2009) who found a significant association between high RDW and high mortality rate in patients with various renal complications, but in this study, the association observed was specifically between high RDW and diabetic nephropathy. Red cell distribution width (RDW) is considered as a prognostic marker which may reflect an underlying renal process (Lippi et al., 2009). It is a measure of variation in size of red cells in a blood sample, which is calculated by both impedance and flow cytometric analyzers as part of the routine complete blood count. RDW is effectively a free test which, is reported alongside a complete blood count without extra cost and has good prognostic value (Al-Najjar et al., 2009).

The reason why RDW is considered to be a predictor in a wide range of conditions is unclear, it was shown to be a strong predictor of mortality in general population of adults aged 45 years and more (Patel et al., 2010). High RDW values were associated with deaths from cardiovascular disease (CVD), cancer, and other causes, however the effect was stronger for CVD Patel et al (2010). It was reported that RDW is a strong predictor of mortality in many other conditions such as obesity, malignancies, and chronic kidney disease (Patel et al 2010).

The motivation for this study was due to the mounting evidence that raised RDW values in some cases could reflect diabetic nephropathy which is potentially associated with increased risk of death mainly from cardiovascular causes (Tilton et al., 2009).

The actual cause of high RDW in diabetic nephropathy is not very clear though Ferruci and his colleagues suggested that it could partly be due to the disruption in the release of the hormone erythropoietin which is produced by the Kidneys and plays a major role in the regulation and normal production of the red blood cells from which the RDW is derived. (Felker et al., 2008) observed that there was a significant high level of RDW in type 2 diabetes mellitus patients with diabetic nephropathy, especially in those patients with long term diabetes and chronic complications, this is consistent with the results of this study. (Cook et al., 2008) found that DN detected by high RDW was independently associated with cardiovascular complications and suggested that high RDW could be considered as a risk marker for cardiovascular diseases.

The mean urea concentration in patients with DN (4.2 ± 2.4) was significantly higher than in the controls (2.2 ± 1.5), P – Value 0.02. These results accord that of (Ziyader et al., 2008) who found increased urea level in a study done on African Americans with DN.
Urea is the major end product of protein nitrogen metabolism (Guder et al., 2009). It is synthesized by the urea cycle in the liver from ammonia which is produced by amino acid deamination. Urea is excreted mostly by the kidneys. Elevations in blood urea nitrogen concentration are seen in inadequate renal perfusion, shock, diminished blood volume such as in patients with Diabetic nephropathy (Mazzachi et al., 2008). Determination of blood urea nitrogen is the most widely used screening test for renal function (Rock et al., 2010).

The creatinine concentration was higher in type 2 diabetes mellitus patients with DN than those without DN (730 + 4.0) and (250 + 2.1), P – Value 0.03 respectively. These results were consistent with those reported in the study done by (Friendman et al., 2008) to find out if creatinine clearance was a reliable index for glomerular filtration rate (GFR). Creatinine is produced endogenously from creatine and creatine phosphate as a result of muscle metabolic processes (Mazzachi et al., 2008). It is excreted by glomerular filtration during normal renal function. An abnormality in the functioning of the kidney such as in diabetic nephropathy will result in elevated amounts of blood creatinine concentration.

When urea is used in conjunction with serum creatinine determinations, it can aid in the differential diagnosis of the three types of azotemia: prerenal, renal, and postrenal (Guder et al., 2009).

The mean urine albumin concentration of participants with DN (12.4 + 3.3) in this study was not significantly higher than the concentration in the control group (12.2 + 2.9), P – Value 0.168. The low urine albumin concentration found in the patients with DN is not consistent with Fersblom’s report (2008) of elevated urine albumin concentration as one of the markers of degenerating kidney function among diabetic patients. The reason for the difference in albumin concentration observed in this study could be as a result of population difference in which this study and others were done.

DN also known as Kimmestiel – Wilson syndrome, is a condition characterized by intercapillary glomerulonephritis (infection of the glomeruli) and is a progressive disease caused by angiopathy of capillaries in the kidney glomeruli and this leads to the compromise in the glomerulus filtration rate (GFR) system leading to the kidney becoming leaky to blood proteins such as albumin.

Levels of creatinine, urea and urine albumin are increased following renal damage such as in DN, resulting in hyper perfusion and hyper filtration leading to the leakage of macro molecules such as proteins from the glomerulus into the urine.
5.7 Suitability of RDW as a marker of diabetic nephropathy in type 2 diabetes mellitus patients

According to this study, 72 (59%) of the participants had diabetic nephropathy (had Creatinine >120µmol/l, Urea >8.3mmol/l and Urine albumin >30mg/l). Out of these results, 67 (55%) participant results were true positives, implying that both the renal profile test results and the RDW results were abnormal. One point six percent (1.6%) of the results were false positive test results with the RDW but the patients did not have diabetic nephropathy because renal profile results were below (Creatinine < 120µmol/l, Urea < 8.3mmol/l and Urine albumin < 30mg/l). 5 (4.1%) of the participant results were false negative, implying that the RDW test results were negative but the participants had diabetic nephropathy in the actual fact. 48 (39%) negative RDW test results were true negatives because renal profile results were also negative, implying that patients had no diabetic nephropathy.

RDW had a sensitivity of 93% (95% CI [88.0 – 96.7]), PPV 97% (95% CI [91.9 – 98.7]), NPV 91% (95% CI [83.6 – 94.5]) which were all acceptable parameters to support the suitability of RDW as a marker for DN.

This study revealed that RDW test had few false negative results leading to high and better sensitivity of 93% (95% CI [88.0 – 96.7]) this implies that RDW test is capable of detecting 93% of DN cases among type 2 diabetes mellitus patients and only 7% will be missed out as this will be reported as negative. Therefore, RDW test has a high probability of detecting diabetic nephropathy in type 2 diabetes mellitus patients.

RDW test also had an acceptable specificity of 96% (95% CI [88.8 – 98.2]). This means that, the probability of type 2 diabetes mellitus patients not having DN was 96% which means 2 (4%) tests gave false positive results.

RDW test had a high PPV 97%(95%CI [91.9 – 98.7]). This can be interpreted to mean that 67 (97%) positive RDW test results were truly DN cases.

91% NPV results obtained for RDW in this study means that 48 (91%) of negative RDW test results were true negatives (no DN) while 5 (9%) were false negatives (had DN). From the available literature searched so far, no diagnostic study has been done to specifically evaluate the suitability of RDW in the detection of DN in type 2 diabetes mellitus patients. (Long T.W., 2009) reported that the acceptable sensitivity, PPV and NPV should be above 90%. From the results obtained in this study, RDW was found to be a suitable diagnostic test for DN in type 2 diabetes mellitus patients because all parameters for testing of reliability were above 90%. Positive and negative predictive values vary according to the prevalence of the condition under study (Loong T.W., 2009). Therefore, it would be wrong for predictive values determined for one population to be applied to another population with a different prevalence. In this case, RDW test results could be used among type 2 diabetes mellitus patients and not the general population because DN may be absent hence low predictive values even if the test is highly sensitive and specific.
6.0 CONCLUSION

The objective of this study was to assess red blood cell distribution width (RDW) as a biomarker of diabetic nephropathy in patients with type2 diabetes mellitus. This study revealed that, type 2 diabetes mellitus patients with DN had higher levels of RDW and abnormal renal profile test results (creatinine >120µmol/l, Urea > 8.3mmol/l and Urine albumin > 30mg/l) than healthy non diabetic control participants. Using diagnostic sensitivity and specificity, PPV, NPV and efficiency, it was found that RDW was a reliable and suitable biomarker for detecting diabetic nephropathy in type 2 diabetes mellitus patients. In addition, RDW is cheap, readily available as part of the full blood count test and easy to perform.
6.1 Study limitations

This study was a cross sectional study, it was therefore unable to determine at what stage diabetic nephropathy preceded the development of type 2 diabetes mellitus. The study did not also include the initial stages of high RDW in the process of diabetic nephropathy development.
6.2 Recommendations

1. RDW should be considered as one of the reliable markers for detecting diabetic nephropathy in type2 diabetes mellitus patients.

2. However, a cohort study involving type2 diabetes mellitus patients needs to be conducted to assess the relationship between RDW and diabetic nephropathy in type2 diabetes mellitus patients by comparing creatinine, urea and urine albumin.
REFERENCES


Loong T.W., (2009) understanding sensitivity and specificity BMJ; 421:618 - 7


APPENDIX 1

INFORMATION SHEET (ENGLISH)

ASSESSMENT OF RED BLOOD CELL DISTRIBUTION WIDTH (RDW) AS A BIOMARKER OF NEPHROPATHY IN DIABETES TYPE2 PATIENTS REVIEWED AT KABWE GENERAL HOSPITAL.

INTRODUCTION

My name is Grant Nombwende, a student of Master of Science in Pathology (Hematology) at the University of Zambia and I am requesting for your participation in the research study mentioned above. The purpose of the study is to determine the association between the variations in the sizes of blood components which carry oxygen to various parts of the body known as red blood cells and the Kidney disease or complications which arise as a result of Sugar disease which commonly affect adults, usually referred to as diabetes type II in patients reviewed at Kabwe General Hospital.

Before you decide whether or not you should participate in the study, I would like to explain to you the purpose of the study, any risk or benefits and what is expected of you. Your participation in this study is purely voluntary or self willing. You are under no obligation to participate therefore, you will not be forced. If you decide that you do not want to participate, no privileges will be taken away from you that is to say, no rights or benefits you are enjoying will be withdrawn from you.

If you agree that you participate, you will be asked to sign this consent form, which is an assurance of agreement in the presence of a witness.

PURPOSE OF THE STUDY

This study will compare the results of variations in the sizes of red blood cells and kidney malfunction in people with sugar disease which usually affect adults (diabetes type II). The information obtained will help health workers to determine type II diabetic patients at risk of developing adverse effects of Kidney complications using samples or tests requested by a doctor or other medical professional that’s gives information about cells in the patient's blood.
PROCEDURES

4ml (about one tea spoon) of blood and urine will be collected from you by a qualified health worker and analyzed. A member of the research team will ask you a few questions and your responses will be recorded on the questionnaire and the interview is expected to last about 15 minutes.

RISKS AND DISCOMFORTS

No major risks are anticipated in this particular study though you will experience slight pain when blood is being collected from you. This lost blood is replenished or replaced by the body within a day also; the bone marrow replaces the lost blood cells within a couple of weeks. It is common to experience slight dizziness or light-headedness after blood is collected from you. However, these effects subside on their own, within a short while. Blood loss side effects are most prevalent among teenagers. Other symptoms commonly reported due to loss of blood are as follows:
- Fainting and falling
- Sweating and chills, this is a feeling of being cold and wet.
- Stiffness in joints
- Stress
- Nausea and convulsions or feeling sick or sensation of unease and discomfort in the upper stomach with an involuntary urge to vomit, convulsions being sudden shaking movements of the body that cannot be controlled
- Tingling sensation in lips or nose which is a slight stinging or un comfortable feeling in the lips or nose.

These side effects are of temporary nature and subside in a day or two. However, if the problem persists, you will be treated or taken care of by the study team.

BENEFITS

There are no immediate benefits to you for participating in this study. However, the information obtained will help policy makers in the ministry of health to take measures in reducing complications or disease affecting tiny blood vessels in patients with sugar disease affecting adults. No gift or money will be given in exchange for the information obtained.
CONFIDENTIALITY

Privacy and confidentiality will be maintained. Your name will not appear anywhere on the forms but will instead be identified by numbers. You will thus be assured that the information you will provide will not be used against you in anyway. Personal information will not be released without your permission except when required by law.

The ministry of Health (MOH), University of Zambia (UNZA) Biomedical Research Ethics committee may review your records again but this will be done with confidentiality.
APPENDIX 2

INFORMATION SHEET (BEMBA TRANSLATION)

ASSESSMENT OF RED BLOOD CELL DISTRIBUTION WIDTH (RDW) AS A BIOMARKER OF NEPHROPATHY IN DIABETES TYPE 2 PATIENTS REVIEWED AT KABWE GENERAL HOSPITAL.

UKUILONDOLA

Ishina lyandi nine Grant Nombwende, umusambi musambililo ayakalamba aya kumona pamalwele yabantu pesukulu likalamba iya University of Zambia. Kabili ndemipusha ukubulamo ulubali mwisambililo ilyo lilumbwilwe pamulu. Ubukankala bwe sambililo ili kwishiba ukwangampa ukwaba pakati ka utusangwa mumulopa na mu balwele ba shuga ilyo isa mukukana bomba bwino ukwa lufyo multi abo ba monwa pa chipatala ichikalamba cha Kabwe General Hospital.

Ilvo tamulatontonkanya nga chakuti mwala ibimbamo multi ili sambililo nagula iyo, kuti natemwa ukumilondolwela ubukankala bwe sambililo ili, ubukumo, nangula ifilambu nefyo tuleenekela ukufuma kuli imwe. Ukuibimbamo kwena umuli ili isambililo kwakuipelesha fye. Tamulepatikishiwa ukusendamo ulubali. Nga ca kuti mwasala ukukanana ukuibimbamo, insambu shenu tashapokwe.

Ngamwasumina ukuibimbamo, mwalaipushwi a uku fwatika pe pepala lyakusuminisha pachinso cabakamboni.

UBUKANKALA BWE SAMBILILO

Ilisambililo lyrondololola ukwangampa ukwaba pakati ka utusangwa mumulopa na mafya ayesa no bulwele bwa shuga elyo kuba ukunana bomba bwino ukwa lufyo ukubomfya fye ukupenda kwa utu tu sangwa mumulopa mumubili.

IFYAKUKONKA

Ababomfi abafikapo abamuchipatala bakasenda utumulopa no tumisu utunono uto tu ngesula pali kasupuni akanono nokupima. Membala wamukabungwe kalelolekesha pali ilisambililo akamipusha amepusho aya noono nokulemba asamuko yenu pepepala. Kabili ukwipusha kukasenda baminiti ikumi lino ne sano.
UBUBI NOKUKANA UMFYWA BWINO
Muli ilisambililo ububi nokukana umfwa bwino mukapitamofye ni pakusendwa umulopa epela. Elyo mukomfwa ifya pala ulunshingwa, amaka ukupwa.

UBUNONSHI
Takuli ubunonshi ubwine bwine imwe palwenu mukanonkelamo ilyo mwa ibimbano muli ili sambililo. Lelo ukwishiba uko ukukafumamo mwi sambililo ili kukafwilisha ba kapanga bamafunde mucitungu ico cilolekesha pa bumi ukumona efyo benga cefyanyako amafya ayo yashingwana naba lwele ba shuga. Ilyo mwa ibimbamo muli ilisambililo, takuli icilambu nangula ulupiya ulukapelwa kuyli imwe.

INKAMA

Akabungwe akalolekesha pabumi, akabungwe akapesukulu likabamba aketwa Biomedical Research ethics committee kuti kalolekesha pelyashi lyenu nakabili lelo ici cikacikwa munkama.
CONSENT FORM

The purpose of this study has been explained to me and I understand the intentions, benefits, risks, discomforts and confidentiality of the study.

I further understand that: if I agree to take part in this study, I have the right to withdraw at any time without having to give an explanation and that taking part in this study is purely voluntary.

I ………………………………………………………………………………………… (Name)

Agree to take part in this study.

Signature………………………… Date……………………………………… (Participant)

Patient’s signature or thumb print.

Signature:……………………….. date ………………………………… (Witness)

Signature:………………………..date…………………………………… (Researcher)
APPENDIX 4

CONSENT FORM (BEMBA TRANSLATION)

Ubukankala bwesambililo ili nabulondololwa kuli ine, kabili ningumfwa nemolilolele, ubunonshi, amafya nenkama ifili mwisambililo ili.

Kabili naumfwikisha ukuti: nga cakuti nasumina ukuibimba muli ilisambililo, ndinensambu shakuleka pa nshita iliyonse ukwabula nokupela umulandu. Kabili ukuibimba muli ili sambililo cili kutemwa kwandi.

Ine………………………………………………………………………………………………… (ishina).

Nasumina ukuibimba muli ilisambililo.

Ukufwatika…………………………………… Ubushiku………………. (ababulilemo ulubali)

Ishina lya mulwele nangula ukufwatika.

Ishina .......................................................... Ubushiku .............................. (kamboni)

Ishina…………………………………………… Ubushiku .............................. (keepusha)
Persons to contact for problems or queries

1. Grant Nombwende
   University of Zambia, School of medicine
   P.O. Box 50110
   Lusaka.
   House No. 10276,
   Luangwa,
   Kabwe
   Mobile: 0977 59 31 38 or 0955 88 2 990

2. The chair person
   University of Zambia Biomedical Research ethics committee
   P.O Box 50110
   Lusaka
   Phone: 256067
Assessment of red blood cell distribution width (RDW) as a biomarker of nephropathy in diabetes type 2 patients reviewed at Kabwe General Hospital.

PARTCIPANT’S CODE: ..................................................................

INSTRUCTIONS

1. No name should appear on/and or in this questionnaire.

2. Answer all the questions.

3. Put the letter “X” in the box next to your choice.

4. Use a pen/pencil in the questionnaire.

5. Information given in this questionnaire will be kept confidential.

SECTION A: BACKGROUND CHARACTERISTICS

1. Age
   a) 20-29
   b) 30-39
   c) 40-49
   d) 50-59
   e) 60-69

2. Sex
   a) Male
   b) Female
3. Ethnicity
   a) African
   b) Indian
   c) Chinese
   d) Other – Specify ……………………

4. Signs and symptoms
   a) Frothing of urine
   b) Swelling of legs
   c) Unintentional weight gain
   d) Poor appetite

5. Recently transfused (within 3 – 4 Weeks)
   a) Yes
   b) No

6. Willing to participate?
   a) Yes
   b) No

7. Cardiovascular complications
   a) Yes
   b) No

8. Above 18 years?
   a) Yes
   b) No
9. History of cancer
   a) yes
   b) No

10. High blood pressure
    a) Yes
    b) No

Interviewer's identification

Data collected by: (state full names)........................................................................
........................................................................................................................................
Date....................................................................................................................................
(Day Month Year)
## APPENDIX 6
### LABORATORY DATA COLLECTION FORMS

### HAEMATOLOGY LABORATORY FORM

<table>
<thead>
<tr>
<th>Participant’s Code</th>
<th>Red blood cell distribution width (RDW)</th>
<th>[   ] %</th>
</tr>
</thead>
</table>

**Name of Technologist/Scientist**

**Signature**

**Counter Checked by**

**Designation**

**Date**

(Day Month Year)
### CLINICAL CHEMISTRY LABORATORY FORM

**Participant's Code** ............................................

1. **Random Blood Creatinine (µmol/l)**  
   - \( \leq 6.0 \text{ µmol/l} \)  
   - 6 - 120µmol/l  
   - \( \geq 120 \text{ µmol/l} \)

2. **Urine Albumin**  
   - \( \leq 5.0 \text{ mg/l} \)  
   - 5.0 – 6.0 mg/l  
   - \( > 30 \text{ mg/l} \)

3. **Random Blood Urea (mmol/l)**  
   - \( \leq 1.0 \text{ mmol/l} \)  
   - 1.0 – 8.3 mmol/l  
   - \( \geq 8.3 \text{ mmol/l} \)

Name of Technologist/Scientist.................................................................

Signature..................................................................................................

Counter Checked by..................................................................................

Designation ...........................................................................................

Date ...........................................................................................................

---

\[ \begin{array}{|c|c|} 
\hline
\text{Range} & \text{Code} \\
\hline \leq 6.0 \text{ µmol/l} & 0 \\
6 - 120\text{µmol/l} & 1 \\
\geq 120 \text{ µmol/l} & 2 \\
\hline
\end{array} \]

\[ \begin{array}{|c|c|} 
\hline
\text{Range} & \text{Code} \\
\hline \leq 5.0 \text{ mg/l} & 0 \\
5.0 – 6.0 \text{mg/l} & 1 \\
\geq 30 \text{ mg/l} & 2 \\
\hline
\end{array} \]

\[ \begin{array}{|c|c|} 
\hline
\text{Range} & \text{Code} \\
\hline \leq 1.0 \text{ mmol/l} & 0 \\
1.0 – 8.3 \text{ mmol/l} & 1 \\
\geq 8.3 \text{ mmol/l} & 2 \\
\hline
\end{array} \]
Appendix 7: Permission letters

REPUBLIC OF ZAMBIA
MINISTRY OF HEALTH
KABWE GENERAL HOSPITAL

All correspondence to be addressed to the Executive Director and not to individuals

Our Ref: P.O. Box 80917
Your Ref: KABWE

Tel: 260-5-222301-6
TelFax: 260-5-223049
Email: kabwegenral@gmail.com

16th October, 2012

The Head of Department
Department of Pathology and Microbiology
Ridgeway Campus
P.O. Box 50110
Lusaka
Zambia

Dear Sir/Madam,

RE: PERMISSION TO CONDUCT RESEARCH AT KABWE GENERAL HOSPITAL – MR GRANT NOMBWENDE.

Reference is made to the above.

This is in response to the letter in which your institution (University of Zambia) requested for permission on behalf of the above named student to conduct his research at Kabwe General Hospital as part of the University requirement to obtain a master’s degree in pathology (Haem) for the said research title “Association between Red blood cell distribution width RDW) and Nephropathy in diabetes type II patients reviewed at Kabwe General Hospital.

I wish to inform you that the hospital has no objection and is willing to allow the student to use the hospital laboratory facilities and offer him any other support deemed possible.

Yours faithfully,

Dr G. Chipulu
MEDICAL SUPERINTENDENT
16th October, 2012

The Senior Medical Superintendent
Kabwe General Hospital
P.O. Box 80917
KABWE

Dear Sir,

Re: PERMISSION TO CONDUCT RESEARCH PROJECT AT KABWE GENERAL HOSPITAL

This is to introduce Mr. Grant Nombwende a Master of Science in Pathology (Haematology) student. It is the programme’s requirement that MSc students do their final year research project in line with their field of study to graduate. It is to this effect that the School of Medicine recommends the above candidate to you for his research and data collection.

In partial fulfillment of this program, he is required to conduct a research study and his topic is “Association between Red Blood Cell distribution with (RDW) and Nephropathy in Diabetes Type II Patients Reviewed at Kabwe General Hospital”.

I write to request your good office to allow him use the laboratories from your institution.

Your support in this matter will be highly appreciated.

Yours faithfully,

Dr. C. Marimo
ACTING HEAD, DEPARTMENT OF PATHOLOGY & MICROBIOLOGY
06th September, 2012

Mr Grant Nombwende
Department of Pathology and Microbiology
School of Medicine
LUSAKA

Dear Mr Nombwende,

RE: GRADUATES PROPOSAL PRESENTATION FORUM (GPPF)

Having assessed your dissertation entitled "Association between red blood cell distribution width (RDW) and nephropathy in diabetes type II patients visiting Kabwe General Hospital". We are satisfied that all the corrections to your research proposal have been done. The proposal meets the standard as laid down by the Board of Graduate Studies.

You can proceed and present to the Research Ethics.

Yours faithfully,

[Signature]

Dr. S. H. Nzala
ASSISTANT DEAN, POSTGRADUATE

CC: HOD – Pathology and Microbiology
THE UNIVERSITY OF ZAMBIA
BIOMEDICAL RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
Telegram: UNZA, LUSAKA
Telex: UNZALU ZA 44370
Fax: + 269-1-250733
E-mail: unzarec@unza.zm
Assurance No. FWA00000338
IRB00001131 of IORG0000774

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

19th February, 2013.

Your Ref: 005-09-12.

Mr. Grant Nombwende
School of Medicine
Department of Microbiology & Pathology
Lusaka

Dear Mr. Nombwende,

RE: RE-SUBMITTED RESEARCH PROPOSAL: “ASSOCIATION BETWEEN RED BLOOD CELL DISTRIBUTION WIDTH AND NEPHROPATHY IN DIABETES TYPE 11 PATIENTS REVIEWED AT KABWE GENERAL HOSPITAL.” (005-09-12)

The above mentioned research proposal was re-submitted to the University of Zambia Biomedical Research Ethics Committee on 30th January, 2013 and was approved.

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this Committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).
- Ensure that a final copy of the results is submitted to this Committee.

Yours sincerely,

Dr. J.C Munthali
CHAIRPERSON

Date of approval: 15 February, 2013

Date of expiry: 14 February, 2014
Appendix 8: Supervisors Curriculum Vitae

Curriculum vitae

PERSONAL DETAILS

Name : Lidiya Korolova
Date of Birth : 9th December, 1953
Place of Birth : Kyiv, Ukraine
Nationality : RUSSIAN
Marital Status : Married
Date of Appointment in University : 4th November, 2000
Present Position : Lecturer I

ACADEMIC QUALIFICATIONS

<table>
<thead>
<tr>
<th>Year</th>
<th>Institution</th>
<th>Qualification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1971-1977</td>
<td>Kyiv State University</td>
<td>Master of Science Chemistry, Biology and Genetics</td>
</tr>
<tr>
<td>1985 - 1989</td>
<td>Scientific Research Institute of Haematology and Blood Transfusion, Kyiv.</td>
<td>PhD in Biology and Haematology</td>
</tr>
</tbody>
</table>

WORK EXPERIENCE

2000 to date - Lecturer I in Department of Pathology and Microbiology
1985 – 1989 - Award Doctorate in Biology and Haematology
1986 – 1993 - Worked as consultant on determining blood related diseases. In conjunction with other research workers
carried out a number of haematological and immunological research on patients exposed to radiation during the Chernobyl Atomic Power Station disaster.

1980 - 1986 Research Engineer in the Clinical and Experimental Laboratory of Haematology and Blood Transfusion. Lecturer II at Kyiv Medical School.

1977 – 1980 Research Engineer in the Clinical and Experimental Laboratory of the Scientific Research Institute of Paediatrics, Obstetrics and Gynaecology.

**ACTIVITIES AND ASSOCIATIONS**


1971 – 1977 Editor in University Newspapers

1977 – 1980 Member of Ukrainian Research Association of Paediatricians, Obstetricians and Gynaecologists.

1980 – 1990 Member of Ukrainian Association of Haematologists and Transfusiologists.

1986 – 1992 Ukrainian Committee of the effects of the Chernobyl Atomic Energy Power Station Disaster and the Effects Elimination

**MEMBERSHIP – OTHER ORGANISATIONS**

1977 – 1992 Member of the Ukrainian Medical Trade Union

1977 – 1992 Honorary Secretary of the Ukrainian Association of Scientists, Inventors and Innovators

1980 – 1992 Advisor of the Ukrainian Red Cross and Red Crescent Societies.

1980 – 1992 Honorary Member of Blood donors
1989 -1992  
Member of the Scientific Council of the Ukrainian 
Institute of Haematology and Blood Transfusion 

PUBLICATIONS 


L. Korolova, Immunological Studies of the Spleen Slides in Patients Suffering from Idiopathic Thrombocytopenia, Symposium of the Scientific Research and Clinical Practice in Kharov, 1986. 


Korolova L., Invention of the Prognosis of the Trend of Post-Surgical period of the Patients Suffering from some Blood diseases of the Splenectomy Moscow, 1992.
Appendix 9: Glucose estimation

Glucose HK
(New Formulation) Hemolysate Application

Order information

<table>
<thead>
<tr>
<th>COBAS INTEGRA®</th>
<th>Cat. No.</th>
<th>System-ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 Tests</td>
<td>20767131</td>
<td>07 6713 1</td>
</tr>
<tr>
<td>Glucose HK</td>
<td>10759500</td>
<td>07 3718 6</td>
</tr>
<tr>
<td>Calibrator (a.s.)</td>
<td>10757131</td>
<td>07 7961 6</td>
</tr>
<tr>
<td>Precipitin® U</td>
<td>10757143</td>
<td>07 7997 0</td>
</tr>
<tr>
<td>Precipitin® U plus</td>
<td>10757178</td>
<td>07 7064 6</td>
</tr>
<tr>
<td>Precipitin® U plus</td>
<td>12140435</td>
<td>07 7969 7</td>
</tr>
<tr>
<td>Hemolyzing Reagent “Fluid”</td>
<td>107570089</td>
<td>07 7009 0</td>
</tr>
</tbody>
</table>

* Indicates analyzer(s) on which cassette can be used.

COBAS INTEGRA 400/700/800

<table>
<thead>
<tr>
<th>Order Information</th>
<th>COBAS INTEGRA 400/700/800</th>
<th>COBAS INTEGRA 700</th>
<th>COBAS INTEGRA 800</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose HK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calibrator (a.s.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitin® U</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitin® U plus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemolyzing Reagent “Fluid”</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* This application is not available in the USA.

Intended use

The COBAS INTEGRA Glucose HK cassette (GLUC2) contains an in vitro diagnostic reagent system intended for use on COBAS INTEGRA systems for the quantitative determination of the glucose concentration in hemolysate.

Glucose measurements are used in the diagnosis and treatment of carbohydrate metabolism disorders including diabetes mellitus and iatrogenic hyperglycemia. This method sheet describes the application for hemolysate (test GLUC2L, 0-313).

The application for serum, plasma, uriné, and CSF are described in the method sheet for Glucose HK.

Summary

Glucose is the major carbohydrate present in the peripheral blood.

Oxidation of glucose is the major source of cellular energy in the body. Glucose derived from dietary sources is converted to glycogen for storage in the liver or to fatty acids for storage in adipose tissue. The concentration of glucose in blood is controlled within narrow limits by many hormones, the most important of which are produced by the pancreas.

The most frequent cause of hyperglycemia is diabetes mellitus resulting from a deficiency in insulin secretion or action.

A number of secondary factors also contribute to elevated blood glucose levels. These include pancreatitis, thyroid dysfunctions, renal failure, and liver disease.

Hyperglycemia is less frequently observed. A variety of conditions may cause low blood glucose levels such as insulinoma, hypoglycemia, or insulin-induced hypoglycemia.

Glucose measurement in hemolysate is a convenient method for routine diabetes monitoring and in cases where only small sample volumes are available.

Test principle

Enzymatic reference method with hexokinase.

Hexokinase (HK) catalyzes the phosphorylation of glucose by ATP to form glucose-6-phosphate and ADP. To follow the reaction, a second enzyme, glucose-6-phosphate dehydrogenase (G6PDH) is used to catalyze oxidation of glucose-6-phosphate by NADP⁺ to form NADPH.

D-Glucose + ATP $\rightarrow$ HK $\rightarrow$ D-glucose-6-phosphate + ADP

D-Glucose-6-phosphate + NADP⁺ $\rightarrow$ D-6-phosphogluconate + NADPH + H⁺

The concentration of the NADPH formed is directly proportional to the glucose concentration. It is determined by measuring the increase in absorbance at 340 nm.

Reagents - working solutions

| R1 | Constitute in vial A and B (liquid). |
| R2 | SR Enzymes in vial C (liquid). |

Active ingredients

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS</td>
<td>100 mmol/L</td>
</tr>
<tr>
<td>ATP</td>
<td>1.7 mmol/L</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>2.4 mmol/L</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>1.0 mmol/L</td>
</tr>
<tr>
<td>G6PDH</td>
<td>3.0 mmol/L</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Please see cassette label for reagent filling volumes.

Precautions and warnings

Pay attention to all precautions and warnings listed in Chapter 1, Introduction.

Reagent handling

Ready for use.

2005-03. V 3 EN
Storage and stability
Storage at 2 to 8°C. See expiration date on cassette.

COBAS INTEGRA 400/400 plus analyzers
On board in use at 10 to 13°C, 8 weeks
COBAS INTEGRA 700/800 analyzers
On board in use at 8°C, 8 weeks

Specimen collection and preparation
For specimen collection and preparation, use suitable tubes or collection containers.

Only the specimens that were tested and found acceptable.
Whole Blood: The samples should be hemolyzed immediately after collection. Collect 20 μL of capillary blood. Take venous blood, or blood from the earlobe or from the fingertip. Earlobe or fingertip should be well supplied with blood at the time of collection. Hemolyze the sample immediately after collection.

Hemolyzate preparation
1. Allow the Hemolyzing Reagent "Fluid" to equilibrate at room temperature before use.
2. Place 1 ml of Hemolyzing Reagent "Fluid" in a test tube.
3. Add the filled 20 μL capillary and close the test tube.
4. Shake well.
5. Allow to stand for at least 5 minutes at room temperature prior to glucose determination. Do not centrifuge.
Glucose values in hemolyzate samples are stable for 14 days at room temperature.

Materials provided
See "Reagents - working solutions" section for reagents.

Materials required (but not provided)
Hemolyzing Reagent "Fluid" 1000 mL, Cat. No. 19750889
For hemolyzate preparation, use Hemolyzing Reagent "Fluid".
See above for sample pretreatment procedure.

Assay
For optimal performance of the assay, follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator manual for analyzer-specific assay instructions.

Application for hemolyzate
COBAS INTEGRA 400/400 plus test definition
Measuring mode
Abs. calculation mode
Reaction mode
Reaction direction
Wavelength A/B
Calc. Blank
Test range
with postdilution
Postdilution factor
Unit
Laboratory correlation factor

Pipetting parameters
R1
Sample
SR
Total volume

COBAS INTEGRA 700/800 test definition
Measuring mode
Abs. calculation mode
Reaction mode
Reaction direction
Wavelength A/B
Calc. Blank
Test range
with postdilution
Postdilution factor
Unit
Laboratory correlation factor

Pipetting parameters
R1
Sample
SR
Total volume

Calculation
COBAS INTEGRA analyzers automatically calculate the analyte concentration of each sample. For more details, please refer to Chapter 7, Data Analysis, User Manual (COBAS INTEGRA 700 analyzers), or to Data Analysis in the Online Help (COBAS INTEGRA 400/400 plus/900 analyzers).

Conversion factor:
mmol/L × 18.62 = mg/dL
Limitations - interference
The hemolytic method using Hemolysis Reagent "Fluid" cannot be used in blood samples with extremely elevated white blood counts (>40,000 leukocytes/mL, for example leukemic patients) since highly viscous hemolysates are obtained which may produce erroneous glucose results. In such cases, it is advisable to perform glucose assays in serum or plasma with or without deproteinization.

Criteria: Recovery within ±10% of initial value.

Icterus  No significant interference.

Lipemia  No significant interference.

Other  In very rare cases gammapathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

Expected values
3.6–5.3 mmol/L (65–99 mg/dL)
Whole blood glucose levels are about 90% of plasma glucose. Hematinocrit level may influence the difference between plasma and whole blood glucose levels due to lower glucose values in erythrocytes compared with plasma concentration. Higher hematinocrit levels lead to an increased plasma glucose level compared to whole blood.

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data
Representative performance data on COBAS INTEGRA analyzers are given below. Results obtained in individual laboratories may differ.

Precision
Reproducibility was determined using human samples and controls in an internal protocol (within run n = 20, between run n = 10). The following results were obtained:

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean</th>
<th>CV within run (%)</th>
<th>CV between run (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>5.54 mmol/L</td>
<td>1.0%</td>
<td>1.6%</td>
</tr>
<tr>
<td>Level 2</td>
<td>19.2 mmol/L</td>
<td>346 mg/dL</td>
<td>0.73%</td>
</tr>
</tbody>
</table>

Analytical sensitivity (lower detection limit)
3.3 mmol/L (60 mg/dL)
Imprecision is ≥20% at <3.3 mmol/L.

Method comparison
Glucose values for hemolyzed samples obtained on a COBAS INTEGRA 700 analyzer with the COBAS INTEGRA Glucose HK assay (GLU2H) were compared to those determined with commercially available reagents for glucose in hemolyzed/denatured whole blood on a COBAS INTEGRA 700 analyzer and an alternative manufacturer's clinical chemistry system. Samples were measured in duplicate. Sample size (n) represents all replicates.

Values ranged from 0.14 to 3.1 mmol/L (2.5 to 688 mg/dL).

COBAS INTEGRA 700 analyzer  Alternative system

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Hemolysis</th>
<th>Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>(n)</td>
<td>250</td>
</tr>
<tr>
<td>Corr. coeff.</td>
<td>(r)</td>
<td>0.997</td>
</tr>
<tr>
<td>(p)</td>
<td></td>
<td>0.998</td>
</tr>
</tbody>
</table>

Litter.

Regression:
y = 1.00x - 0.18 mmol/L  y = 0.96x + 0.74 mmol/L
Passing–Bablok:
y = 1.00x - 0.23 mmol/L  y = 0.96x + 0.75 mmol/L

References
6. Data on file at Roche Diagnostics.

FOR US CUSTOMERS ONLY: LIMITED WARRANTY
Roche Diagnostics warrants that this product will meet the specifications stated in the labeling when used in accordance with such labeling and will be free from defects in material and workmanship for 90 calendar days from the shipment date if purchased directly from Roche Diagnostics. THIS LIMITED WARRANTY IS IN LIEU OF ANY OTHER WARRANTIES, EXPRESS OR IMPLIED, INCLUDING ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR PARTICULAR PURPOSE. IN NO EVENT SHALL ROCHE DIAGNOSTICS BE LIABLE FOR INCIDENTAL, INDIRECT, SPECIAL OR CONSEQUENTIAL DAMAGES.

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Roche Diagnostics GmbH, D-68453 Mannheim
Roche Diagnostics, Indianapolis, IN
US Customer Technical Support 1-866-428-2316
Appendix 10: Creatinine estimation

Creatinine plus ver.2

Order Information

<table>
<thead>
<tr>
<th>Product</th>
<th>Cat. No.</th>
<th>System-ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>CORAS INTEGRA®</td>
<td>0343991</td>
<td>07 66627</td>
</tr>
<tr>
<td>Creatinine plus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ver.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calibrator f.a.s.</td>
<td>10 x 3 mL</td>
<td>10759350</td>
</tr>
<tr>
<td>Preci@s® U</td>
<td>20 x 5 mL</td>
<td>10171783</td>
</tr>
<tr>
<td>Preci@s® U plus</td>
<td>10 x 3 mL</td>
<td>12149435</td>
</tr>
<tr>
<td>Preci@s® Albumin</td>
<td>5 x 3 mL</td>
<td>12109546</td>
</tr>
<tr>
<td>Preci@s® Albumin</td>
<td>3 x 3 mL</td>
<td>11208838</td>
</tr>
</tbody>
</table>

Intended use

The cassette CORAS INTEGRA Creatinine plus ver.2 (CREP2) contains an in vitro diagnostic reagent system intended for use on CORAS INTEGRA systems for the quantitative determination of the creatinine concentration in serum, plasma, and urine. This method sheet describes the Applications for serum, plasma (test CRE12, 0-612), and urine (test CRE2U, 0-512).

Summary

Creatinine is produced endogenously from creatine and creatine phosphate as a result of muscle metabolic processes. It is excreted by glomerular filtration during normal renal function. Creatinine assays are conducted for diagnostic purposes, for therapeutic monitoring of acute and chronic renal diseases, and for monitoring kidney dialysis. The urinary creatinine concentration can also be used as a reference parameter for analysis of scraties (albumin, u-analect). Numerous methods have been described for determining creatinine, including the Jaffé alkaline picrate method in various modifications, as well as an enzymatic test which involves measuring ammonia after cleavage of creatinine by creatinine-iminohydrolase.

Test principle

Enzymatic, colorimetric method. The enzymatic method is based on the established determination of hydrogen peroxide after conversion of creatinine with the aid of creatasees, creatasees, and sarcosine oxidase. The liberated hydrogen peroxide reacts with 4-amino-phenazone and HTOH to form a quinone imine chromogen.

\[ \text{creatinine} + \text{H}_2\text{O}_2 \xrightarrow{\text{creatinase}} \text{creatinine} \]

\[ \text{creatinine} + \text{O}_2 \xrightarrow{\text{sarcosine oxidase}} \text{sarcosine} + \text{H}_2\text{O} \]

\[ \text{sarcosine} + \text{H}_2\text{O}_2 \xrightarrow{\text{glycine + HCHO + H}_2\text{O}} \]

HOT + 4-amino-phenazone + \xrightarrow{\text{POO}} \text{quinone imine chromogen} + \text{H}_2\text{O} + \text{H}_1

\[ \text{HTOH} \rightarrow \text{5,4,6-triols-aldehyde acid} \]

The color intensity of the quinone imine chromogen formed is directly proportional to the creatinine concentration. It is determined by measuring the increase in absorbance at 552 nm.

Reagents - working solutions

R1 = Buffer, enzymes, and HTOH in vial A (liquid).
R2 = SR buffer, enzymes, and 4-amino-phenazone in vial C (liquid).

Active ingredients

Component | Concentrations | R1 | SR | Test |
-----------|----------------|----|----|------|
TAPS®      | 30 50 34.5 mmol/L |
Creatinase | ≥312 µkat/L (≥12.6 IU/L) |
Sarcosine oxidase | ≥132 µkat/L (≥5.9 IU/L) |
Ascorbic oxidase | ≥33 µkat/L (≥1.2 IU/L) |
HTOH       | 1.2 0.76 g/L |
Creatinase | ≥498 µkat/L (≥9.9 IU/L) |
Peroxidase | ≥16.6 5.2 µkat/L (≥0.31 IU/L) |
4-Aminophenazone | 0.4 0.13 g/L |
Potassium  | 60 38.7 mg/L |
Hexacyanoferrate (II) | 0.1 0.8 8.1 |
H1 = N-nitro-phenazonemethoxyl-3-aminophenazone acid

Both reagents contain non-ionic detergents and preservatives. Please see cassette label for reagent filling volumes.
Precautions and warnings
Pay attention to all precautions and warnings listed in Chapter 1, Introduction.

Reagent handling
Ready for use.

Storage and stability
Séñl life at 2 to 8°C
INTEGRA 400
On-board in use at 10 to 15°C
INTEGRA 760/800
On-board in use at 8°C
See expiration date on cassette
8 weeks
8 weeks

Specimen collection and preparation
Only the specimens listed below were tested and found acceptable.
Serum: Collect serum using standard sampling tubes.
Plasma: Li-heparin or K3-EDTA plasma.
Urine: Collect urine without additives. Urine samples are automatically preserved 1:20 (1:19) with water by the instrument.
When processing samples in primary tubes, follow the instructions of the tube manufacturer.

Stability in serum/plasma
7 days at 20-25°C
7 days at 4-8°C
3 months at -20°C

Stability in urine
2 days at 20-25°C
6 days at 4-8°C
6 months at -20°C

Cartridge samples containing precipitates before performing the assay.

Materials provided
See "Reagents - working solutions" section for reagents.

Assay
For optimal performance of the assay, follow the directions given in this document for the analyzer concerned. Refer to the appropriate operators manual for analyzer specific assay instructions.

Applications for serum, plasma and urine

INTEGRA 400 test definition
Measuring mode
Absorbance

Absorptivity
Endpoint

Wavelength A/B
552/659 nm

Calc. flint
350/5

Serum, plasma

Reaction mode
R1-S-R

Test range
0-2700 μmol/L (0-30.5 mg/dL)

Postdilution factor
10 recommended

Urine

Reaction mode
D-R1-S-R

Test range
0-40 mmol/L (0-452 mg/dL)

Postdilution factor
5 recommended

Pipetting parameters

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Diluent (H2O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>77 μL</td>
</tr>
<tr>
<td>Sample</td>
<td>2 μL</td>
</tr>
<tr>
<td>SR</td>
<td>38 μL</td>
</tr>
<tr>
<td>Total volume</td>
<td>122 μL</td>
</tr>
<tr>
<td>Urine</td>
<td>77 μL</td>
</tr>
<tr>
<td>Sample</td>
<td>2 μL</td>
</tr>
<tr>
<td>SR</td>
<td>38 μL</td>
</tr>
<tr>
<td>Total volume</td>
<td>122 μL</td>
</tr>
</tbody>
</table>

INTEGRA 760/800 test definition
Measuring mode
Absorption

Absorptivity
Endpoint

Wavelength A/B
552/659 nm

Calc. flint
46/98

Serum, plasma

Reaction mode
R1-S-R

Test range
0-2700 μmol/L (0-30.5 mg/dL)

Postdilution factor
19 recommended

Urine

Reaction mode
D-R1-S-R

Test range
0-40 mmol/L (0-452 mg/dL)

Postdilution factor
20

Pipetting parameters

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Diluent (H2O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>77 μL</td>
</tr>
<tr>
<td>Sample</td>
<td>2 μL</td>
</tr>
<tr>
<td>SR</td>
<td>38 μL</td>
</tr>
<tr>
<td>Total volume</td>
<td>122 μL</td>
</tr>
<tr>
<td>Urine</td>
<td>77 μL</td>
</tr>
<tr>
<td>Sample</td>
<td>2 μL</td>
</tr>
<tr>
<td>SR</td>
<td>38 μL</td>
</tr>
<tr>
<td>Total volume</td>
<td>122 μL</td>
</tr>
</tbody>
</table>

Calibration
Calibrator f.a.s.

Calibration mode
Linear regression

Calibration interval
Each lot

Traceability: This method has been standardized against Id-MS.

Quality control
Quality control serum, plasma Precinorm U or Precinorm U plus Precipad U or Precipad U plus

Quality control urine Precinorm Albumin, Precipad Albumin

Control interval 24 hours recommended

Control sequence User defined

Control after calibration Recommended
**Calculation**
COBAS INTEGRA analyzers automatically calculate the analyte concentration of each sample. For more details please refer to Chapter 7, Data Analysis, User Manual (COBAS INTEGRA 700i), or to Data analysis in the online Help (COBAS INTEGRA 400i/800i).

**Conversion factor**
\[ \text{µmol/L} = \times 0.0133 = \text{mg/dL} \]

**Limitations - Interference**
Criterion: Recovery within ± 10% of initial value.

- **Serum, plasma**
  - Hemolysis: No significant interference up to a hemoglobin level of 500 µmol/L (20 mg/dL).
  - Isosmolar: No significant interference up to a bilirubin level of 340 µmol/L (20 mg/dL).
  - Lipemia: No significant interference up to a triglyceride level of 1000 mg/dL and to an Interilip level of 1000 mg/dL. There is poor correlation between turbidity and triglyceride concentrations.
  - Ascorbic acid: No significant interference up to an ascorbic acid level of 1.76 mmol/L (0.8 mg/dL).
  - Drugs: In vitro therapeutic drug interference on the assay was tested according to the recommendations of the Symposium on Drug Effects in Clinical Chemistry Methods (1990). Of the drugs tested in vitro, levodopa and calcium debrisoquine cause artificially low creatinine levels at the tested drug levels while DL-propranol at a concentration of >1 mmol/L causes falsely high results. Refer to Chapter 1, Introduction for a list of tested drugs and their concentrations.
  - Other: In very rare cases monoclonal gammopathy can lead to incorrect results.
  - Urine: No significant interference up to a creatinine level of 1.51 mmol/L (20 mg/dL).

- **Urine**
  - Drugs: Levodopa causes artificially low results.
  - Other: No significant interference up to a creatinine level of 0.05 mmol/L (1.4 mg/dL).

**Estimation of the Glomerular Filtration Rate (GFR) on the basis of the Schwartz Formula can lead to an overestimation.**

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

**Expected values**

- **Serum, plasma**
  - Adults:
    - Females: 45-84 µmol/L (0.51-0.95 mg/dL)
    - Males: 59-104 µmol/L (0.67-1.17 mg/dL)
  - Children:
    - Neonates (premature): 27-77 µmol/L (0.31-0.88 mg/dL)
    - Neonates (full term): 14-32 µmol/L (0.16-0.39 mg/dL)
    - 1-3 y: 15-35 µmol/L (0.18-0.93 mg/dL)
    - 3-5 y: 23-37 µmol/L (0.26-0.84 mg/dL)
    - 5-7 y: 25-42 µmol/L (0.29-0.87 mg/dL)
    - 7-9 y: 30-47 µmol/L (0.34-1.03 mg/dL)
    - 9-11 y: 39-56 µmol/L (0.44-1.04 mg/dL)
    - 11-13 y: 39-66 µmol/L (0.44-1.08 mg/dL)
    - 13<15 y: 40-64 µmol/L (0.46-0.77 mg/dL)

- **Urine**
  - 1st morning urine:
    - Females: 2.55-20.0 mmol/L (29.26-226 mg/dL)
    - Males: 3.54-24.6 mmol/L (40.27-276 mg/dL)
  - 24h urine:
    - Females: 6.13 mmol/24 h (720-1510 mg/24 h)
    - Males: 9.19 mmol/24 h (980-2200 mg/24 h)
  - Creatinine clearance:
    - 46-143 mL/min

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

**Specific performance data for serum and plasma**
Representative performance data on the COBAS INTEGRA analyzers are given below. Results obtained in individual laboratories may differ.

**Precision**
Reproducibility was determined using human samples and controls in an internal protocol (within run n = 21, between run n = 21). The following results were obtained:

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean µmol/L</th>
<th>CV within run %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99</td>
<td>1.6%</td>
</tr>
<tr>
<td>2</td>
<td>329</td>
<td>0.7%</td>
</tr>
</tbody>
</table>

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

**Expected values**

- **Serum, plasma**
  - Adults:
    - Females: 45-84 µmol/L (0.51-0.95 mg/dL)
    - Males: 59-104 µmol/L (0.67-1.17 mg/dL)
  - Children:
    - Neonates (premature): 27-77 µmol/L (0.31-0.88 mg/dL)
    - Neonates (full term): 14-32 µmol/L (0.16-0.39 mg/dL)
    - 1-3 y: 15-35 µmol/L (0.18-0.93 mg/dL)
    - 3-5 y: 23-37 µmol/L (0.26-0.84 mg/dL)
    - 5-7 y: 25-42 µmol/L (0.29-0.87 mg/dL)
    - 7-9 y: 30-47 µmol/L (0.34-1.03 mg/dL)
    - 9-11 y: 39-56 µmol/L (0.44-1.04 mg/dL)
    - 11-13 y: 39-66 µmol/L (0.44-1.08 mg/dL)
    - 13<15 y: 40-64 µmol/L (0.46-0.77 mg/dL)

- **Urine**
  - 1st morning urine:
    - Females: 2.55-20.0 mmol/L (29.26-226 mg/dL)
    - Males: 3.54-24.6 mmol/L (40.27-276 mg/dL)
  - 24h urine:
    - Females: 6.13 mmol/24 h (720-1510 mg/24 h)
    - Males: 9.19 mmol/24 h (980-2200 mg/24 h)
  - Creatinine clearance:
    - 46-143 mL/min

**Method comparison**
Creatinine values for human serum and plasma samples obtained on COBAS INTEGRA 400 with the cassette COBAS INTEGRA Creatinine plus ver.2 were compared to those determined with the commercially available Creatinine plus reagent on Roche/Hitachi 917.

Values ranged from 53 to 2300 µmol/L (0.66 to 26.1 mg/dL).

**Specific performance data for urine**
Representative performance data on the COBAS INTEGRA analyzers are given below. Results obtained in individual laboratories may differ.

**Precision**
Reproducibility was determined using human samples and controls in an internal protocol (within run n = 21, between run n = 21). The following results were obtained:

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean µmol/L</th>
<th>CV within run %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>96</td>
<td>0.8%</td>
</tr>
<tr>
<td>2</td>
<td>20.4</td>
<td>1.8%</td>
</tr>
</tbody>
</table>
INTEGRA 400/700/800

Creatinine 9.6 mmol/L 21.1 mmol/L
(104 mg/dL) (388 mg/dL)

CV between runs 2.0%

Analytical sensitivity (lower detection limit) 0.06 mmol/L (0.88 mg/dL)
The detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying three standard deviations above that of a zero sample (zero sample + 3 SD, within run precision, n = 30).

Method comparison
Creatinine values for human urine samples obtained on COBAS INTEGRA 400 with the cassette COBAS INTEGRA Creatinine plus ver.2 were compared to those determined with the commercially available Creatinine plus reagent on Roche/Hitachi 917.

Roche/Hitachi 917
Method Creatinine plus
Sample size (n) 54
Corr. coefficient (r) 0.998
Lin. regression y = 0.96x + 0.63 mmol/L
Passing Bablok y = 0.96x + 0.31 mmol/L

References
5. Data on file at Roche Diagnostics.

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Roche Diagnostics Corporation, Indianapolis, IN
US Customer Technical Support 1-800-829-7374

CREP2 4/4 2003-09, V 2 EN
Appendix 11: Urea estimation

**Order information**

- **COBAS INTEGRA** 400/700/800
- **Cat. No.** 20763039 322
- **System-ID** 07 6303 9
- **Calibrator** (USA) 12 + 3 ml.
- **Calibrator** (for USA) 12 + 3 ml.
- **Prewarmer U** 20 + 5 ml.
- **Prewarmer U plus** 20 + 5 ml.
- **Prewarmer U plus** 10 + 3 ml.
- **Prewarmer U plus** 10 + 3 ml.
- **COBAS INTEGRA Cleaner Cassette** 150 Tests

**Intended use**

The cassette COBAS INTEGRA Urea/BUN (UREAL) contains an in vitro diagnostic reagent system intended for use on COBAS INTEGRA systems for the quantitative determination of the urea/BUN (blood urea nitrogen) concentration in serum, plasma, and urine. This method should not be used for the analysis of urine samples containing urea or other substances known to interfere with the assay. This method is not intended for the determination of urea in serum or plasma samples containing high levels of urea or other substances known to interfere with the assay. This method is not intended for the determination of urea in serum or plasma samples containing high levels of urea or other substances known to interfere with the assay. This method is not intended for the determination of urea in serum or plasma samples containing high levels of urea or other substances known to interfere with the assay. This method is not intended for the determination of urea in serum or plasma samples containing high levels of urea or other substances known to interfere with the assay.

**Summary**

Urea is the major end product of protein nitrogen metabolism. It is synthesized in the urea cycle in the liver from ammonia which is produced by amino acid deamination. Urea is excreted mostly by the kidney but minimal amounts are also excreted in sweat and degraded in the intestines by bacterial action. Determination of blood urea nitrogen is the most widely used screening test for renal function. When used in conjunction with serum creatinine determinations, it can aid in the differential diagnosis of the three types of asymptomatic: prerenal, renal, and postrenal.

**Test principle**

Kinetic test with urease and glutamate dehydrogenase. Urea is hydrolyzed by urease to form ammonium and carbonate. In the second reaction, 2-oxoglutarate reacts with ammonium in the presence of glutamate dehydrogenase (GLDH) and the coenzyme NADH to produce L-glutamate. In this reaction, two moles of NADH are oxidized to NAD for each mole of urea hydrolyzed.

**Urea + 2 H2O → 2 NH4+ + CO32-**

**2 NH4+ + 2-oxoglutarate + NADH → L-glutamate + NAD+ + H2O**

The rate of decrease in the NADH concentration is directly proportional to the urea concentration in the specimen. It is determined by measuring the absorbance at 340 nm.

**Reagents - working solutions**

- R: Mono reagent in vial A and B (liquid).

**Active ingredients**

- **Components**
  - TRIS 220 ± 5 mmol/L
  - 2-Oxoglutarate 73 ± 5 mmol/L
  - NADH 2.5 ± 0.5 mmol/L
  - ADP 5.1 ± 1 mmol/L
  - Ureaase (fish extract) 2.00 ± 0.51 mmol/L (± 0.25 mmol/L)
  - GLDH (bovine) 2.80 ± 0.20 mmol/L (± 0.05 mmol/L)
  - Sodium azide 0.09 ± 0.02 %
  - pH 8.6 ± 0.6

Both reagents contain nonreactive stabilizers.

**Precautions and warnings**

Pay attention to all warnings and precautions listed in Chapter 1, Introduction, particularly point 6 (sodium azide).

**Reagent handling**

Ready for use.
Storage and stability

Stabilty at 2 to 8°C: See expiration date on cassette
INTEGRA 400: On-board in use at 10 to 35°C: 8 weeks
INTEGRA 700/800: On-board in use at 8°C: 8 weeks

Specimen collection and preparation

For specimen collection and preparation, only use suitable tubes or collection containers.
Only the specimens listed below were tested and found acceptable.

*Serum

Plasma

Li-heparin, EDTA or fluoride plasma. Do not use ammonium heparin.

Urine

Bacterial growth in the specimen and high atmospheric ammonia concentration as well as contamination by ammonium ions may cause erroneously elevated results.

When processing samples in primary tubes, follow the instructions of the tube manufacturer.

Stability in serum/plasma:

- 7 days at 20-25°C
- 7 days at 4-8°C
- 1 year at -20°C

Stability in urine:

- 3 days at 20-25°C
- 7 days at 4-8°C
- 1 month at -20°C

Centrifuge samples containing precipitates before performing the assay.

Materials provided

See "Reagents - working solutions" section for reagents.

Materials required (but not provided)

COBAS INTEGRA Cleaner Cassette, Cat. No. 20784337.
System-ID 07 6435 7. We recommend the use of extra wash cycles when certain test combinations are run together on COBAS INTEGRA systems. For information about test combinations requiring extra wash cycles, please refer to Chapter 1, Introduction, Part III.

Assay

For optimal performance of the assay follow the directions given in this document for the analyser concerned. Refer to the appropriate operator manual for analyser-specific assay instructions.

Application for serum, plasma and urine

INTEGRA 400 test definition

Measuring mode: Absorbance
Abs. calculation mode: Kinetic
Reaction direction: Decrease
Wavelength A/B: 340/409 nm
Calc. fixed/fast: 25/28
Unit: mmol/L
Serum, plasma
Reaction mode: R-S
Test range: 0-40 mmol/L (0-240 mg/dL)
with postdilution: 0-400 mmol/L (0-2400 mg/dL)
Postdilution factor: 10 recommended

Urine

Reaction mode: D-R-S
Test range: 0-2000 mmol/L (0-12 mg/dL)
with postdilution: 0-6000 mmol/L (0-36 mg/dL)
Postdilution factor: 50
Postdilution factor: 5 recommended

Pipetting parameters

Serum, plasma, urine
R: 50 μL, 95 μL
Sample: 2 μL, 43 μL
Total volume: 245 μL

INTEGRA 700/800 test definition

Measuring mode: Absorbance
Abs. calculation mode: Kinetic
Reaction direction: Decrease
Wavelength A/B: 340/409 nm
Calc. fixed/fast: 25/28
Unit: mmol/L
Serum, plasma
Reaction mode: R-S
Test range: 0-40 mmol/L (0-240 mg/dL)
with postdilution: 0-400 mmol/L (0-2400 mg/dL)
Postdilution factor: 10 recommended
Urine
Reaction mode: D-R-S
Test range: 0-2000 mmol/L (0-12 mg/dL)
with postdilution: 0-6000 mmol/L (0-36 mg/dL)
Postdilution factor: 50
Postdilution factor: 5 recommended

Pipetting parameters

Serum, plasma, urine
R: 50 μL, 95 μL
Sample: 2 μL, 43 μL
Total volume: 245 μL

Calibration

Calibrator: Calibrator F.a.S.
Use deionized water as zero calibrator.

Calibration mode: Linear regression
Calibration replicate: Each cassette, every 6 weeks, and as required following quality control procedures
Calibration interval

Traceability: This method has been standardized against SMB 9056.

Quality control

Quality control serum, plasma

Quality control urine

Control interval: 24 hours recommended
Control sequence: User defined
Control after calibration: Recommended
Calculations

COBAS INTEGRA analyzers automatically calculate the
analyte concentration of each sample. For more details,
please refer to Chapter 7, Data Analysis, User Manual
(COBAS INTEGRA 700), or to Data Analysis in the Online
Help (COBAS INTEGRA 400/800).

Conversion factors:

- mg/dL \times 0.024 = \text{mmol/L}
- mg/dL \times 0.0047 = \text{mg/dL}
- \text{mmol/L} \times 0.024 = \text{mg/dL}
- \text{mmol/L} \times 0.0047 = \text{mg/dL}
- mg/dL \times 0.024 = \text{mg/dL}
- mg/dL \times 0.0047 = \text{mg/dL}

Limitations - interference

Criterion: Recovery within ±10% of initial value.

Serum, plasma

Hemolysis

No significant interference. Hemolysis
specimens may cause high absorbance
fluctuating. Choose diluted sample treatment
for automatic rerun.

Icterus

No significant interference.

Lipemia

No significant interference. Lipemic
specimens may cause high absorbance
fluctuating. Choose diluted sample treatment
for automatic rerun.

Anticoagulants

Do not use amomunium heparin as an
anticoagulant.

Other

Ammonium ions may cause erroneously
elevated results.

In very rare cases gammopathy, in
particular type IgM ( Waldenstrom's)
macroglobulinemia, may cause unreliable
results.

For diagnostic purposes, the results should always be
reviewed in conjunction with the patient's medical history,
clinical examination and other findings.

Expected values

- Urea

  | Serum, plasma | Serum, plasma |
  | Adults | Adults |
  | Urea (mmol/L) | Urea (mg/dL) |
  | <8.3 | <112 |
  | <8.3 | <112 |

  | Urine | Urine |
  | Urea | Urea |
  | 24 h urine | 100-200 mg/dL |
  | 150-300 mg/dL |

Specific performance data for serum and plasma

Representative performance data on the COBAS INTEGRA
analyzers are given below. Results obtained in individual
laboratories may differ.

Precision

Reproducibility was determined using human samples and
controls in an internal protocol (within run n = 20, between
run n = 20). The following results were obtained:

<table>
<thead>
<tr>
<th>Level 1</th>
<th>Level 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>6.1 mmol/L</td>
</tr>
<tr>
<td></td>
<td>35.0 mmol/L</td>
</tr>
<tr>
<td>CV within run</td>
<td>2.3%</td>
</tr>
<tr>
<td></td>
<td>(24.6 mg/dL)</td>
</tr>
<tr>
<td></td>
<td>(190 mg/dL)</td>
</tr>
<tr>
<td>CV between run</td>
<td>3.9%</td>
</tr>
<tr>
<td></td>
<td>0.99%</td>
</tr>
</tbody>
</table>

Analytical sensitivity (lower detection limit)

0.30 mmol/L (1.80 mg/dL)

The detection limit represents the lower measurable analytic
level that can be distinguished from zero. It is calculated as the
value lying three standard deviations above that of a zero sample.

Method comparison

Urea values for human serum and plasma samples obtained
on COBAS INTEGRA 700 with the cassette COBAS INTEGRA
Urea/BUN were compared to those determined with commercially
available reagents for urea on COBAS INTEGRA 700 and an
alternative manufacturer's clinical chemistry system. Samples were
measured in duplicate. Sample size in parentheses represents all replicates.

<table>
<thead>
<tr>
<th>Method</th>
<th>Sample size</th>
<th>Corr. coefficient (r)</th>
<th>(r²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>236</td>
<td>0.999</td>
<td>0.999</td>
</tr>
<tr>
<td>BUN</td>
<td>236</td>
<td>0.999</td>
<td>0.999</td>
</tr>
</tbody>
</table>

Specific performance data for urine

Representative performance data on the COBAS INTEGRA
analyzers are given below. Results obtained in individual
laboratories may differ.

Precision

Reproducibility was determined using human samples and
controls in an internal protocol (within run n = 20, between
run n = 20). The following results were obtained:

<table>
<thead>
<tr>
<th>Level 1</th>
<th>Level 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>421 mmol/L</td>
</tr>
<tr>
<td></td>
<td>679 mmol/L</td>
</tr>
<tr>
<td>CV within run</td>
<td>1.3%</td>
</tr>
<tr>
<td></td>
<td>(2.35 mg/dL)</td>
</tr>
<tr>
<td></td>
<td>(4.08 mg/dL)</td>
</tr>
<tr>
<td>CV between run</td>
<td>1.8%</td>
</tr>
<tr>
<td></td>
<td>1.8%</td>
</tr>
</tbody>
</table>

Analytical sensitivity (lower detection limit)

9.32 mmol/L (50.8 mg/dL)

The detection limit represents the lower measurable analytic
level that can be distinguished from zero. It is calculated as the
value lying three standard deviations above that of a zero sample.

Each laboratory should investigate the transferability of the
expected values to its own patient population and if
necessary determine its own reference ranges.

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Method comparison

Urea values for human urine samples obtained on COBAS INTEGRA 700 with the cassette COBAS INTEGRA Urea/RUN were compared to those determined with commercially available reagents for urea on COBAS INTEGRA 700.

Values ranged from 56 to 790 mmol/L (0.34 to 4.78 g/dL).

COBAS INTEGRA 700

Sample size (n) 120
Corr. coefficient (r) 0.999

Lin. regression

\[ y = 1.01x + 1.3 \text{ mmol/L} \]

Passing Bablok

\[ y = 1.01x + 3.5 \text{ mmol/L} \]

References

7. Data on file at Roche Diagnostics.

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Significant additions or changes are indicated by a change bar in the margin.
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Roche Diagnostics, Grenzstraße, 9, 4008 Basel, Switzerland
Roche Diagnostics, 4001 M pioneer, USA
Roche Diagnostics, 2227 Sunset, CA

UREAL
Appendix 12: Red blood cell distribution width (RDW)

**Micros 60**

The Accuracy performance was proven by analyzing approximately (200) patient specimens on the *ABX MICROS 60* along with a commercially available Reference Analyzer, located in (3) different locations throughout the United States. The following table summarizes the data:

<table>
<thead>
<tr>
<th>Site 1</th>
<th>Site 2</th>
<th>Site 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameters</strong></td>
<td><strong>n</strong></td>
<td><strong>R²</strong></td>
</tr>
<tr>
<td>WBC (10⁴ mm⁻³)</td>
<td>198</td>
<td>0.992</td>
</tr>
<tr>
<td>RBC (10⁶ mm⁻³)</td>
<td>198</td>
<td>0.995</td>
</tr>
<tr>
<td>HGB (g/dl)</td>
<td>188</td>
<td>0.994</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>198</td>
<td>0.980</td>
</tr>
<tr>
<td>MCV (µm³)</td>
<td>198</td>
<td>0.988</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>188</td>
<td>0.969</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>188</td>
<td>0.311</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>198</td>
<td>0.950</td>
</tr>
<tr>
<td>PLT (10⁵ mm⁻³)</td>
<td>169</td>
<td>0.994</td>
</tr>
<tr>
<td>MPV (µm³)</td>
<td>191</td>
<td>0.639</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>98</td>
<td>0.975</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>98</td>
<td>0.552</td>
</tr>
<tr>
<td>Granulocytes (%)</td>
<td>98</td>
<td>0.969</td>
</tr>
</tbody>
</table>

Reference Analyzers Baker System 9000 Coulter S Plus IV Coulter JT

*n*: Number of specimens analyzed

*R²*: Correlation coefficient from the regression curve Reference/*ABX MICROS 60*

As mentioned above, this clinical study was performed at (3) different sites throughout the United States.

**Specifications**

RDW (Red cell Distribution Width)

The Red blood cell distribution width is determined, according to the RBC count. The red blood cells pass through a Micro-aperture that will generate electronic pulses, as the cells pass through it. These pulses are then Grouped according to size, Threshold, and calculated to form a Histogram (Distribution curve). This distribution curve is then used to calculate the distribution of the Red blood cells as a percentage of the curve. This curve is then used in determining the RBC size abnormalities as in Anisocytosis.

**Agglutinated Red Blood cells** - May cause a falsely low RBC count and erroneous RDW’s. Blood samples containing the agglutinated RBC’s may be detected by observing abnormal MCH and MCHC values, as well as examination of a stained blood smear.